NOTTINGHAM TRENT UNIVERSITY

CARNOSINE IN SKELETAL MUSCLE: BIOLOGICAL ACTION AND THERAPEUTIC IMPLICATIONS

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Abstract

The worldwide prevalence of diabetes has risen to 8.5% among adults, which represents a staggering rise in prevalence from 4.7% in 1980. More than 90% of these individuals have type 2 diabetes (T2DM), a disease typically characterized by peripheral insulin resistance and underpinned by pancreatic β-cell dysfunction. Importantly, more than 80% of those with T2DM are either overweight or obese, which results in chronic elevated fatty acid and glucose concentrations in these individuals. The ensuing glucolipotoxic (GLT) environment drives much of the pathogenesis of T2DM (β-cell dysfunction and insulin resistance) and contributes to the development of mitochondrial stress, generation of reactive species, proinflammatory cytokines, and altered gene expression. There are currently a limited number of options to treat T2DM, and oral and injectable medications often become less effective over time. Thus, there is an urgent need to better understand the causes of diabetes and to identify new targets for the development of novel treatment strategies. Carnosine (β-alanyl-L-histidine) is an endogenously synthesised dipeptide that is widely and abundantly distributed in the skeletal muscles. Beneficial actions that have been credited to carnosine include, but are not limited to, intracellular buffering, metal-ion chelation, antioxidant, anti-glycating, and free-radical scavenging. This PhD project is focussed on investigating the biological actions and therapeutic potential of carnosine to combat T2DM, through targeted action to improve insulin resistance in skeletal muscle cells, and to augment insulin secretion from pancreatic β-cells. A diabetic model of glucolipotoxicity was generated by incubating pancreatic β-cells or myotubes in standard tissue culture media supplemented with 28mM glucose, 200μM palmitic acid, and 200μM oleic acid. Intracellular reactive species content was assayed using 2, 7-dichlorofluorescein diacetate dye (DCFDA), whereas 3-nitrotyrosine (3-NT) and 4-hydroxynonenal (4-HNE) content, as well as insulin secretion, were assayed and quantified using respective ELISA assays. SDS-PAGE in conjunction with immunoblotting and semi-quantitative densitometry analysis was employed to determine protein expression. Glucose uptake was determined through 2-deoxy glucose-6-phosphate (2-DG6P) luminescence. Immunoprecipitation-mass spectrometry tandem techniques were utilised to study GLT-mediated protein adduction. Seahorse XF Cell Mito Stress Test kit was employed to preliminarily investigate the functional capacity of mitochondria in GLT-exposed skeletal muscle cells. Using carnosine as a starting material and template, both synthetic and computational chemistry approaches were utilised to generate carnosine mimetics and putative carnosinase inhibitor molecules, respectively. Carnosine supplementation resulted in protection of cells against GLT-mediated generation of reactive species, and thereby enhanced glucose uptake into skeletal muscle and increased insulin secretion from pancreatic β-cells. Further investigation showed that carnosine prevented adduction or modification of between 65-90% of protein by 4-HNE or 3-NT in GLT-treated pancreatic islets and muscle cells. Analysis using Panther software showed that many of these proteins are involved in catalytic and binding activities, with the leading cellular function affected being metabolic processes. Importantly, and consistent with the aforementioned findings, addition of carnosine to GLT-treated cells significantly improved mitochondrial respiration in both mouse C2C12 muscle cells and a human skeletal muscle cell-line. By contrast, in human serum donated (with informed consent) by individuals who are either obese or type 2 diabetic, and are both diabetic and obese, several proteins associated with the immune system were detected to have formed adducts with both 3-nitrotyrosine and 4-hydroxynonenal. Screening of carnosine analogs identified 5 candidate drugs that were effective at scavenging reactive species whilst having no impact on cell viability. Subsequent *in vivo* experiments, carried out with collaborators, showed that one of these molecules reduced obesity in high-fat fed mice, whereas one was effective at improving glucose tolerance in these animals. This strategy offers potential therapeutic benefit to patients with obesity and diabetes. In summary, this body of work provides new insights into the biological actions and therapeutic implications of carnosine and associated analogues.

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List of Abbreviations

2DG - 2-deoxyglucose
2DG6P - 2-deoxyglucose-6-phosphate
3-NT - 3-nitrotyrosine
4- HNE - 4-hydroxy-2-nonenal
AA - amino acid
AGEs - advanced glycation end products
ALDH - aldehyde dehydrogenase
ALES - advanced lipoxidation end products
AMPK - adenosine monophosphate activated protein kinase
APS - ammonium persulfate
ATP - adenosine triphosphate
ATPGD - ATP-grasp domain-containing protein
BCA - bicinchoninic acid
Calcein-AM – calcein acetoxy methyl
CAMK - calcium-activated protein kinase
CARNS - carnosine synthase
CE3 - carnosine ester number 3
CN2 - carnosinase-2
CNDP - cytosolic non-specific dipeptidase
CPT-1 - carnitine-palmitoyl transferase-1
DCFDA - 2′7′-dichlorofluorescien diacetate
DMEM - Dulbecco's modified eagle medium
DMSO - dimethylsulfoxide
DPP-4 - dipeptidyl peptidase – 4
DTT - dithiothreitol
ECM - extracellular matrix
EDTA - ethylenediaminetetraacetic acid
ELISA - enzyme-linked immunosorbent assay
ESI - electrospray ionization
FAs - fatty acids
FCCP - carbonyl cyanide-4-trichloromethoxy phenylhydrazone
FFA - free-fatty acid
FGF - fibroblasts growth factor
GDM - gestational diabetes mellitus
GLP-1 - glucagon like peptide
GLT - glucolipotoxicity
GLUT - glucose transporter
GOLD - protein ligand docking software
GPCRs - G-protein coupled receptors
GST - glutathione s-transferase
GSVs - glucose storage vesicles
GTT - glucose tolerance test

HCD - histidine-containing dipeptide
HPLC - high-performance liquid chromatography
HSkM - human skeletal myoblast
iNOS - inducible nitric oxide synthase
IP - immunoprecipitation
IR - insulin receptor
IRS - insulin receptor substrate
JNK - c-Jun-N-terminal kinase
KREBS - Krebs-Ringer buffer solution
LCFA-CoA- long-chain fatty acyl CoA
M28 - maybridge molecule number 28
MAPK - mitogen-activated protein kinase
MG - methylglyoxal
MS - mass spectrometry
NEFA - non-esterified fatty acids
NF-κB - nuclear factor kappa B
NO - nitric oxide
NTU - nottingham trent university
OCR - oxygen consumption rate
o-PA - ortho pthaldialdehyde
OS - oxidative stress
PANTHER - protein analysis through revolutionary relationship
PBS - phosphate buffered saline
PI3K - phosphoinositide 3 kinase
PKB - protein kinase B
PPAR - peroxisome proliferator activated receptor
PTB - phosphotyrosine binding
PTEN - phosphatase and tensin homologue
PUFA - polyunsaturated fatty acid
RCS - reactive carbonyl species
RER - rough endoplasmic reticulum
RIPA - radioimmunoprecipitation buffer
RNS - reactive nitrogen species
RONCS - reactive oxygen nitrogen carbonyl species
RONS - reactive oxygen and nitrogen species
ROS - reactive oxygen species
RPMI - roswell park memorial institute
SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERCA - sarcoplasmic/endoplasmic reticulum calcium-ATPase
SGLT2 - sodium-glucose cotransporter 2
T2DM - type 2 diabetes
TBST - tris-buffered saline tween-20
TEAB - triethylammonium bicarbonate
TEMED- tetramethylethylenediamine
TFA - trifluoroacetic acid

Chapter 1:

Introduction

1.1 Glucose Homeostasis and Health Implications

Glucose is the major energy source for many cell types, making it an essential nutrient of great importance for the organism. Glucose is a hydrophilic molecule and cannot freely cross the plasma membrane, therefore, a carrier-mediated system is required to promote entry of glucose into body cells (Yamamoto, et al., 2015). Glucose transport into most tissues is achieved by the action of molecules called glucose transporters (Leto and Saltiel, 2012). These molecules transport glucose by facilitative diffusion down concentration gradients, in contrast to energy-dependent uptake of glucose in the gut or kidney. In the basal (unstimulated) state, glucose transport is very low and thus most of the glucose transporters are internally sequestered, and their movement, or "trafficking," from this intracellular pool to the cell surface and back is low (Leto and Saltiel, 2012). However, in the presence of insulin, glucose transport is rapidly stimulated through exocytosis of glucose transporter vesicles from the intracellular pool (Leto and Saltiel, 2012). Vesicles move and fuse with the plasma membrane, exposing glucose transporters to the extracellular substrate and thereby effecting glucose transport into the cell. On termination of the insulin stimulus, glucose transporters are recycled from the plasma membrane to the intracellular pool ready for the next insulin stimulus (Wardzala et al., 1978).

The regulation of blood glucose level is essential for the human body to ensure that energy requirements of vital organs are met, and thereby to facilitate normal body functions. This is achieved by a highly complex network of signalling events involving hormone and neuropeptide crosstalk between the brain, pancreas, liver, intestine as well as adipose and muscle tissues (Han et al., 2016, Roder et al., 2016). Regulation of the peptides and hormones involved in the pathways controlling glucose homeostasis is therefore of paramount importance, and failure to maintain this may lead to metabolic disorders such as type 2 diabetes.

1.1.1 Glucose Metabolism in Skeletal Muscle

Glucose transport activity is instrumental in regulating skeletal muscle glucose metabolism, and thus contributes to maintaining whole-body glucose homeostasis. Consequently, this is also a potential therapeutic target for reversal or improvement of insulin resistance in skeletal muscle (Garvey et al., 1998; Wallberg-Henriksson and Zeirath, 2001).

Skeletal muscle energy utilisation is tightly controlled. This is dependent on energy availability and requirements, and skeletal muscle can adapt to utilise different substrates to generate ATP. Depending on the metabolic state of an individual, skeletal muscles can either utilise glucose (fed state) or fatty acid (fasting state) as a source of ATP production (Cahova and Kazdova, 2007). Therefore, switching ability between FFA and glucose in the fasted and fed state is very important, otherwise metabolic inflexibility could result in impaired metabolism of these substrates and potentially lead to development of insulin resistance (DeFronzo, 2009).

1.1.2 Skeletal Muscle Glucose Transport

After a meal, glucose is actively transported across the plasma membrane by carrier proteins belonging to the glucose transporter (GLUT) family. There are several types of glucose transporters located in the plasma membrane, however glucose transport into skeletal muscles occurs via GLUT1 and GLUT4 (Klip et al., 1996). GLUT1 is the most ubiquitously distributed transporter isoform, it has high affinity to glucose and thus is believed to be responsible for constitutive glucose uptake or in regulating basal glucose. It is concentrated in endothelial cells of blood-tissue barriers, and therefore acts as a vehicle of glucose between blood and organs that have limited access to small solutes via passive diffusion (Mueckler, 1994).

Whilst GLUT1 is restricted to the cell surface, the more abundant GLUT4 is largely located intracellularly in the basal state. However, it can rapidly translocate to the plasma membrane in response to insulin stimulation or exercise, which leads to increased glucose uptake into the muscle. GLUT4 is primarily expressed in adult tissues that exhibit insulin-stimulated glucose transport such as adipose tissue, skeletal, and cardiac muscle (Klip et al., 1996; Mueckler, 1994).

The amino acid sequence of both GLUT1 and GLUT4 are highly conserved, with 95%-98% identity among the sequences of human, rat, mouse, rabbit, or pig transporters (James et al., 1988 in Simmons, 2003). GLUT3 was first isolated from human foetal skeletal muscle and is not as highly conserved as that of GLUT1 or GLUT4. Whilst GLUT3 can be found in all human tissues, it is most abundant in the brain, kidney, and placenta, and is considered the major neuronal glucose transporter (Thorens and Mueckler, 2010). The glucose transporter isoform GLUT2 is expressed in the liver, intestine, kidney and pancreatic islet β-cells, as well as in the central nervous system, neurons, and astrocytes. GLUT2 is required for glucose-stimulated insulin secretion and its inactivation in the liver could lead to an impaired glucose-stimulated insulin secretion (Thorens, 2015). In addition, GLUT2-dependent glucose sensing controls feeding, thermoregulation and pancreatic islet cell mass and function, as well as sympathetic and parasympathetic activities (Thorens, 2015).

1.2 Skeletal Muscle as Target for T2DM Therapy

Skeletal muscle is the largest (by mass) organ of the human body and is the primary site of glucose uptake, disposal, and storage, accounting for approximately 75% of the entire body's glucose uptake under insulin stimulation (Smith and Muscat, 2005). During physiological hyperinsulinemia, leg muscle capacity for glucose uptake increases linearly with time, reaching a plateau value of around 10 mg/kg leg weight per minute after 60 min. By contrast, in type 2 diabetic subjects the onset of insulin action is delayed and the ability of insulin to maximally stimulate glucose uptake is markedly blunted by almost 50% (DeFronzo et al., 1981).

Peripheral insulin resistance is the central pathogenesis of major metabolic disorders, and thus insulin resistance in skeletal muscle impacts whole-body glucose homeostasis (DeFronzo and Tripathy, 2009). It has also been argued that an interaction between skeletal muscle and pancreas occurs, and that this crosstalk may regulate insulin secretion to coordinate intracellular glucose utilization (Mizgier et al., 2014; Hartwig et al., 2014). Skeletal muscle also plays a major role in insulin sensitivity through interactive crosstalk with hepatic and adipose tissues (Gancheva et al., 2018).

T2DM is increasingly becoming viewed as a disease with an underlying autoinflammatory component (Gonzalez et al., 2018). Studies of skeletal muscle response to cytokines or other proinflammatory molecules have been useful in this regard, as they have helped to elucidate mechanisms and pathways associated with insulin sensitivity, glucose metabolism and the role of these molecules on the pathophysiology of obesity and diabetes (Jian et al., 2013; Poelkens et al., 2013). This also suggests that anti-cytokine biologics might also have beneficial actions in T2DM through improved glucose uptake and general muscle health.

Impairments in insulin action on non-oxidative glucose metabolism in this tissue are among the earliest metabolic defects in T2DM (DeFronzo and Tripathy, 2009). Proteomic and genomic studies indicate that there are intrinsic differences in the profile of proteins involved in energy metabolism, cellular oxidative stress, protein dynamics, and gene regulation in myotubes between T2DM patients and individuals with normal glucose tolerance [NGT] (Al-Khalili et al., 2014). As the only known insulin-responsive glucose transporter, GLUT4 plays a key role in insulin-mediated regulation of glucose metabolism in vivo (Liu et al., 2009). Studies indicated that GLUT4 function is reduced in diabetic states and because it has an important role in insulin action in vivo, therapeutics that promote insulin-stimulated GLUT4 translocation could increase postprandial glucose uptake into skeletal muscle, thereby consequently improving insulin-sensitivity (Henriksson, 2001).

These are just a few of the many studies to support the notion that resistance to the action of insulin in its metabolic target tissues, and particularly in skeletal muscles, occurs in almost all patients with type 2 diabetes. This forms the pathophysiologic basis of the visceral obesity-linked metabolic syndrome, and improving tissue sensitivity to insulin is a major clinical goal to help ameliorate not only abnormal glucose metabolism, but also some of the complications like cardiovascular that are associated with this syndrome. Therefore, skeletal muscle is an essential target tissue for therapy of type 2 diabetes.

1.3 Skeletal Muscle Insulin Resistance

A major pathological defect in diabetes is insulin resistance, which is characterized by the impaired capacity of peripheral tissues to utilize glucose effectively in the face of hyperinsulinemia (Lann and LeRoith, 2007). In skeletal muscle, impaired glucose homeostasis results from suboptimal insulin signaling that mediates various events of glucose metabolism. The underlying pathogenic mechanism can however occur at various stages in the signal transduction pathway, including the ligand-receptor interaction (Carnagarin et al., 2015).

It has been reported that in the early stages of development of type 2 diabetes, impaired glycogen synthesis in muscle is the primary defect responsible for insulin resistance (Krook et al., 2000). In addition, elevated plasma free fatty acid concentrations are typically associated with many insulin-resistant states, including obesity and type 2 diabetes (Boden et al., 1994). Indeed, an acute increase in plasma FFA via intravenous lipid infusion has been shown to induce skeletal muscle insulin resistance in non-diabetic and diabetic subjects, while acute lowering of elevated plasma FFA levels lowered insulin resistance in both diabetic and non-diabetic patients (Boden, 2011), thereby demonstrating the major role played by skeletal muscle in the pathogenesis of insulin resistance.

Fatty acid metabolites may also have an important role in insulin resistance, with diacylglycerol, fatty acyl CoA's, or ceramides activating a serine/threonine kinase cascade (possibly initiated by protein kinase C), that leads to phosphorylation of serine/threonine sites on insulin receptor substrates. Serine-phosphorylated forms of these proteins fail to associate with or to activate PI3-kinase, resulting in decreased activation of glucose transport and other downstream events (Schulman, 2000). Insulin stimulation of PI3-kinase activity is also a requisite for the activation of glucose transport and glycogen synthesis.

Using magnetic resonance spectroscopy, a study conducted on the offspring of T2DM parents observed lower mitochondrial density in their muscles as compared to control subjects (Morino et al., 2005). This was associated with a decreased rate of insulin-stimulated glucose uptake, increased IRS serine phosphorylation and reduction in Akt activation. Thus, reduction in mitochondrial content and/or function might be an early defect responsible for impaired insulin signaling and action in muscle (Morino et al., 2005). This study also supports the hypothesis that reduction in mitochondrial density and function could then eventually inhibit intracellular insulin signaling pathways and ultimately result in insulin resistance (Abu Bakar et al., 2015). Therefore, by elucidating the cellular and molecular mechanisms responsible for insulin resistance, potential new targets for the treatment and prevention of type 2 diabetes might be identified.

1.4 Structure, Function, and Secretion of Insulin

The discovery of insulin by Banting, Best, and colleagues in 1921 at the University of Toronto, Canada was legendary and became one of the most important events in both the study and treatment of people with diabetes. The circulating and biologically active form of this hormone is composed of 51 amino acid residues in two chains, A and B, connected by two disulphide bridges, A7-B7 and A20-B19. This is secreted by pancreatic β-cells of the Islets of Langerhans (De Meyts, 2004).

The hormone insulin is the post-translational product of a single-chain precursor called proinsulin, which is itself processed from preproinsulin at the point of insertion into the rough endoplasmic reticulum (RER) through signal sequence cleavage by a signal peptidase. In RER, the folded and stable 3D configuration of proinsulin will then link the semi helical A domain and the helical B domain through the formation of three disulphide bonds. The properly folded proinsulin is subsequently sorted into immature secretory granules, following post-translational modifications and transit through the Golgi apparatus (Hutton, 1994).

These granules bud away from the trans-Golgi network, whereupon they acidify. This in turn activated the prohormone convertases (PC1/3, and PC2) which cleave the C-peptide from proinsulin. Carboxypeptidase E removes the C-terminal basic amino acids of the resulting peptide chains to yield the mature insulin, with A and B chains linked by disulfide bonds (Tokarz et al, 2018). The synthesis of insulin is generally rapid and efficient in less than 2 hours, and with only about 2% remaining as proinsulin within mature secretory granules. Insulin then forms a hexameric crystal with a central zinc ion during granule maturation (Figure 1.1), the zinc having been transported into secretory granules through ZnT8 zinc transporters (Tokarz et al, 2018).

After glucose ingestion, insulin secretion is stimulated much more than it is when infused intravenously (Perley and Kipniss, 1967). This effect (the incretin effect) plays a major role in the regulation of glucose metabolism in healthy subjects and is estimated to be responsible for 50 to 70% of the insulin response to glucose (Nauck et al., 1986). The incretin effect implies that carbohydrate (glucose) ingestion causes the release of gut-derived hormones that enhance insulin secretion beyond the release caused by the absorbed glucose itself (McIntyre et al., 1965). The two most important candidates responsible for incretin effects are the glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) (Vilsboll and Holst, 2004). In patients with type 2 diabetes, however, the incretin effect is lost or greatly impaired. It is hypothesized that this loss explains an important part of the impaired insulin secretion in patients (Holst and Gromada, 2004). In recent years, there has been a great interest in developing effective methods to regulate glucagon-like peptide-1 secretion (MacDonal et al., 2002; Ellingsgaard et al., 2011; Wang et al., 2015).

Insulin plays a central role in the regulation of glucose homeostasis through stimulation of glucose uptake into peripheral cells and tissues, and suppression of hepatic gluconeogenesis. Hyperglycemia results however when there is either insufficient insulin release, and/or decreased insulin sensitivity, and this then leads to the development of type 2 diabetes. Chronic exposure of tissues to the resulting elevated glucose concentrations can in turn result in the development of both macro- and microvascular complications and diseases including cardio-and cerebrovascular, retinopathy, nephropathy, and neuropathy (Weiss et al, 2000).

Multiple defects in insulin secretion can lead to the development of type 2 diabetes. These include downregulation (Marshall et al, 2007) and mislocalisation (Somanath et al., 2009) of the SNARE machinery that regulates exocytosis in pancreatic β-cells, and the consequent impaired release of insulin in diabetic individuals (Gandasi et al., 2017). By contrast, impaired vasoactive effects of insulin can occur during insulin resistance, including capillary recruitment (de Jong et al,2004). There is also reduced sensitivity to insulin in skeletal muscle of obese individuals, which in part explains the close association between obesity and T2DM (Broussard et al, 2017). This then results in decreased GLUT4 translocation to the muscle membrane in diabetic patients (Czech, 2017). In addition, defective kidney function in patients with T2DM may further alter insulin bioavailability (Kanasaki et al., 2013).

Figure 1.1. Insulin biosynthesis and secretion. (A) Preproinsulin mRNA from the INS gene is transcribed and translated to preproinsulin peptide and then processed to proinsulin in RER and after transit to the Golgi network, proinsulin is then sorted into immature secretory granules. Following release from the TGN, granules acidify and proinsulin is cleaved to its mature form, then stored as hexameric crystals coupled with Zn^{2+} within mature secretory granules [TGN-Trans-Golgi Network]. (B) Glucose sensing leads to a series of metabolic and electrical signals that culminate in closure of ATP-dependent K^{+} channels, membrane depolarisation, then entry of Ca^{2+} through Lc type channels. This then triggers the exocytotic release of insulin from the pancreatic β-cells [GK-Glucokinase, VDCC - voltage-dependent Ca2+ channels, SNARE-Soluble N–ethylmaleimide sensitive factor (NSF) attachment protein receptor, GLUT-Glucose Transporter]. (C) Insulin released from the pancreas will be transported to the liver. During the first pass, over 50% of insulin is cleared by the hepatocytes in the liver and the rest will exit and proceeds to the heart. Through arterial circulation, it can be distributed to the rest of the body for metabolic use in the liver and after its final clearance in the liver during the second

pass, it exits from the circulation and travels to muscle and fat cells where it exerts metabolic actions including GLUT4 translocation and glucose uptake. The remaining circulating insulin is delivered to and finally degraded by the kidney (Figure Source: Tokarz et al., 2018).

1.5 Insulin Receptor and Insulin Receptor Substrate-1 (IRS-1)

The insulin receptor (IR) has a key role in the regulation of glucose homeostasis, and therefore when its function is impaired this can lead to a range of clinical manifestations including diabetes mellitus, cancer and Alzheimer's disease (Kidmose, 2016). Insulin mediates its biological effects via the insulin receptor (IR), and like type-I insulin-like growth factor receptor (IGF-1R) and the insulin-related receptor (IRR), they all belong to the large family of cell surface receptors that possess an intrinsic tyrosine kinase activity that is essential for their biological activity and action.

Homologous with IGF-1R, insulin receptor's architecture (Figure 1.2) is in homodimeric form ($\alpha_2\beta_2$) and is composed of two hormone-binding extracellular α-subunits (130 kDa each) and two membrane-spanning tyrosine kinase β-subunits (95 kDa each), wherein each of this αβ protomer is covalently linked by disulfide bond (Kidmose et al, 2016, Kahn et al, 2014, and Obberghen et al, 2001). The ectodomain monomer of the receptor (α chain) is composed of two leucine-rich repeat (L1 and L2) domains with a cysteine-rich region (CR) between them, then two fibronectin type III domains (FnIII-1 and 2) where about half of FnIII-2 belongs to the β chain, a region designated as insert domain (ID), and finally a C-terminal peptide domain (αCT). The β chain also contains an insert domain, FnIII domain (FnIII-3), the remaining part of FnIII-2, regulatory regions trans- and juxtamembrane domain (TM and JM), a tyrosine kinase (TK) domain where phosphotyrosine-binding sites for signaling molecules are located, and finally a C-terminal domain (Lawrence et al, 2007; Kidmose et al., 2016; Ye et al., 2017).

The autophosphorylation sites of the insulin receptor are three tyrosine residues (Y1146, Y1150 and Y1151) in the kinase catalytic domain, and one key tyrosine residue (Y960) in the juxtamembrane domain. These residues contribute to the recognition motif for insulin receptor substrates once phosphorylated, and there are two autophosphorylation sites (Y1316 and Y1322) in the C-terminus of the insulin receptor (Obberghen et al, 2001). A conformational change results when the ligand, insulin, binds to the α-subunits, thereby stimulating the β-subunits intrinsic tyrosine kinase activity. This activation process allows the receptor to initiate a cascade of phosphorylation events which in turn leads to the activation of enzymes with pivotal roles in many aspects of metabolism and growth (Obberghen et al, 2001).

At the time of insulin binding to the α-subunits of IR, a transphosphorylation occurs among β-subunits, which in turn further activates the kinase to recruit insulin receptor substrates. There are six (IRS-1 to IRS-6) well-characterised members of the insulin receptor substrate (IRS) family of proteins, which act as scaffolds to organize and mediate signaling complexes. This utilises the pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains in the amino terminus of IRS proteins to couple to its receptor and uses its tyrosine phosphorylation sites in the COOH-terminal as on-off switches or docking sites for recruiting and regulating several downstream src homology-2 (SH2) containing-signalling proteins. These include PI3-kinase cascades, of which activation is an important insulin-regulated pathway (Lee and White, 2008; Obberghen et al, 2001).

Figure 1.2. Schematic representation of the insulin receptor. Leucine-rich repeat domain (L1 and L2); cysteine-rich region (CR); fibronectin type III domains (FnIII-1/2/3); insert domain (ID- α/β); transmembrane and juxtamembrane domains (TM/JM); C-terminal domains for alpha chain (αCT); C-terminal domains for beta chain (C). Disulphide bonds are depicted as blue dashed lines. (Figure Source: Kidmose et al., 2016).

1.6 Insulin Signaling in Healthy and Insulin Resistant Skeletal Muscle

Insulin signaling is complex, and elucidating the molecular mechanisms involved in insulin resistance is a major challenge. Insulin is the most potent anabolic hormone known and is essential for appropriate tissue development, growth, and maintenance of whole-body glucose homeostasis. It regulates glucose homeostasis at many sites, reducing hepatic glucose output (via decreased gluconeogenesis and glycogenolysis) and increasing the rate of glucose uptake, primarily into striated muscle and adipose tissue (Pessin and Saltiel, 2015).

The insulin receptor is an α2β2 heterodimeric transmembrane protein that possesses intrinsic tyrosine kinase activity. When insulin binds to the receptor, it induces conformational changes of the receptor, resulting in autophosphorylation and activation of receptor tyrosine kinases, which in turn recruits and stimulates insulin receptor substrates (IRS) including IRS1. IRS1 then binds to the regulatory subunit of phosphoinositide 3-kinase (PI3-K) via Src homology 2 domains (SH2) which leads to activation of PI3-K. PI3-K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) (a membrane phospholipid) on the 3′ position. The resulting complex activates the 3-phosphoinositide-dependent protein kinases-1 (PDK-1) resulting in activation of Akt/ protein kinase B (PKB) and atypical protein kinase C isoforms (aPKC ζ/λ), each of which is serine/threonine kinases (Desmukh, 2006). The physiological regulation of insulin action is controlled by the balance between phosphorylation and dephosphorylation events. For example, a negative effect on insulin-mediated glucose metabolism is observed when protein tyrosine phosphatases (PTPs) dephosphorylate and thus inactivate IR and when other phosphatases like phosphatase and tensin homologue (PTEN) inhibit the PI3K pathways (Desmukh, 2006; Taniguchi et al., 2006; Vinciguerra and Foti, 2006).

Once glucose enters the cell, it is phosphorylated by the enzyme hexokinase. Glucose-6-phosphate is then either utilized in the glycolytic pathway or else incorporated into glycogen by glycogen synthase. However, insulin signaling defects that lead to impaired GLUT4 translocation are believed to be the major cause of skeletal muscle insulin resistance, and thus a major risk factor in developing type 2 diabetes. Studies have reported that skeletal muscle GLUT4 expression in type 2 diabetic subjects is normal (Pedersen et al., 1990; Kahn et al., 1991). In conclusion, impaired insulin-stimulated glucose uptake most likely results either from an inability to correctly signal to or translocate GLUT4 to the plasma membrane, or from impaired function of GLUT4.

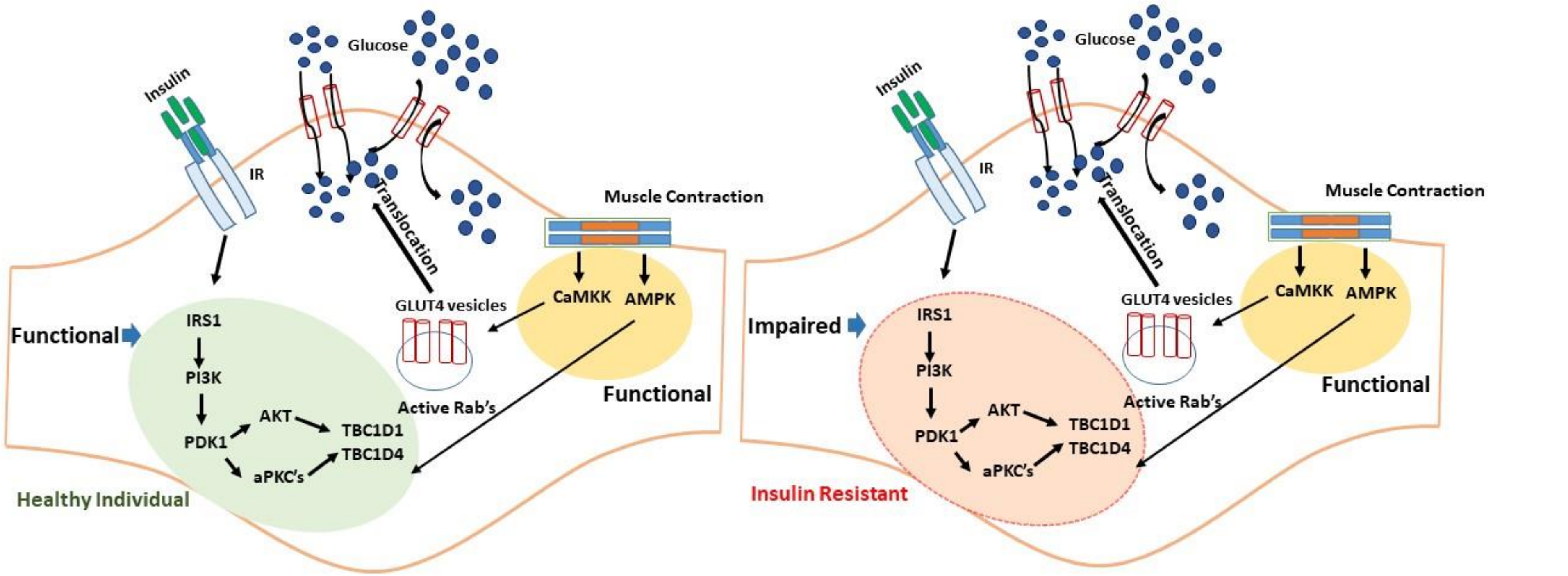


Figure 1.3. Insulin signaling cascade regulating glucose uptake in healthy and insulin resistant skeletal muscle. (**Left**)Both insulin and muscle contraction-mediated signaling pathways to GLUT4 are functional in healthy skeletal muscle. (**Right**) Insulin signaling leading to GLUT4 is impaired in insulin resistant skeletal muscle while muscle contraction-induced signaling pathways are intact. (Adapted from Deshmukh, 2016)

1.6.1 Negative and Positive Regulators of Insulin Signalling

The attenuation of insulin-induced glucose uptake in muscle and fat cells via GLUT4 and the reduced ability of insulin to suppress glucose production by the liver is believed to be the earliest abnormality observed in insulin resistance. The hyperglycaemic effect of insulin resistance is initially compensated by pancreatic β-cell hypersecretion of insulin to maintain euglycemia, however, when this becomes chronic, hyperinsulinemia exacerbates insulin resistance which leads to ß-cell failure and eventually to the development of clinical overt type 2 diabetes (Gual et al., 2005).

There are several ways in which insulin signaling can become defective, including reduced concentration and phosphorylation of IRS-1, decreased PI(3)K expression or activity, and inhibited translocation of glucose transporters (Saltiel and Kahn, 2001; Pessin et al., 2000). Type 2 diabetes is a polygenic disorder, with diabetes predisposition polymorphisms in several genes encoding proteins associated with insulin signaling or insulin secretion such as hepatocyte nuclear factor-1A (HNF1A) and the glucokinase (GCK), Calpain10 (CAPN10), transcription factor 7-like 2(TCF7L2), peroxisome proliferator-activated receptor gamma (PPARG), insulin receptor substrate (IRS) (Ali, 2013; Stern, 2000). In addition, there are insulin receptor mutations linked to Donohue syndrome or leprechaunism, Rabson Mendenhall Syndrome, and type A syndrome of insulin resistance and although these genetic disorders are relatively rare, they represent the most severe forms of insulin resistance (Taylor and Arioglu, 1998).

Obesity increases the risk of developing type 2 diabetes initiated through insulin resistance. In this section, relevant obesity-related factors that are causally linked to insulin resistance are described briefly. Elevated plasma FFA are often found in obese individuals due to increased or enlarged mass of fat or adipose tissue. The increased circulating level of FFA is believed to cause insulin resistance because of an altered and impaired insulin signaling through activation of JNK, IKK and PKC. Although the mechanism of kinases activation by FFA is unclear, this may include FFA-mediated production of reactive oxygen species (ROS), activation of the Toll-like receptor 4 (TLR4) pathway, or endoplasmic reticulum stress. Alternatively, there may be impaired insulin signaling following reduction of tyrosine phosphorylation of the insulin receptor substrates and decreased activity within the IRS-PI3 kinase-Akt axis – an important pathway which regulates the metabolic action of insulin like glucose uptake (Schenk et al., 2008; Shi et al., 2006; Yu et al., 2002; Inoguchi et al., 2000).

A study conducted in healthy men and women (irrespective of age) showed that raising plasma FFA by acutely infusing heparinised lipid emulsion resulted in a significant reduction in glucose-uptake and the development of insulin resistance within 4-hours of the increase. These changes then reverted back after normalisation of FFA levels (Boden and Chen, 1995). Furthermore, it has been demonstrated that lowering FFA could reduce insulin resistance in a study of obese (non-diabetic and T2DM) individuals, where after 12h normalisation of elevated FFA levels this resulted in normalised insulin-stimulated glucose uptake and 25-50% improvement of insulin sensitivity. This therefore suggests that high circulating levels of FFA may have been the cause of insulin resistance in these subjects. Interestingly, similar findings were also reported in individuals who are genetically predisposed to T2DM (Santomauro et al., 1999; Cusi et al., 2007).

The expansion of adipose tissue that results from caloric overload and obesity results in the release of increasing amounts of cytokines and chemokines (collectively referred as adipokines) that are associated with inflammation (Gonzalez et al., 2018), and these are considered a major causes of obesity-associated insulin resistance (Osborn and Olefsky, 2012). For example, the increased generation of the proinflammatory cytokines TNF-α, IL-6, and C-reactive protein (CRP) have been demonstrated in insulin-resistant and diabetic subjects (Hotamisligil et al., 1993), and normalisation of TNF-α levels in adipose tissue and blood from obese rodents improves insulin sensitivity in these animals (Ellies et al., 2007). Similarly, in a high-fat-fed mouse model of obesity there was also chronic inflammation associated with the increased production and secretion of cytokines (Xu et al., 2003).

FFA-associated secretion of cytokines like TNF-α, IL1-β, IL6 not only activates pro-inflammatory pathways, but also results in peripheral and hepatic insulin resistance. This is associated with phosphorylation of certain IRS serine residues , which then prevents its interaction with the insulin receptor (Gual et al., 2005). Thus consumption of a high-fat diet is likely to have a negative impact on glucose homeostasis, by affecting normal glucose transport as a consequence of inflammatory-induced insulin resistance.

The adipocyte-derived hormones such as leptin and adiponectin have been recognised to have major influence on energy balance. Leptin levels has been shown to be higher in individuals with higher BMI and higher per cent total body fats (Schwartz et al., 1996). Interestingly, despite leptin's functions to signal key regulatory centres in the brain to inhibit food intake and to regulate body weight and energy homeostasis, and even though it increases proportionally with adiposity, the increased leptin fails to curtail the progression of obesity (Halaas et al., 1995; Widdowson et al., 1997; Levin and Dunn-Meynell, 2002). It is believed that in essence, the augmented leptin accompanying obesity contributes to leptin resistance, and this leptin ineffectiveness promotes further obesity, leading to a vicious cycle of escalating metabolic devastation (Zhang and Scarpace, 2006). It has been recognised that the accumulation of fat in obesity has an important role in the altered expression of several hormones, growth factors, and adipokines (Nigro et al., 2014). Among the adipokines, adiponectin shows protective activity in various processes such as energy metabolism, inflammation, and cell proliferation (Lihn et al., 2005). Adiponectin is an adipokine that is specifically and abundantly expressed in adipose tissue and directly sensitizes the body to insulin (Kadowaki et al., 2006). Genetic and environmental factors causing obesity is believed to lead the occurrence of a condition called hypoadiponectinemia which appears to play an important causal role in insulin resistance and type 2 diabetes (Kondo et al., 2002). Expression enhancement of adiponectin and its receptors may represent as therapeutic approach against obesity and obesity-related diseases or could be potentially prevent the establishment and/or progression of lethal conditions related to obesity.

Chronic hyperglycaemia is a hallmark of type 2 diabetes, and glucotoxicity is known to reduce the capacity of pancreatic β-cells to secrete insulin (Marshall et al, 2007), and to increase the risk of developing insulin resistance (LeRoith, 2002). Similar to FFA, glucose infusion has been found to impair insulin sensitivity by reducing skeletal muscle glucose uptake by affecting post-receptor signaling cascades, and the hyperglycaemia-induced formation of advanced glycation end products and hyperglycaemia-mediated PKC activation are known to inhibit insulin-stimulated phosphorylation of several proteins along the insulin signaling pathway (Nishikawa, 2000). In a study where sustained hyperglycaemia was employed in human and rat primary skeletal muscle cells, C2C12 muscle cells, L6 muscle cells, and adipocytes, impaired insulin signalling was associated with elevated production of diacylglycerols, ceramides, and from increased oxidative stress. This resulted in a decreased capacity of insulin to activate of Akt/PKB (Tomas et al., 2006), which in turn can lead to defective GLUT4 translocation (Van Cromphaut, 2009). Catecholamines, excessive glucocorticoids, and growth-hormones have also been reported to induce insulin resistance through inhibiting insulin binding to the insulin receptor, impaired GLUT4 translocation, reduced insulin receptor expression, decreased tyrosine kinase activity, and abolished insulin-induced PI3K activation (Haring et al., 1986; Dimitriadis et al. 1997; Dominici et al., 1999).

Nutrient overload can also affect important organelles which regulate energy homeostasis (Qiu and Schlegel, 2018). This can result in increased generation of ROS by the mitochondria, production of unfolded protein responses (UPRs) by the endoplasmic reticulum (ER), and the activation of the mechanistic target of rapamycin. The resulting mitochondrial and ER stress are known to negatively affect insulin signalling, and thus insulin resistance (Wellen and Thompson, 2010). Mitochondrial oxidant production activates JNK which in turn phosphorylates IRS1, whereas ER stress affects protein kinase RNA (PKR)-like ER kinase (PERK) and the inositol-requiring protein-1 (IRE1). JNK and IKK activation further impairs insulin signalling via phosphorylation of Ser-307 (Ozcan et al., 2004; Laybutt et al., 2007). In addition, a study by Koh et al., 2013 using C2C12 and mouse skeletal muscles demonstrated that overexpression of Tribble 3 protein (TRB3) in this tissue mediates endoplasmic reticulum stress and impairs insulin signaling by inhibiting IRS phosphorylation and decreasing Akt activation.

Physical inactivity and nutrient overconsumption are major risk factors for the development of insulin resistance and thus type 2 diabetes. The ideal intake of calories varies depending on age, metabolism and levels of physical activity, among other things (National Health Service UK, 2019). Generally, the recommended daily calorie intake is 2000 calories a day for women and 2500 for men (Institute of Medicine, 2002). The NHS guidelines on physical activity also recommend at least 150 minutes of moderate-intensity aerobic activity and muscle-strengthening activities per week (National Health Service UK, 2019). A study shown that adults in the UK are consuming 50% more than they realised (Office for National Statistics UK, 2018). In another study, it has shown that 44% of UK adults never do any moderate physical activity and 13% of UK adults are sedentary for longer than 8.5 hours a day (British Heart Foundation, 2015). Epidemiological studies have indicated that regular or increased physical exercise can reduce the risk of developing type 2 diabetes (Helmrich, 1991; Tuomehlito et al., 2001). With this, exercise or muscle contraction is believed to be a positive regulator of insulin signalling, which then improves the rate of whole-body glucose disposal and glucose uptake (Goodyear and Kahn, 1998; Holloszy, 2005).

Exercise and insulin have an independent signaling route, although both lead to translocation of glucose transporter and increased GLUT4 expression. Muscle contraction or exercise mediate glucose uptake through activation of signaling proteins, including AMP-activated protein kinase (AMPK), calcium-activated protein kinase (CAMK), Akt and nitric oxide pathways which are linked to the translocation of GLUT4 to the plasma membrane (Fujii et al., 2006; Witczak et al., 2010). The observed effects of exercise-mediated improvement of insulin sensitivity were not however linked with the IRS1-PI3K-AkT signaling cascade (Wojtaszewski et al., 1997). Instead a study demonstrated that this effect was mediated most likely at more distal signaling component namely the TBC1 Domain Family Member 1 and 4 (TBC1D1 and TBC1D4) (Pehmoller et al., 2012) which corroborated with an earlier observation by Maarbjerg and colleague (Maarbjerg et al., 2011).

The mammalian target of rapamycin (mTOR) is a highly conserved serine–threonine kinase that regulates numerous functions essential for cell homeostasis and adaptation in mammalian cells such as the insulin signalling cascade, and has also been described as an insulin-independent nutrient sensor that may represent a critical mediator in obesity-related impairments of insulin action in skeletal muscle (Rivas et al., 2009). Signaling through the mTOR pathway could have both favourable and unfavourable consequence in the maintenance of β-cell function and glucose metabolism in response to overnutrition (Jia et al., 2014). When mTOR is chronically activated via overnutrition, it increases insulin resistance by inhibiting IRS-1 through increased JNK phosphorylation and induces hyperinsulinemia which then could eventually lead to a decreased β-cell survival and increased apoptosis (Fraenkel et al., 2008). On the other hand, Nutrients, hormonal, and contractile stimuli often converge at this protein, suggesting that mTOR is an important modulator of protein synthesis (Wang and Proud, 2006). Studies have indicated that a combination of nutrients (leucine-enriched essential amino acids in particular) and resistance exercise could stimulate human muscle protein synthesis probably through enhanced mTOR signalling pathway (Fujita et al., 2007; Drummond et al., 2009; Dickinson et al., 2011).

Long-term caloric restriction (CR) has been proposed as a possible intervention to improve the quality of health. For instance, early studies in rats showed augmented insulin-stimulated glucose transport via increased plasma membrane GLUT4 after CR (Dean et al., 1998; Cartee et al., 1994) A later study by Argentino et al., 2005 reported that long-term CR significantly increased the abundance of IRS-1 which might be linked to the animals' adaptation mechanism to enhance insulin sensitivity in light of reduced availability of glucose. A study also demonstrated that even a brief period (20 days) of CR resulted in an increased whole-body insulin sensitivity due to enhanced glucose transport mediated by insulin (Gazdag et al., 1999). CR could also improve insulin-stimulated glucose transport through enhanced phosphorylation of Akt, as an increase in the ratio of PI3-kinase catalytic to regulatory subunits has been observed, which indicates that PI3-K signaling is favoured and thus likely to result in greater Akt phosphorylation and consequently improved skeletal muscle insulin sensitivity (McGurdy, Davidson and Cartee, 2005).

1.7 Structure, Properties, and Functions of Skeletal Muscle

The contractile property of muscle may strongly influence its function and is the basis for most skeletal muscle studies on its important functional role particularly in response to disease and injury. Skeletal muscle is one of the most dynamic tissues in the human body. It is composed of 75% water, 20% protein and 5% other substances including inorganic salts, minerals, fats, and carbohydrates. Muscle constitutes approximately 40-45% of total body weight and contains 50–75 % of all body protein (Frontera and Ochala, 2015). The muscle mass of an individual is directly affected by the balance between muscle protein synthesis and degradation, which in turn is influenced by several factors including injury and disease, hormonal imbalance, physical activity or exercise, and nutritional status (Frontera and Ochala, 2015).

Skeletal muscle is composed of bundles of muscle fibres called fascicles (Figure 1.4), which in turn are composed of muscle cells, surrounded by sarcolemma. The sarcoplasm in the muscle contains the cellular proteins, the organelles, and the myofibrils. The myofibrils contain the contractile apparatus of the muscle, namely the thin filament (actin) and the thick filament (myosin) which is organised into repeating contractile units called sarcomeres (Rivas and Fielding, 2012).

The size of the whole muscle is primarily determined by the number and size of the individual muscle fibres. Each muscle fibre has an approximate diameter and length of 100 μm and 1 cm, respectively that is surrounded by a layer of connective tissue referred to as the epimysium, and within each muscle fibre are bundles surrounded by another layer called perimysium. Between the sarcolemma and the basal lamina are adult stem cells of skeletal muscles called satellite cells which have an essential role for its regeneration, growth, and repair, wherein upon activation by myogenic factors these will proliferate and differentiate into new muscle fibres (Gopinath and Rando, 2008).

The main function of skeletal muscle from a mechanical point of view is to convert chemical energy into mechanical energy in order to generate force and power, and thereby movement and posture which influence the body to perform physical activities and maintain or enhance health (Rivas and Fielding, 2012). The metabolic role of skeletal muscle includes contribution to the basal energy metabolism, maintenance of body core temperature, and oxygen and fuel consumption during physical activity. It also serves as a reservoir for amino acids and carbohydrates, which are needed by other tissues including brain, heart, and skin for the synthesis of organ-specific proteins (Wolfe, 2006).

Skeletal muscle has recently been identified as an endocrine organ, with cytokines and other peptides that are produced, expressed or released from muscles referred to as myokines. These have an essential role in metabolism in health and disease (Lightfoot and Cooper, 2016, Pedersen and Febbraio, 2008). Importantly, a loss of muscle mass and strength reduces the body’s ability to respond to stress, and may in turn lead to reduced quality of life, increased morbidity and mortality resulting from chronic illnesses that may then develop (Cohen et al, 2014 and Ebner et al, 2015).

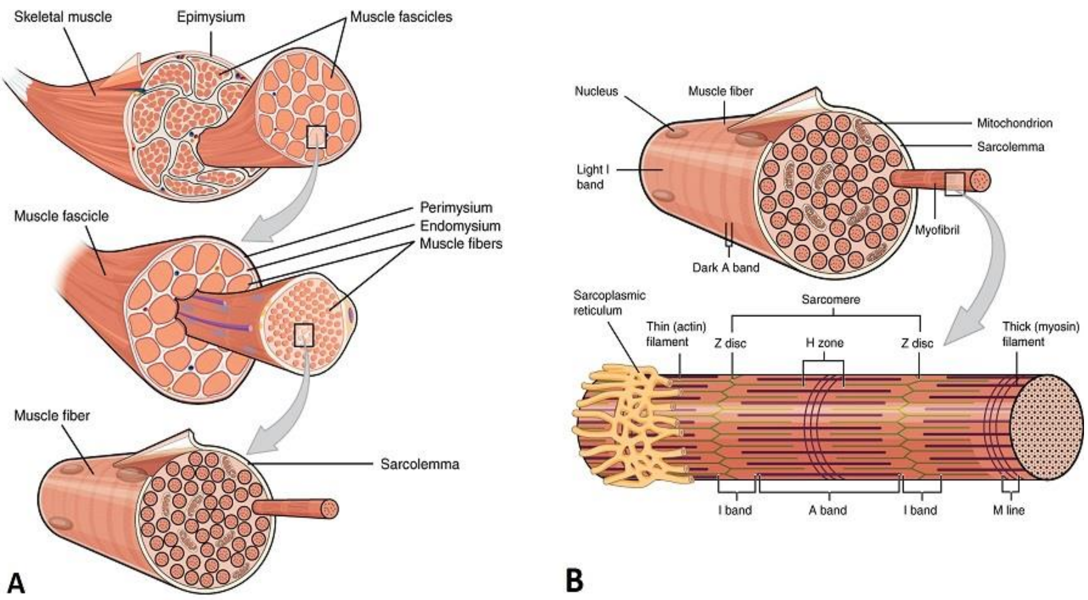


Figure 1.4 The structure of skeletal muscle. (A) Each skeletal muscle has three layers of connective tissue enclosing it and provides the structure to the muscle. The epimysium wrapped each muscle, the bundles of muscle fibres called fascicles are covered by the perimysium and the muscle fibres are covered by the endomysium. (B) The muscle fibre. Each fibre in the skeletal muscle is surrounded by a plasma membrane called the sarcolemma, which contains the cytoplasm of muscle cells (sarcoplasm). A muscle fibre is composed of many fibrils, which give the cell its striated appearance. (Figure from OpenStax, Anatomy & Physiology)

1.8 Adaptation of Skeletal Muscle

Skeletal muscles provide elements vital for human mobility and function, and this has a crucial role in regulating and preserving global metabolic homeostasis of the body. Substrate utilization including carbohydrates, proteins and lipids, contractile activity, ageing, and chronic illnesses are some of the processes which determine their ability to adapt and affect normal function (Eigan and Zeirath, 2013).

1.8.1 Diet and Exercise

Lipids, carbohydrates, and amino acids (AA) are all important fuels for metabolism under aerobic conditions, although AA contribution is only minimal at resting energy metabolism. The transport of glucose into the cell is the rate-limiting step for carbohydrate metabolism which is facilitated via glucose transporters (1-12) during postabsorptive and postprandial conditions. In skeletal muscles, this can be accomplished through both insulin- and exercise-mediated signaling pathways involving glucose transporter 4, which in turn promotes the metabolic actions of insulin to utilise plasma glucose for energy use by the cell (Ryder et al, 2001 and Rivas and Fielding, 2012). During fasting and strenuous exercise (low-energy status), the level of insulin decreases considerably and circulating free-fatty acids (FAs) become the predominant fuel at this stage that is readily taken up by the skeletal muscle (Horowitz and Klein, 2000). Exercise, either resistance or endurance type, has been observed to improve insulin sensitivity and shown to stimulate key signals that positively impact major pathways associated with skeletal muscles' mitochondrial biogenesis and growth, fusion and metabolism (Russel et al, 2014).

Obesity and excess nutrient intake (high-sugar and high-fat diet) are strongly associated with the development of insulin resistance. This results in part from the inhibition of glucose transport and phosphorylation and is associated with the development of T2DM (Yu et al., 2002). The skeletal muscle's adaptation to the metabolic action of insulin is sensitive to the dynamics of circulating nutrients, and because this tissue is responsible for the majority of insulin-mediated glucose disposal, significant scientific effort has been devoted to understanding the mechanisms of diet-induced metabolic dysfunction, particularly by which overnutrition leads to impaired insulin signal transduction in skeletal muscle (Rivas et al., 2012).

1.8.2 Ageing and Chronic Diseases

Skeletal muscle has a reduced ability to incorporate amino acids to synthesise protein in response to advancing age, sedentary lifestyle, and obesity. The loss of muscle mass usually begins at the age of 50 years and results in diminished muscle function, power and strength. This in turn has been linked to several age-related conditions, including osteoporosis, diabetes, and arthritis (Descheness, 2004).

As tissues of the muscle change with age, the loss of muscle mass is accompanied by increased accumulation of intramuscular fat, and therefore the incidence of metabolic disorders such as impaired lipid metabolism and insulin resistance (Volpi et al, 2004). Furthermore, the genetic architecture involved in energy metabolism and mitochondrial protein synthesis has been shown to be negatively impacted in ageing muscle. In addition, a potent antianabolic regulator of muscle mass called myostatin was observed to be significantly higher in extremely obese human myotubes, and this is postulated to have contributed in the systemic metabolic deterioration of skeletal muscles during progression of insulin resistance and type 2 diabetes (Welle et al, 2003 and Hittel et al, 2009).

A study has shown that the metabolic profile of peripheral skeletal muscle from patients with chronic obstructive pulmonary disease (COPD), and particularly uncoupling protein -3 (UCP-3) (which contributes to energy metabolic regulation), was found to be decreased (Gosker et al., 2003). UCP-3 is an isoform of uncoupling protein that is predominantly expressed in skeletal muscles and adipose tissues of rodents and humans (Boss and Lowell, 2000). It is located in the inner mitochondrial membrane and believed to play a role in energy expenditure, and in mitochondrial fatty acid oxidation (Bugge et al., 2010; Schrauwen et al., 2001). It has been demonstrated that aging skeletal muscles could profoundly modify UCP-3 expression and thus affect its energy expenditure and ATP production (Barazzoni and Nair, 2001). Interestingly, it has been suggested that the primary function of UCP3 is to limit the production of reactive oxygen species associated with respiration (Bouillaud, 2009).

Skeletal muscle wasting was thought to have a pathogenic role in altered protein turnover, and this has been demonstrated in a study amongst critically ill patients in which muscle wasting occurred early and rapidly during the first week of critical illness and was more severe among those with multiorgan failure (Lodeserto and Yende, 2014). Muscle dysfunction is also evident in cancer patients, wherein 50% of these patients suffer from progressive atrophy of skeletal muscle and adipose tissue (cachexia) that leads to weight loss and reduced survival, partly as a consequence of concomitant increased lipolysis by tumour or host products and protein degradation in adipose tissues and skeletal muscles, respectively (Tisdale, 2009).

1.9 Activation, Proliferation, and Differentiation of Skeletal Muscle

Skeletal muscle is composed of postmitotic multinucleated muscle fibres that contain its contractile elements. Skeletal muscle fibres form in development by migration of muscle precursor cells (myoblasts) from the somites into the nascent muscles, then they fuse to form multinucleated muscle fibres after morphological, biochemical and molecular modifications (Morgan and Partridge, 2003).

Muscle precursor cells are called satellite cells that are known to be the main, if not only, cell type that serves as a reserve population of cells able to expand in number repopulating the host muscle with new satellite cells. In response to an injury these cells can extensively proliferate to regenerate compact clusters of myofibers (Collins et al, 2005). Initially, satellite cells are mitotically quiescent and have limited capacity for gene expression and protein synthesis at this point. However, they can become activated in response to stress by trauma or injury. Although this transition is still poorly understood, production of sphingosine-1-phosphate, which is intrinsic to these cells, is required for the satellite cell to gain entry to the cell cycle, and thus inhibiting this process could dramatically abrogate muscle regeneration (Nagata et al, 2006).

Intracellular signals can be influenced by extrinsic mechanical stretch, including nitric oxide synthesis (NO) which is thought to release hepatocyte growth factor (HGF) and induce expression of the fusigenic-secreted molecule known as follistatin – a process that triggers satellite cell activation. The latter molecule is known to antagonise a negative regulator of myogenesis such as myostatin and myogenic regulatory factor (MyoD), and thus may contribute to the satellite cells' exit from quiescence (Wozniak and Anderson, 2007 and Pisconte et al, 2006).

Microenvironment-secreted growth factors like fibroblast growth factors (FGF) are also reported to be another stimulus for satellite cell activation by inducing pro-myogenic mitogen-activated protein kinase (MAPK) signaling cascades. The p38α/β MAPK functions as a molecular switch for satellite cell activation and regulates the quiescent state of these cells (Jones et al, 2005). Several reported growth factors are implicated in the chemotaxis, proliferation, and differentiation of satellite cells that are mitogenic for muscle precursor cells namely, basic-fibroblast growth factor (basic FGF), platelet-derived growth factor (PDGF-BB), transferrin, and hepatocyte growth factor (HGF). These growth factors, including transforming growth factor beta (TGFβ) beta and insulin-like growth factor (IGF1), also promote chemotaxis of satellite cells in tissue culture (Morgan, 2003).

The myogenic potential of the satellite cells mostly depends on the expression of Pax genes (Pax3 and Pax7) and myogenic regulatory factors (MRFs) like MyoD, Myf5, myogenin, and MRF4 (Figure 1.5). The activated satellite cells migrate from their niche and transfer outside of the basal lamina, initiate to cycle and concomitantly express Pax7 and MyoD. The resulting formation of skeletal myoblasts then undergo multiple rounds of division, whereupon most of them downregulate Pax7 and express myogenin, differentiate to fuse and form the multinucleated myofibre (Kuang and Rudnicki, 2008).

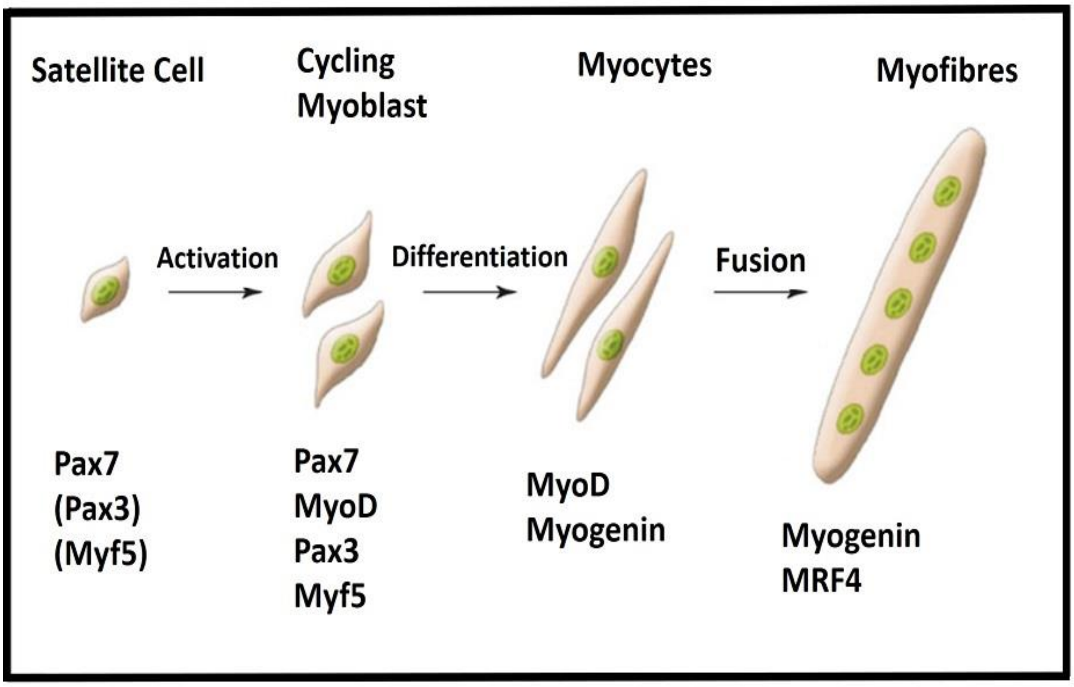


Figure 1.5. Schematic representation of adult myogenesis. Activated satellite cells start to cycle and become skeletal myoblasts expressing paired-box transcriptions factors Pax7 and Pax3, as well as the myogenic regulatory factors Myf5 and MyoD. Once committed to differentiation, myoblasts stop cycling and lose expression of Pax7, Pax3, and Myf5. The myogenin-containing myocytes will then align and fuse to form multinucleated myofibers.

1.10 Diabetes Mellitus (*Diagnosis, Classification of the major types and Epidemiology*)

Diabetes Mellitus (Greek *diabetes* – siphon; Latin *mellitus* – honeyed or sweet) is a group of metabolic diseases that are categorised by chronic and persistent elevated levels of blood glucose, referred to as hyperglycaemia. This hyperglycaemia is a consequence of defective insulin secretion, insulin action, or both. This chronic dysfunction is associated with long-term complications including failure of different organs particularly the eyes, kidneys, nerves, heart, and blood vessels. Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the β-cells of the pancreas with consequent insulin deficiency, to abnormalities that result in resistance to insulin action.

The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action. Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycaemia (American Diabetes Association). For decades, the diagnosis of diabetes has been based on glucose criteria which include fasting blood glucose ≥ 126 mg/dL (7 mmol/L), blood glucose level ≥200 mg/dL (11.1 mmol/L) or an abnormal glucose test. However, an additional test for diagnosing diabetes is glycated haemoglobin, or HbA1c, with a threshold of ≥6.5% (World Health Organisation). There are three major types of diabetes (Type 1 diabetes, Type 2 diabetes, and gestational diabetes), and the causes and risk factors are different for each type.

Type 1 diabetes results from a cellular-mediated autoimmune destruction of the β-cells of the pancreas due to the development of islet autoantibodies and accounts for only 5-10% of those with diabetes. In this form of diabetes, the rate of β-cell destruction is variable, being rapid in some infants and children, and slow in others mainly adults (Alberti and Zimmet, 1998, Atkinson et al, 2014 and American Diabetes Association).

Type 2 diabetes mellitus (T2DM) accounts for 90-95% of those with diabetes making it one of the most common chronic disorders in older adults, and the prevalence is growing worldwide (Dardano et al, 2014 and American Diabetes Association). Genetic, environmental, influences of poor dietary and exercise habits or physical inactivity, are believed to have mutually contributed to the development of type 2 diabetes mellitus (Fletcher et al, 2002) which then leads to the gradual decline of β-cell function and insulin insensitivity (Stumvol et al, 205).

Gestational diabetes mellitus (GDM) is any abnormal glucose tolerance first detected during pregnancy which occurs in 3-5% of pregnancies (Spaight et al, 2016 and Diabetes UK). This observation might be due to the β-cells trying to compensate the observed 50-70% decreased in insulin sensitivity that develops during pregnancy (Kuhl, 1998). In addition to known risk factors like increased glycaemic load and fat consumption, excessive gestational weight gain, a low vitamin D level, psychological stress, and negative mood are risk factors for GDM (Radesky et al, 2008 and Spaight et al, 2016) and so the risk of this disease is found to be significantly increased among overweight, obese, and extremely obese women with varying proportion that is associated with ethnicity and racial groups (Cavicchia et al, 2014 and Kim et al, 2010).

Type 3C diabetes mellitus or pancreatogenic diabetes is a type of diabetes that develops when another disease causes damage to the pancreas. The conditions related to type 3c are pancreatic cancer, pancreatitis, cystic fibrosis or haemochromatosis. This type of diabetes can also happen when the pancreas stops producing enough insulin for the body (Diabetes UK, 2019). It is a clinically relevant condition with a prevalence of 5%-10% among all diabetic subjects in Western populations. In nearly 80% of all type 3c diabetes mellitus cases, chronic pancreatitis seems to be the underlying disease (Ewaldt and Hardt, 2013). You can also develop type 3c if you have part or all of your pancreas removed because of other damage. Some of the key features of medical therapy for patients who have this type of diabetes include fat-soluble vitamins (Vitamin D) and restoring impaired fat hydrolysis (Ewaldt and Hardt, 2013).

Diabetes is a global pandemic which continues to be a growing problem responsible for an escalating human and financial cost annually, and for several life-threatening complications. It is predicted that over 1 billion people will be living with or at high risk of diabetes in 2045 (Figure 1.6), of which about 80% of these patients will appear to be in low-middle income countries, with the majority of them being 45-64 years old (Wild et al, 2004, International Diabetes Federation and Harvard T.H. School of Public Health). While there is a clear association between advancing age and greater prevalence of T2DM, there is also an increasing incidence of obesity that has resulted in a dramatic rise of T2DM among children, teenagers and adolescents. This has made them at high risk for later health complications, and thus represents a significant new public health issue with potentially major personal and societal cost (Pulgaron et al, 2014 and Silverstein et al, 2001).

Figure 1.6; The 2019 Diabetes Atlas. Indicating the worldwide and regional prevalence of diabetes as of 2019 and the projected number of cases in 2045, human and financial cost of diabetes with respect to age and corresponding income per country or region. (Figure Source: International Diabetes Federation, 2019).

1.11 Prediabetes and Type 2 Diabetes (Pathogenesis, Risk Factors, Symptoms and Health Implications)

Impaired fasting glucose (IFG – with fasting glucose value of 6.1-6.9 mmol/L) and impaired glucose tolerance (IGT – with 2-hour oral glucose test value of 7.8 – 11.1 mmol/L) are the intermediate metabolic states between normal and diabetic glucose homeostasis (World Health Organization and International Diabetes Federation, 2006). These conditions are thought to be the precursors of T2DM, but the progression to overt disease is not straight-forward (Santaguida et al., 2005). Both β-cell dysfunction and insulin resistance are thought to have also contributed to the progression of IGT and IFG to T2DM (Kanat et al., 2015). Therefore, improving insulin sensitivity and/or preserving β-cells functions could be a rational way to normalise the glucose tolerance and to prevent the conversion of IGT and IFG to T2DM which is possibly achievable through pharmacological interventions combined with behavioural changes such as diet and exercise. Although not everyone with prediabetes would go on to develop diabetes, about 25% of prediabetic individuals developed full-blown diabetes over the short term (three to five years) and this number would be significantly larger over the long term (Komaroff, 2013). For instance, one study has reported that amongst 5450 American subjects who have IFG, it took 29-41 months for the 8.1-24.3% of them to have developed T2DM (Nichols et al., 2007).

Type 2 diabetes mellitus is a metabolic disorder that results in hyperglycaemia due to the body failing to secrete enough insulin (Figure 1.7). In addition, there is also often insulin insensitivity and an inability to metabolise blood glucose. Together this can lead to damage the organs of the body over time (Diabetes UK). The prevalence of T2DM is increasing dramatically in all age groups, sexes, racial/ethnic group, and in all education categories, which consequently poses a major health and socio-economic burden. Much of the increase in the prevalence of T2DM is due to the increasing prevalence of obesity, for instance, a study conducted in three and six year-periods with American subjects observed a trend that about 85% of people with T2DM are obese or overweight (Cnop, 2018, Bhupathiraju, 2016 and Centers for Disease Control and Prevention, 2004), and data also suggests that in England about 90% of adults with T2DM aged 16-54 years are obese or overweight, and 12.4% adults aged 18 years or over with obesity were diagnosed with diabetes - five times that of people with a healthy weight (Public Health England).

The prevalence of T2DM is also attributed to some lifestyle factors which include physical inactivity, unhealthy diet, cigarette smoking and alcohol consumption, all of which are modifiable where preventive measures could be developed on these habits to reduce the steadily increasing prevalence of this chronic disease (Deepa et al, 2017 and Shi et al, 2013). A study showed that first-degree relatives of patients with T2DM disease are at high risk of developing this disease and observed to have early metabolic defects or impaired glucose metabolism, indicating that this disease has also heritable genetic correlation (Eriksson et al, 1989, Shaw et al, 1998 and Wu et al, 2014).

Several susceptibility loci have been shown to be associated with T2DM using genome-wide association studies (GWAS) (Wu et al, 2014). There are also data to suggest that the gut metagenome is an essential player in the development of T2DM where altered gut microbiota is strongly linked to the disease, and that the gut bacterial populations of patients with T2DM are different from those who are non-diabetic. Data indicates various opportunistic pathogens and butyrate-producing bacteria being increased and decreased, respectively, suggesting that T2DM patients may have a gut environment that is incapable of stimulating protective mechanisms against microbes and oxidative stresses (Larsen et al, 2010, Qin et al, 2012, and Tai et al, 2015).

T2DM often develops over a period of years, and therefore symptoms can also develop gradually. Symptoms of T2DM include excessive thirst (polydipsia) and hunger (polyphagia), frequent urination (polyuria), feeling very tired (fatigue), losing weight without trying to, regular yeast infection (thrush), blurred vision and cuts or wounds taking longer to heal (National Health Service and Diabetes UK).

T2DM patients have higher susceptibility to various forms of acute and chronic complications, both of which could lead to serious damage and other complications. These complications may include some of the macrovascular diseases like hypertension, hyperlipidaemia, myocardial infarction, coronary artery disease, stroke, cerebral vascular disease, and peripheral vascular disease, and microvascular diseases namely, retinopathy, nephropathy, and neuropathy (Forbes, 2013, Wu et al, 2014 and Evans, 2015). Epidemiologic evidence clearly indicates that the risk of several types of cancer including colorectal, breast, pancreatic and liver is also increased in patients who are diabetic (Gallagher, 2015 and Noto, 2017).

The cost of diabetes to the NHS is greater than £1.5 million per hour, or 10% of the NHS budget. In total, an estimated £14 billion pounds is spent per year treating diabetes and its complications, and analysis showed that people with diabetes in England has more likely to experience these debilitating complications (e.g. additional risk of >50% for heart attack, >70% for Angina and heart failure, >30% for stroke, >300 and 200% for minor and major amputations, respectively). Also, a 2016 study indicated that the global cost of diabetes has reached 825 billion dollars a year (Diabetes UK and Harvard School of Public Health).

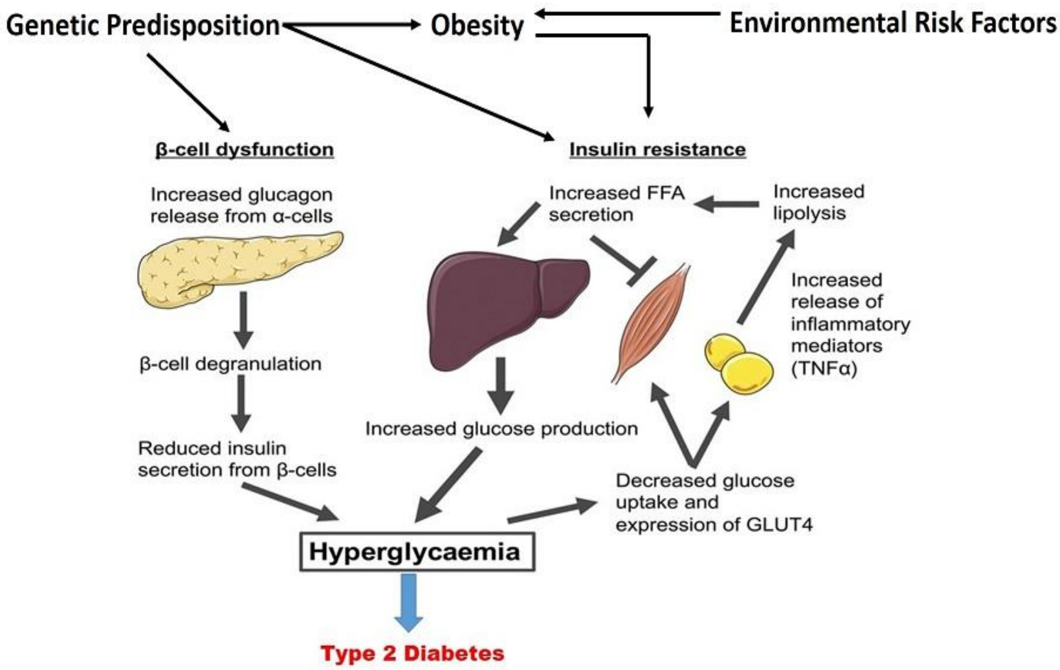


Figure 1.7 The Pathogenesis and Pathophysiology of Type 2 Diabetes. Genetic predisposition, obesity, insulin resistance, and inflammation are factors that could affect normal β-cell function. Also, increased fat accumulation in adipocytes will lead to increased production of proinflammatory cytokines and lipolysis both of which could trigger the liver to produce more glucose. When β-cells are no longer capable of increasing insulin output along with the occurrence of insulin resistance, it could result to persistently elevated level of glucose concentration circulating which is initially manifested as impaired glucose tolerance. Since β-cell failure progresses, further elevations of glucose occur and thus preserves the hyperglycaemic environment that will ultimately lead to type 2 diabetes. (Figure from Riddy et al., 2018 with slight modifications)

1.12 Current Treatment Options for Type 2 Diabetes

The alarming increase in prevalence of type 2 diabetes has led to the development of several new approaches (lifestyle modification and pharmacologic agents) to safely treat hyperglycaemia. The basic risk factor in the progression of prediabetes (IGT and IFG) to diabetes is obesity along with sedentary lifestyle (Hafner et al., 1990).

Consequently, gaining weight could potentially trigger insulin resistance and force the capacity of β-cell to secrete insulin (Kanat et al., 2015). On the other hand, weight loss by means of lifestyle intervention had been proven to improve whole body insulin sensitivity and to preserve β-cell function, and therefore an effective approach in preventing IGT or IFG conversion to T2DM (Tuomilheto et al., 2001; Muscelli et al., 2005). For instance, studies showed that when individuals reduced 5% of their body weight, total body insulin sensitivity improved by 30% and decreased in their IGT-T2DM progression nearly by 58% (Knowler et al., 2002; Kitabchi et al., 2005). However, interventions aiming weight loss if not practical in real life, is difficult or unsustainable (Wing et al., 2001). In cases where this first line of therapy is insufficient and not satisfactory for insulin sensitivity improvement, then pharmacological interventions are available alternative ways.

Pharmacological interventions include therapies either in the form of oral and injectable drugs help patients mainly to reduce and maintain their blood level of glucose concentrations as close to normal for as long as possible after being diagnosed. Consequently, they aim to either prevent or delay the patients from developing complications. Despite some of these drugs having been unsuccessful due to adverse effects or negligible therapeutic efficacy, several are very well accepted and are being used worldwide. Many have different modes of action, partly due to the heterogeneous nature of the pathophysiology of T2DM and partly because individuals have different responses towards these drugs (Kahn et al, 2014). Some of these drugs are described below.

Biguanides are one of the major classes of antidiabetic drugs, among which metformin has been used since the 1950s as the first-line therapy to treat people with type 2 diabetes. Metformin has been proven to be effective in lowering blood glucose by reducing hepatic glucose output and increasing insulin-stimulated glucose uptake and glycogenesis in skeletal muscle. When used as a monotherapy, it can lower HbA1c by around 1.5%. It has also been demonstrated that metformin activates hepatic and muscle adenosine monophosphate-activated protein kinase (AMPK) which has important roles in regulating of lipid and glucose metabolism. The use of metformin, however, is shown to cause lactic acidosis and gastrointestinal (GI) effects such as nausea, vomiting, flatulence and diarrhea, the reduction of calorie intake, and weight loss (Cheng, 2005 and Holman, 2007).

Sulfonylureas are also widely used as antidiabetic agents. They act by binding to the specific receptor for sulfonylureas on β-pancreatic cells, blocking the inflow of potassium (K+) through the ATP-dependent channel. This allows the cell membrane to depolarise, thus allowing the diffusion of calcium into the cytosol. The increased flow of calcium into β-cells causes the contraction of the filaments of actomyosin responsible for the exocytosis of insulin, which is therefore promptly secreted in large amounts (Ashcroft and Rorsman, 2013). The higher rate of hypoglycaemia especially in older adults with hepatic dysfunction, impaired kidney functions, and alcohol abuse are the major acute side effects reported with the use of sulfonylureas and may become worse when taken in combination with other drugs such as aspirin, oxidase inhibitors, and phenylbutazone. In addition, sulfonylureas can also cause weight gain, and may elevate the risk of cardiovascular disease among patients with diabetes (Scheen et al, 2005 and Phung et al, 2013).

Thiazolidinediones (TZDs) are classified as insulin sensitizers. They are referred to as peroxisome proliferator-activated receptor γ (PPAR-γ) ligands which exert insulin-sensitizing effects on the liver, skeletal muscles and adipose tissues (Hevener et al, 2007). In combination with insulin or metformin, TZDs are effective to improve glycemic control in T2DM. However, TZDs exhibit several negative effects in the treatment for T2DM, including an increased risk of bladder, liver and colorectal cancer

(Chang et al, 2012 and Mamtani et al, 2012). Rosiglitazone and troglitazone are types of TZDs that have been withdrawn from the market as they are associated with the increased risk of myocardial infarction and idiosyncratic hepatotoxicity, respectively (Galasko, 2016 and Bailey, 2011). Another TZD option available is the pioglitazone which has been found to reduce the risk of IGT conversion to T2DM by 72% but was associated with significant weight gain and edema (DeFronzo et al., 2011), and in one study involving high-risk patients with type 2 diabetes and previous myocardial infarction (MI), pioglitazone significantly reduced the occurrence of fatal and nonfatal MI and acute coronary syndrome (Erdmann et al., 2007).

α-Glucosidase inhibitors (AGIs): *α*-glucosidase inhibitors, such as acarbose, voglibose, and miglitol, are markedly effective for postprandial hyperglycemia. They decrease or delay carbohydrate absorption in the gut by inhibiting the intestinal mucosal enzyme (α-glucosidase) which converts complex polysaccharides into monosaccharides (Baron, 2012 and van de Laar, 2008). Adverse effects such as abdominal bloating, diarrhea and flatulence are often observed after the use of this class of drugs, and hence they should be cautiously administered in older adults due to gastrointestinal side effects and in patients with significant renal impairment (Kim et al, 2012).

Incretins are hormones that stimulate insulin secretion and suppress postprandial glucagon secretion in a glucose-dependent manner. This effect is estimated to be responsible for 50-70% of the insulin response to glucose caused by glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) that are secreted from intestinal endocrine cells (Vilsbol, 2004). Incretin-based drugs are now used routinely for T2DM and their use is associated with good efficacy and tolerability, and low risk of hypoglycaemia and weight loss (Lovshin, 2009).

GLP-1 receptor agonist also called incretin mimetics, are incretin-based medicine for the treatment of T2DM. Its actions include potentiation of insulin secretion, suppression of glucagon release, delay nutrient absorption, and appetite, thus making it effective in the regulation of glucose metabolism (Lund et al, 2014). GLP-1 receptor agonists such as exenatide and liraglutide are classified as antidiabetic agents that were observed to reduce levels of hemoglobin A1c (HbA1c) by 0.8% to 1.5% (Kurukulasuriya, 2010). These GLP-1 receptor agonists can be categorized as either short-acting compounds (exenatide and lixisenatide), which offer short-lived receptor activation or as long-acting compounds (albiglutide, dulaglutide, and liraglutide), which at recommended dose provide continuous GLP-1 receptor activation. The pharmacodynamic profiles are different from each other – the former primarily lower postprandial blood glucose levels via inhibition of gastric emptying whereas the latter compounds have a stronger effect on fasting glucose levels, which is mediated predominantly through their insulinotropic and glucagonostatic actions Meier, 2012).

Dipeptidyl peptidase-4 (DPP-4) **inhibitors** are agents that block the action of DPP-4 enzyme on the endogenous active GLP-1 and GIP, hence they are effective in the protection of pancreatic β-cells to potentiate glucose-dependent insulin secretion and in the promotion of normal glucagon secretion, thus inhibiting the progression of T2DM. DPP-4 inhibitors are able to maintain inhibitory action throughout treatment, and thus are observed to prolong GLP-1 half-life. They are also well tolerated and have few gastrointestinal adverse effects, exert cardiovascular protection and have anti-arteriosclerotic action, and no increase in hypoglycaemic episodes (Duez et al, 2012).

Insulin and insulin analogues initiated-therapy are usually required for T2DM patients when lifestyle changes and oral antidiabetic agents are insufficient to achieve and maintain glycemic control. The mechanism by which this treatment option could regulate level glucose concentrations is observed to be suppressing hepatic glucose production, increasing postprandial glucose utilization, and improving abnormal lipoprotein composition (Buse, 2001 and Wu et al, 2014). Analogue insulin is available in three forms, rapid acting (Humalog, NovoRapid) and long acting (Lantus, Levemir, Tresiba) as well as premixed combinations (Humalog Mix 25, Humalog Mix 30 and Humalog Mix 50). Rapid acting insulins initiate their effects immediately after injecting with peak of action within the 1st hour from injection and has a duration of up to 4 hours. On the other hand, the long acting version of insulin analogues has no peak activity so as to act uniformly after 2 hours from injection and action lasts up to 12 hours. In the case of a premixed analogue insulins, this is simply a combination of rapid acting and long acting insulin. For example, Humalog Mix 25 consists of 25% rapid acting and 75% long acting insulin (Diabetes UK, 2019).

Sodium-glucose co-transporter type 2 (SGLT2) inhibitors are a relatively new class of glucose-lowering agents that have been proposed as a novel therapeutic strategy for diabetes. Blocking these transporters will elevate renal glucose excretion and prevent the reabsorption of glucose from the kidney back into the circulation, thus lowering blood glucose levels and potentially reducing weight (Chao and Henry, 2010).

T2DM is a progressive condition and so the need for additional agents might be essential to tailor patients' preferences whilst improving glycaemic control. In this case, clinicians of all levels of expertise has now practiced a form of **combination therapy** as rational approach in the treatment or management of T2DM (Bailey, 2013). For instance, GLP-1 receptor agonists are recommended in combination with metformin for patients who do not achieve HbA1c goals with metformin alone (Garber et al., 2018). For patients with persistent hyperglycaemia and/or those who are trying to control their weight, a triple therapy might be required which is a combination of

GLP-1 receptor agonists, metformin and a SLGT2 inhibitor (Hinnen, 2017). Another combination therapeutic approach that has become important for diabetic patients is the use of DPP-4 inhibitors and GLP-1 receptor agonists which could improve their glycaemic control whilst reducing bodyweight and blood pressure with relatively low chance of hypoglycaemia (Garber, 2011).

1.13 Glucolipotoxicity

Under physiological levels, glucose and lipids are vital requirements for normal cellular and tissue functions. However, when levels of these become elevated, pathological conditions, damage, and abnormalities within tissues occurs. Thus the term glucolipotoxicity has resulted (Poitout et al., 2007). The damaging effects of glucolipotoxicity and its role towards the pathogenesis of T2DM (including β-cell dysfunction and insulin resistance) can be ascribed from the ability to initiate and mediate pathways leading to mitochondrial stress, generation of reactive species, proinflammatory cytokines, and altered gene expression (Bagnati et al., 2016; Akash et al., 2018).

1.13.1 Glucotoxicity

Glucotoxicity is exacerbated by poor control or management of T2DM, with hyperglycaemia reducing the capacity of pancreatic β-cells to secrete insulin and increasing the risk of developing insulin resistance. This in turn is likely to lead to further hyperglycaemia, and this vicious circle eventually results in dysfunctional β-cells and reduced β-cell mass (LeRoith, 2002). Glucotoxicity is also the main cause of diabetic complications which are mostly only manifested several years after the illness has begun.

The molecular signaling mechanisms of how hyperglycaemia activates cellular injury involve PKC activation via diacylglycerol, increased hexosamine pathway flux, elevated formation of advanced glycation end product (AGE), and increased polyol pathway flux, pathways which could potentially disrupt normal glucose metabolism. The unifying mechanism integrating these pathways is the increased production of reactive oxygen species, in particular, superoxide by the mitochondrial electron transport chain (ETC) (Nishikawa et al., 2000). This is further exacerbated by the activation of PARP that inhibits or inactivates GAPDH, delivering more glycolytic intermediates to the mitochondrion that would in turn increase ROS production (Reusch, 2003).

Oxidative stress reduces biosynthesis and secretion of insulin (Sakai et al., 2003), in part through decreased DNA binding capacity of pancreatic duodenal homeobox-1 (PDX-1). This a transcription factor that is a master regulator of gene transcription in β-cells, including key metabolic enzymes and the insulin gene (Matsuoka et al., 2001). It is thought that due to the extremely weak manifestation of antioxidant defence system present in pancreatic islet cells, they are also more susceptible to glucose toxicity and thus oxidative stress compared to other tissues and organs (Tiedge et al., 1997). A study showed that glucolipotoxic conditions upregulated UCP2 expression and its increased activity was negatively correlated with insulin secretion (Chan et al., 2004). Beta cells express a mitochondrial uncoupling protein, UCP2, which is thought to lower the efficiency of oxidative phosphorylation, and thus could impair glucose-stimulated insulin secretion (GSIS) (Affourtit et al., 2008). In contrast, a study by Produit and colleagues showed that increased UCP2 levels decreased cytokine-induced production of reactive oxygen species and indicated that this might have a potential protective effect on beta cells (Produit et al., 2007), and similarly increased expression of this protein conferred protective effects on ß-cells against glucotoxic conditions via reduction of Cell death and partially preserving the secretory response to glucose stimulation (Li et al., 2017).

The negative impact of glucotoxicity does not only affect pancreatic beta cells but is also involved in insulin resistance of insulin-sensitive tissues, which include liver, skeletal muscle, and adipose tissue. It is thought that before the onset of chronic hyperglycaemia insulin resistance is typically present and that glucotoxicity may potentially aggravate this defect under diabetic conditions (Figure 1.8). In particular, glucotoxicity-driven oxidative stress has been shown to inhibit translocation of GLUT4 to the plasma membrane, and to have induced insulin resistance in skeletal muscle and adipose tissues (Zhao et al., 2004; Dokken et al., 2008).

The potentially harmful effects of chronic hyperglycemia have also been linked to both microvascular complications such as retinopathy, neuropathy or nephropathy and macrovascular complications like cardiovascular diseases (Manucci et al., 2013; Yamagisi and Imaizumi, 2005).

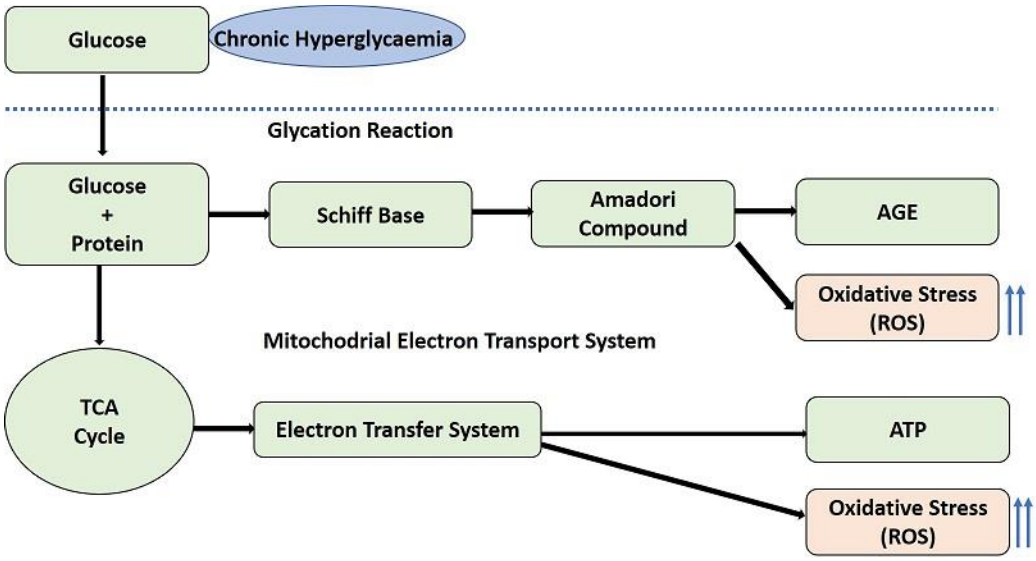


Figure 1.8. Chronic hyperglycaemia in diabetic state induces oxidative stress by overproduction of ROS. Glycation is enhanced in the hyperglycaemic state which leads to the generation of ROS from intermediate metabolite known as Amadori product which also forms the advanced glycosylation end products. Under hyperglycaemic state, oxidative stress is also detected as a result of ROS being generated from the electron transfer system in the mitochondrial inner membrane. (Figure Adapted from Kawahito et al., 2009)

1.13.2 Lipotoxicity

Lipotoxicity is a term used to describe the functional impairment and damaging effects of excessive fat accumulation or elevated circulating levels of lipids on several metabolic pathways, both in adipose tissues and peripheral organs like the liver, heart, pancreas, and muscle (Yazici and Sezer, 2017). In response to an increasing energy demand where glucose supply is not adequate, lipolysis of fat stored (triglycerides) in adipose tissue occurs to release long-chain non-esterified fatty acids NEFAs or free fatty acids (FFAs) and glycerol. A study by Moro et al., 2009, showed that DAG accumulates in muscle of high-fat-fed rodent animals, and also of obese individuals. This related to a study by Timmers et al., 2008 which reported that DAG accumulates after esterification of excess LCFA-CoA, whereby these lipid metabolites have been demonstrated to activate PKC and be linked with inhibition of early steps in the insulin signal cascade.

Elevated levels of NEFA observed in type 2 diabetes has been reported to cause severe muscle and liver insulin resistance, and to inhibit insulin secretion. It impairs insulin signalling and multiple intracellular steps of glucose metabolism (Figure 1.9) through intracellular toxic metabolites of FFA, including diacylglycerol (DAG), ceramides and the long-chain fatty acyl CoA (LCFA-CoA) (Kashyap et al., 2003; Belfort et al., 2005). Dietary fatty acids can be in the form of saturated or mono/polyunsaturated fatty acids, with the former being associated with obesity, insulin resistance, and cardiovascular disease. Upon entry to the cell, fatty acids are immediately esterified to fatty acyl-CoAs, and upon transfer of this to a glycerol backbone can form mono-triacylglycerols that in turn form the basis for ceramide biosynthesis upon esterification with sphingosine. These intracellular lipid intermediates are thought to be responsible in the pathogenesis of insulin resistance (Samuel and Schulman, 2012).

The oxidation of fatty acid is a mitochondrial aerobic process where it breaks down into acetyl-CoA units utilizing nicotinamide adenosine dinucleotide (NAD) and flavin adenine dinucleotide (FAD). Once the FFA is activated and converted to a long-chain acyl-CoA, it then enters to mitochondria via carnitine-palmitoyl transferase-1 (CPT-1) to undergo β-oxidation through a series of enzymatic reactions to yield acetyl-CoA. This is then directed to the citric acid cycle for further oxidation – a process that provides ATP (Kumari, 2017). At a physiological level, fatty acids entry to the cells via CPT-1 for beta-oxidation has no detrimental functional effects, however when glucose concentration is elevated, the result would be the formation of metabolites like citrate and malonyl-CoA inhibiting CPT-1 which in turn block fatty acid oxidation and therefore accumulation of LCFA-CoA that has a negative effect on insulin signaling and beta-cell function. The deleterious effect of lipotoxicity as a consequence of the altered lipid partitioning is dependent upon elevated glucose levels and so glucotoxicity is a prerequisite for lipotoxicity to occur and the combined damaging effects of both is the concept of glucolipotoxicity (Poitout and Robertson, 2008).

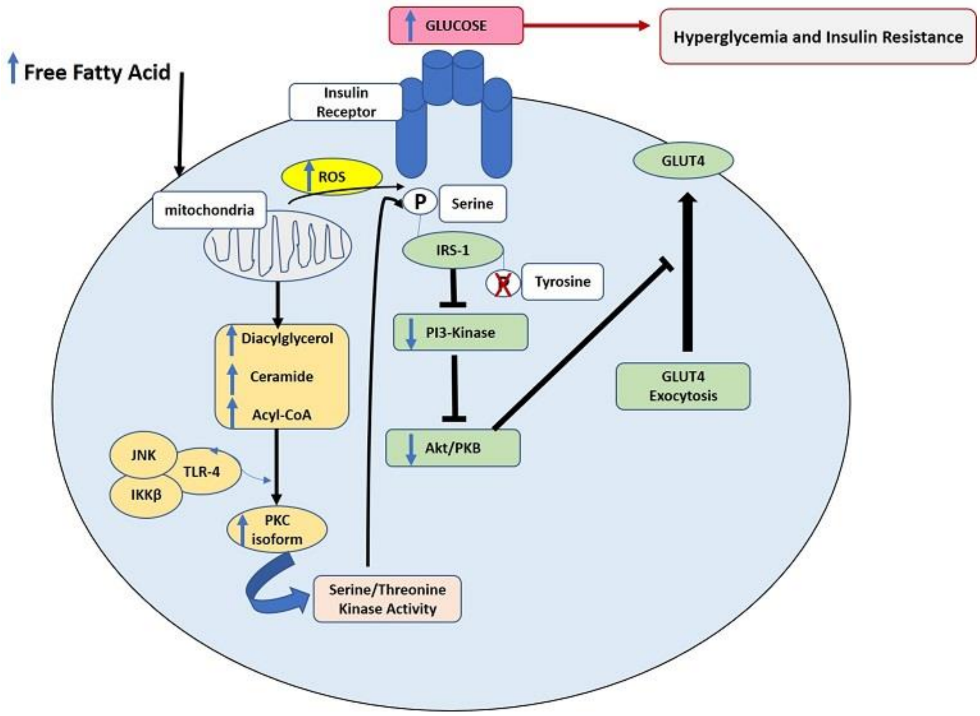


Figure 1.9. Possible mechanism of Fatty-induced insulin resistance in skeletal muscle. Elevated fatty acid may favour increased formation of intramuscular ceramide and diacylglycerol metabolites which in turn could inhibit the insulin signaling pathway. Increased FFA promotes proinflammatory events and ROS generation which could in turn impair insulin signaling pathways. (Figure Adapted from Abu Bakar et al., 2015).

1.14 Oxidative Stress and Diabetes

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a dual role as both harmful and beneficial species. Overproduction of these species results to oxidative stress which is an important mediator of damage to cell structures, including lipids and membranes, proteins, and DNA. Persistent elevation of glucose level in an organism activates ROS formation from a variety of sources which include oxidative phosphorylation, glucose autooxidation, NAD(P)H oxidase, lipoxygenase, cytochrome P450monooxygenases, and nitric oxide synthase (NOS). However, ROS-RNS occurring at low to moderate concentrations are beneficial, for example in cellular defence systems against infectious agents, and in the induction of a mitogenic response (Valko et al., 2006).

Under diabetic conditions, mitochondria and NAD(P)H oxidases are the most important sources of ROS. Hyperglycaemia leads to decreased activity of glyceraldehyde-3-phosphate dehydrogenase via increased generation of mitochondrial superoxide, with a concomitant increase in hexosamine pathway activity (Du et al., 2000). Complex II is the primary source of electrons that contribute to superoxide formation under hyperglycaemic conditions (Nishikawa, et al., 2000). A significant increase in superoxide production has been observed in the blood vessels of type 2 diabetic subjects, and this is associated with endothelial dysfunction and increased risk of atherosclerosis (Gruzik et al., 2002).

Glucose autooxidation is another mechanism of ROS production, with glucose able to react with hydrogen peroxide in a metal-catalysed process or in the presence of iron and copper ions to form hydroxy radicals (Wolff and Dean, 1987). Xanthine oxidases (XO) and lipoxygenases are also proposed to be major sources of ROS in diabetes. When XO delivers electrons to molecular oxygen it generates superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), and in the presence of iron gives rise to the hydroxy radical ($HO^{\cdot}$) (Batelli et al., 2016).

In a study (Suzuki et al., 2015) on streptozotocin (STZ)-induced diabetic mice and exposure of neonatal cardiomyocytes to high glucose, an increased expression of lipoxygenases and monooxygenases was observed. This in turn catalyses the conversion of free fatty acids like arachidonic acid to leukotrienes and eicosanoids.

This is believed to then drive oxidative stress and inflammation, and to be involved in the development of diabetic cardiomyopathy. Hypergylcemia is also linked with increased formation of advanced glycation end products which result from the reaction between the free amino groups of proteins or lipids and glucose (Ling et al., 2001). They in turn can damage the functions of DNA, proteins, and lipids, thereby triggering cellular responses including activation of inflammatory pathways, which are thought to be involved in the pathogenesis of several diabetic complications.

1.15 Application of Antioxidants to Correct Oxidative Stress

Oxidants are molecules that can have either unpaired electrons (free-radicals) or paired electrons (non-radicals). Both are produced from either an endogenous source like those that are of physiologically significance including superoxide anion, hydroxyl, and hydrogen peroxide, or from an exogenous source that can be obtained from cigarette smoke, ozone exposure, hyperoxia, and ionising radiations (Birben et al., 2012).

The formation of oxidative stress (OS), due to increased levels of reactive oxygen species and excess activation of free radical species, is known to be involved in the pathogenesis of most diseases and thereby could potentially disturb the organism's antioxidant protection system, homeostasis and redox processes (Birben et al., 2012; Prokopieva et al., 2016). Under conditions of OS, biomolecules such as proteins, lipids, carbohydrates, and nucleic acids are the main target sites susceptible to damage, which consequently affects the intrinsic properties of these biomolecules. These vital properties include protein cross-linking, enzyme activity, ion transport, fluidity and protein synthesis (Sharma et al., 2012). However, under low to moderate concentrations, ROS/RNS were shown to have beneficial effects and carry out important physiological roles. This included defence against infectious diseases, and functioning as messengers in intracellular signaling cascades (Valko et al., 2007).

Markers of oxidative stress include nitrosylated and carbonylated proteins, lipid peroxidation products like isoprostanes, malondialdehyde, acrolein and hydroxynonenals, and other adducts like advanced glycation end products. The accumulation of these products was shown to have direct effects on the functions of biomolecules (Baraibar et al., 2013; Ho et al., 2007; Ames et al., 1993). Indeed, this damage can ultimately result in cell death. Thus, attempts to neutralise these deleterious substances could play a significant role in correcting oxidative stress.

Antioxidants are natural or synthesised molecules that are believed to be effective in neutralising ROS. These have been shown to counteract oxidative stress, and therefore considered for use in many clinical situations to prevent a patient against several diseases induced by these harmful free-radicals or reactive metabolites (Ames et al., 1993). The enzymatic scavengers (superoxide dismutase, catalase, glutathione peroxidase, thioredoxin, peroxiredoxin, and glutathione transferase) and non-enzymatic scavengers (Vitamin A, E and C, β-carotene, and glutathione) are two categories of antioxidant defence and repair system that the human body could utilize to regulate and counterbalance the detrimental effects of oxidants and redox modulations (Prokopieva et al., 2016; Valko et al., 2007; Birben et al., 2012).

Despite the potential and efficiency of these scavengers, the use of antioxidants remains a challenge in medicine wherein some reported antioxidants did not always show positive results, changed mechanism of actions or may even show prooxidant action more likely attributed to its chemical structure, bioavailability, severity of oxidative stress or might be due to the improper adherence to the correct dosage or the ways these have been administered (Palozza, et al., 2003; Bowry et al., 1992). Exploring for molecules with maximum antioxidant action with minimum side effects for oxidative-driven diseases remains to be a significant challenge in medicine. The antioxidants that exhibit strong protective potential should have nontoxic behaviour, good bioavailability, have no negative effects in case of overdose, would not form side products from its reaction with other molecules or reactive species, compatible with other medications, natural and show protective actions at a broad range of dose (Akbarirad et al., 2016).

1.16 Mitochondrial Dysfunction in Skeletal Muscle and Insulin Resistance

Skeletal muscles strongly rely upon oxidative phosphorylation in order to produce energy and thus perform its intended function. However, insulin-resistant skeletal muscle in type 2 diabetic individuals is ineffective in regulating the metabolism of both glucose and fatty acids (Kelley et al., 2002). Some manifestations of this abnormality include reduced glucose transport and phosphorylation along with reduced glycogen synthesis, elevated accumulation of triglycerides and other lipid metabolites, and dysregulated oxidation of lipids during fasting and insulin-stimulated conditions (Blaak et al., 2000; Schulman et al., 1990).

Another factor that has the potential to perturb the normal metabolism of substrates is dysfunctional mitochondria and impaired functional capacity of mitochondria is thought to be an important aspect of the pathogenesis of insulin resistance. For instance, in the skeletal muscles of obese and type 2 diabetic individuals, it has been found that the activity of marker enzymes involved in oxidative pathways are significantly reduced, and fatty acid oxidation is impaired (Simoneau and Kelly, 1997). These observations resulted in the accumulation of lipid intermediates and led to abnormal glucose metabolism that was correlated with severe insulin-resistant glucose metabolism. It has also been postulated that the direct contribution of dysfunctional mitochondria to the abnormal glucose metabolism was because of the limited availability of ATP for use by hexokinase, along with other reactions where phosphorylation is necessary (Gerbitz et al., 1996; Goodpaster and Sparks, 2017).

In a study by Kelly et al., 2002, skeletal muscle mitochondria were found to be smaller in obese and T2DM, and this is believed to be a hallmark for a disturbed biochemical function of the mitochondria. In the same study, the activity of the electron transport chain was also reduced, as indicated by an almost 40% decrease in the activity of rotenone-sensitive NADH:O_2 oxidoreductase. This observation was in line with an earlier report indicating an impaired activity of mitochondria tricarboxylic acid cycle enzymes in skeletal muscle in type 2 diabetic subjects (Vondra et al., 1997). In a later study using cultured cells from T2DM reported that the activity of oxidative enzyme citrate synthase form these patients was observed to be defective (Ortenblad et al., 2005).

The effects of reduced skeletal mitochondrial functional capacity in insulin resistance is an exciting area for future research. In humans and animals, methodologies to assess mitochondrial capacity include quantitation of mitochondrial density, analysis of enzyme content, and functional evaluation of either isolated or in situ mitochondria and the use of magnetic resonance spectrometry to evaluate *in vivo* mitochondrial function (Chow et al., 2010). More recently, technology has been developed that employs an analyser to measure oxygen consumption rate and extracellular acidification rate of live cells. This allows investigators to interrogate key cellular functions like mitochondrial respiration where basal respiration, ATP production and maximal respiration capacity of live cells are quantified, which could provide insight into the mechanism of mitochondrial dysfunction (Seahorse XF Analyser, www. Agilent.com).

Reduced mitochondrial functional capacity has been demonstrated in the context of type 2 diabetic patients, and in obese/insulin resistant individuals who are not diabetic. Some reported specific observations (as described earlier) include smaller mitochondrial size, lower ATP generation and synthetic rates, decreased level of mitochondrial enzyme activity, reduction of genes involved in oxidative metabolism, and reduced rate of TCA cycle flux rates (Asmann et al., 2006; Patti et al., 2003; Befroy et al, 2007; Szendroedi et al., 2007)

Metabolic inflexibility is a phenomenon that is implicated in insulin resistance, and is believed to have a causal link to reduced mitochondrial function as a result of an organism's failure to adapt or respond according to changes in metabolic or energy demand (Goodpaster and Sparks, 2017). Metabolic flexibility is mediated by cellular and organelle processes, most particularly the mitochondria. In a situation of metabolic inflexibility, muscle cells have an impaired capacity for substrate switching that is from higher rates or predominant fatty acid oxidation during fasting, to greater glucose utilization during fed (insulin-stimulated) conditions. As such this could contribute to the accumulation of lipid metabolites within the cell that has been observed in insulin resistance (Kelly and Mandarino, 2000). On top of the reported evidence described above, mitochondrial dysfunction also takes the form of metabolic inflexibility as evaluated in both *in vivo* and *in vitro* studies has been correlated with the degree of insulin insensitivity in healthy subjects and those that are predisposed to T2DM (Upropkova et al., 2005 and 2007).

1.17 Muscle Myokines in Insulin Resistance and Type 2 Diabetes

Skeletal muscle accounts for about 40% of body mass and for a non-obese individual it constitutes the largest organ (by mass). Skeletal muscle is also now recognised as being an endocrine organ where proteins expressed by and released from skeletal muscle are termed as myokines (Febbraio and Pedersen, 2005). Myokines are believed to participate in the communication between skeletal muscle and other tissues including gut, adipose tissue, liver and pancreas. Their autocrine, paracrine and endocrine effects have been regarded to have roles in metabolic regulation, and thereby make this as a relevant focus in understanding their contribution to the impairment, dysregulation and protection in the above tissues, particularly in insulin resistance or T2DM-associated conditions. Proteomic approaches have allowed researchers to identify hundreds of myokines. Three comprehensive profiling studies of the human skeletal muscle secretome led to the identification of more than 300 potential myokines which are believed to affect muscle physiology, and again might exert endocrine effects on other tissues and organs or work locally as paracrine or autocrine effects (Norheim et al., 2011; Raschke et al., 2013; Hartwig et al., 2014).

A study which investigated the impact of type 2 diabetes on myokine secretion of a skeletal muscles showed that altered secretion, which is increased expression as compared to healthy subjects, of some myokines such as IL6, IL8,IL15, monocyte chemotactic protein (MCP) -1, follistatin, TNFα, and growth-related oncogene (GRO) is an intrinsic property of a type 2 diabetic skeletal muscles (Ciaraldi et al., 2016). Another study profiling human myotubes also revealed an intrinsic proteomic signature associated with T2DM. In this study, 47 abundant proteins were found to be significantly different in myotubes from T2DM patients as compared to healthy donors. Mostly, these proteins are involved in energy metabolism, cellular oxidative stress, protein dynamics and gene regulation (Al-Khalili et al., 2014).

Quantitative determination of the muscle secretome offers the potential to explore not only the biology of the muscle itself, but to understand how crosstalk through release and secretion of proteins between muscles and other tissues could be utilised in the regulation of biological functions and signaling pathways that are relevant in different pathological conditions like type 2 diabetes. A study which used combined experimental and bioinformatics workflow identified 1073 putative secreted proteins from lipid-induced insulin-resistant C2C12 skeletal muscle cells, and from this list about 40% were influenced by insulin-resistant conditions. This included insulin-like growth factor 1 (IGF-1), which has an important role in regulating growth and metabolism and is known to have beneficial impact on glucose homeostasis due to its glucose-lowering and insulin-sensitizing effects. Additionally, because of its glucose-lowering and insulin-sensitizing actions, IGF-1 is down-regulated under palmitate-treated insulin-resistant conditions (Deshmukh et al., 2015).

1.18 Carnosine: Properties, Biological Effects, Metabolism and its Therapeutic Potential as a Histidine-containing Dipeptides

Carnosine and related compounds (anserine, balenine or ophidine, homocarnosine, and acetyl carnosine) are collectively referred to as histidine-containing dipeptides (HCD). Histidine is among the proteinogenic amino acids which play manifold roles due to the reactivity of its imidazole ring that characterizes its side chain. This reactivity is mostly ascribable to its amphoteric nature that can be seen as a combination of pyridine and pyrrole or acidic and basic in character. The imidazole basicity renders it an optimal buffer at physiological pH and several studies showed that the buffering capacity in human tissues is mostly related to the histidine concentration (Li et al., 2011).

The total concentrations of HCD content between different mammals varies, although it is believed that almost all mammals have carnosine, along with one of the methylated carnosine analogs, anserine or ophidine. The exception to this are humans, which are the only species that does not have any of the two methylated carnosine analogs (Boldyrev et al., 2013). In mammals, the skeletal muscle and the olfactory bulb are the two tissues with highest concentration of carnosine, and here this can reach up to the millimolar range. Although 99% of carnosine present in an organism is found in the skeletal muscle tissue, the brain regions have 10-1000-fold lower measurable concentration as compared to the muscles (Kamal et al., 2009).

The carnosine content of muscle in humans is variable, ranging from a lowest value of 10mmol/kg dry weight and highest to about 40mmol/kg dry weight. This variation is linked to individual's muscle fibre type, sex, age, diet and exercise training (Tallon et al., 2005; Harris et al., 2007; Everaert et al., 2010). In addition to $Mg^{2+}$and ATP, L-histidine and β-alanine are two required amino acids for the synthesis of carnosine - a reaction catalysed by carnosine synthase (Kalyankar and Mesier, 1959). The proton-coupled oligopeptide transporter family are found to shuttle carnosine and its methylated analogs across the cellular membrane, this includes particularly the mammalian oligopeptide transporter 1 and 2 (PEPT1 and PEPT2) and the peptide/histidine transporter 1 and 2 (PHT1 and PHT2) (Yamashita et al., 1997).

Nutritional supplementation and hormonal stimuli through a complex interaction amongst related transporters and enzymes are believed to play an important role in the regulation of carnosine dynamics in the muscles (Everaert et al., 2013). For instance, increased muscle carnosine through β-alanine supplementation was found to be associated with increased expressions of TauT (taurine transporter – a β-alanine transporter), CARNS and ABAT (aminobutyrate aminotransferase - a carnosine precursor) in the muscles (Everaert et al., 2013). The hydrolysis of carnosine is mainly due to tissue carnosinase or serum carnosinase (Lenny, 1990; Jackson et al., 1991). Compared to its natural derivatives such as homocarnosine, N-acetylcarnosine and carcinine, carnosine hydrolysis by serum and tissue carnosinases were found to be 3-4 times higher (Pegova et al., 2000). In addition, modification of carnosine through one of many processes such as methylation, acetylation or decarboxylation not only changed its biological activity (Boldyrev and Abe, 1999) but also observed to decrease the rate of hydrolysis by both forms of carnosinase, an observation suggesting that these compounds could have a longer half-life in the blood stream (Pegova et al., 2000).

The interest in carnosine and other imidazole dipeptides, such as anserine (β-alanyl-L-3-methyl-histidine), homocarnosine (γ-aminobutyryl-L-histidine) and balenine (-alanyl-L-3-methylhistidine), has recently increased. A study has reported carnosine as an effective antioxidant, intracellular buffer, immune modulator, neurotransmitter, metal-ion chelator, and free-radical scavenger (Boldyrev, 2012). It has been proposed also that carnosine could act as a naturally occurring scavenger of harmful reactive aldehydes or reactive carbonyl species (RCS) from the degradative oxidative pathway of endogenous compounds such as sugars, polyunsaturated fatty acids (PUFAs), and proteins (Hipkiss et al., 1998). Further, it has been demonstrated that carnosine reacts with 4-hydroxy-trans-2,3-nonenal (4-HNE), a toxic aldehyde involved in the pathogenesis of a number of diseases such as atherosclerosis, diabetes, and neurodegenerative diseases, in biological systems through a sacrificial mechanism mimicking the preferred HNE addition sites in proteins. This does not only further define the biological role of carnosine but more importantly to carnosine–HNE adducts as specific and unequivocal markers of lipid peroxidation in those biological districts where carnosine is specifically located at high concentrations, such as cardiac and skeletal muscles (Aldini et al., 2002). Recently, carnosine has been reported to be a highly effective scavenger of glucolipotoxic free radicals such as reactive oxygen and nitrogen species (RONS), resulting in beneficial actions on glucose homeostasis through both increased insulin secretion and skeletal muscle glucose uptake (Cripps et al., 2017).

Reactive carbonyl detoxification has been suggested as a new potential therapeutic strategy due to its pathogenic role (Shapiro, 1998). The use of antioxidants and/or carbonyl trapping agents (nucleophilic compounds) are among the most important proposed pharmacological approaches for this purpose and these reagents should possess some basic requirements such as high and specific reactivity towards cytotoxic aldehydes, safety, bioavailability, be non-toxic and easily or rapidly excreted. Interestingly, the dipeptide carnosine possesses a number of these basic requirements, since it is safe, and reacts through an autocatalytic mechanism with unsaturated aldehydes such as HNE (Carini et al., 2003). However, the rapid hydrolysis of carnosine in blood limits its application as a detoxifying agent towards RCS involved in the pathogenic mechanisms of many diseases.

The positive biological effects of carnosine are not only confined to its scavenging action towards reactive species, but might also be attributed to its ability to function as a mobile pH buffer (Skulachev, 1992; Parkhouse et al., 1985) not only against hydrogen ions but also for mixed-valence metal ions particularly iron, cadmium, copper, manganese and cobalt (Hartman and Hartmann, 1992; Brown and Antholine, 1979). These ions are known to have biological roles in many metabolic processes including the pathogenesis of some neurodegenerative and other age-related diseases (Sigel et al., 2006), and there are different pathological conditions involving critical metabolic processes such as protein misfolding and free radical driven-oxidative stress that requires the presence of these metal ions (Nishida, 2012) and so the ability of carnosine to regulate the level of mixed-valence metal ions is another important property of carnosine that supports its antioxidant status. There are some reported putative physiological roles of carnosine, particularly in tissues or organs where it is more concentrated. For example, it plays an important role in the contractile function of skeletal muscles where it acts to offset muscle fatigue as an intracellular pH buffer in the acidosis-associated muscular contractile fatigue (Pedersen et al., Sale et al., 2013). Given the high concentrations of carnosine in the olfactory neurons, it was thought that this dipeptide might be involved in neurotransmission, either as a neurotransmitter or neuromodulator. This assumption was based on the idea that glutamate as the main excitatory neurotransmitter has been found to be colocalized with carnosine in the synaptic terminals of mouse olfactory neurons in an immunohistochemical study (Sassoe-Pognetto et al., 1993). The neuroprotective effect of carnosine in certain brain structures has also been hypothesized to be mainly through its antioxidant action, and its ability to chelate transition metals like copper and zinc that have been identified as modulators in neurotransmission (Kawahara, Tanaka and Kato-Negishi, 2018; Boldyrev, 2001).

Carnosine has also been found to have beneficial effects on cardiovascular function. The vasorelaxant effect of carnosine regulates the vascular tone which then helps to maintain normal haemodynamics or blood pressure and the mechanism involved was proposed to be increased pH buffering capacity and improved calcium handling, as carnosine is not only capable of facilitating the release of calcium in the sarcoplasmic reticulum it also helps improve tension of the contractile proteins in response to calcium (Zaloga et al., 1997; Ririe et al., 2000). The protection afforded by carnosine in ischemia and reperfusion tissue damage is associated with its ability to scavenge reactive oxygen species. For example, in a study involving a model of permanent focal cerebral ischemia in mice, carnosine treatment prior to ischemia significantly reduced the neuronal damage and infarct size paralleled with reduction of ROS levels in ischemic brains and a sparing effect on the depletion of glutathione (Rajanikant et al., 2007).

Other therapeutic applications of carnosine include neurological disorder like Alzheimer's disease where it has been observed to inhibit β-amyloid polymerization and protect the brain from the neurotoxic effect of β-amyloid peptide (Preston et al., 1998). In ocular diseases, carnosine can modulate the aggregation and disaggregation dynamics of α-crystallin – insoluble aggregates that induces lens opacity (Seidler et al., 2004). Carnosine has been also applied for faster wound healing - where it could promote collagen biosynthesis through its β-alanine component and through improved activity of the fibroblast growth factor leading to macrophage recruitment and angiogenesis which is associated to the L-histidine-regulated histamine synthesis during trauma (Nagai et al., 1986; Numata et al.,2006). These observations were confirmed in a study whereby administration of carnosine enhances wound healing in db/db mice that might be ascribable to the increased expression of growth factors and cytokines involved in wound healing (Ansurudeen et al., 2012).

The beneficial action of carnosine in diabetes was first observed in a study involving mice where 4-week oral supplementation of the dipeptide in a dose-dependent manner reduced plasma glucose and fibronectin levels, increased insulin levels, and reduced oxidative damage (Lee at al., 2005). This observation was also consistent with a later study which demonstrated that carnosine treatment (100-200mg/kg) reduced hyperglycaemia, and normalised dyslipidaemia in a streptozotocin diabetic-induced model (Soliman et al., 2007). Carnosine has therefore attracted much attention as a naturally occurring antioxidant and thereby considered to be a possible therapeutic agent. This versatile antioxidant's ability in controlling oxidative stress, suppressing glycation, and sequestering metal ions, make it as a champion in reducing harmful sequelae such as DNA damage, a known causal of many diseases.

1.19 Carnosine in Foods

The occurrence of aging had been associated with carnosine decline, and so carnosine-rich diets become increasingly relevant for human aging (Stuerenburg, 2000). The nutritional interest in carnosine and other imidazole dipeptides stemmed from the idea that these antioxidant molecules are significantly present in meat products for human consumption (Peiretti and Meineri, 2015). In addition to endogenous source of carnosine through carnosine synthase, a diet such as a portion of beef, pork or chicken daily could provide approximately 250 mg per day of carnosine (Peiretti and Meineri, 2015). Carnosine is not available in plants and so practically absent from vegetarian diets (Krajcovicova-Kudlackova et al., 2002). In fact, a study showed that the levels of AGEs were found to be higher in the plasma of vegetarians than in those of omnivorous people (Krajcovicova-Kudlackova et al., 2002). Vegetarian diets are of course considered to be very healthy, however, the potential protective effects (controlled secondary diabetes complications) of meat observed amongst omnivorous diabetic patients as compared to exclusively vegetarian patients, (Peiretti and Meineri, 2015) remained to be poorly known among public opinion.

Carnosine may be useful to improve the quality of meat for human consumption most likely via preserving it from oxidation, and thus maintaining its colour and taste. The level of polyunsaturated fatty acids in meat is a major player in the rate and extent of lipid oxidation in muscle tissue (Buckley et al., 1995). Lipid oxidation or the deterioration of its nutritional value or quality and affects taste, is caused by a substance called thiobarbituric acid (Fernandez et al., 1997). Different antioxidant systems such as α-tocopherol, and antioxidant glutathione peroxidase, superoxide dismutase, catalase and dipeptides containing histidine such as carnosine are present in muscle tissues that could potentially act as defensive mechanism to prevent or retard oxidation reactions (Meineri at al., 2013). It has been shown that the oxidative stability of skeletal muscle is largely influenced by dipeptides containing histidine, such as carnosine and anserine. These dipeptides, which are found in meat, are regarded as bioactive food components (Gardner *et al.*, 1991). Imidazole dipeptides are also linked to the reduction of some aromatic compounds generated in meat which are responsible for its rancidity and colour change (Jimenez-Colmenero et al., 2010). In addition, carnosine is highly efficient at maintaining an acceptable red colour in ground beef during storage and has therefore been suggested as a useful antioxidant in foodstuffs of animal origin (Badr, 2007). Thus, carnosine either as a component of meat or as an additive, may be essential in preventing the formation of anomalous flavours, in increasing the shelf life of meat and meat products, and most importantly in preserving its nutritional quality.

1.20 Aims and Objectives of the Study

The global epidemic of diabetes continues to be a huge and growing problem and is responsible for an escalating human and financial cost annually. Importantly, existing treatments often become less effective over time. The first phase of this thesis details mechanistic experiments to investigate the potential of carnosine to scavenge, neutralise or detoxify glucolipotoxicity driven generation of reactive oxygen, nitrogen and carbonyl species and whether or not this effect could improve insulin signalling or glucose uptake of glucolipotoxicty-induced insulin resistant skeletal muscle cells. Secondly, this work utilises immunoprecipitation-mass spectrometry tandem techniques in order to identify proteins that are adducted by known biomarkers of oxidative stress, namely 3-nitrotyrosine and 4-hyroxynonenal, in cells and tissues central to the control of glucose homeostasis and that are subject to metabolic stress. The extent of adduction protection to these proteins by carnosine is also determined. Thirdly, the effectiveness of supplementation by β-alanine and slowly-hydrolysable carnosine analogs is assessed, and their potential to induce therapeutic benefit through enhanced glucose uptake or increased insulin secretion determined. Through these combined approaches, this PhD project aims to establish novel treatment strategies that can potentially ameliorate high sugar and high fat-induced insulin resistance in skeletal muscle cells, and thereby provide more effective treatment or prevention of type 2 diabetes.

Chapter 2: Materials and Methods

2.1 Reagents and Solutions

2.1.1 Materials

Unless otherwise stated, all chemicals or reagents were purchased from Sigma Aldrich (Dorset, UK) and all plasticwares from VWR International Ltd (Lutterworth, UK). The luminescence-based glucose uptake kits were purchased from Promega (Southampton, UK). Antibodies were purchased from Abcam (Cambridge, UK) unless otherwise stated.

2.1.2 Solutions and Buffers

The following table indicates the list of prepared solutions commonly used in one or more of the experiments conducted in this project.

Table 2.1; List of Solutions and Buffers with its Corresponding Components.

SOLUTION	COMPOSITION
Krebs-Ringer Buffer Solution (KREBS)	125 mM NaCl, 1.2 mM KH_2PO_4, 5 mM KCl, 2 mM Mg SO_4, 1 mM $CaCl_2$, 1.67 mM glucose, 0.1% Bovine Serum Albumin (BSA), 25 mM HEPES, pH7.4
PBS (Phosphate buffered saline) 10X	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2mM KH_2PO_4, pH7.4
Sample Buffer	4X Laemmli sample buffer (BioRad):2-mercaptoethanol (9:1)
Running Buffer	25 mM Tris base, 190 mM glycine, 0.1% SDS, pH 8.3
Transfer buffer	20% 5X Trans-Blot Transfer buffer (BioRad), 60% distilled H_2O, 20% of pure Ethanol
TBS (Tris Buffered Saline)	50mM Tris-Cl, 150mM NaCl pH7.6
TBST	TBS + 0.1% TWEEN20
RIPA Buffer	150mM NaCl, 0.5% Deoxycholate, 0.1% SDS, 50mM Tris Base, 1% Triton 100, 1x protease inhibitor tablet pH=8.0

Paraformaldehyde	1 % PFA in PBS with 2-3 drops of 1N NaOH, pH= 7.4
Immunoprecipitation Lysis/Wash Buffer	25mM Tris, 150mM NaCl, 1mM EDTA, 1% NP40, 5% glycerol. pH=7.40
Coupling Buffer	10mM sodium phosphate, 150mM NaCl, pH=7.20

2.2 Cell Culture

2.2.1 Cell Line and Primary Cells Used

Table 2.2; List of Different Cell Types Used in this Project.

Cell Types and Species	Characteristics or Description
C2C12 - Mouse	The C2C12 (ECACC 91031101) used in this project was purchased from Public Health England and as supplied by the European Collection of Authenticated Cell Cultures (ECACC). This is a sub-clone from myoblast line established from normal adult C3H mouse leg muscle. It proliferates readily in high serum and differentiates rapidly at low serum and produces extensive contracting myotubes expressing characteristic muscle proteins. C2C12 cells express GLUT 4 transporters that are translocated and activated in

	response to insulin stimulation resulting in glucose uptake (Yaffe and Saxel 1977; Fisher and Williams, 2011).
Human Skeletal Muscle Myoblasts (HSkM)	Cells were purchased from Lonza Bioscience, Switzerland in the second passage. Clonetics^{TM} Human Skeletal Muscle Myoblasts (HSkM) are isolated from the upper arm or leg muscle tissue of a healthy donor (20-year old, Caucasian male, BMI = 21, non-smoker). Using the conditions described below, HSkMs can be differentiated to form multinucleated myotubes in culture. The use of this cell in this project involved population doublings not more than 10. As provided in its certificate of analysis, this cell is negative for mycoplasma, bacteria, yeast, and fungi and HIV-1, hepatitis B and hepatitis C are not detected for this cell lot.
Primary Mouse Muscle Cells	Five, young, male, 19-21g five male mice (CD-1 IGS strain, source: Charles River) were purchased and maintained by staff in the Biological Services Unit, NTU. The muscle isolation was

	conducted by Dr. Craig Doig (NTU) using the cell culture conditions and protocol detailed below. In contrast to the use of immortalised myogenic cell lines, primary myoblasts have been suggested and highly recommended as the most physiologically relevant *in vitro* model of myogenesis because they are devoid of some degree of variability observed in cell lines associated with the origin of the cells, culture conditions and passage number (Hindi et al., 2017).
Primary Adipocytes - Human	In collaboration with Prof McTernan's group in NTU, these adipocytes obtained as passage 1 from a female healthy donor (age -37, BMI- 23.5) were provided and cultured until passage 3 through the assistance of Dr. Alice Murphy and Dr. Adaikala Antonysunil. Along with other human samples used in this project, including serum and fat from different human patients, these were obtained with informed consent and local Ethical Approval ((IRAS No. 81368).

INS-1 - Rat	INS-1 is one of the most widely used insulin-secreting cell lines and was derived from a rat insulinoma induced by X-ray irradiation. These cells exhibit several relevant characteristics of primary pancreatic beta cells, including high insulin content and responsiveness to glucose within the physiological range, thus making them a suitable β-cell model. These cells do however require 2-mercaptoethanol in their culture media and without this component, the cells cannot be propagated and lose many important functional characteristics (Asfari et al., 1992; Skelin et al., 2010).

2.2.2 Media Components and Preparation

Table 2.3; Media Components for Different Cell Types Used in this Project.

Cell and Media Type	Components
For C2C12: - Growth Media	DMEM-high glucose (Dulbecco's Modified Eagle Medium; 41966029,ThermoFisher Scientific, UK), 10% hi Fetal Bovine Serum (FBS; F9665,

	Sigma, UK), 10% Newborn Calf Serum (NCS; 26010074, ThermoFisher Scientific, UK), 1% penicillin-streptomycin (Pen-Strep; 15140122, ThermoFisher Scientific, UK), 1%L-glutamine(25030024; ThermoFisher Scientific, UK). Aliquoted and stored in 4^0C up to not more than 8 weeks.
- Differentiation Media	DMEM-high glucose (Dulbecco's Modified Eagle Medium; 41966029, ThermoFisher Scientific, UK), 2% hi Horse Serum (HS; 26050088, ThermoFisher Scientific, UK), 1%L-glutamine(25030024; ThermoFisher Scientific, UK), 1% penicillin-streptomycin (Pen-Strep; 15140122, ThermoFisher Scientific, UK). Aliquoted and stored in 4^0C up to not more than 8 weeks.
For HSkM: - Growth Media	Ready to use Human Skeletal Muscle Growth Media (C-23060; PromoCell, Germany) with supplement pack containing fetal calf serum, fetuin (bovine), dexamethasone, and epidermal growth factor, insulin, basic fibroblast growth factor (recombinant human). After adding the supplements to the medium, this was then aliquoted and stored in 4^0C up to not more than 6 weeks.
	DMEM-F12, HEPES (11330032; ThermoFisher Scientific, UK), 2% hi

- Differentiation Media	Horse Serum (HS; 26050088, ThermoFisher Scientific, UK), 1% penicillin-streptomycin (Pen-Strep; 15140122, ThermoFisher Scientific, UK). Aliquoted and stored in 4^0C up to not more than 6 weeks.
For Primary Mouse Muscle Cells: - Satellite Cell Culture Media	DMEM-high glucose (Dulbecco's Modified Eagle Medium; 41966029, ThermoFisher Scientific, UK), 30% FBS F9665, Sigma, UK), 10% not hi HS (16050-122, ThermoFisher Scientific, UK), 1% Chick Embryo Extract (C3999-USB, Stratech, UK), 2% L-glutamine, 1% Penicillin-Streptomycin, 10 ng/mL recombinant murine fibroblast growth factor-basic (450-33, Peprotech, UK). Freshly prepared and to be used on the day of the muscle isolation.
- Proliferation Media	DMEM-high glucose, 10% horse serum, 0.50% Chick Embryo Extract, 2% L-glutamine, 1% Penicillin-streptomycin. Prepared as required and stored at 4^0C.
- Differentiation Media	DMEM-high glucose, 2% horse serum, 2% L-glutamine, 1% Penicillin-streptomycin. Prepared as required and stored at 4^0C.
For Primary Adipocytes: - Growth Media	DMEM/Ham's F-12 no phenol red (11039047, ThermoFisher Scientific, UK), 1% Penicillin-Streptomycin, 10% FBS (F9665, Sigma, UK), 5 ng/mL FGF-basic recombinant human (VXPHG0026, Fisher Scientific, UK), 5 μg/mL Transferrin, human (VX0030124SA , Fisher Scientific, UK). Aliquoted and

	stored in 4^0C up to not more than 6 weeks.
- Differentiation Media	DMEM/Ham's F-12 no phenol red (11039047, ThermoFisher Scientific, UK), 3% FBS (F9665, Sigma, UK), Preadipocyte Supplement Mix (C39436, PromoCell, Germany). Aliquoted and stored in 4^0C up to not more than 6 weeks.
- Nutrition Media	DMEM/Ham's F-12 no phenol red (11039047, ThermoFisher Scientific, UK), Adipocyte Nutrition Supplement Mix (C39439, PromoCell, Germany).
For INS-1 β-cells: - Growth Media	Pre-prepared 10.30g of RPMI powder (11Mm glucose) (ThermoFisher, UK) in 1L distilled H_2O, 26mM $NaHCO_3$, 10mM HEPES, 50μM mercaptoethanol, 10% FBS (Life Technologies, UK), 1% sodium pyruvate, 1% penicillin-streptomycin. Sterile-filtered, pH adjusted to 7.4. Stored at 4^0C.

2.2.3 Cell Culture, Propagation and Differentiation

C2C12 and HSkM. Mouse C2C12 and human skeletal myoblasts were maintained in their growth media specified in Table 2.2 in a humidified atmosphere with 5% CO_2 at 37^oC. At ~ 80% confluency, cells were washed twice with PBS and thereafter, the medium was then switched to differentiation medium to facilitate the myocytic differentiation prior to performing the relevant assay and was replaced after 2, 4, and 6 days of culture.

Primary Mouse Skeletal Muscles. Briefly, isolated muscles placed in DMEM-1% penicillin-streptomycin and glutamine were disaggregated in 0.2% collagenase/DMEM in an incubator at 37ºC for 90-120 mins with gentle agitation. After trituration of the muscles to liberate the myofibres, these were then serially transferred to 6-well plates coated with 1/40 Matrigel in DMEM (354234; Corning, UK) containing the satellite culture media and stored in the incubator at 37°C with 5% CO_2 for the next 96 hours. After this time, adhered fibres were gently removed from the surface of the well leaving satellite cell-derived myoblasts. When about 30-50% confluency was reached, the media were then changed to Proliferation media as detailed in the table above. When wells became about 90% confluent, the media were replaced with Differentiation medium and maintained media replacement every 48 hours for the next 5-6 days prior to any use for assay.

Primary Adipocytes. Isolated primary human preadipocytes were cultured in T75 flasks using its growth media described above and changing this every 48 hours and once 80-90% confluent was reached, cells were further incubated in its growth media for 2 days, then initiated (day 0) the differentiation by incubating these in differentiation media. During day 0 to day 6 of adipogenesis, media were maintained and replaced every 48 hours and placed in nutrition media from day 6 to day 14.

2.2.4 Cell Passage and Amplification

Cells were passaged or split once the desired confluency (e.g. 80-90% for C2C12 cells, or 70% for HSkM) was reached. Growth media was aspirated and cells washed three times with pre-warmed sterile phosphate-buffered saline (PBS), and then incubated in 5mL (for T75 flask) Trypsin-EDTA (Life Technologies, UK) for 5 minutes at 37°C and with moderate tapping of the flask thereafter, this allows cells to be completely detached. The dislodged cells were then added an equal volume of growth media and centrifuged at 1300 RPM for 5 minutes at room temperature to form a pellet of cells. Cell pellets were then resuspended in an appropriate volume of complete growth media at desired seeding density in new tissue culture treated flasks of plates.

2.2.5 Cell Counting and Plate Seeding

All types of cells used in this project were initially grown in T75 culture flasks and after existing media was aspirated, cells were washed three times with pre-warmed sterile phosphate-buffered saline (PBS) and were detached by incubating in 4ml of Trypsin EDTA (Life Technologies). Trypsin was then neutralised with an equal volume of complete growth media and the cell suspension collected and centrifuged at 1300 RPM for 5 minutes. The supernatant was discarded, and the resulting cell pellet was resuspended in 6-8ml of complete growth media. Cells were either counted manually using haemocytometer or automated cell counter Lumina II (Vita Scientific, USA). For manual counting, a 10µl of cell suspension (1:1 mixture of cell suspension and 0.4% Trypan Blue) was loaded into a Neubauer chamber then the number of cells was determined from four big squares (counting clockwise and Top-Left) highlighted in Figure 2.1 to quantify the estimated cell concentration using the following formula:

$$\boldsymbol{Cell\ Concentration}\ \left(\frac{\boldsymbol{cells}}{\boldsymbol{mL}}\right) = \frac{\boldsymbol{Number\ of\ cells}}{\boldsymbol{Number\ of\ squares}}\ \boldsymbol{x\ 10000\ x\ Dilution\ Factor}$$

For experiments using C2C12, ~20000 cells in 1mL media or ~40000 cells in 2mL media grow to ~80% confluency in 2 days when grown in 6-well and 12-well plates, respectively. At the same density, HSkM take a day or two more to reach the same confluency. After each desired confluency, cells were differentiated for 5-6 days prior to any particular treatment for further experimental assay.

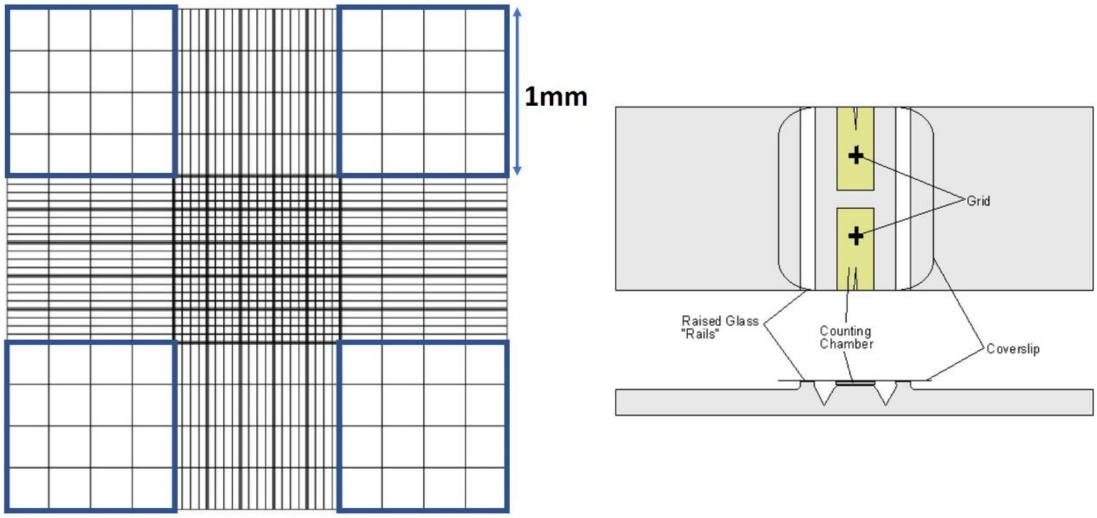


Figure 2.1; Haemocytometer gridlines and counting chamber for cell counting. A 10µL cell suspension is introduced to a space of known depth (0.1mm) beneath a coverslip and counted within a grid of area $1mm^2$. This gives the number of cells per $0.1mm^3$ (or 0.1µL). Multiplying by 10 gives the number of cells per µL and multiplying by 10,000 gives the number of cells per mL.

2.2.6 Cell Cryopreservation and Recovery of Frozen Cells

Cells grown in T75 culture flasks were collected when 80-90% confluent. The growth medium was then aspirated, and cells were washed thrice in sterile PBS and then incubated in 5 ml Trypsin-EDTA (Life Technologies, UK) for 3-5 minutes at 37°C to allow dispersal of cells. Cells were then harvested using an appropriate volume of complete growth media and gently mix before being collected by centrifugation at 1300 rpm for 5 minutes at room temperature. Cell pellets were then resuspended at 1 mL per cryovial in freezing media called Synth-a-Freeze (A1254201; ThermoFisher Scientific, UK), a liquid cryopreservation medium containing 10% dimethylsulfoxide (DMSO). These cells were then stored at -80°C using a cell freezing container (BCS405; Biocision, USA) with standardized controlled-rate -1^{o} C/minute cell freezing in a $-80^{o}C$ freezer without alcohol or any fluids and uses a thermo-conductive alloy core and highly-insulative outer material to control the rate of heat removal and provide reproducible cell cryopreservation. After 24 hrs – 2 months cells were then transferred to liquid nitrogen facility for long-term storage.

Cells recovered from long term storage were defrosted then added gently with 3-5 mL of complete growth media, centrifuged for 5 minutes, the supernatant removed then washed twice with growth media. Thereafter, cells were then resuspended in 3-5 mL of growth media and seeded appropriately in a T75 culture flask and incubated at 37°C in a 5% CO2 atmosphere.

2.2.7 Mycoplasma Screening

The Bioscience department of NTU and/or the Turner laboratory group conducts biannually (or when necessary) screening for mycoplasma infection for all cells used. The screening utilised a highly sensitive and specific polymerase chain reaction (PCR)-based assay mycoplasma detection kit (K10210; Geneflow Ltd., UK). The system excludes the amplification of DNA originating from other sources, such as tissue samples or bacteria, which affect the detection result. Instead, the primer sets used enhance not only the sensitivity but also the specificity of detection which is the amplification of the 16S rRNA gene region of mycoplasma and produces a PCR reaction product of 270bp band when run on an agarose gel. About 1mL of cell culture supernatant from cultured cells growing for a minimum of 24 hours was used for this analysis, which is processed for PCR amplification and then analysed the amplified products by gel electrophoresis.

In order to prevent mycoplasma contaminations, aside from observation of proper laboratory practice in cell culture (e.g. always wearing proper protective equipment in the cell culture lab and using proper sterile techniques), the Turner group also utilises non-toxic pharmacidal spray (K10335; Geneflow, UK), as part of keeping the laboratory regularly tidy, containing quaternary benzyl ammonium compounds as active ingredients for disinfecting benchtops and incubators to protect against multiple types of contaminants including bacteria, fungi, virus and mycoplasma.

2.3 Preparation of Healthy Control and Glucolipotoxicity Media

For C2C12 and primary mouse muscle cells, the media for the control or normal condition ("healthy") are composed of DMEM (11mM of glucose) with 2% heat-inactivated horse serum whilst for HSkM healthy control is DMEM-F12 (5mM glucose media) with 2% horse serum and for primary adipocytes this is DMEM-F12 Nutrition media (5mM glucose). Each prepared healthy control media was then sterilely filtered using a 0.20 µm syringe filter. On the other hand, to mimic diabetic extracellular glucolipotoxic conditions or GLT (high glucose and high fatty acids) *in vitro,* cells were incubated for 5-days in similar media components as healthy except that glucose concentration is 28mM for mouse muscle cells, 17mM for HSkM and all were supplemented with 200µM palmitic acid and 200µM oleic acid in combination. Stock solutions (100mM) of oleic acid in 50% ethanol-H_2O and palmitic acid in absolute ethanol were previously prepared, and appropriate volume to obtain each a final concentration of 200 µM was then added to the GLT media containing 2% of fatty-acid free BSA and placed this mixture in 37^0C for a minimum of 1 hour to allow the fatty-acid and BSA conjugation and thereafter this was then sterilely-filtered using 0.20 µm syringe filter (UY02915-60; Cole Parmer, UK). All experimental condition media were freshly prepared as required and were used only within the treatment period.

Table 2.4; Volume of Experimental Media (Control or GLT) Added for Cell Treatment

Cell Culture Plates or Flasks	Volume of Control or GLT Media, mL (replacement on Day 3)
12-Well	1
6-Well	2
T25	5
T75	13

2.4 Protein Expression Analysis

2.4.1 Protein Sample Preparation

Cells were cultured under the desired conditions, then washed thrice with PBS and subsequently added with an appropriate amount (1mL/T75 flask or 0.25 mL for 6-well plates) ice-cold RIPA buffer containing 1X protease and phosphatase inhibitors tablets (Roche Applied Science, Switzerland). Scrapers were used to detach the cells from the plates and destroy cellular integrity. Lysates were collected in 1.5 ml tubes, placed in an ice bath for 30 minutes with regular vortexing to disaggregate protein from cell debris and then centrifuged 13000 x g at 4^0C for 12 minutes. The supernatants were transferred into a clean 1.5 ml tube and protein concentration was quantified using the Pierce™ BCA Protein Assay Kit (Pierce Biotechnology Inc., ThermoFisher Scientific, UK) ready for analysis. For later use, lysates are stored in either -20^0C or -80^0C whilst avoiding multiple freeze/thaw cycles as much as possible.

2.4.2 Protein Quantification (BCA Assay)

The quantification of total protein was via colorimetric detection based on bicinchoninic acid using Pierce™ BCA Protein Assay Kit. The principle of this method entails the well-known reduction of Cu (II) to Cu (I) by the proteins in the sample in an alkaline medium using a unique reagent containing bicinchoninic acid. The purple-coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000μg/mL). Accordingly, protein concentrations were determined and reported with reference to standards of a common protein which in this analysis used bovine serum albumin (BSA). A series of dilutions of known concentration are prepared from the protein standards and assayed alongside the sample solutions and hence determined the concentration of each sample based on the standard curve using the concentration of standards as the x-axis and the absorbance as y-axis.

The required volume of Working Reagent (WR) was prepared following the manufacturer's instructions that is using the (50:1, Reagent A:B) mixing ratio. A 200-μL of WR is required for each 10-μL of samples and standards. After about 30 seconds of thorough mixing on an orbital shaker, the microplate was covered and incubated at 37°C for 30 minutes then absorbance was read at 595 nm using the iMark™ microplate absorbance reader (BioRad, UK). Table 2.5 indicates the serial dilution steps for the preparation of different standard concentrations of BSA.

Table 2.5; BSA Standards Preparation

Solution	BSA Source, µL	Diluent (RIPA + Inhibitors), uL	BSA Standard Concentration, µg/mL
A	300 of Stock	0	2000
B	375 of Stock	125	1500
C	325 of Stock	325	1000
D	175 of B dilution	175	750
E	325 of C dilution	325	500
F	325 of E dilution	325	250
G	325 of F dilution	325	125
H	100 of G dilution	400	25
I	0	400	0 = blank

2.4.3 Western Blotting

2.4.3.1 SDS Gel Preparation

BioRad Mini-PROTEAN® tetra hand-cast systems were used. Preparation of a 10.0% polyacrylamide resolving gel mixture and a 4% stacking gel mixture is shown in Table 2.6 mixed in the order indicated. This was then poured between one short and one spacer plate assembled in a casting stand up to a level about 2cm below the comb for the stacking gel, and aside from ensuring gel has an equal level or set, bubbles should be removed and this was achieved by layering the top of the gel with 100% ethanol. After the gel has completely polymerised for about 30-45 minutes, the ethanol was completely removed by washing out the traces with distilled water or with a use of filter paper carefully dipped into it, and thereafter a stacking gel is poured on top of the resolving gel then a comb was inserted to make wells and then allowed to set for about 30 minutes.

Table 2.6; SDS-PAGE Gel Recipe

Gel Composition	Resolving Gel (10%), 10mL	Stacking Gel (4%), 5mL
Distilled Water	4.0 mL	3.1mL
30% Acrylamide/Bis-acrylamide	3.3 mL	0.65mL
Tris Buffer (1.5M, pH=8.8)	2.5 mL	-
Tris Buffer (0.5M, pH=6.8)	-	1.25mL
10% SDS	100 µL	100 µL
N,N,N′,N′-tetramethylethylene-diamine (TEMED)	15 µL	10 µL
10% Ammonium Persulfate (APS)	50 µL	100 µL

2.4.3.2 Sample Loading, Separation, and Transfer

From the previously determined concentration of each cell lysate by BCA assay, for example each 20µg equivalent amount of protein is diluted to 30µL using RIPA buffer and added with 5µL of 4X Laemmli sample buffer (90µL 4X Laemmli:10µl β-mercaptoethanol) (BioRad, UK). This mixture was then placed in a heating block at 95°C for 5 minutes. After gels have been placed in an assembled electrophoretic chamber containing an adequate volume of running buffer, an equal amount of samples alongside a molecular weight marker (5-10 µL) were then carefully and gently loaded using gel loading tips into the wells of the gel ensuring samples to settle evenly on the bottom of the well. Gels were then run at 50V for ~20 minutes and afterward the voltage was then increased to 120V and separation is judged complete until the sample front approached the bottom of the gel.

After complete separation, the Cassette protein were then transferred to nitrocellulose membrane (immersed and equilibrated 2-3 minutes in Trans-Blot Turbo Transfer Buffer) (BioRad, UK) by having the gel placed on top of the membrane and sandwiched this between transfer packs previously saturated in same transfer buffer and ensuring that no bubbles are trapped in the sandwich. Finally, the transfer was carried using a pre-programmed transfer protocol (e.g. mixed molecular weight; 1.3A, 25V, 7minutes) of the Trans-Blot Turbo™ Transfer system (BioRad, UK).

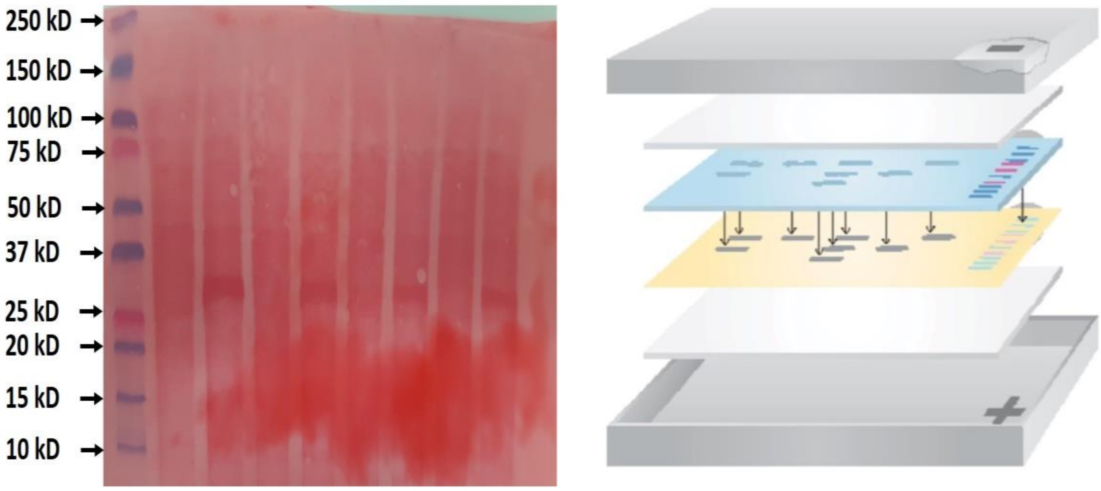


Figure 2.2; Example of protein transfer and the proper layering of the assembled transfer pack. Separated protein samples were efficiently transferred to nitrocellulose membrane using Trans-Blot Turbo™ (right) with preprogrammed setting for mixed molecular weights; 25 volts constant for 7 minutes and the dual-color Precision Plus Protein Standards (BioRad, UK). Successful transfer was confirmed using Ponceau S solution (left) before immunoblotting. Briefly, the membrane was incubated in 5 ml of Ponceau solution for one minute with gentle rocking until bands were visible, then solution was decanted and the remaining adhered stain were washed with TBST.

2.4.3.3 Antibody Incubation and Chemiluminescent Detection

In order to check the transfer quality as shown in Figure 2.2, the blot was briefly rinsed first in distilled water then was stained in Ponceau solution (Sigma, UK). Afterwards, the Ponceau S stain was rinsed off with three or more washes with TBST, then the membrane was blocked in 5% (w/v) milk (Marvel Original dried skimmed milk powder) or 3% BSA in TBST for 1-2 hours. Subsequently, the membrane was incubated with gentle agitation overnight with a primary antibody against the target protein (e.g. iNOS, GLUT4, ATPGD-1, CNDP2) diluted according to the manufacturer's recommended ratio in 5% (w/v) milk or 3% BSA in TBST at 4°C. The membrane was rinsed 3-5 times with TBST for 5 minutes before incubation in appropriate horse radish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit or mouse IgG) diluted in 5% milk in TBST for ~1 hour at room temperature. Similarly, the membrane was washed three times in TBST for 5 minutes each before being developed for imaging and data analysis using ECL plus mix solution (GE Healthcare, UK) and the chemiluminescence sensitive Image reader LAS4000 (GE Healthcare, UK).

Densitometric analysis was performed using gel electrophoresis image analysis software GelAnalyzer where the ratio between the band intensity of protein of interest to its corresponding housekeeping protein is calculated and the data is represented as fold change between conditions in comparison to control.

2.5 Cellular Function Analysis

2.5.1 Cell Viability

Cell viability was assessed using the Calcein-Acetoxymethyl ester kit (ThermoFisher, UK). Calcein AM is a non-fluorescent, hydrophobic compound that easily permeates intact, live cells. The hydrolysis of Calcein AM by intracellular esterases of viable eukaryotic cells produces calcein, a hydrophilic, strongly fluorescent compound that is well-retained in the cell cytoplasm. In the assay, conditioned media of cells that were previously incubated in either control or experimental conditioned growth media for 5-days were removed and cells were then washed 3 times in KREBS to ensure that carry-over media were completely removed. Afterwards, the cells were then incubated for 1 hour at 37^0C under 5% CO_2 with 5µM of Calcein AM cell viability dye solution freshly prepared using KREBS. Cell viability was measured via fluorescence, with excitation and emission at 490nm and 520nm respectively and viability results are presented as percentage change compared to standard control conditions.

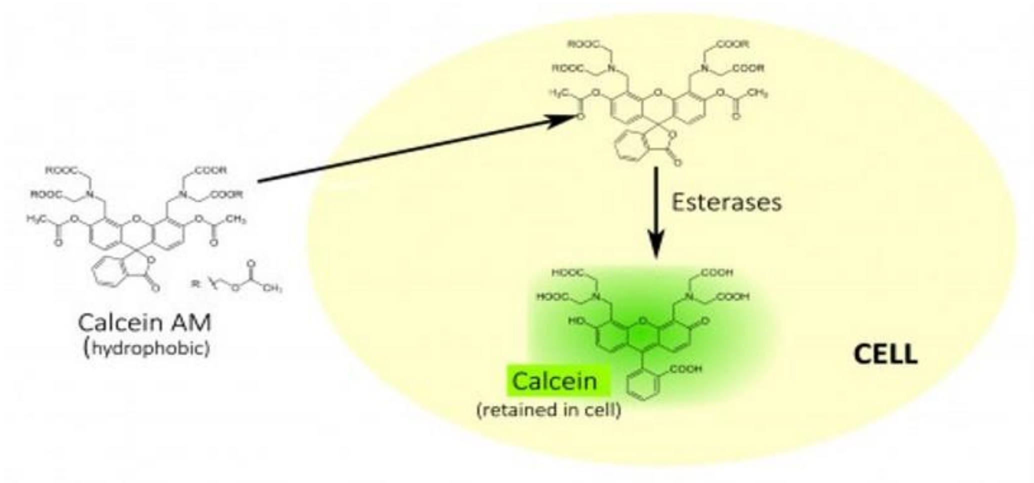


Figure 2.3; Principle of Calcein-AM Cell Viability Assay

2.5.2 Reactive Species Detection Assay

The level of intracellular reactive species were estimated using 2′,7′-Dichlorofluorescin diacetate (DCFDA), a non-fluorescent cell-permeable probe that is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2′, 7′ –dichlorofluorescein (DCF) – a highly fluorescent compound. The DCFDA is not only applicable for the measurement of reactive oxygen species, however, this can also be used to assess ROS activity mediated by other free-radicals or oxidizing reactive nitrogen or carbonyl species too.

In the assay, old media of cells that have been previously incubated in either control or experimental conditioned growth media for the desired period of time were removed and cells were then washed 3 times in KREBS to ensure that carry-over media were completely removed. Afterwards, the cells were then incubated for 1 hour at 37^0C under 5% CO_2 with 20µM of light-protected DCFDA solution freshly prepared using KREBS. The intracellular reactive species or reactive oxygen and nitrogen species (RONS) activity was then measured via fluorescence, with excitation and emission at 490nm and 530nm respectively and quantifications are presented as percentage change compared to standard control conditions.

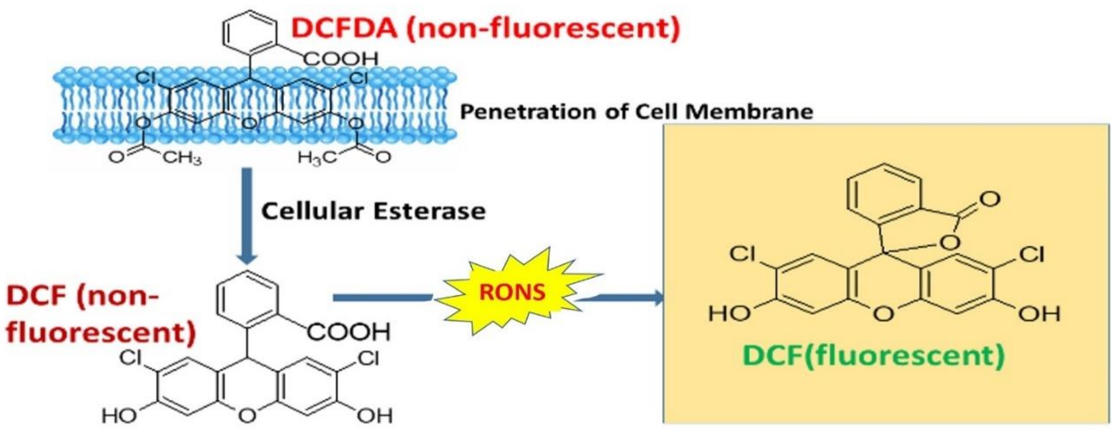


Figure 2.4; Principle of Reactive Species Detection assay using DCFDA

2.5.3 3-Nitrotyrosine (3-NT) Detection Assay

A 3-nitrotyrosine Enzyme-Linked Immunosorbent Assay (ELISA) (Abcam, UK) was used for the quantitative measurement of 3-nitrotyrosine. The assay employs an antibody specific for 3-nitrotyrosine coated on a 96-well plate. Briefly, after sample preparation from cell lysates of previously treated cells (control, carnosine, glt, and glt + carnosine), 50-µL of each standard or sample solution were added to each well and incubated at room temperature for 2-hours with shaking at 300 rpm. After two washes with 300-µL of 1X Wash Buffer, a 50- µL of 1X Detector antibody was added to each well and incubated for another 1 hour, then washed again before adding 50-µL of 1X HRP-labelled secondary antibody followed by another 1-hour incubation at room temperature. After this, 100-µL of HRP Development Solution to each empty well was added, the reaction was stopped by adding 1 N HCl and immediately record the absorbance at 450 nm. The cellular protein content of the sample that was previously quantified using BCA protein assay (PierceTM BCA Protein Assay Kit – ThermoFisher Scientific, UK) was then used to normalise 3-NT data.

2.5.4 4-Hydroxynonenal (4-HNE) Detection Assay

The 4-HNE ELISA kit (Universal Biologicals, UK) was used for the quantitative measurement of 4-HNE. The microtiter plate provided in this kit has been pre-coated with 4-HNE. During the reaction, 4-HNE in the sample or standard competes with a fixed amount of 4-HNE on the solid phase supporter for sites on the Biotinylated Detection Antibody specific to 4-HNE. Briefly, after sample preparation from cell lysates of previously treated cells (control, carnosine, glt, and glt + carnosine), 50-µL of each standard or sample solution were added to each well with 50-µL of Biotin-detection antibody working solution and then incubated at 37^{0}C for 1 hour. After three washes, 100-µL of HRP-Streptavidin Conjugate (SABC) Working Solution was added and the plate was then incubated for 30 minutes at 37^{0}C. After another round of washing, 90-µL of 3,3,5,5-tetramethybenzidine (TMB) Substrate was added to each well incubated for about 20 minutes then this reaction was stopped using sulfuric acid-containing Stop Solution in the kit and thereafter obtained the absorbance reading at 450 nm. The cellular protein content of the sample that was previously quantified using BCA protein assay (PierceTM BCA Protein Assay Kit – ThermoFisher Scientific, UK) was then used to normalise 4-HNE data.

2.5.5 Glucose-Uptake Assay

A non-radioactive bioluminescent Glucose Uptake-Glo™ Assay (Promega, UK) was employed to measure the glucose transport in cells. This assay is based on the uptake of 2-deoxyglucose (2-DG) and the enzymatic detection of 2-deoxyglucose-6-phosphate (2-DG6P) that accumulates. Following the desired treatment, cells (in 12-well format) were serum-starved overnight in DMEM supplemented with 5 mM glucose, the media were then removed and washed twice with DPBS before incubating this for 1 hour at 37^0C under 5% CO_2 in glucose-free DMEM +/− 100 nM insulin to stimulate GLUT4 translocation. Immediately after, the medium was then replaced with PBS + 2-deoxy glucose (2-DG) and allowed for the uptake reactions to take place for 30 min at 37^0C under 5% CO_2. Afterwards, the reaction was then terminated by addition of Stop Buffer provided in the kit containing 0.4 M HCl + 2% dodecyl trimethyl ammonium bromide, and added with the Neutralisation Buffer (1M Trizma, pH>10). A 100μL mixture (2:1:1, sample: stop buffer: neutralisation buffer) of the above in a white 96-well plate was added with 100μL of 2-DG6P Detection Reagent (prepared and equilibrated for 1 hour before use) was loaded and after 1 hour reaction, luminescence data were then acquired using a CLARIOStar luminometer (BMG Labtech, Ortenberg, Germany) with user-defined parameters or with Infinite® 200 Pro multimode plate reader (Tecan Life Sciences, Switzerland).

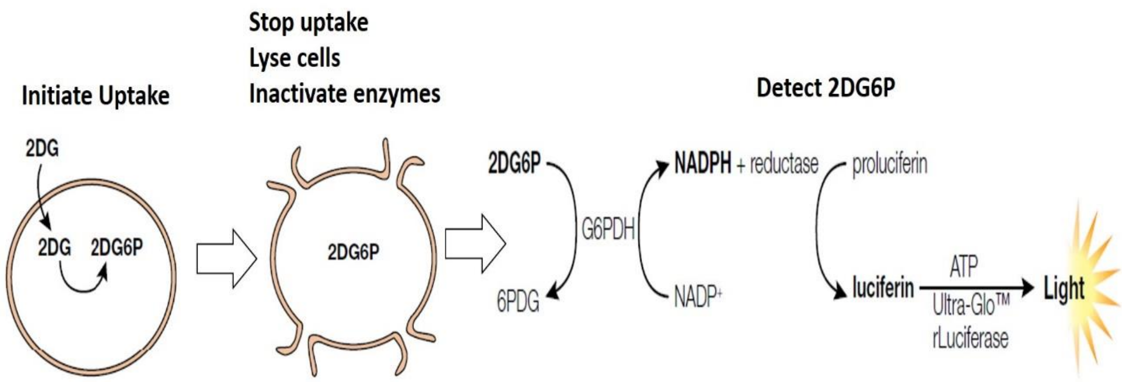


Figure 2.5. **Principle of Bioluminescent Glucose Uptake Assay**. [2-deoxyglucose(2DG), 2-deoxyglucosephosphate (2DG6P), 6-phosphodeoxygluconate (6PDG), glucose-6-phosphate dehydrogenase (G6PDH), nicotinamide adenine dinucleotide phosphate (NADPH).

2.5.6 GLUT-4 Translocation Assay

The GLUT4 translocation assay was conducted following the protocol of Koshy et al., with modifications. Following the desired treatment, cells were serum-starved overnight in DMEM supplemented with 5 mM glucose. The media were then removed, and cells washed twice with DPBS. Each well containing the cells (24-well format) was added with 0.50 mL of glucose-free DMEM +/−100nM insulin and with 6μL of antibody mix (prepared by mixing 5μL of primary anti-GLUT4 antibody (Abcam, UK) and 1μL of secondary antibody conjugated to AlexaFluor 488 (Abcam, UK), and was previously incubated for 10 minutes at room temperature) and incubated back for 30 minutes in the dark (37^{0}C, 5% CO_2). The cells were then fixed by adding 0.50mL of 1% paraformaldehyde in PBS and incubated for 20 minutes at room temperature in the dark. Using a cell scraper or cell lifter, cells (1 mL total) were then transferred to a flow cytometer tube and centrifuged to pellet the cells, washed twice with 1mL PBS and resuspended in 0.4mL of 1% PFA in PBS. The cells were wrapped in foil and immediately taken to the Flow cytometer for data acquisition. Data acquired with the help of Dr. Gemma Foulds of NTU using the Beckman Coulter Gallios™ flow cytometer and analyzed using Beckman Coulter Kaluza™ software.

2.6 Antigen Immunoprecipitation and Mass Spectrometry

The following samples were used for this analysis:

- Mouse C2C12 muscle cells
- Human skeletal muscle cell-line (HSkM)
- Mouse primary muscles
- Mouse primary β-islet cells
- Human primary adipocytes
- Human serum samples
- Human adipose tissues (abdominal subcutaneous)

The antigen immunoprecipitation process for this analysis employed the Pierce Crosslink Magnetic IP/Co-IP Kit (88805; ThermoFisher Scientific, UK) with DynaMag™-2 magnet (12321D; Life Technologies, UK). For all immunoprecipitation steps, there were two primary antibodies used namely: 3-nitrotyrosine and 4-hydroxynonenal.

2.6.1 Sample Preparation

Following desired treatment of the cell samples mentioned above (excluding human serum and adipose tissues) in either control or GLT ± carnosine for 5 days, cell lysates of C2C12, human skeletal muscle, and mouse muscle cells were obtained from each of these sample conditions using the IP Lysis buffer (25mM Tris, 150mM NaCl, 1mM EDTA, 1% NP40, 5% glycerol + protease inhibitor, pH=7.40) provided in the kit. The protein concentration for each of this sample was then quantified by BCA assay for further use in the subsequent steps of the analysis.

Human serum and adipose tissues are obtained with informed consent and local Ethical Approval (IRAS No. 81368) and this work is in collaboration with Prof. McTernan's group in NTU. Samples used from this are from lean, obese, and diabetic patients ages 25-60 years old (Females) with BMI ranging from 17-54. Cell lysates of both adipose and primary adipocytes were initially subjected to clean-up steps, prior to its use in the subsequent steps of the analysis, following the protocol and using the ReadyPrep^{TM} 2-D Cleanup Kit (BioRad, UK in order to wash away substances in these samples particularly lipids that would interfere and not ideal for the efficiency of mass spectrometer column.

For primary mouse islets, these samples were isolated and provided by Paul Caton of King's College London. Briefly, pancreata were inflated with 1mg/ml collagenase solution (Sigma-Aldrich, Poole, U.K.) followed by density gradient separation (Histopaque-1077; Sigma-Aldrich). Islets were re-suspended in RPMI media (10% FBS, 1% P/S, 1% L-Glutamate) and, after recovery at 37°C, were handpicked and placed in fresh medium. After incubating at 37^{0}C overnight, islets were treated with either RPMI media, 10mM carnosine in RPMI media, glucolipotoxicity (GLT) conditions, and both 10mM carnosine and GLT conditions. For GLT 100mM palmitate was made up by dissolving palmitate in sterile water and incubating at 70^{0}C and vortexing until completely dissolved. 100mM palmitate was further diluted to 5mM in serum-free DMEM and 5% NEFA free BSA and incubated for 1 hour at 40^{0}C and shaking at 140RPM, 28mM glucose was added. After 5 days incubation at 37 degrees in each condition islets were picked and spun at 500RCF for 3 mins. Any remaining media was removed, and islets were placed in 20 ul PBS and were snap-frozen into liquid nitrogen and stored at -80^{0}C.

Following quantification of protein content and depending on the amount available about 0.4mg – 1.0 mg of samples with a similar amount for each batch was then used later for antigen immunoprecipitation as described below.

2.6.2 Binding of Antibody to Protein A/G Magnetic Beads

The beads (supplied as 10 mg/mL in water containing 0.05% NaN_3) were initially vortexed to obtain homogenous suspension and 25µL in a microcentrifuge tube was used for each reaction and placed on a magnetic stand to collect the beads. Each of these bead-containing tubes were then washed twice with 500µL of previously prepared 1X Modified Coupling Buffer (10mM Na_3PO_4, 150mM NaCl; pH=7.20). 100µL containing 5µg of antibody (3-nitrotyrosine or 4-hydroxynonenal) solution previously prepared in 1:20 20X coupling buffer and 1:20 lysis/wash buffer was added to the beads and allowed to mix gently using a revolver tube rotator (Lightlabs, USA) for 1 hour at room temperature and with gentle agitation every 10 minutes. Afterwards, the beads were then collected by discarding the supernatant using the magnetic stand and washed twice with 300µL of the 1X coupling buffer and beads collected for the next step in 2.6.3.

2.6.3 Crosslinking the Bound Antibody

Conventional IP can be performed by omitting crosslinking, however, if this is omitted, the bound antibody during the elution steps will co-elute with the antigen. Crosslinking of the bound antibody was done using disuccinimidyl suberate (DSS). For each IP reaction, a mixture of previously prepared 4.0µL of 0.25mM DSS in DMSO, 2.5µL of 20X Coupling Buffer, and 43.5µL of ultrapure water were then added to the beads. The crosslinking reaction was incubated at room temperature for 1 hour with gentle mixing using a revolver tube rotator. After collecting the beads with a magnetic stand, 100µL of elution buffer was then added and mixed gently for 5 minutes at room temperature, and liquid removed before two serial washes with 200µL of cold IP wash buffer. The beads with the crosslinked antibody were then collected for antigen immunoprecipitation described in the next step.

2.6.4 Antigen Immunoprecipitation

Equal initial protein amount was used among the different batches of samples processed. Each sample lysate was diluted up to 1mL using IP lysis/wash buffer, then this solution was then added to the tube containing crosslinked magnetic beads and incubated overnight at 4^0C using a revolver tube rotator. Afterwards, the beads were collected and then washed with IP wash buffer, then supernatant discarded.

2.6.5 On-bead Reduction, Alkylation, and Tryptic Digestion

The beads collected in 2.6.4 steps were washed thrice with 50mM triethylammonium bicarbonate buffer (TEAB). Then 92µL of TEAB with 1µL of 0.50M dithiothreitol (DTT) was added onto the beads, incubated for 20 minutes at 56^0C with constant shaking. To this solution, was added 2.7µL of 0.55M iodoacetamide (IAA), incubated at room temperature in the dark for 15 minutes before adding 4µL of 1mg/mL proteomics grade trypsin. This was then incubated at 37^0C overnight with constant shaking. Afterwards, the solution was then collected and transferred to a new tube, added with trifluoroacetic acetic acid (TFA) to inactivate the trypsin, and incubated at room temperature for 5 minutes. The solution was then vacuumed dry as soon as possible and forwarded to the Mass Spectrometry facility of NTU for MS analysis.

2.6.6 Mass Spectrometry (MS) Analysis

Samples that had been previously vacuum concentrated to dryness were transferred to NTU John Van Geest Research Center for MS analysis and subsequently resuspended with appropriate amount of 5% (v/v) acetonitrile/0.1% (v/v) formic acid. The analyses were conducted by Dr. Amanda Miles and Dr. Clare Coveney and employed the reverse-phase high-performance liquid chromatography-electrospray ionization tandem mass spectrometry (RP-HPLC-ESI-MS) using TripleTOF 6600+ mass spectrometer coupled to Eksigent ekspert nano LC 425a pump system and autosampler (SCIEX, Canada). Data were acquired via two tools namely data-dependent acquisition (DDA) of sample pools to initially generate a spectral library and then the data independent acquisition (DIA) to measure the relative quantity of each protein within each individual sample. The two mobile phases used were A [0.1% (v/v) formic acid in LC/MS grade water] and B [LC/MS grade acetonitrile containing 0.1% (v/v) formic acid]. The two stationary phases used were firstly a 5 x 0.3µm YMC Triart C18 trap column followed by a YMC Triart-C18 analytical column 15 cm, 3µm, 300 um i.d at 5 µL/min. The samples were injected into the column above using an increasing linear of solvent B. The instrument was also routinely auto-calibrated every 2 samples using PepCal mix supplied directly from SCIEX.

2.7 Preparation of Carnosine-Related Compounds

Dr. Christopher Garner and his team in the NTU Chemistry department utilised both synthetic organic chemistry and computational chemistry approaches for the design, synthesis, and screening of prospective drug candidates against diabetes. In collaboration with this group, one major aim of this project is to design and screen potential carnosine related compounds that are most likely resistant to hydrolysis by carnosinase.

2.7.1 Putative Carnosinase Inhibitors

Briefly, a library of carnosine-based molecules (~50000) from Maybridge (www.maybridge.com) were screened to identify top 500 hits with shape, stereochemical and physical properties similar with that of carnosine. The selected hit compounds were then docked with the binding site of carnosinase-2 (CN2) particularly in the region involved in the hydrolysis using the software GOLD (https://www.ccdc.cam.ac.uk/solutions/csd-discovery/components/gold). Employing the Tanimoto scoring function, the resulting docking scores obtained above were used as basis for selecting 50 candidate molecules for further bioactivity screening. The top 50 hits were re-docked to the active site of CN1 and CN2 with increased search efficiency in order to determine which of these have the most similar shape to carnosine. The molecule bestatin, an analogue of carnosine, was used as an internal control to validate and to identify any malfunction and error of the method. Carnosine binding to the active site of the enzyme gave a ChemPLP score of 87 and based from this measurement, 14 of these molecules labelled as M4, M8, M14, M17, M21, M28, M36, M37, M38, M43, M44, M47, M48, M49, were purchased and initially evaluated for biological assays relevant to glucose homeostasis, namely insulin secretion and glucose uptake assays.

2.7.2 Carnosine Esters (Carnosine Mimetics)

Different ester derivatives, namely methyl-, ethyl- and isopropyl-, were prepared at the Organic Synthesis Chemistry laboratory in NTU under the supervision of Dr. Christopher Garner and Prof. John Wallis. Briefly, a mixture of 10mL alcohol (methanol, ethanol, and isopropanol for methyl, ethyl and isopropyl ester, respectively) with 0.5mL trimethylsilyl chloride was allowed to react for 20 minutes, then 0.20g of L-carnosine was added after which the resulting solution was refluxed at 95^{o}C overnight to yield about 0.30g of the desired product. The purity of the compound was validated using magnetic resonance spectroscopy at the NMR facility of NTU.

2.8 Cytosolic Non-specific Dipeptidase-2 (CNDP2) Activity Assay

For each reaction, 10ng/µL of recombinant human CNDP2 in Assay Buffer (50mM Tris, 0.10mM $MnCl_2$, pH=9.00) was used. In a 2mM substrate (carnosine or carnosine mimetics) dissolved in assay buffer, the above rhCNDP2 was added and incubated for 1 hour at room temperature and protected from light. This project used and evaluated some selected carnosine-shaped compounds designed and aimed to inhibit CNDP2 enzyme and as resistant to hydrolysis, and so in the presence of inhibitor (carnosine analogue), rhCNDP2 was allowed to react first with this compound for 30 minutes prior to addition of the substrate. This resulting mixture was then added with a 1% aqueous solution of trichloroacetic acid (TCA) to stop the reaction. In the case of a blank sample containing only rhCNDP2, the substrate was added after the TCA. All sample solutions were then centrifuged at 13000 rpm for 10 minutes, afterwards 180µL of supernatant was transferred to a new tube with 60µL of 5mg/mL of ortho-pthaldialdehyde (o-PA) in 2M NaOH, vortexed and incubated at room temperature for 30 minutes, protected from light. Then finally, 200µL of the reaction mixture including the standards (0-1000pmol L-Histidine) was loaded to a black 96-well plate and obtained fluorescence reading at 360 nm (excitation) and 460 nm (emission), top read and in endpoint mode using the Infinite® 200 Pro multimode plate reader (Tecan Life Sciences, Switzerland).

2.9 Seahorse XF Cell Mito Stress Test

In order to investigate the functional capacity of mitochondria in GLT-exposed skeletal muscle cells, and to assess what impact GLT has upon the activity of the mitochondrial electron transport chain, the Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies, USA) which uses different bioenergetic modulators (Oligomycin, FCCP and Rotenone/Antimycin A) was used. A day prior to the assay, the sensor cartridge (XFe24 Flux Assay Kit) was hydrated by filling each well with Seahorse XF calibrant solution and placed in a non-CO_2 incubator at 37^0C overnight.

Figure 2.6; Cartridge hydration procedure. This was performed 1 day prior to the Assay. Sensor cartridge is an essential component in the assay platform, and for the sensors to function correctly, they must be thoroughly hydrated and placed in the correct manner. The Hydro Booster and Cartridge Lid should be removed prior to placing the sensor cartridge and utility plate in the XFe24 Analyzer.

For C2C12 and mouse muscle cells, prior to the day of the assay cells that have been previously treated either with control or GLT ± carnosine for 5 days were harvested from T75 culture flasks. Cells were then seeded at 30,000 per well in a 24-well Agilent Seahorse XF24 Cell Culture microplate in its corresponding media and were incubated into 37°C humidified incubator with 5% CO_2 to allow the cells to adhere. On the other hand, human skeletal myotubes were treated for 3 days directly in 24-well Seahorse plate. On the day of the assay, the following conducted steps are briefly described as follows: Previously used media were removed and cells were washed twice with the 300μL Seahorse media (Seahorse XF-DMEM based medium supplemented with 1mM Pyruvate, 10mM glucose, and 2mM glutamine) before finally replacing it with 500μL of the same media. This was then incubated in a non-CO_2 incubator at 37^0C for 1 hour. During this incubation period, previously hydrated sensor cartridge was then taken out to have the different bioenergetic modulators (103105-100; Agilent, USA) namely Oligomycin, FCCP and Rotenone/Antimycin-A (freshly prepared on the day of the assay) loaded into its ports with its final concentration indicated in Table 2.8. Afterwards, cartridge (with lid and hydro booster removed) was then loaded to the Seahorse XFe24 Analyzer for calibration and equilibration that would take 20-25 minutes. Then, the utility plate was replaced with the seahorse plate containing the cells and was run for Mito Stress test using the pre-programmed or pre-prepared template, and the calculated results from the Wave data obtained from the Seahorse XF Mito Stress Test Report Generator were used for data analysis.

The tables below described the preparation of stock solutions of the different compounds as modulators of mitochondrial respiration, and its preparation for loading to the sensor cartridges port.

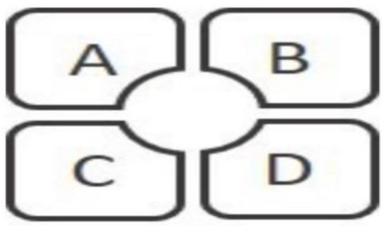
Table2.7; Preparation of Stock Solutions

Compound	Quantity per tube, (nmol)	Volume of Assay Medium, (µL)	Stock Concentration, (µM)
Oligomycin	63	630	100
FCCP	72	720	100
Rotenone/Antimycin-A	27	540	50

Table2.8; Preparation of compounds for loading to XFe24 sensor cartridges.

Compound	Port	Stock Solution Volume (µL)	Assay Media Volume (µL)	Volume Added to Port (µL)	Final Well (µM)
Oligomycin	A	300	2700	56	1.0
FCCP	B	300 720 (HSkM)	2700 2280	62 100	1.0 4.0
Rotenone/Antimycin-A	C	300	2700	69	0.5

Each well in a cell plate has a starting 500µL of assay medium**.** The location and numbering of injection ports on sensor cartridges are indicated in the figure below.



2.10 Statistical Analysis

Depending on the objective, one may show error bars in the form of confidence intervals, standard errors, standard deviations, or other quantities. Different types of error bars give quite different information (Cumming et al., 2007). All error bars associated in each figure (where applicable) here are shown as SEM from 3 or 4 independent experiments, and by purpose, this is also indicated in its respective figure legend. In addition, SEM was used in all of the bar charts in order to draw inference from the data obtained such as to evaluate whether a treatment (GLT or carnosine) could have a significant effect when compared with a particular control group.

For analysis which involved only two independent conditions, a two-tailed unpaired T-test was carried out using Microsoft Excel – for instance to determine whether GLT treatment could significantly affect cell viability from both directions (either to increase or decrease) when being compared to its healthy control counterpart. On the other hand, where analysis involved more than two conditions, first an ordinary Analysis of Variance (ANOVA) was carried out using Excel or GraphPad Prism Software Version 9.0.0 (Trial Subscription), and when result turned to be significant ($P<0.05$), an appropriate post hoc test (Tukey's test or Dunnett's test both using critical values at $\alpha = 0.05$) was then performed where $P<0.05$ values are considered as significant.

The ANOVA test could tell if results are significant overall, but it won't indicate exactly where those differences lie. For instance, Tukey's test was used to find out which specific group's means (compared with each other) are different. This test compares all possible pairs of means.

Another multiple comparison test employed was Dunnett's test. This is particularly useful when comparing means from several experimental groups against a fixed control group mean (e.g Figures 5.17 and 5.20) to see if there is a difference. Again, this can only be performed when ANOVA has shown ($P<0.05$) result.

The above version of GraphPad Prism Software was also used for normality test (Shapiro-Wilk test, with $P > 0.05$ ($\alpha=0.05$) passed for normality test).

Chapter 3:

Carnosine Scavenging of Glucolipotoxic Free Radicals Enhances Glucose Uptake and Insulin Secretion

3.1 Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterised by an elevated level of blood glucose due to impaired insulin secretion and/or increased cellular insulin resistance (Eid et al., 2015). T2DM is often associated with individuals who are either overweight or obese, and is often, although by no means always, characterised by over-nutrition and lack of exercise, set against a background of genetic pre-disposing factors (Chellan et al., 2012 and Balasubramanian et al., 2014). Glucose uptake by peripheral tissues, such as the skeletal muscles, has an important role in the maintenance of glucose homeostasis. When this tissue is no longer responsive to the action of insulin (insulin resistance) then insulin-stimulated glucose uptake is severely impaired. Initially the pancreas compensates by increasing insulin secretion, but over time this compensatory hypersecretion fails, resulting in hyperglycaemia. Skeletal muscle is considered to be the primary site of insulin resistance during the development of T2DM, as over 80% of glucose disposal occurs in this tissue, plus its mass is >40% of total body mass. As such, this tissue is crucial in controlling the level of blood glucose and in treating T2DM (Aguer and Harper, 2012).

The insulin receptor and its substrates, the phosphatidylinositol 3-kinase (PI3K) and the AMP-activated kinase (AMPK) are amongst the components of the insulin signalling pathways involved in glucose transport and uptake systems that are well-studied and reported to be potential molecular targets in the development of drugs for T2DM treatment (Lee et al., 2010; Schultze et al., 2012; Mor et al., 2011). Importantly, increased levels of plasma free fatty acids (FFA) has been shown to impair insulin action and, thus, play a central role in the pathophysiology of skeletal muscle insulin resistance (Ragheb et al., 2009; Silveira et al., 2008; Samuel et al., 2010).

The activation of protein phosphatase 2A (PP2A) and various serine/threonine kinases such as protein kinase C (PKCs) isoenzymes, nuclear factor kappa B (NF-κB) kinase, inhibitory- κB kinase β (IKK β), c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase (p38 MAPK) by the presence of elevated fatty acid levels are proposed to inhibit insulin signalling. When activated, these enzymes catalyse phosphorylation of serine residues in IRS-1, which in turn, could lead to a reduction in the phosphorylation of tyrosine residues of IRS-1, and in the activity of downstream signaling pathways activated by insulin (Li et al., 2015; Sears and Perry, 2015; Haasch et al., 2006).

Another factor that has gained recognition in causing a reduction of insulin sensitivity of skeletal muscle is sustained hyperglycaemia, also referred to as glucotoxicity. The possible mechanisms by which hyperglycaemia causes insulin resistance include impaired glycogen synthesis, enhanced apoptosis, and increased level of ceramides that could potentially inhibit several proteins along the insulin signalling cascade to glucose transport (Tomas et al., 2002).

Glucose-induced insulin resistance is also linked to other mechanisms including tissue damage implicated in diabetes micro-and macrovascular complications caused by the irreversible formation of advanced glycation end (AGE) products (Riboulet-Chavey et al., 2006). Overproduction of reactive oxygen species (ROS), which could be important mediators of damage to cellular components such as lipids, proteins, or DNA, is also directly linked to chronic hyperglycaemia. This indicates that oxidative stress is an important process in the development and progression of T2DM and its associated complications (Kawahito et al., 2009; Pittoco et al., 2003).

Metabolic reactions continuously produce reactive oxygen species (ROS), including superoxides (O_2^-), hydroxyl radicals (OH^-), peroxyl radicals (ROO^-) or nitric oxide, all of which can be either deleterious or beneficial to living systems (Valko et al., 2006). Some beneficial effects of ROS at low-moderate concentrations include defence against infectious agents and induction of a mitogenic response (Valko et al., 2006). On the other hand, a harmful effect causing potential biological damage occurs when there is either overproduction of ROS or a deficiency of antioxidant systems, resulting in inhibition of normal cellular function, which is, in turn, implicated in inflammation, carcinogenesis, ageing, and atherosclerosis (Kawahito et al., 2009; Birben et al., 2012).

T2DM is the most common form of diabetes. Pathophysiology is associated not only with the skeletal muscle and pancreas, but also with other organs including the kidney, liver, adipose tissue, brain, and gastrointestinal tract. Whilst there are several oral agents (e.g., metformin, sulfonylurea, meglitinides, sodium-glucose co-transporter 2 (SGLT-2) inhibitors) and injectable agents (e.g., various analogs of insulin and glucagon-like peptide 1 (GLP-1) receptor agonists (Cornell, 2015)), their effectiveness decreases over time. Therefore, it is imperative not only to understand the underlying mechanism of how glucolipotoxicity contributes to the development of diabetes, but also to develop new treatment strategies aimed at increasing insulin sensitivity or enhancing insulin secretion. This could be accomplished with drugs that can be administered either alone or in combination with available pharmacotherapies for improving comorbidities and disease outcomes.

Carnosine is a naturally occurring histidine-containing dipeptide (HCD) that is formed by bonding of the two amino acids β-alanine and L-histidine. Carnosine is found in several tissues, although most notably in skeletal muscle (Quinn et al., 1992). Many claims have been made of the therapeutic actions of carnosine, for instance, a review

(Artioli, Sale and Jones, 2019), reported that carnosine was observed to have renoprotective and nephroprotective effects, as well as being used as a treatment for neurologic and mental disorders, wound healing and cardiovascular disease. These different diseases all have oxidative stress in their respective pathophysiology, so the benefit of carnosine could extend beyond just the treatment for type 2 diabetes.

This PhD project initially focuses on the impairment of glucose uptake by muscle cells in response to a glucolipotoxicity cellular model of type 2 diabetes. This Chapter investigates whether carnosine supplementation in C2C12, human skeletal myoblasts, and primary muscle cells, could protect these cells against the oxidative stress driven by the reactive species generated from chronic exposure to high glucose and free fatty acid concentrations and thus to identify whether this protection could elicit insulin-sensitising effects or an enhancement of insulin-dependent glucose uptake, using a combination of different spectrophotometric-based techniques including luminescence, fluorescence, and absorbance. This chapter will also address the beneficial action of carnosine upon insulin secretion using INS-1 β-cells.

3.2 Results

3.2.1 Myogenin Expression in Myotubes

Myogenesis or formation of the skeletal muscles involves the proliferation of precursor cells or myoblasts then after morphological, biochemical and molecular modifications they fuse to form multinucleated myotubes, and at this point, these myotubes have then enough capacity to express genes or synthesise proteins. So in all experiments, unless stated otherwise, experimental activities were conducted on myotubes. In order to induce myoblast differentiation, all muscle cells (C2C12, HSkM, and primary mouse) used in this study were cultured in their corresponding differentiation medium (detailed in Chapter 2) containing 2% horse serum for a period of 6 days. As shown in Figure 3.1 A, C, and D, it can be seen that the cells morphologically changed in terms of the alignment, elongation, and fusion of mononucleated myoblasts into multinucleated myotubes. In addition to these morphological changes, a representative experiment with C2C12 by western blotting analysis showed a significantly increased expression of myogenin, a specific differentiation marker (Lee et al., 2011). With this, a 6-day differentiation period for C2C12 and HSkM was chosen in this study to induce the formation of myotubes.

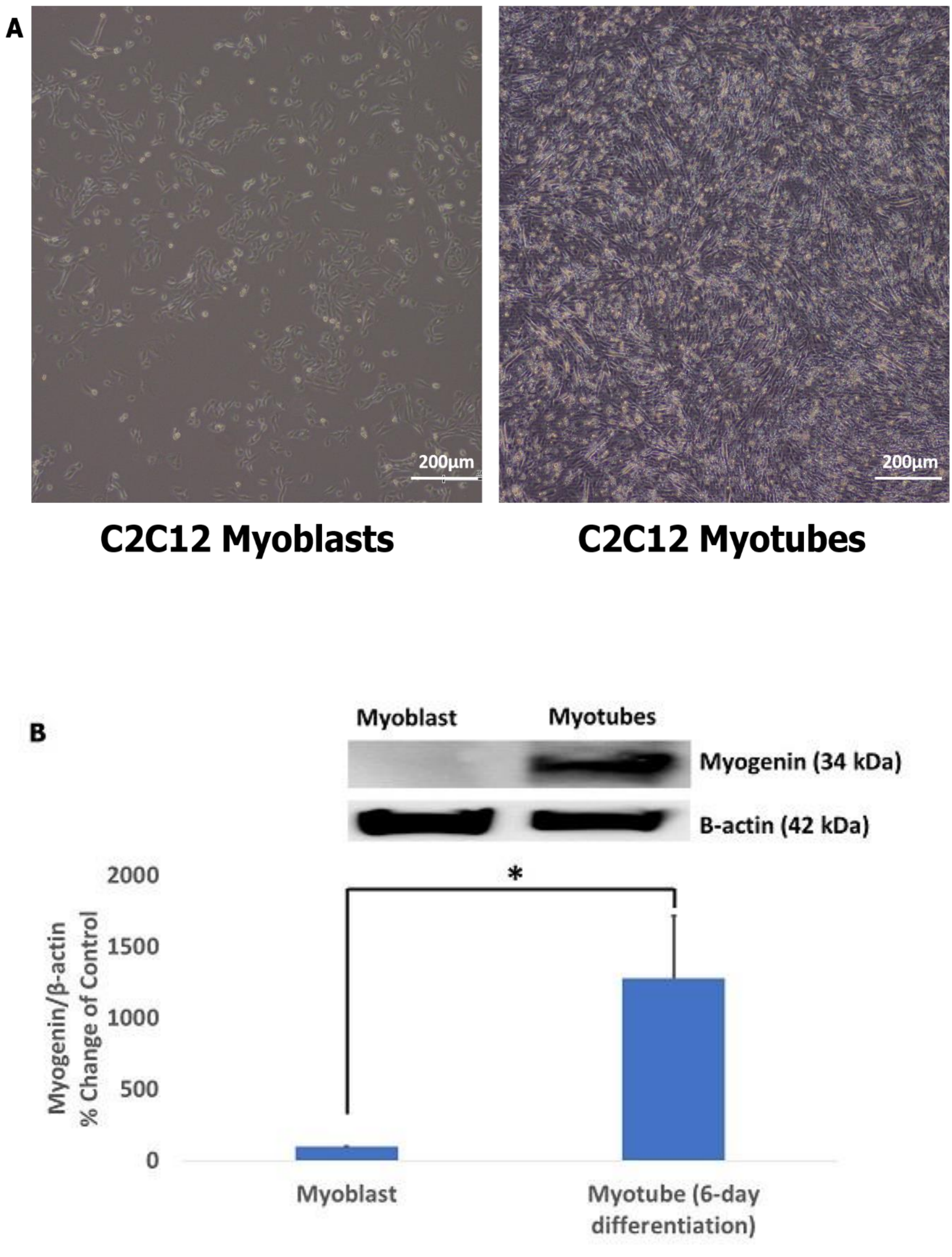

A
200µm
200µm
C2C12 Myoblasts
C2C12 Myotubes
B
Myoblast
Myotubes
Myogenin (34 kDa)
B-actin (42 kDa)
Myogenin/β-actin
% Change of Control
2000
1500
1000
500
0
*
Myoblast
Myotube (6-day differentiation)

Figure 3.1

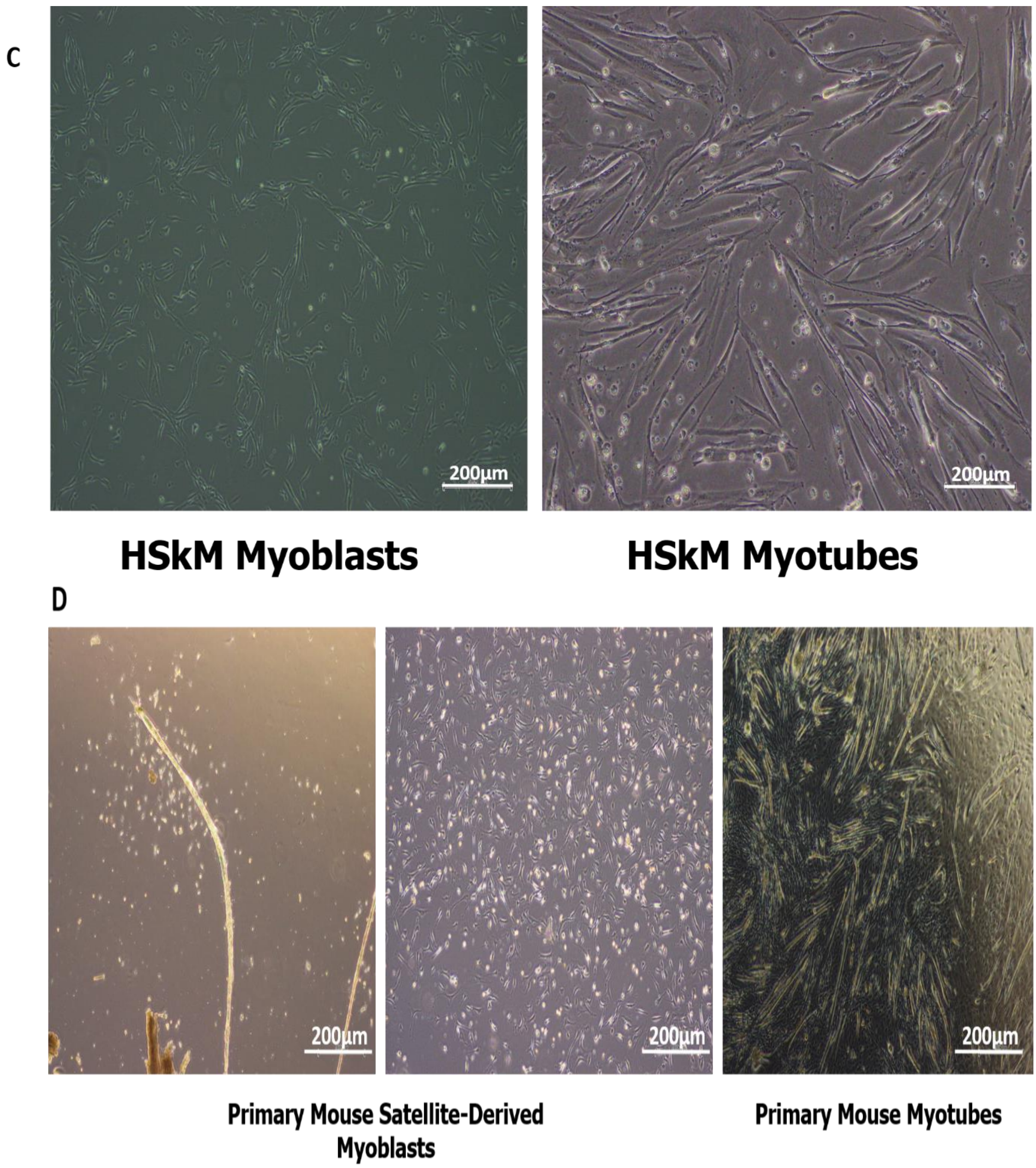


Figure 3.1 (Continuation); 6-day incubation in low-serum media induces the formation of myotubes. (A) C2C12 myoblasts were incubated with 2% horse serum media for 6 days to induce myotube differentiation, and representative photographs are shown. (B) Cell lysates from myoblasts and myotubes were subjected to western blot analysis using antibodies against myogenin. $^{*}p < 0.05$ from 3 independent experiments. (C) HSkM myoblasts and myotubes. (D) Primary mouse satellite cell-derived myoblasts and myotubes

INS-1 cells and muscle cells used in this PhD work were incubated in its respective standard growth media in the absence or presence of 10mM carnosine for 5 days. The choice of this concentration was based on the initial finding in the Turner laboratory group which indicated that between 1 and 10 mM concentrations, the latter was found to be the optimum and more effective amount enough to significantly increase, and more importantly reversed GLT-inhibited insulin secretion. In addition, this value is within the physiological range of carnosine level in the skeletal muscles of many vertebrates (Begum et al., 2005). For instance, in wet human skeletal muscles it could reach up to 20 mM (Hipkiss, 2002), and about 2.25 mmol/kg dm for rat muscles (Naderi et al., 2017).

3.2.2 Effect of Carnosine on Skeletal Muscle Cells

The following data indicated here present the protective effects and beneficial actions of carnosine upon glucose uptake and insulin secretion in skeletal muscle myotubes and pancreatic β-cells, respectively.

3.2.2.1 Scavenging Activity of Carnosine towards Glucolipotoxicity-Mediated Reactive Oxygen, Nitrogen and Carbonyl Species on C2C12 and on Human Skeletal Myotubes

Carnosine has been shown to be effective quencher of reactive and cytotoxic carbonyl compounds (RCS), such as the lipid peroxidation by-product 4-hydroxy-2-nonenal (4-HNE), and this endogenous compound has been used for oxidative stress reduction in different pathologies (Aldini et al., 2012; Bellia et al., 2012; Prokopieva et al., 2015). Therefore, this study sought to investigate the scavenging potential of carnosine towards intracellular reactive oxygen and nitrogen species (RONS), which are postulated to contribute to muscle tissue dysfunction and are, therefore, linked to the negative regulation of the insulin signalling pathway.

In order to determine the level of cellular reactive species generated from each treatment condition, a fluorescence-based assay was employed using a cell-permeant fluorogenic dye called 2′7′-dichlorofluorescein diacetate (DCFDA) which will be deacetylated by cellular esterases and upon the presence of oxidizing species, is converted into 2′, 7′ –dichlorofluorescein (DCF) – a highly fluorescent compound, which can be detected by fluorescence spectroscopy. Briefly, differentiated myoblasts of C2C12 and HSkM were further incubated in control or GLT media (DMEM supplemented to 28 mM glucose, 200μM Palmitic acid, and 200μM Oleic acid) for 5 days with media replaced on the 3rd day, then a final incubation for 1h with or without 10mM carnosine supplemented to fresh experimental condition media. To each condition, 20 μM of DCFDA was then added for 1h; the results are shown in Figure 3.2. GLT media increased the reactive species significantly by 169.6 ± 23.9% compared to control, and importantly this was reduced significantly by 147.5 ± 37.1% with the addition of 10mM carnosine. Also, a 1h dose of 10mM carnosine significantly reduced reactive species in control conditions. A similar quantification procedure was also conducted using human skeletal muscle cell-line (HSkM). As indicated in Figure 3.3, incubation of cells in GLT media also showed a significantly increased level of reactive species to 179.25± 6.88%. Supplementation with 10mM carnosine significantly reduced this level to 123.13 ± 7.44%, with there being no significant difference in the level of cellular reactive species between control conditions with carnosine added.

In this DCFDA assay, the detection of ROS activity or cellular reactive species detection was not normalised to either cell number or protein content, and so it was necessary to investigate the effect of GLT on cell number and viability to ensure that the response shown in GLT was not an artefact of either glucolipotoxic-driven cell death or glucose-driven cell proliferation. Using a cell-permeant dye called Calcein

AM, which can only fluoresce upon the action of intracellular esterases through acetoxymethyl ester hydrolysis, it is possible to determine the cell viability of most eukaryotic cells. All viable eukaryotic cells will have the esterases necessary to drive the hydrolysis reaction forward and the use of Calcein AM is thus an accepted model for cell viability and number measurement (Bratosin et al., 2005). Since there was a non-statistically significant change observed in the cell viability and number of cells exposed to GLT in 5 days with respect to control (Figure 3.4.), it can be inferred that the significant changed detected in the level of reactive species in chronic exposure of GLT conditions were not due to muscle cell viability.

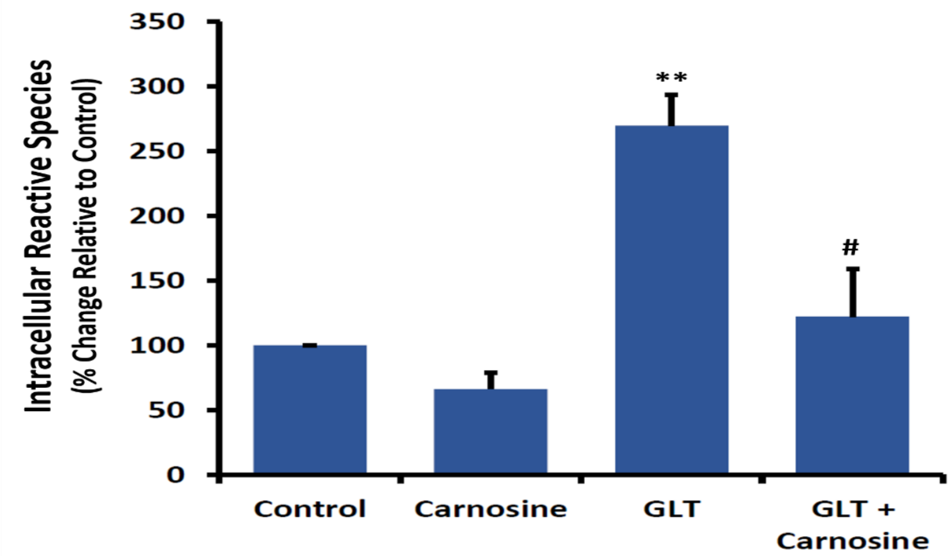


Figure 3.2; **Carnosine effectively scavenges reactive species in C2C12 skeletal muscle cells.** C2C12 myotubes were cultured in control or GLT media for 5 days. Corresponding media were then replaced supplemented ± 10mM carnosine for 1h. A 20μM DCFDA was loaded in KREBS buffer for 1h and reactive species detected via fluorescence with excitation and emission of 495nm and 530nm. Reactive species are expressed as a percentage change in comparison to control from 4 independent experiments ± SEM. (**p<0.01 vs Control, $^{\#}p$<0.05 vs GLT; Tukey's test)

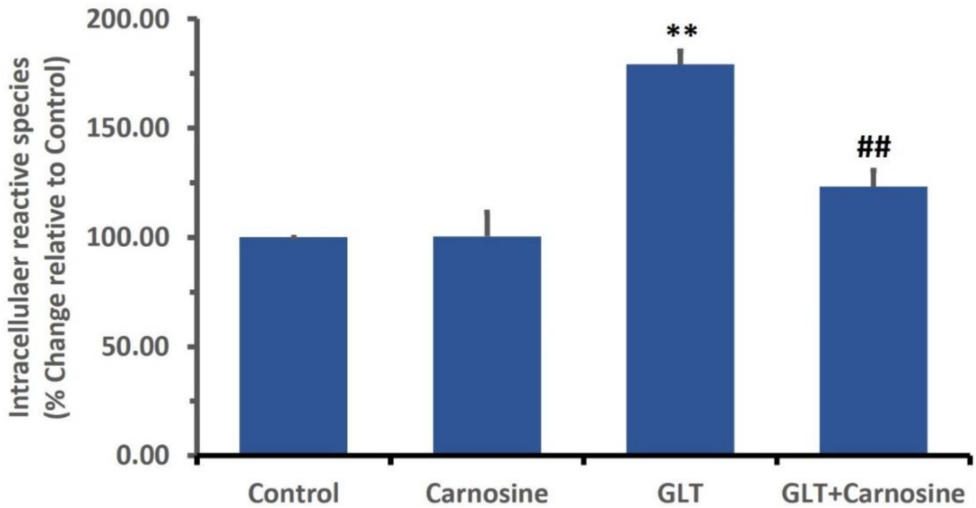


Figure 3.3; **Carnosine effectively scavenges reactive species in human skeletal muscle cells (HSkM).** HSkM myotubes were cultured in control or GLT media for 5 days. Corresponding media were then replaced supplemented ± 10mM carnosine for 1h. A 20µM DCFDA was loaded in KREBS buffer for 1h and reactive species detected via fluorescence with excitation and emission of 495nm and 530nm. Reactive species are expressed as a percentage change in comparison to control from 4 independent experiments ± SEM. (** versus control $p<0.01$; $^{\#\#}$ $p<0.01$ versus GLT; Tukey's test)

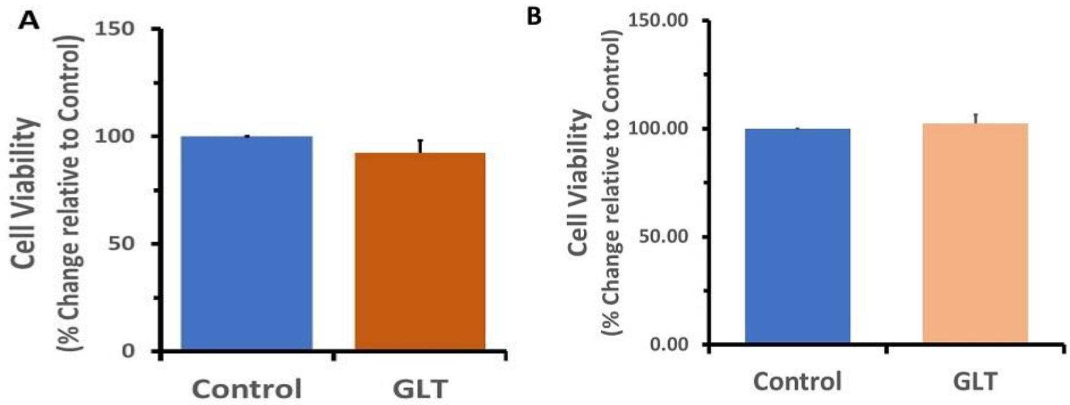


Figure 3.4; GLT treatment does not significantly affect cell viability. (A) C2C12 and (B) HSkM myotubes were cultured in control or GLT media for 5 days. After 1h incubation with 5µM solution of Calcein AM, fluorescence intensity was measured using excitation and emission of 490nm and 520nm. Results shown are expressed as percentage change compared to control from 4 independent experiments ± SEM. ($p>0.05$; t-test)

Increased expression of inducible nitrogen oxide synthase (iNOS) has been implicated in the pathophysiology of inflammatory diseases, including a potential role in insulin resistance. Upregulation of this enzyme can also be inferred with an increased level of reactive nitrogen species (RNS), due to its role in the production of nitric oxide (NO) from the oxidation of L-arginine by using oxygen NADPH electrons (Lowenstein and Padalko, 2004; Soskic, 2011). Therefore, in order to determine whether exposure of C2C12 cells to GLT media could lead to an overexpression of iNOS, and to determine whether carnosine could influence this formation, myotubes were incubated in control or GLT media for 5 days supplemented ±10mM carnosine before lysates were collected from this, and protein contents were then separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were subsequently transferred to nitrocellulose membrane and immunoblotted with an anti-inducible nitric oxide synthase (iNOS) antibody.

Figure 3.5 is an image of the immunoblot analysis to indicate the iNOS band intensity quantified by densitometry and 5-day incubation with GLT shown to cause a 1.97 ± 0.30 fold upregulation in iNOS expression, and that the addition of 10mM carnosine to GLT media showed carnosine is also able to supress the GLT-mediated formation of RNS thus inhibiting the GLT upregulation of iNOS by 78%. From this data, carnosine demonstrates protective effects against the formation of a key enzyme like iNOS which participates in the production of deleterious reactive species.

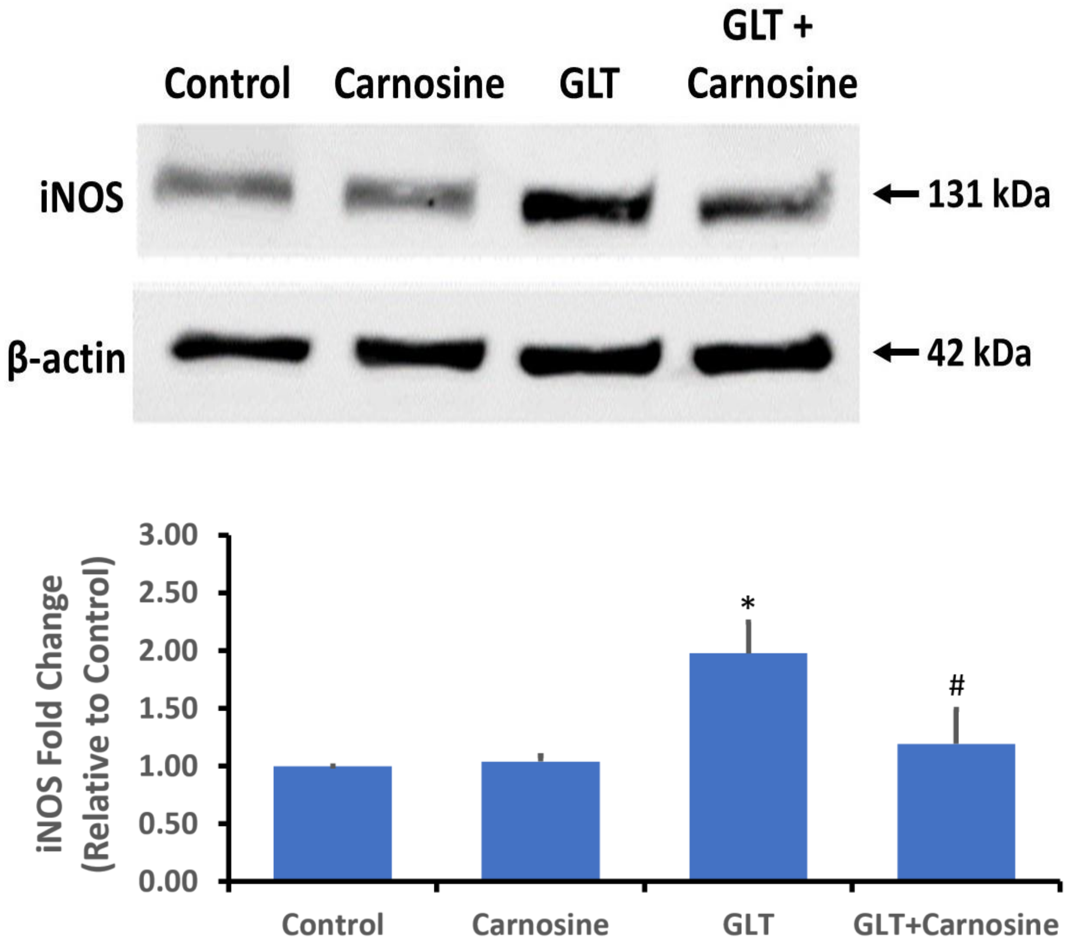


Figure 3.5; **Inducible nitric oxide synthase (iNOS) is upregulated in GLT-exposed cells.** (a) C2C12 myotubes were incubated in control or GLT media supplemented with or without 10mM carnosine for 5 days. Cells were then lysed to extract proteins and then separated via SDS-PAGE, transferred to nitrocellulose and detected using anti-iNOS or anti-actin antibody. Data expressed as mean ± SEM from 3 independent experiments. (* versus control $p<0.05$; $^{\#}p<0.05$ versus GLT; Tukey's test)

A simultaneous flux of nitric oxide and superoxide anion overproduction leads to the formation of a potent oxidant in the biological system called peroxynitrite, which has been implicated in several important diseases, including but not limited to, cancer, neurodegeneration, stroke, inflammatory conditions, cardiovascular problems, and diabetes mellitus (Reiter et al., 2000; Stadler, 2011). The oxidative reaction through nitration of key functional parts in active sites of enzymes, receptors and other proteins by peroxynitrite produces 3-nitrotyrosine. This 3-nitrotyrosine is an established biomarker of cell, tissue, and systemic nitroxidative stress that again resulted from incorporating the nitro group into the tyrosine residue of the protein which caused to modify its functional and structural properties and thus contribute to altered cell and tissue homeostasis (Radi, 2012). This product is indicative of the level of peroxynitrite that is present in the cell and can also be used to assess the degree of reactive nitrogen species generated; the formation of 3-nitrotyrosine can be quantified using ELISA.

In order to determine the level of this marker of cell damage and inflammation, C2C12 and HSkM myotubes were incubated in control or GLT media supplemented with or without 10mM carnosine for 5 days. The sample lysates were then obtained using the extraction buffer supplied in the kit, and the extracts were then analysed according to the protocol. As shown in Figure 3.6, a 5-day treatment of GLT media resulted in a significant increase of 3-NT species formation (40.91 ± 7.73% and 30.34 ± 4.64%) in C2C12 and HSkM cells compared to their respective controls. Importantly, carnosine was able to prevent this 3-NT adduct formation and for C2C12 and HSkM, respectively.

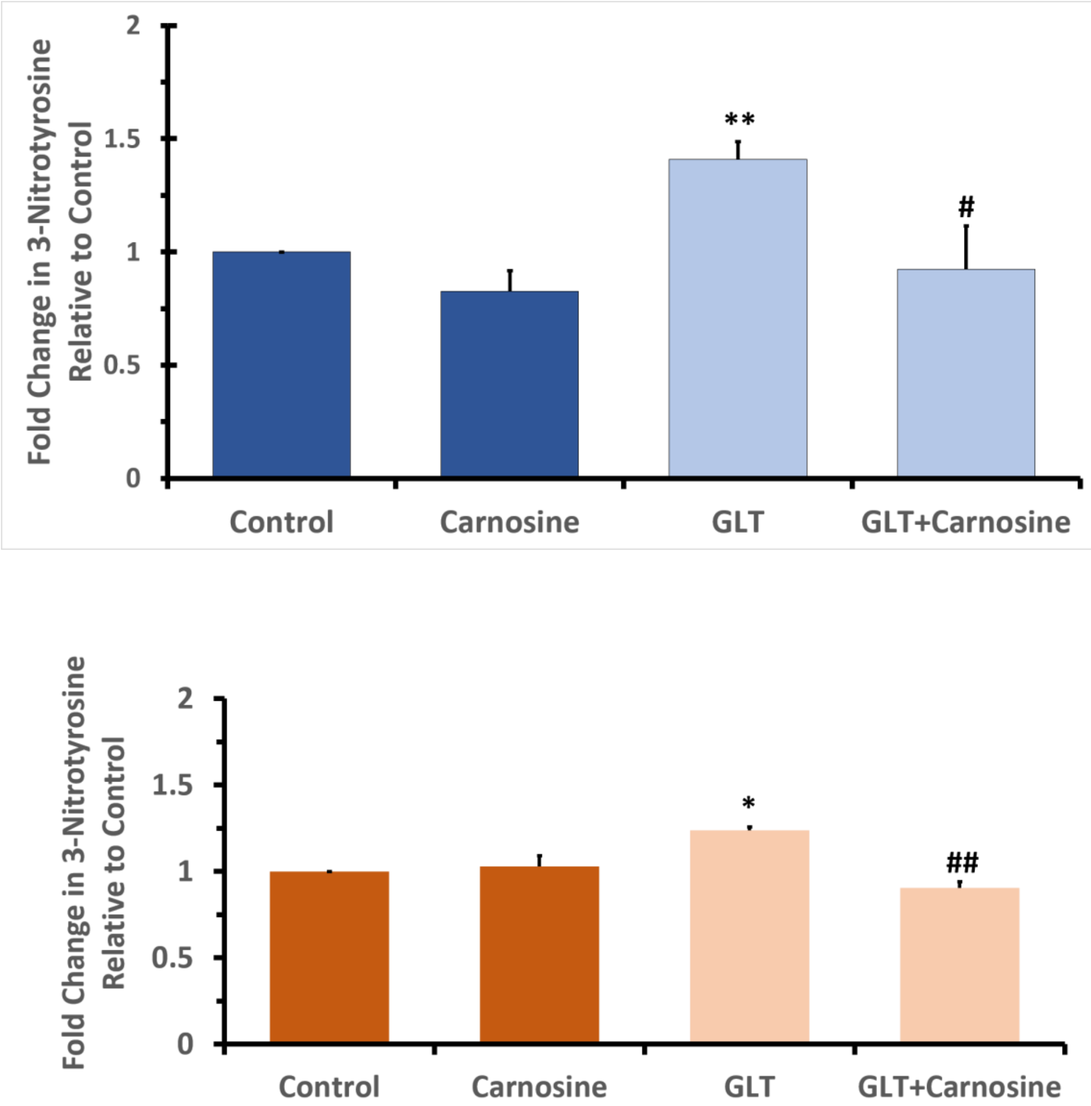
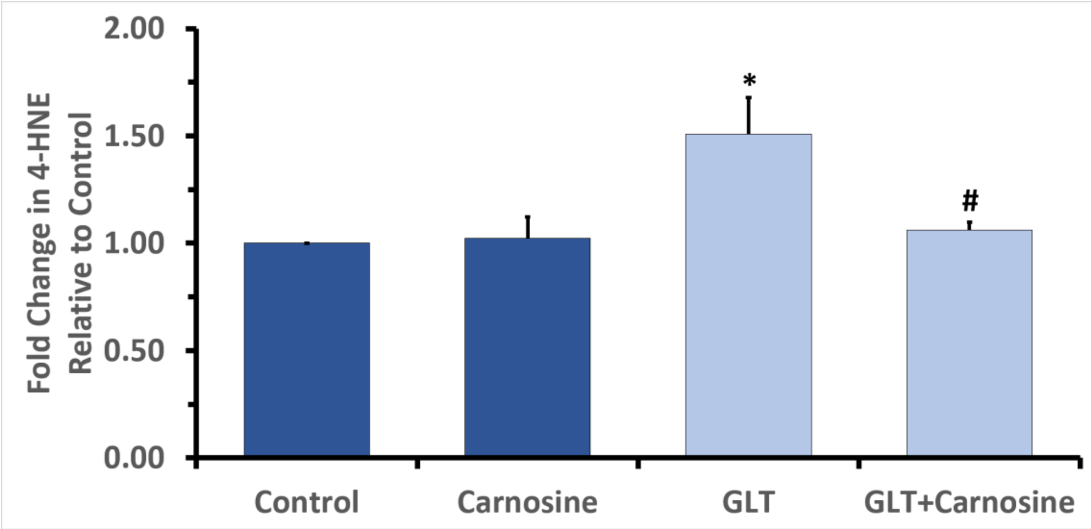


Figure 3.6; Carnosine is an effective antioxidant against nitroxidative stress markers. (A) C2C12 and (B) HSkM myotubes were incubated in control or GLT media supplemented ± 10mM carnosine for 5 days. Cells lysates were then determined for 3-NT using ELISA with absorbance measured at 450nm. Concentrations detected were normalised to protein content. Results are expressed as fold change compared to control from 3 independent experiments ± SEM. (*p<0.05 **p<0.01 vs Control, # p< 0.05 ## p<0.01 vs GLT; Tukey's test).

A consequence of established oxidative stress is formation of reactive carbonyl species or aldehydes, including 4-hydroxynonenal (4-HNE), which is formed through enhanced ROS-induced lipid peroxidation (Yang et al., 2003). Excessive levels of 4-HNE are believed to induce insulin resistance and desensitisation of insulin signaling pathways, and has been associated with metabolic defects present in obesity (Pillon et al., 2007; Ingram et al., 2012). Similarly, the present study then sought to determine the effect of GLT exposure of muscle myotubes on 4-HNE species generation. After C2C12 and HSkM myotubes were incubated in control or GLT media in the presence or absence of 10mM carnosine treatment, sample lysates were collected using RIPA buffer and immediately assayed for 4-HNE using ELISA kits. Indicated in Figure 3.7, an increase of (50.77 ± 17.02%) and (38.56 ± 6.38%) in 4-HNE were detected in GLT-treated cells of C2C12 and HSkM, respectively as compared to their control. However, in the presence of carnosine supplementation at 10mM concentration, a significant amount of this generated oxidant molecule was being significantly neutralised by this dipeptide in both types of myotubes used.



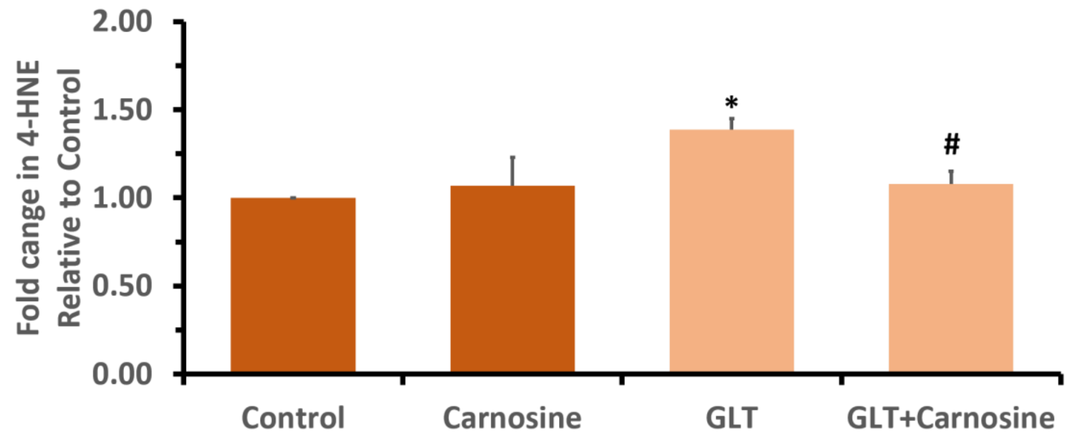


Figure 3.7; Carnosine is effective in sequestering reactive aldehyde (4-HNE) generated in GLT-exposed muscle cells. (A) C2C12 and (B) HSkM myotubes were incubated in control or GLT media supplemented ± 10mM carnosine for 5 days. Cell lysates were then determined for 4-HNE using ELISA. Concentrations detected were normalised to protein content. Results are expressed as fold change compared to control from 3 independent experiments ± SEM. (* versus control p<0.05; # p<0.05 versus GLT; Tukey's test)

3.2.2.2 Carnosine Improves Glucose-Uptake in Glucolipotoxicity-induced insulin-resistant C2C12 Myotubes, Primary Mouse and Human Skeletal Myotubes

As shown earlier, carnosine has a protective effect on muscle cells against GLT-mediated reactive species. Therefore, it was next sought to determine whether this, as a result, could have a beneficial action upon glucose uptake. In order to answer this, a luminescence-based glucose uptake assay was conducted. C2C12 myotubes were incubated either in control or GLT media for 5 days. After cells were serum-starved overnight with low-glucose DMEM, cells were then incubated for 1-hr in glucose-free DMEM ±100 nM insulin. Medium was then replaced with PBS + 2-deoxyglucose (2-DG), followed by an uptake reaction for 30-minutes. Glucose uptake was then measured based on 2-deoxyglucose-6-phosphate using a luminometer.

As shown in Figure 3.8, GLT- exposed cells have reduced capacity to uptake glucose both in basal and in insulin-stimulated conditions. For instance, GLT significantly reduced uptake by 36.89 ± 5.91% and 74.19 ± 7.37% as compared to control basal and insulin-stimulated glucose uptake, respectively. Importantly, carnosine scavenging the glucolipotoxic reactive species resulted in a significant enhancement of glucose uptake by 44.14 ± 12.79% in the stimulated condition. This finding is not due to significantly altered C2C12 cell viability, as in C2C12 myotubes treated with GLT media for 5 days, cell viability was found to be 93.67+/−3.61%. Therefore, carnosine scavenging is likely to exert a beneficial action on glucose homeostasis through enhanced skeletal muscle glucose uptake.

In order to validate the findings obtained through C2C12 *in vitro* experiments, it was essential to conduct similar studies using primary mouse muscle cells (isolated by Dr. Craig Doig, NTU), and a human skeletal muscle cell line (with passage not later than 6), employing the same treatment conditions and methodology. Results obtained in these experiments (Figure 3.9) showed a similar response as found previously with C2C12. When comparing all values to control basal, a significant reduction of glucose uptake was shown from 170.30% to 102.20% under insulin- stimulated conditions, and importantly was enhanced when supplemented with 10mM carnosine by about 57%. In the case of using primary muscle cells from mouse as shown in Figure 3.10, only 20.72 ± 7.16 % of glucose uptake compared to control basal was observed in GLT-treated cells under insulin-stimulated conditions. In the presence of 10mM carnosine, this resulted to 77.93%.

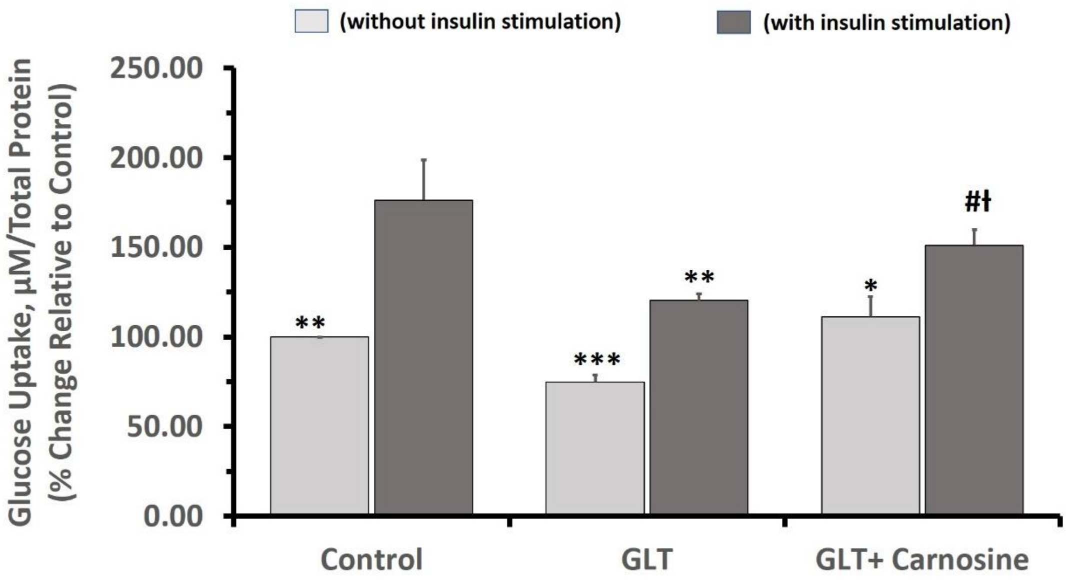


Figure 3.8; Carnosine enhances glucose uptake in GLT-exposed C2C12 muscle cells. C2C12 myotubes were cultured in DMEM media, or DMEM GLT media for 5 days ± 10mM Carnosine. Cells were serum-starved overnight in DMEM supplemented with 5 mM glucose, then incubated for 1 h in glucose-free DMEM +/−100nM insulin. Medium was replaced with PBS + 0.125 mM 2-deoxy glucose (2-DG). Glucose uptake reactions were conducted for 30 min. 2DG6P was detected using a luminometer. Data are expressed as means ± SEM of 3 or more independent experiments. (*$p < 0.05$,**$p<0.005$,***$p<0.001$ vs Control with insulin stimulation; $^{\#}p<0.05$ vs GLT with stimulation, $^{ŧ}p<0.01$ vs GLT without stimulation; Tukey’s test)

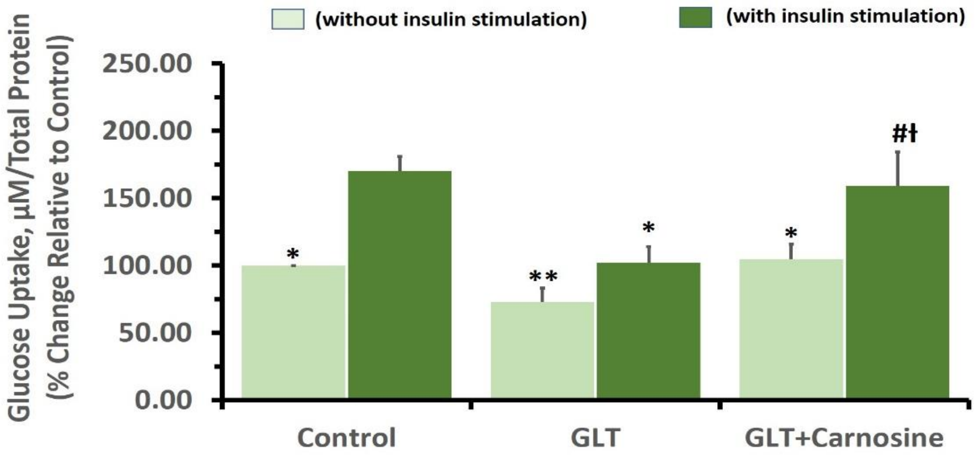


Figure 3.9; Carnosine enhances glucose uptake in GLT-exposed human skeletal muscle cells. Human skeletal myotubes (HSkM) were cultured in DMEM-F12 media, or DMEM-F12 GLT media (17mM glucose, 200 µM Palmitic acid, 200 µM Oleic acid) for 5 days ± 10mM Carnosine. Cells were serum-starved overnight in DMEM supplemented with 5 mM glucose, then incubated for 1 h in glucose-free DMEM +/− 1µM insulin. Medium was replaced with PBS + 0.150 mM 2-deoxy glucose (2-DG). Glucose uptake reactions were conducted for 30 min. 2DG6P was detected using a luminometer. Data are expressed as means ± SEM of 3 independent experiments. (*$p < 0.05$,**$p<0.005$, vs Control with insulin stimulation; $^{\#}p<0.05$ vs GLT with stimulation, $^{ƚ}p<0.01$ vs GLT without stimulation; Tukey's test)

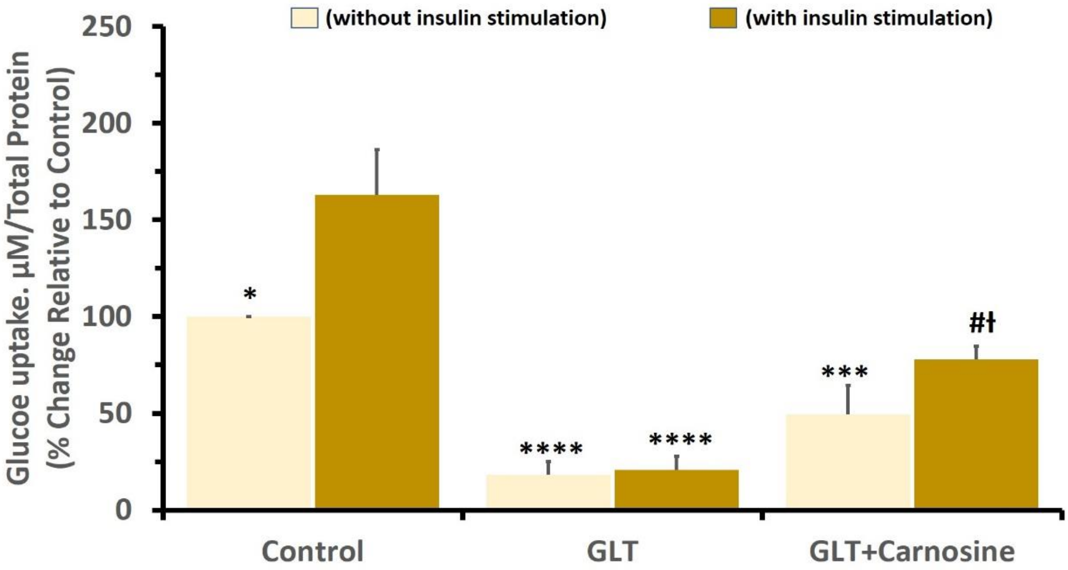


Figure 3.10; Carnosine improves glucose uptake in GLT-treated muscle cells isolated from mice. Primary mouse myotubes were cultured in DMEM media, or DMEM GLT media (28mM glucose, 200 µM Palmitic acid, 200 µM Oleic acid) for 5 days ± 10mM Carnosine. Cells were serum-starved overnight in DMEM supplemented with 5 mM glucose, then incubated for 1 h in glucose-free

DMEM +/− 100nM insulin. Medium was replaced with PBS + 0.150 mM 2-deoxy glucose (2-DG). Glucose uptake reactions were conducted for 30 min. 2DG6P was detected using a luminometer. Data are expressed as means ± SEM of 3 independent experiments (*$p < 0.05$,***$p<0.005$, **** $p<0.0001$ vs Control with insulin stimulation; #$p<0.05$ vs GLT with stimulation, ǂ$p<0.05$ vs GLT without stimulation; Tukey's test)

3.2.3 Effects of Glucolipotoxicty and Carnosine on GLUT4 Translocation in C2C12 myotubes

Glucose is an important fuel for the body and therefore normal regulation of its concentration in the blood is vital for the health. Insulin released by the pancreas stimulates uptake of glucose by insulin-sensitive tissues such as skeletal muscle, and so T2DM and/or obese patients who often develop insulin resistance are unable to control their glucose homeostasis (Koshy et al., 2010). In the body, there are 13 known members of glucose transporters (GLUT) that are distributed in different tissues, and glucose transporter type 4 (GLUT4) is the major transporter in skeletal muscles that upon its translocation from intracellular storage depots to the plasma membrane, facilitates the diffusion of glucose entry into the cell (Zao and Keating, 2007; Richter et al., 2013). Reduced glucose transport activity is believed to be one of the causes of insulin resistance in type 2 diabetes (Graham et al., 2007).

Pedersen et al., (1990) and Garvey et al. (1998) reported that the levels of GLUT4 in the skeletal muscles of obese, type 2 diabetic patients, individuals with gestational diabetes, individuals with impaired glucose tolerance, and those with insulin-resistant states were normal. Although both studies above have established data for this, this Ph.D. work also sought to determine whether the GLT model of T2DM would have influenced the level of GLUT4. In order to do this, immunoblot analysis on GLUT4 expression was conducted on C2C12 myotubes incubated in control or GLT media.

As shown in Figure 3.11, there was no significant difference in GLUT4 levels between control and GLT conditions.

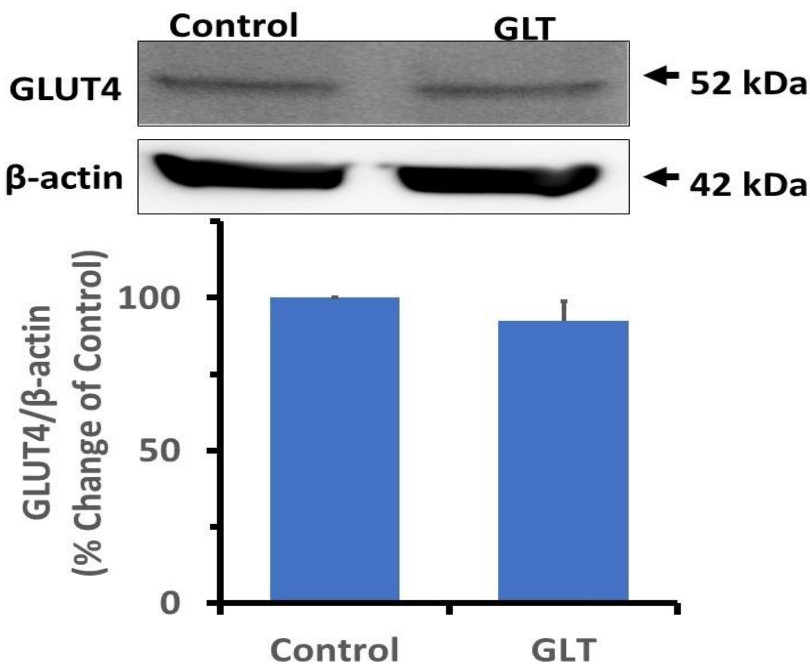
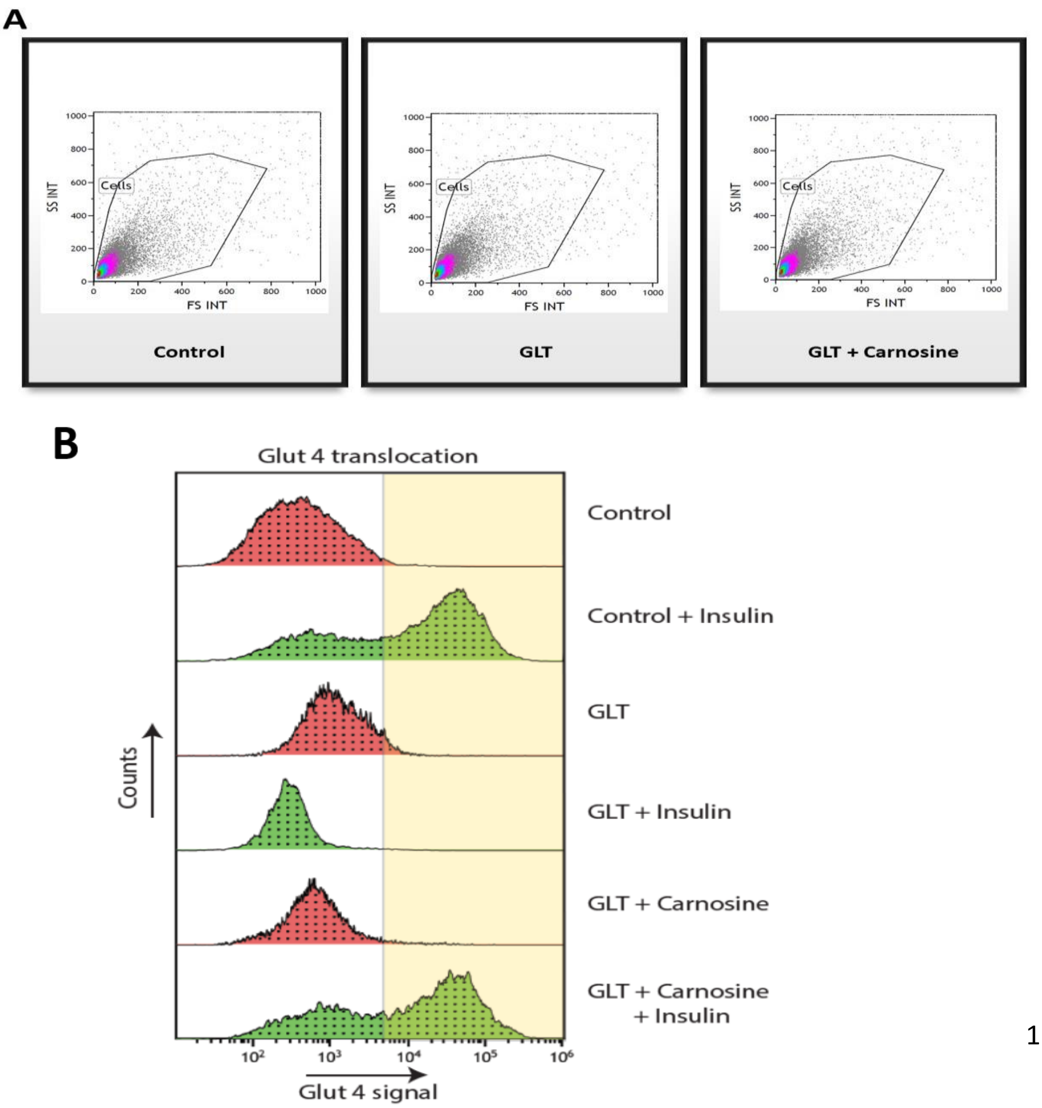


Figure 3.11; Level of glucose transporter (GLUT4) expression between normal and GLT-treated muscle cells shows no significant difference. C2C12 myotubes were incubated in control or GLT media supplemented with or without 10mM carnosine for 5 days. Cells were then lysed to extract proteins, and then separated via SDS-PAGE, transferred to nitrocellulose and detected using anti-iNOS or anti-actin antibody. Data expressed as mean ± SEM from 4 independent experiments. (p>0.05; t-test)

In order to determine whether glucolipotoxicity could lead to defective GLUT4 translocation, and, if so, to investigate whether or not carnosine could provide a protective effect against this. In collaboration with Dr. Gemma Foulds (NTU), GLUT4 translocation was quantified using flow cytometry. This utilised a primary anti-GLUT4 antibody directed to an external epitope of the transporter that binds to it as soon as it is exposed after being translocated and a corresponding secondary antibody conjugated to AlexaFluor 488. Although GLUT4 is endocytosed due to it only being transiently expressed at the plasma membrane of the cell, the fluorescence signal measured still accounts for the total amount of GLUT4 exposed at the surface of the cell in the presence of insulin at particular incubation time because the bound antibodies have remained to be attached to the GLUT4 even during the endocytosis (Koshy et al., 2007).

Figure 3.12 indicates that under the normal condition there is a significant increase of translocation upon insulin stimulation (100nM) relative to its basal. However, the transport activity of GLUT4 was found to be significantly reduced by 3.27 ±0.67 fold upon exposure of cells to glucolipotoxicity, indicating the potential negative regulation of GLT upon insulin signalling. Importantly, supplementation of 10mM carnosine of this GLT-treated cells showed a significant improvement of GLUT4 translocation by 2.15 ±0.80 fold.



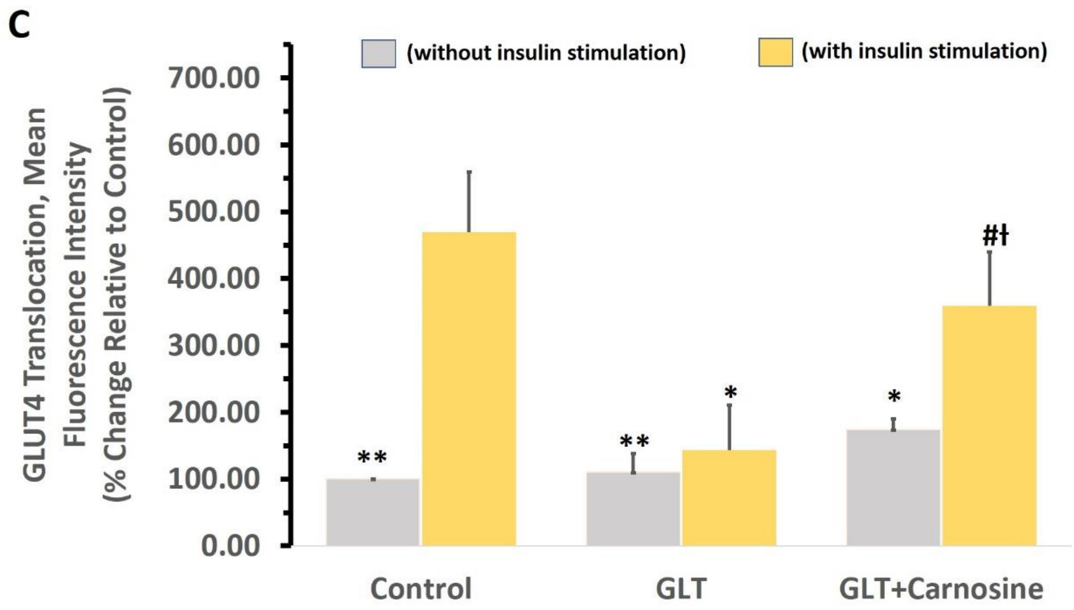


Figure 3.12; Translocation of GLUT4 is impaired in GLT-treated C2C12 muscle cells and enhanced by carnosine supplementation. (A) Representative typical dot plots of side scatter intensity (SC INT) versus forward scatter intensity (FS INT) with a gate around the live cells. (B) Representative images for cells staining intensity with the antibody against GLUT4. (C) Average fluorescence intensity of each indicated condition normalised to control unstimulated. Myotubes were incubated in control or GLT media supplemented ± 10mM carnosine for 5 days. Cells were serum-starved overnight in DMEM supplemented with 5 mM glucose, then simultaneously stimulated with insulin and stained using the prepared antibody mix (primary anti-GLUT4 antibody and Goat Anti-Rabbit IgG H&L conjugated to Alexa Fluor 488) for 30 minutes, fixed in 1% PFA for 20 minutes and data acquisition using flow cytometry. Data are expressed as means ± SEM of 3 independent experiments. (*$p < 0.05$,**$p<0.01$, vs Control with insulin stimulation; $^{\#}p<0.05$ vs GLT with stimulation, $^{ƚ}p<0.05$ vs GLT without stimulation; Tukey's test)

3.2.4 Effects of Carnosine on Pancreatic β-cells

Pancreatic β-cells are also very important in regulating glucose concentrations in the blood and altered function and viability of these insulin-producing cells underlies type 2 diabetes pathophysiology. Oxidative stress is believed to be a major cause of pancreatic islet death, and compared to other tissues, β-cells have low levels of antioxidant enzymes. Due to their lack of a robust antioxidant system, beta cells are therefore more susceptible to oxidative damage (Miceli et al., 2018; Harmon et al., 2010). In collaboration with a colleague in the Turner laboratory (Michael Cripps), a parallel activity was thus conducted in β-cells in order to determine the effect of glucolipotoxicity on cell viability, generation of intracellular reactive oxygen and nitrogen species, and finally on insulin secretion. At the same time, we also determined the potential protective effects of carnosine against GLT-driven oxidative stress.

INS-1 cells were incubated in control or GLT media for 5 days, then replaced with fresh experimental condition media supplemented with or without 10mM carnosine for 1 hour. Similarly, the amount of intracellular reactive species was determined using DCFDA. As shown in Appendix 1, (obtained and produced by Cripps), GLT-exposed cells were shown to have significantly increased reactive species to 180.4 ± 8.2% normalised to control, whilst addition for 1h with 10mM carnosine did reverse the upregulation back to within control limits (77.5 ± 19.7%). Again, the observed increase in reactive species by chronic GLT exposure conditions were not associated with the glucolipotoxic changes in β-cell viability as INS-1 cells treated for 5 days in GLT media showed only a small and non-statistically significant increase in viability (data not shown).

Carnosine significantly protected β-cells from deleterious adduction resulting from GLT-mediated generation of reactive nitrogen and carbonyl species - namely iNOS, 3-nitrotyrosine, and 4-hydroxynenal. The data for these have been published in our paper (Cripps et al., 2017) and reported in Michael Cripps' PhD thesis.

As carnosine has been shown to be effective at scavenging these reactive species and can inhibit potentially harmful adduct formations, it was also determined whether chronic treatment with carnosine could lead to an improved β-cell function particularly on insulin secretion. INS-1 cells were incubated in standard growth media in the absence or presence of 10mM carnosine for 5 days. After a 5-day period of incubation in each condition, cells were washed and incubated in either KREBS or a secretagogue cocktail (containing 1mM Tolbutamide, 10mM Leucine, 10mM Glutamine, 1mM 3-isobutyl-1-methylxanthine (IBMX), 1µM phorbol 12-myristate 13-acetate (PMA) and 10mM glucose) for 2h. The amount of insulin produced in each treatment condition was quantified using an ELISA and values were normalised to protein content. As shown in Appendix 2, GLT significantly reduced insulin stimulated secretion by 63.1 ± 0.4%. The addition of 10mM carnosine fully reversed the GLT-mediated loss of insulin production. Data obtained by the Turner group indicates that carnosine is not only able to enhance insulin secretion, but it can reverse and inhibit the damaging effects to beta cells that have resulted from high-sugar and high-fatty acid exposure.

3.3 Discussion

There is growing evidence to indicate that oxidative stress (OS) is a common denominator for the pathogenesis of several diseases, including cancer, diabetes, obesity, neurodegenerative disorders, among others (Son, 2012; Devi, 2015; Reuter, 2010). OS dysregulates cell metabolism and cell-cell homeostasis and plays an important role in the pathogenesis of the two most relevant aspects of T2DM, namely insulin resistance and β-cell dysfunction (Pitocco et al., 2013; Poitout et al., 2008). OS ensues when oxidant production in the living system exceeds that of the cell's antioxidant machinery, in other words, a disease results when there is an imbalance of the redox system of the cell.

When production of both ROS and RNS are carefully regulated, they participate and have functional effects in normal physiology such as hormone action, immune response, cell growth, and cell adhesion. By contrast, ROS and RNS can also become toxic agents and thereby participate in pathophysiological processes, causing irreversible modifications via inducing damage to cellular components (lipids, DNA, carbohydrates) and thereby altering their normal function (Chiarugi et al., 2003; Yang et al., 2013; Sisein, 2014).

There are two specific reactive species focused on this study, namely 3-nitrotyrosine (3-NT) and 4-hydroxynonenal (4-HNE). The former is a useful biomarker of peroxynitrite-driven OS and has been implicated in diabetic neuropathy and nephropathy (Thuraisingham, 2000; El-Remessy et al., 2003). Data presented in this work showed that glucolipotoxicity mediated the excessive generation of reactive oxygen and nitrogen species which in turn generated 3-NT protein adducts. The presence of 3-NT suggests that peroxynitrite is just one of the reactive species in GLT-exposed cells. On the other hand, 4-HNE, an α-β-unsaturated alkenal, is a reactive carbonyl specie that could easily react with the nucleophilic sites of proteins such as Lys, His, and Cys, and DNA, thereby causing cellular dysfunction, is the reactive carbonyl species (RCS). HNE is a product of oxidative stress and is most likely produced following peroxidation of intracellular lipids. This work has also shown that GLT leads to damaging 4-HNE adduct formation.

HNE is the most intensively investigated and quantitatively most important product of lipid peroxidation due to its highly cytotoxic role in inhibiting gene expression. It also enhances the development and progression of several pathological states, including diabetes, Alzheimer's diseases, cancer, cardiovascular diseases, liver diseases, and Parkinson's disease (Ayala, Muńoz and Arguelles, 2014).

There are three HNE metabolising enzymes namely, glutathione-S-transferase (GST), alcohol dehydrogenases (ADH) and aldehyde dehydrogenases (ALDH), (Castro, et al., 2017) however these antioxidant defence systems are reduced in liver microsomes and mitochondria in diabetic conditions. Therefore, it has been suggested that hyperglycaemia and excessive ROS generation could lead to HNE accumulation, or deficiency of its removal, and thus produce a cycle amplifying the damage (Traverso et al., 2002). As such, this lipid peroxidation product is very important in tackling T2DM, particularly in those organs involved in glucose homeostasis, because for instance pancreatic islets have one of the least antioxidant defence systems and are hence very susceptible to oxidative damage. At the same time intracellular glutathione pools in insulin-resistant states are depleted, with negative effects on the skeletal muscle insulin sensitivity and decreased insulin-induced glucose uptake (Pillon et al., 2012).

Nitric oxide synthases (NOS) are enzymes involved in reactions generating reactive oxygen or nitrogen species called nitric oxide (NO). The most important isoform of

NOS is the inducible nitric oxide synthase (iNOS) which has an important role in the regulation of insulin resistance (Soskic et al., 2011). iNOS was reported to have a link with insulin resistance, and upregulation of this was also observed with several inducers of insulin resistance (IR) including obesity, hyperglycemia, oxidative stress, and tumor necrosis factor-α. It is also elevated in skeletal muscles of type 2 diabetic patients (Ceriello et al., 2002; Tannous et al., 1995; Torres et al, 2004). The Turner group previously reported that INS-1 β-cells incubated in GLT media for 5 days have significant fold upregulation in iNOS expression (Cripps et al., 2017), and together with what has been indicated earlier (Figure 3.5), this clearly shows the potential role of GLT-driven increased expression on T2DM.

The damaging effects of glucolipotoxicity and its role towards the pathogenesis of T2DM (β-cell dysfunction and insulin resistance) can be ascribed from the ability to initiate and mediate pathways leading to mitochondrial stress, generation of reactive species, proinflammatory cytokines, and altered gene expression (Bagnati et al., 2016; Akash et al., 2018; Van Raalte et al., 2011). These pathways are most likely responsible for the defective glucose regulatory mechanism (insulin secretion and insulin resistance) observed in T2DM individuals. The Turner group initially generated data to indicate that the cellular model condition employed in this project could both initiate islet cell inflammation and pancreatic β-cell death (Bagnati et al., 2016), as well as inhibit insulin secretion (Marshall et al, 2007) and glucose uptake (Cripps et al., 2017). The GLT condition employed in this project uses a combined high-glucose, palmitic acid, and oleic acid supplementation to the cell culture media. The choice of these fatty acids and the corresponding concentrations are based on consultation and collaboration with diabetologists. In addition, these two fatty acids were also found to be most abundant in the human diet (Orsavova et al., 2015).

In this work it has been demonstrated that excessive production of reactive species mediated by glucolipotoxicity has deleterious effects on the pancreas and skeletal muscle cells. For instance, in the skeletal muscles, the GLT-induced OS might have contributed to the impairment of the main signalling pathways involved in insulin action such as the IR-IRS-PI3K-Akt axis. As a consequence, these pathways could not collectively function properly to translate the signal generated from the interaction between insulin and its receptor into relevant physiological actions, including stimulation of GLUT4 for glucose transport and utilization this for other biologically important macromolecules in target tissues. As mentioned somewhere in this thesis, β-cells have low level of antioxidant enzyme expression. With this, it is tempting to speculate that these cells are at greater risk for oxidative damage or are easy target for ROS. Exposure of β-cells to GLT, might have inhibited the ATP-dependent cascade of events responsible for insulin secretion, release, and action probably through altered mitochondrial shape, volume, and function. Other possible reason for the decreased in insulin secretion by GLT is that there should be activated proinflammatory pathways (e.g. JNK) that might have suppressed insulin gene expression. Therefore, the ability of oxidative stress to damage mitochondria and markedly blunt insulin secretion is not surprising.

Given the implication of oxidative stress in the onset of the disease, it is possible that antioxidant strategies would be effective in the prevention or treatment of diabetes – as these are molecules that have the ability to scavenge, quench or even neutralise these excess reactive species, thereby preventing cellular damage and preserving function. This work has shown that a natural dipeptide, carnosine, has the ability to offset the negative effects these reactive species have upon β-cells and muscle cells, and by so doing offered significant improvement respectively on insulin secretion and glucose uptake by these cells exposed to glucolipotoxic conditions.

The antioxidant and scavenging action of carnosine towards radicals and reactive species could potentially be attributed to multiple possible actions. One is its buffering capacity not only for protons but also for regulating the level of mixed-valence metal ions (copper, cobalt, manganese, iron, and cadmium) that take an active part in many metabolic processes activating free-radical processes. Another one is its antiglycation or anti-crosslinking properties which could block oxidative damage of biomolecules (Prokopieva et al., 2015). In the case of RNS and ROS, carnosine could form a charge-transfer complex (e.g. superoxide radical and hydroxyl radical). This reaction yields a stable intermediate or unreactive molecule (Boldyrev et al., 2013). In the case of RCS like 4-HNE, the imidazole ring of L-histidine and the amino group of β-alanyl in carnosine act through a synergistic way in trapping this cytotoxic aldehyde. In other words, carnosine has been suggested to react with HNE in biological systems through a sacrificial mechanism in lieu of the target substrate by acting like the preferable site of addition by HNE (Liu, Xu and Sayre, 2003). The functional properties of carnosine are associated to its L-histidine component particularly through its imidazole moiety, however histidine-containing dipeptide like carnosine still offers more advantages compared to free L-histidine. For instance, it has been observed that that carnosine could sequester HNE 10 times more and could react two- to fourfold faster with ROS than free L-histidine whilst β-alanine was totally inactive (Boldyrev et al., 2013; Aldini et al, 2002).

Based on the findings above, the next step being sought was to investigate the role of reactive species further by identifying specific proteins that form adducts with 4-HNE and 3-NT, and to evaluate the extent to which carnosine intervention could offer prevention from GLT-mediated adduction. In addition, it was also necessary to investigate what impact glucolipotoxicity has upon muscle mitochondrial bioenergetics, and to identify whether carnosine could offer protective effects on mitochondrial function. These research questions formed the basis for work in Chapter 4.

In conclusion, data presented here indicate that exposure of muscle cells and β-cells to persistently elevated levels of glucose and fatty acids will have negative effects on glucose homeostasis by dysregulating pathways associated with insulin signalling, as evidenced by impaired translocation of glucose transporter (GLUT4) and decreased glucose uptake, and decreased secretion of insulin. Importantly, treatment with carnosine showed protective effects against GLT-mediated induction of reactive species (RNS, ROS, RCS) that are believed to be responsible for impaired cellular function. The scavenging action and the use of carnosine as a blocking agent against those deleterious species could therefore potentially offer a novel treatment and therapeutic perspectives for T2DM patients.

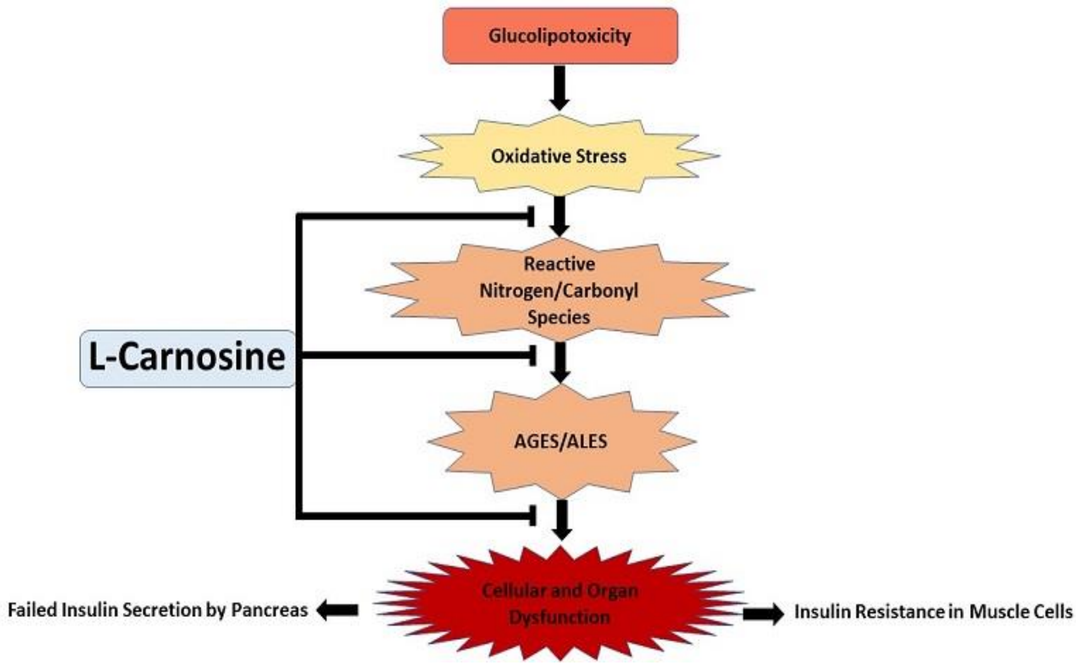


Figure 3.13; A schematic representation of how carnosine is beneficial against the oxidative stress cascade mediated by glucolipotoxicity in two key tissues involved in glucose homeostasis, and hence protective by directly quenching these reactive species.

Chapter 4:

Carnosine prevents damaging protein adduction and preserves mitochondrial function in cells under metabolic stress

4.1 Introduction

The accumulation of excess ROS as a result of oxidant production, when higher than the available antioxidant defences in the cell, can induce damage to biological molecules, such as unsaturated fatty acids in membranes, thiol groups in proteins and nucleic acids in DNA (Valko et al., 2007). Therefore, oxidative stress may lead to the development and progression of various chronic diseases. One particular group of compounds that have a crucial role in the progression of metabolic disorders, such as diabetes, are the reactive carbonyl species – products that result from the oxidation of polyunsaturated fatty acids and sugars (Hwang et al., 2016). The electrophilic nature of these carbonyl compounds allows them to favourably react with the nucleophilic regions of amino acids, such as lysine, histidine, and cysteine. This, in turn, leads to the formation of protein adducts that can cause irreversible cellular dysfunction (Dalle-Donne et al., 2006).

The focus of this chapter includes one of the most abundant and toxic reactive carbonyl species, 4-hydroxynonenal (4-HNE). 4-HNE is believed to be an indicator of oxidative stress and could easily form covalent adducts with nucleophilic side chains of proteins such as the thiol group of cysteine, the lysine ε-amino group, and the imidazole ring of histidine. When this happens, this could potentially cause the protein to undergo a conformational change, thereby generating a distorted catalytic site and impaired function. Consequently, 4-HNE is a leading contributory agent to the development of several diseases, including diabetes (Carini et al., 2004). As part of this PhD project, it was therefore sought to identify those proteins that both interact and form adducts with 4-HNE in cells, tissue and serum samples under metabolic stress.

In addition to 4-HNE, another biomarker of oxidative stress that is also capable of forming damaging adducts with protein is 3-nitrotyrosine. A combination of superoxide radical anion ($O_2^{\cdot -}$) and nitrogen monoxide (NO·) drives the formation of 3-nitrotyrosine through generation of the intermediate, peroxynitrite. In addition to this, the peroxidase enzyme-catalysed reaction using hydrogen peroxide and nitrite is another possible route for the formation of 3-NT (Radi, 2013). L-tyrosine and protein-bound tyrosine are prone for attack by reactive nitrogen species (RNS), including peroxynitrite, and form either free or protein-3-NT adducts. Thus, the formation of nitrotyrosine and the detection of this molecule in proteins may not only signify RNS-mediated protein modifications, but could also be an important indicator of endogenous peroxynitrite activity, which can lead to the development of diverse pathologic conditions (Stadler, 2011). Elevated circulating levels of 3-nitrotyrosine and other cellular oxidative stress markers are shown in patients with metabolic syndrome (Ruiz-Ojeda et al., 2018) and diabetes (Pop-Busui, 2007). As with 4-HNE, it was also aimed to identify those proteins that are adducted by 3-nitrotyrosine.

Data presented in Chapter 3 demonstrated that the naturally occurring dipeptide, carnosine, could sequester the aforementioned reactive species. This PhD research next sought to determine the extent to which carnosine could prevent these individual damaging adduction events. By so doing, an accurate picture of the effectiveness of carnosine as a scavenging agent can be constructed, and its potential as a future therapeutic agent further interrogated.

4.2 Results

4.2.1 Impact of Obesity and Diabetes on Serum Protein Adduction

In order to test the validity of our experimental procedures to human obesity and diabetes, this PhD also determined the extent to which serum proteins form adducts with 4-hydroxynonenal and 3-nitrotyrosine as a consequence of metabolic stress. Following a 10 hour overnight fast, blood was collected from lean controls, obese non-diabetic, obese gestational diabetic, and obese type 2 diabetic non-menopausal women with informed consent. Serum samples were immunoprecipitated using either 4-HNE or 3-NT primary antibodies and a Pierce crosslink magnetic immunoprecipitation kit. Mass-spectrometry analysis was conducted using a SCIEX TripleTOF 6600 mass spectrometer, with data acquisition processed using PEAKS studio 8.5 software in conjunction with the SwissProt database.

Table 4.1. Characteristics of Study Patients (provided by Dr. Alice Murphy, Bioscience Department, NTU).

Obese-Non-Diabetic Patients (All Female) [W-white, B-black]						
Patient ID	Age	Ethnicity	BMI (kg/m^2)	HbA1c (%)	Surgery Date	Smoking Status
AT179	29	W	37	*	24.07.12	2-3 per day
AT281	38	W	37.1	*	25.02.13	3 per day
AT185	26	W	37.7	*	16.08.12	15 per day
AT294	28	W	43	*	25.03.13	Non-smoker
AT277	43	B	47.7	*	20.02.13	Non-smoker
AT242	33		47	*		
• (* No data - diabetic status was taken from their medical records)						

Obese-Type 2 Diabetic Patients (All Female)[W-white]						
Patient ID	Age	Ethnicity	BMI (kg/m^2)	HbA1c (%)	Surgery Date	Smoking Status
AT025	59	W	37.7	8.1	14.01.11	Smoker
AT036	60	W	50	10.4	24.02.11	Non-smoker
AT028	54	W	57.3	9.4	27.01.11	Non-smoker
AT405	59	W	44.3	**	13.01.15	Non-smoker
AT407	67	W	37.8	**	09.04.15	Non-smoker
** No data - diabetic status was taken from their medical records						

***Obese-Gestational Diabetic Patients [W-white,B-black, A-Asian]						
Patient ID	Age	Ethnicity	BMI kg/m^2		Surgery Date	Smoking Status
AT270	36	W	38.6		08.02.13	Non-smoker
AT335	24	W	39		18.07.13	Non-smoker
AT263	38	A	40.9		08.01.13	Non-smoker
AT245	31	W	42.5		30.11.12	Non-smoker
AT303	34	W	46		16.04.13	Non-smoker
AT317	37	B	52.9		23.05.13	Non-smoker
*** diagnosed by an oral glucose tolerance test						

As can be seen from the Tables below, there is increased damaging serum protein adduction associated with both 4-HNE and 3-NT species in each of the metabolic conditions tested (relative to healthy control individuals). However, there is more adducted protein (>60%) in the serum of obese-type 2 diabetic individuals than in the other serum samples (~30%).

Table 4.2. 4-Hydroxynonenal-Associated Proteins Identified in Different Clinical Serum Samples.

Obese-nondiabetic Serum (BMI=41.60±4.99; Age = 32.80±6.50)		
Apolipoprotein(a)	Histone H2B type 1	Immunoglobulin lambda-like polypeptide 5
Extracellular matrix protein 1	Histone H2B type 2	Myeloperoxidase
Fibrinogen beta chain	Histone H2B type 3	Pregnancy-specific beta-1-glycoprotein 3
Galectin-3-binding protein	Histone H3	Putative pregnancy-specific beta-1-glycoprotein 7
Histone H2A type 1	Histone H4	Putative transmembrane protein encoded by LINC00477
Histone H2A type 2	Immunoglobulin heavy variables	Putative trypsin-6
Histone H2A type 3	Immunoglobulin kappa variables	Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1
Histone H2A.V	Immunoglobulin lambda variables	Trypsin-2
Obese – Type 2 Diabetes (BMI=45.42±8.38; Age = 59.80±4.65)		
BPI fold-containing family A member 1	Immunoglobulin heavy variables	piRNA biogenesis protein EXD1
BTB/POZ domain-containing protein 9	Immunoglobulin heavy variable 3-30-5	Putative transmembrane protein encoded by LINC00477
Dermcidin	Immunoglobulin kappa variables	Putative trypsin-6
Extracellular matrix protein 1	Immunoglobulin lambda constant 6	Statherin
Fibrinogen beta chain	Immunoglobulin lambda variables	Sulfate anion transporter 1
Fibrinogen gamma chain	Immunoglobulin lambda-like polypeptide 5	Trypsin-2
Immunoglobulins heavy constant alpha 2	Keratin, type I cytoskeletal 9	
Obese – Gestational Diabetes (BMI=43.31±5.41; Age = 33.33±5.20)		
Histone H2A type 1	Immunoglobulin heavy variables	Putative trypsin-6
Histone H2A type 2	Immunoglobulin kappa variables	Ribonucleoside-diphosphate reductase large subunit
Histone H2A type 3	Immunoglobulin lambda variables	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform
Histone H2A	Immunoglobulin lambda-like polypeptide 5	Trypsin-2
Histone H2A.Z	Lethal(3)malignant brain tumor-like protein 1	Urea transporter 2
Histone H2AX	Protein HID1	

Table 4.3 3-Nitrotyrosine-Associated Proteins Identified in Different Clinical Serum Samples.

Obese-nondiabetic Serum (BMI=41.60±4.99; Age = 32.80±6.50)		
Afamin	Fibrinogen beta chain	Immunoglobulin mu heavy chain
Apolipoprotein	Haptoglobin-related protein	Keratin
Apolipoprotein A-II	Hemoglobin subunit	Leucine-rich alpha-2-glycoprotein
C4b-binding protein beta chain	Histidine-rich glycoprotein	Plasminogen
Coagulation factor XII	Histone H3	Polymeric immunoglobulin receptor
Complement component C8 gamma chain	Immunoglobulin heavy variables	Pregnancy zone protein
Complement factor H-related protein 5	Immunoglobulin kappa variables	Putative uncharacterized protein RUSC1-AS1
Complement factor I	Immunoglobulin lambda constant	REVERSED SHC-transforming protein 1
Dermcidin	Immunoglobulin lambda variables	Vascular endothelial growth factor C
Dermokine	Immunoglobulin lambda-like polypeptide 1	Vitamin K-dependent protein S
	Zinc-alpha-2-glycoprotein	
Obese – Type 2 Diabetes (BMI=45.42±8.38; Age = 59.80±4.65)		
Abnormal spindle-like microcephaly-associated protein	Dermcidin	Insulin-like growth factor-binding protein complex acid labile subunit
Afamin	DNA polymerase zeta catalytic subunit	Inter-alpha-trypsin inhibitor heavy chain H3
Alpha-1-antichymotrypsin	E3 ubiquitin-protein ligase MYCBP2	Kallistatin
Apical junction component 1 homolog	Ecotropic viral integration site 5 protein homolog	Keratin
Apolipoprotein A-II	Extracellular matrix protein 1	Keratinocyte proline-rich protein
Apolipoprotein A-IV	Fanconi-associated nuclease 1	Kinesin-like protein KIF1B
Apolipoprotein C-I	Fibrinogen beta chain	Leucine-rich alpha-2-glycoprotein
Apolipoprotein C-II	Fibrinogen gamma chain	Lumican
Apolipoprotein C-III	Gelsolin	N-acetylmuramoyl-L-alanine amidase
Apolipoprotein D	Granulins	pdb|1FNI_A| A Chain A, Crystal Structure
Apolipoprotein L1	Haptoglobin-related protein	Phosphatidylinositol-glycan-specific phospholipase D
Apolipoprotein(a)	HEAT repeat-containing protein 1	Pigment epithelium-derived factor
Brefeldin A-inhibited guanine nucleotide-exchange protein 2	Hemoglobin subunit alpha	Plasma kallikrein
Carboxypeptidase B2	Hemoglobin subunit beta	Plasminogen
Carboxypeptidase N subunit 2	Heparin cofactor 2	Platelet basic protein
Coagulation factor IX	Histidine-rich glycoprotein	Pregnancy zone protein

Coagulation factor XII	Histone H4	pre-rRNA processing protein FTSJ3
Coagulation factor XIII A chain	Homeobox protein Mohawk	Putative transmembrane protein encoded by LINC00477
Cohesin subunit SA-3	Hornerin	Retinol-binding protein 4
Complement component C6	Immunoglobulin heavy variables	REVERSED Serine/threonine-protein kinase PRP4 homolog
Complement component C7	Immunoglobulin kappa variables	REVERSED Supervillin
Complement component C8 gamma chain	Immunoglobulin lambda constant 1	RuvB-like 1
Complement factor I	Immunoglobulin lambda variables	Ryanodine receptor 2
Corticosteroid-binding globulin	Immunoglobulin lambda-like polypeptide 5	Ryanodine receptor 3
C-reactive protein	Immunoglobulin mu heavy chain	Serum amyloid A-1 protein
Serum amyloid A-4 protein	Structural maintenance of chromosomes protein 5	Thyroxine-binding globulin
Serum amyloid P-component	Thrombospondin-1	Vitamin K-dependent protein S
Serum paraoxonase/arylesterase 1		Zinc-alpha-2-glycoprotein
Obese – Gestational Diabetes (BMI=43.31±5.41; Age = 33.33±5.20)		
Alpha-1-acid glycoproteins	Haptoglobin	Immunoglobulin lambda variables
Alpha-1-antichymotrypsin	Haptoglobin-related protein	Immunoglobulin lambda-1 light chain
Alpha-1-antitrypsin	Hemopexin	Immunoglobulin lambda-like polypeptide 5
Alpha-2-macroglobulin	Immunoglobulin heavy constants	Immunoglobulin mu heavy chain
Apolipoprotein A and B	Immunoglobulin kappa constants	Inter-alpha-trypsin inhibitor heavy chains
Complement C1q	Immunoglobulin kappa variables	Inter-alpha-trypsin inhibitor heavy chain H2
Complement C4-A and B	Immunoglobulin lambda constants	Inter-alpha-trypsin inhibitor heavy chain H4
Keratin		Vitamin D-binding protein

Protein adduction data were then analysed using PANTHER software (www.pantherdb.org). This facilitates classification of uploaded protein data from the Uniprot Knowledgebase (UniprotKB) via several available parameters. Figures 4.1-4.2 show the relative proportion of biological functions affected by protein adduction from 4-HNE and 3-NT in each condition.

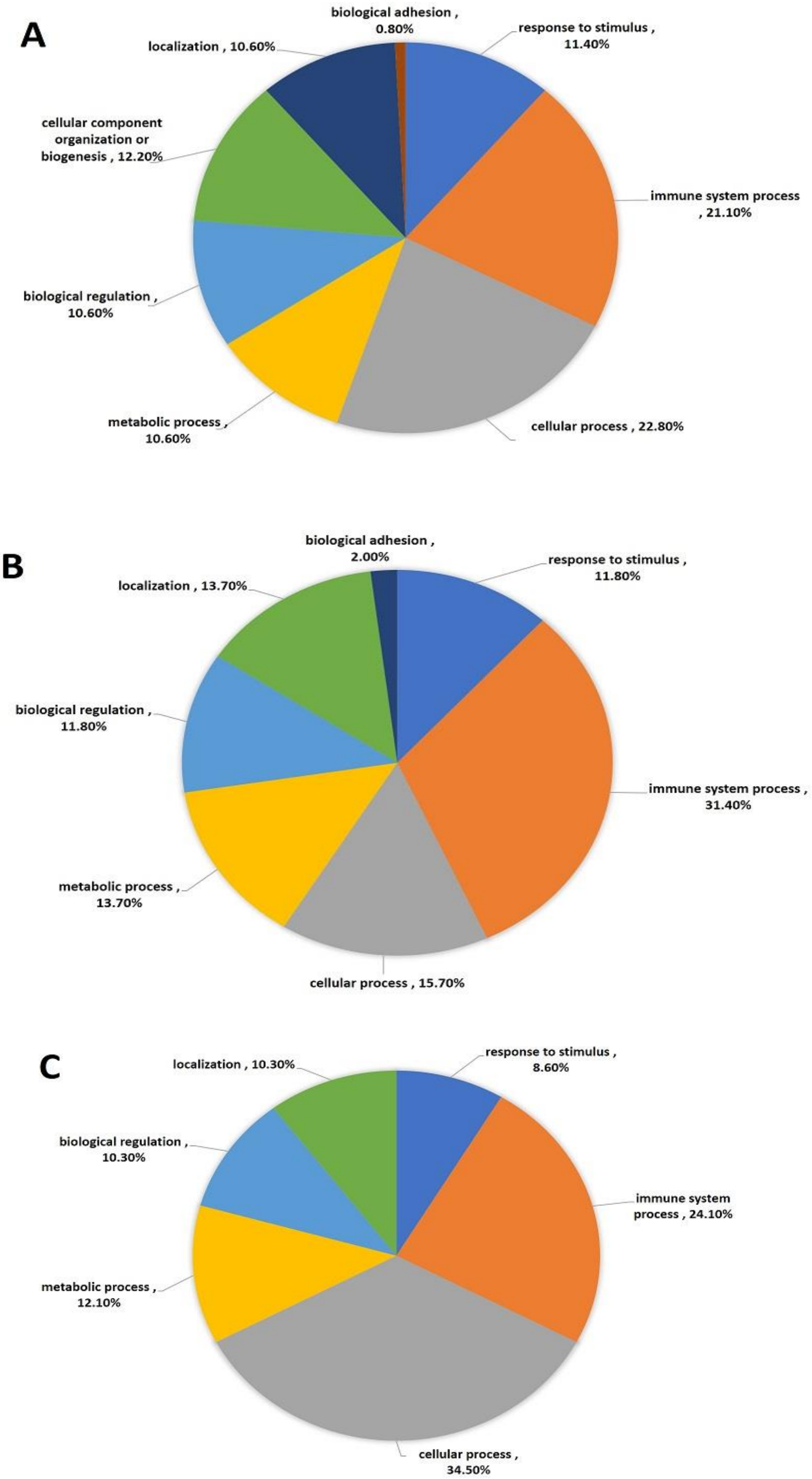


Figure 4.1. Biological process classification of proteins identified and associated with 4-hydroxynonenal in (A) obese (B) obese – type 2 diabetes and (C) obese-gestational diabetes serum samples. Generated using the Panther Classification System.

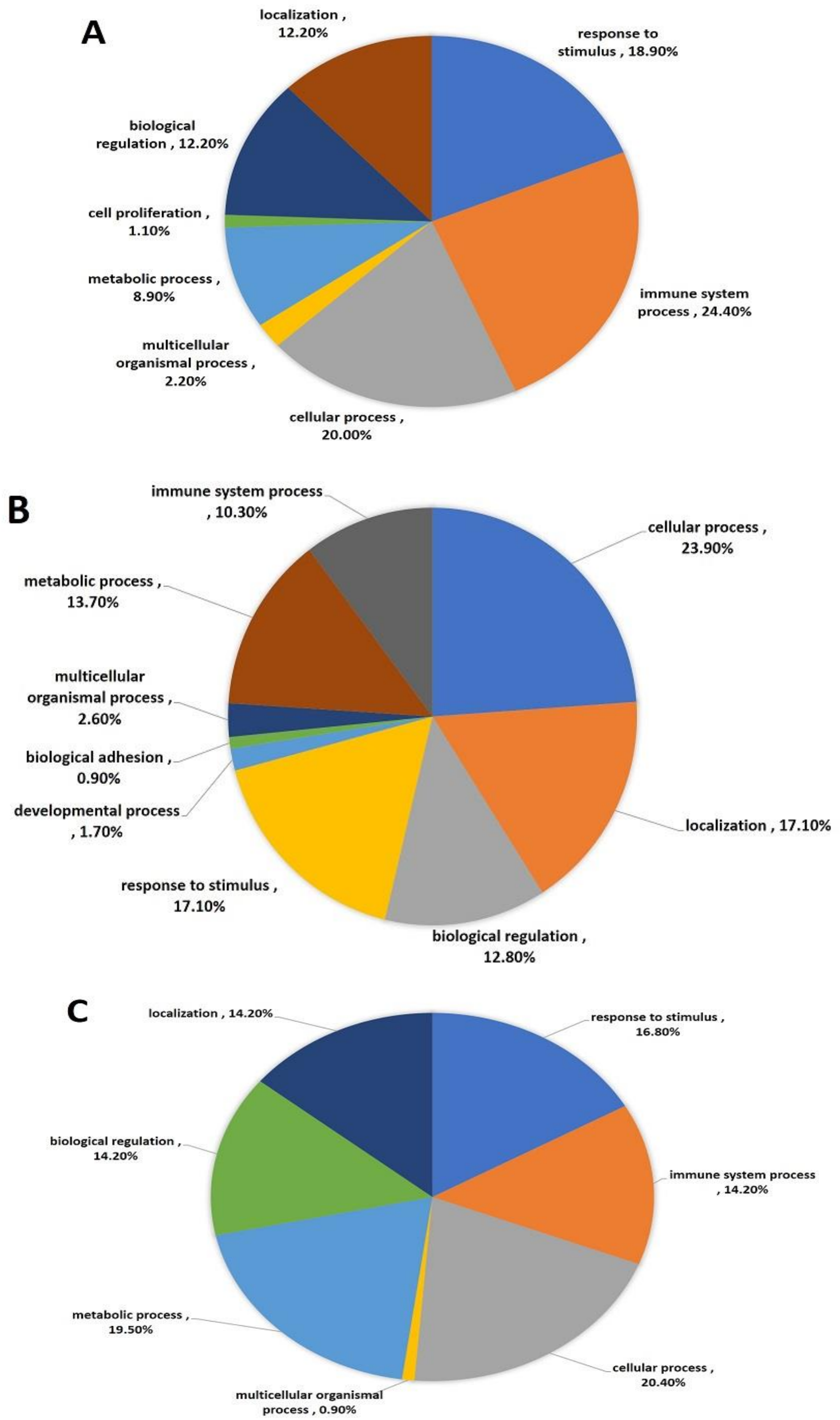


Figure 4.2. Biological process classification of proteins identified and are adducted with 3-nitrotyrosine in (A) obese (B) obese – type 2 diabetes and (C) obese-gestational diabetes serum samples. Generated using the Panther Classification System.

Consistent with the hypothesis that obesity and diabetes drive metabolic stress (Evans et al, 2002), data here indicated that there are increased levels of serum protein adduction in all of the investigated metabolic conditions relative to healthy lean controls. Having established the veracity of our approach, the next aim was to determine how protein adduction, which is driven by high levels of glucose and fatty acids, might affect function in cells and tissues central to the regulation of glucose homeostasis, namely pancreatic islets and skeletal muscle cells. Importantly, the extent to which this damage could be prevented by carnosine was also determined.

4.2.2 4-hydroxynonenal (4-HNE) and 3-nitrotyrosine (3-NT) Protein Adduction in C2C12 Myotubes

In order to identify those proteins that form adducts with 4-HNE and 3-NT as a consequence of exposure to metabolic stress, cells were incubated in DMEM media, or DMEM media supplemented to 28mM glucose and 200μM palmitic acid and 200μM oleic acid (GLT media) for 5-days ± 10mM carnosine. Cells were lysed and protein concentration quantified using a BCA assay. Lysates were immunoprecipitated against either 4-HNE or 3-NT using appropriate primary antibodies (detailed methodology in Chapter 2). Mass-spectrometry analysis was then conducted with assistance from collaborators (John van Geest Cancer Research Center, NTU) using the mass spectrometer SCIEX TripleTOF 6600, and data acquisition processed using PEAKS studio 8.5 software along with the SwissProt database.

Tables 4.4 and 4.5 indicate proteins that were adducted in diabetic GLT conditions, but not in healthy control conditions. In all cases, proteins shown in green are those where adduct formation was prevented by carnosine supplementation. By contrast, carnosine supplementation did not prevent adduction of those proteins shown in black. Data are generated from three independent experiments.

In this analysis, it was found out that carnosine prevented 90% (4-HNE) and 65% (3-NT) of protein adduction in C2C12 cells.

Table 4.4. 4-HNE protein adduction in C2C12 myotubes incubated in GLT media for 5 days.

• [Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 3, mitochondrial	• Bifunctional purine biosynthesis protein PURH
• 182 kDa tankyrase-1-binding protein	• cAMP-dependent protein kinase catalytic subunit beta
• 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-3	• Canalicular multispecific organic anion transporter 1
• 26S protease regulatory subunit 8	• Carnitine
• 39S ribosomal protein L28, mitochondrial	• Centrosomal protein of 170 kDa
• 7-dehydrocholesterol reductase	• Ceruloplasmin
• Actin-related protein 3B	• Clathrin interactor 1
• Acyl-coenzyme A thioesterase 13	• Coagulation factor V
• Adenylosuccinate synthetase isozyme 1	• Coiled-coil domain-containing protein 114
• Adipocyte plasma membrane-associated protein	• Cold shock domain-containing protein E1
• ADP-ribosylation factor-like protein 1	• Collagen alpha-1(VI) chain
• Alanine--tRNA ligase, cytoplasmic	• CTP synthase 1
• Alpha-methylacyl-CoA racemase	• Cytoplasmic dynein 1 intermediate chain 2
• Amine oxidase [flavin-containing] A	• Cytosolic acyl coenzyme A thioester hydrolase
• Ankyrin repeat domain-containing protein 17	• DDB1- and CUL4-associated factor 8
• AP-2 complex subunit alpha-1	• Gamma-enolase
• AP-3 complex subunit sigma-1	• GAS2-like protein 1
• Apoptosis-inducing factor 1, mitochondrial	• Glycine--tRNA ligase
• Aspartyl/asparaginyl beta-hydroxylase	• Glycogen [starch] synthase, muscle
• ATP-binding cassette sub-family F member 1	• Glycogen phosphorylase, muscle form
• ATP-citrate synthase	• Hemoglobin subunit alpha
• ATP-dependent RNA helicase	• Immunity-related GTPase family M protein 1
• Band 4.1-like protein 2	• Integrator complex subunit 3
• Beta-galactosidase-1-like protein 3	• Integrin alpha-7
• Deoxynucleoside triphosphate triphosphohydrolase SAMHD1	• Inter-alpha-trypsin inhibitor heavy chain H3
• DNA replication licensing factor MCM5	• IQ domain-containing protein F1
• DNA-binding protein SMUBP-2	• Isoleucine--tRNA ligase, cytoplasmic
• DnaJ homolog subfamily B member 6	• Isoleucine--tRNA ligase, mitochondrial
• Dystrophin	• Kelch-like protein 40
• E3 ubiquitin/ISG15 ligase TRIM25	• Keratin
• EH domain-containing protein 3	• Kinesin

• Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial	• Myosin light chain 3
• ELKS/Rab6-interacting/CAST family member 1	• Myosin-6
• Elongator complex protein 3	• NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial
• ER degradation-enhancing alpha-mannosidase-like protein 1	• NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial
• Eukaryotic peptide chain release factor subunit 1	• Nascent polypeptide-associated complex subunit alpha
• Eukaryotic translation initiation factor 3 subunit C	• Nesprin-1
• Ezrin	• Neutral alpha-glucosidase AB
• FACT complex subunit SSRP1	• Nodal
• Fatty acid desaturase 3	• Nuclear factor 1 B-type
• FERM, RhoGEF and pleckstrin domain-containing protein 1	• Nucleolar protein 14
• Ferrochelatase, mitochondrial	• Peptidyl-prolyl cis-trans isomerase C
• Fibrous sheath-interacting protein 2	• Peptidyl-prolyl cis-trans isomerase FKBP1A
• Phosphoglycerate kinase 2	• Phenylalanine--tRNA ligase beta subunit
• Platelet-derived growth factor receptor beta	• Phosphoglucomutase-like protein 5
• Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	• Sodium- and chloride-dependent GABA transporter 3
• Prolyl 4-hydroxylase subunit alpha-2	• Sodium- and chloride-dependent taurine transporter
• Prolyl endopeptidase	• Sodium channel protein type 11 subunit alpha
• Proteasomal ubiquitin receptor ADRM1	• Sodium/glucose cotransporter 5
• Proteasome subunit beta type-2	• Spliceosome RNA helicase Ddx39b
• Protein arginine N-methyltransferase 1	• Splicing factor U2AF 26 kDa subunit
• Protein FAM98B	• Stomatin-like protein 2, mitochondrial
• Protein kinase C and casein kinase substrate in neurons protein 2	• Striatin-interacting proteins 1/2
• Protein NipSnap homolog 2	• TBC1 domain family member 9B
• Protein prune homolog 2	• Thioredoxin domain-containing protein 5
• Protein transport protein Sec61 subunit alpha isoform 2	• Transcription elongation factor SPT6
• Protein VAC14 homolog	• Transcription initiation factor TFIID subunit 4B
• Pyruvate dehydrogenase protein X component, mitochondrial	• Transcription intermediary factor 1-beta
• Ras-related GTP-binding protein C	• Transforming growth factor beta-1-induced transcript 1 protein
• Ras-related GTP-binding protein D	• Translocon-associated protein subunit delta
• Ras-related protein Rab-6/8/11/15	• Trimeric intracellular cation channel type A
• Receptor-type tyrosine-protein phosphatase beta	• Tripartite motif-containing protein 2
• REVERSED Arylsulfatase B	• Triple functional domain protein
• REVERSED Hepatoma-derived growth factor-related protein 2	• Type 1 phosphatidylinositol 4,5-bisphosphate 4-phosphatase
• Rho GTPase-activating protein 11A	• U8 snoRNA-decapping enzyme

• Rho-associated protein kinase 2	• UDP-glucose:glycoprotein glucosyltransferase 1
• Serine beta-lactamase-like protein LACTB, mitochondrial	• Uncharacterized protein C11orf87 homolog
• Serine/threonine-protein kinase MRCK beta	• Vacuolar protein sorting-associated protein 18 homolog
• Serine/threonine-protein kinase NIM1	• Valine--tRNA ligase
• Serine/threonine-protein phosphatase	• Vesicle-associated membrane protein-associated protein B
• Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial	• Zinc transporter SLC39A7
• Signal recognition particle subunit SRP68	• GRIP and coiled-coil domain-containing protein 2
• Small nuclear ribonucleoprotein Sm D2	• Heterogeneous nuclear ribonucleoprotein A0
• 26S protease regulatory subunit 10B/6B	• Heterogeneous nuclear ribonucleoprotein D-like
• 26S protease regulatory subunit 6B	• Importin-4
• 26S proteasome non-ATPase regulatory subunit 3	• Interferon-activable protein 202
• 5'-AMP-activated protein kinase subunit gamma-1	• Interferon-inducible double-stranded RNA-dependent protein kinase activator A
• 60S ribosomal protein L39	• Keratin
• Adenine phosphoribosyltransferase	• Leucine--tRNA ligase, cytoplasmic
• Alcohol dehydrogenase [NADP(+)]	• Lysine--tRNA ligase
• Aminoacyl tRNA synthase complex-interacting multifunctional protein 1	• Macrophage-capping protein
• Aminoacyl tRNA synthase complex-interacting multifunctional protein 2	• Malate dehydrogenase, cytoplasmic
• AP-3 complex subunit	• MAP7 domain-containing protein 1
• Beta-actin-like protein 2	• Methionine--tRNA ligase, cytoplasmic
• Biglycan	• Microtubule-associated protein 1B
• Casein kinase I isoform alpha	• Myosin-14
• Cathepsin Z	• Myosin-8
• Chloride intracellular channel protein 4	• NADH dehydrogenase
• Chromodomain-helicase-DNA-binding protein 2	• Sorting nexin-9
• CLIP-associating protein 1	• Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial
• Coatomer subunit	• Suppression of tumorigenicity 5 protein
• Nucleosome assembly protein 1-like 1	• TBC1 domain family member 1
• Peptidyl-tRNA hydrolase 2, mitochondrial	• Tetratricopeptide repeat protein 30B
• Peroxiredoxin-2	• Thimet oligopeptidase
• Platelet-activating factor acetylhydrolase IB subunit alpha	• Threonine--tRNA ligase, cytoplasmic
• Potassium-transporting ATPase alpha chain 1	• Transmembrane 9 superfamily member 1
• Pre-mRNA-processing factor 19	• Tubulin beta-4A chain
• Procollagen galactosyltransferase 1	• Ubiquitin carboxyl-terminal hydrolase 5
• Proteasome subunit alpha type-6	• UDP-N-acetylhexosamine pyrophosphorylase-like protein 1
• Protein transport protein Sec23A	• Xaa-Pro aminopeptidase 1

• Protein-lysine 6-oxidase	• Septin-2
• Putative ATP-dependent RNA helicase	• Serine/arginine-rich splicing factor 5
• Selenocysteine-specific elongation factor	• S-formylglutathione hydrolase
• Sodium/potassium-transporting ATPase subunit	• Short-chain specific acyl-CoA dehydrogenase, mitochondrial

Table 4.5. 3-NT protein adduction in C2C12 myotubes incubated in GLT media for 5 days.

• 10 kDa heat shock protein, mitochondrial	• Citrate synthase, mitochondrial
• 14-3-3 protein beta/alpha/gamma/sigma	• Collagen alpha-1(I) chain
• 26S proteasome non-ATPase regulatory subunit 11/13	• Complement component 1 Q subcomponent-binding protein, mitochondrial
• 40S ribosomal protein S21	• Copper transport protein AT
• Acyl-CoA-binding protein	• Creatine kinase B-type
• Acyl-coenzyme A thioesterase 13	• Cullin-associated NEDD8-dissociated protein 1
• Annexin A4	• Cystatin-B
• Argininosuccinate synthase	• Cytochrome b-c1 complex subunit 1, mitochondrial
• ATP synthase protein 8	• Cytochrome c oxidase subunit 7A2, mitochondrial
• Brain acid soluble protein 1	• Dihydrolipoyl dehydrogenase, mitochondrial
• Calreticulin	• Dihydropyrimidinase-related protein 2
• Cathepsin B	• DnaJ homolog subfamily A member 2
• Cathepsin D	• Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit
• Cell division control protein 42 homolog	• Glutathione S-transferase P 1/2
• Chloride intracellular channel protein 4	• GTP-binding protein SAR1a/SAR1b
• Electron transfer flavoprotein subunit alpha, mitochondrial	• Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-3
• Endoplasmic reticulum resident protein 44	• Hepatocyte growth factor-regulated tyrosine kinase substrate
• Enoyl-CoA hydratase, mitochondrial	• Isocitrate dehydrogenase [NADP], mitochondrial
• Epidermal growth factor receptor kinase substrate 8-like protein 1	• Kelch-like protein 41
• Eukaryotic translation initiation factor 6	• Lactoylglutathione lyase
• Fibronectin	• Macrophage-capping protein
• Fragile X mental retardation syndrome-related protein 1	• Malate dehydrogenase, cytoplasmic
• Galectin-3	• Myoglobin
• Glucosidase 2 subunit beta	• N6-adenosine-methyltransferase subunit METTL3
• Glutathione S-transferase Mu 1/3	• N-acetylglucosamine-6-sulfatase
• Glutathione S-transferase Mu 3	• NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2
• Parathymosin	• Phosphoglycerate mutase 2
• Peptidyl-prolyl cis-trans isomerase C	• PRA1 family protein 3
• Peptidyl-prolyl cis-trans isomerase FKBP1A	• Prohibitin 1/2
• Peroxiredoxin-5, mitochondrial	• Stress-induced-phosphoprotein 1
• Peroxiredoxin-6	• Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial
• Prolyl 4-hydroxylase subunit alpha-1	• Superoxide dismutase [Cu-Zn]

• Prosaposin	• Synaptic vesicle membrane protein VAT-1 homolog
• Prostaglandin E synthase 3	• Thioredoxin
• Proteasome subunit alpha type-5 / 7	• Thioredoxin-dependent peroxide reductase, mitochondrial
• Protein disulfide-isomerase A4	• Thymosin beta-10
• Protein S100-A13	• Transcription elongation factor B polypeptide 2
• Protein SEC13 homolog	• Transgelin
• Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	• Translationally-controlled tumor protein
• Rab GDP dissociation inhibitor beta	• Tripartite motif-containing protein 72
• Ras-related protein Rab-5A/B/C	• Tubulin beta-4A chain
• Ras-related protein Rab-5B	• Ubiquitin-conjugating enzyme E2 N
• Ras-related protein Rab-5C	• Ubiquitin-like modifier-activating enzyme 1
• Receptor expression-enhancing protein 5	• Vacuolar protein sorting-associated protein 35
• Reticulon-4	• Voltage-dependent anion-selective channel protein 1
• REVERSED Protein phosphatase methylesterase 1	• Activated RNA polymerase II transcriptional coactivator p15
• Rho-related GTP-binding protein RhoQ	• Cofilin-2
• Ribonuclease inhibitor	• Destrin
• Septin-7	• Filamin-B
• Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	• Flotillin-1
• S-formylglutathione hydrolase	• Heat shock 70 kDa protein 1A /1B
• Sideroflexin-1	• Histone H1t
• Small ubiquitin-related modifier 2/3	• Myosin-6
• Spectrin beta chain, non-erythrocytic 1	• Peroxiredoxin-4
• Splicing factor U2AF 26 kDa subunit	• Protein S100-A6
•	• Tubulin beta-3 chain

4.2.3 PANTHER Classification System of 4-HNE and 3-NT Associated Proteins in GLT-exposed C2C12 muscle cells

Using the program PANTHER (Protein Analysis Through Evolutionary Relationships), the proteins identified as being associated with 4-HNE and 3-NT under glucolipotoxic conditions were classified according to their molecular function, biological process, and protein class. The classification analysis was generated using the protein's corresponding UniprotKB via the program available free from (www.pantherdb.org). The indicated percentage of representation in the following pie charts are considered and based on over the total class hits and not on over the total amount of proteins detected since a particular protein annotation number might fit more than one class hits.

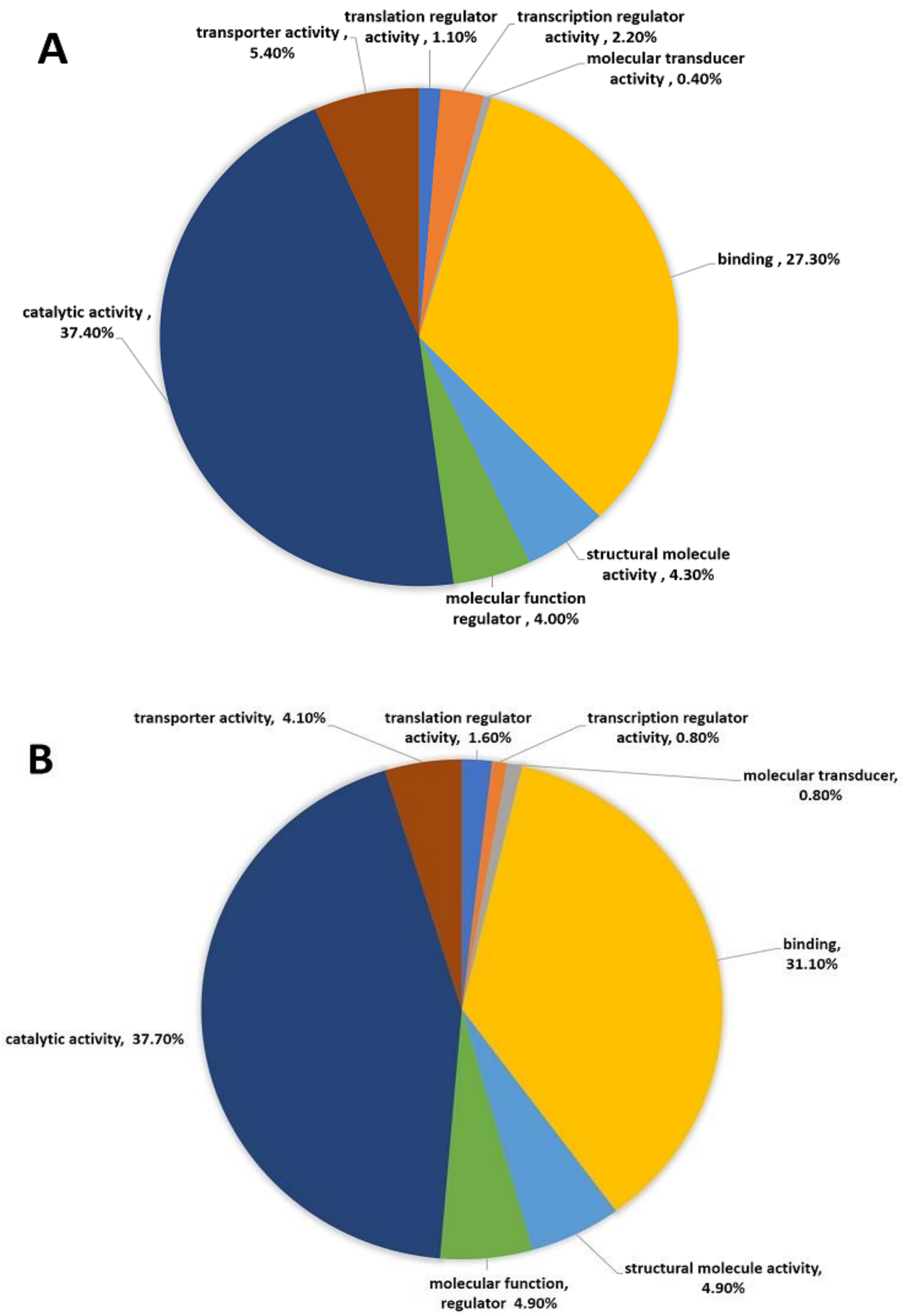


Figure 4.3. Molecular function of 4-HNE (A) and 3-NT (B) adducted proteins. Proteins that are adducted by 4-HNE or 3-NT in C2C12 myotubes under glucolipotoxic conditions for 5 days and are protected by the presence of carnosine. Generated using the Panther Classification System.

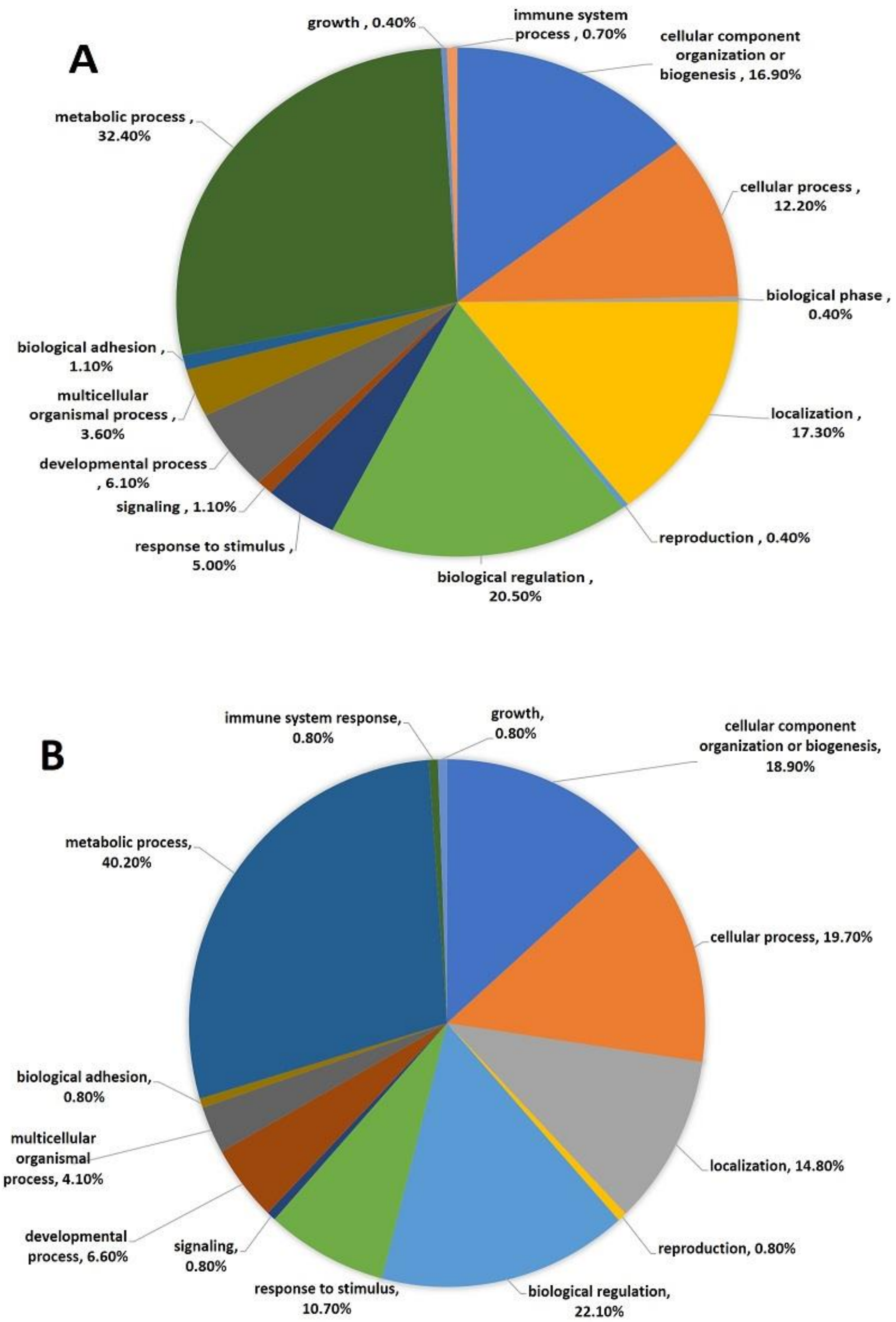


Figure 4.4. **Biological process of 4-HNE (A) and 3-NT (B) adducted proteins**. The chart shows the biological process of associated proteins that formed adducts with 4-HNE or 3-NT in C2C12 under GLT conditions and in which formation is prevented by the addition of carnosine. Generated using the Panther Classification System.

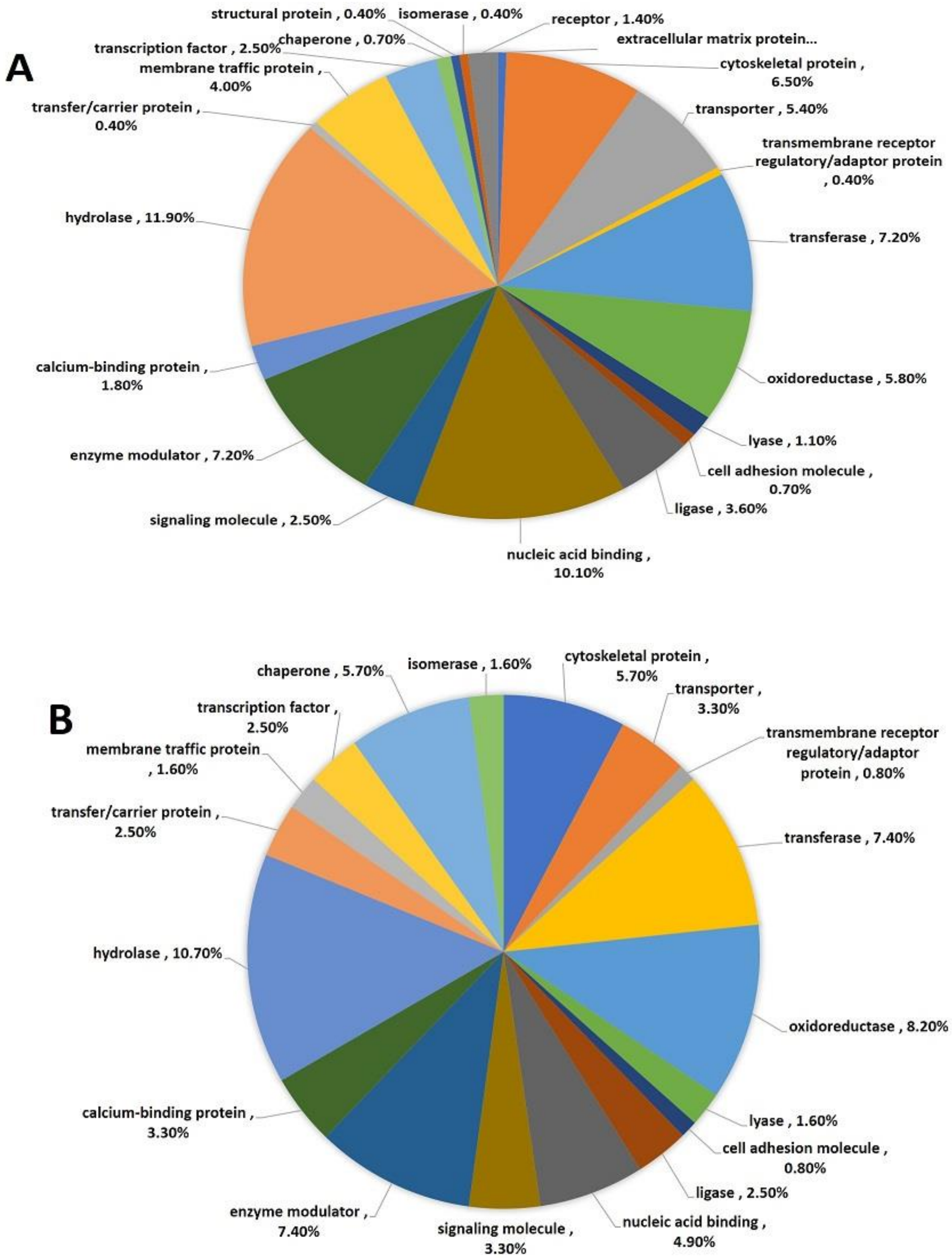


Figure 4.5. Protein class of 4-HNE (A) and 3-NT (B) adducted proteins. The chart depicts the protein class of associated proteins that formed adducts with 4-HNE or 3-NT in GLT-treated C2C12 cells and in which formation is prevented by the addition of carnosine. Generated using the Panther Classification System.

4.2.4 4-HNE and 3-NT Protein Adduction in Human Skeletal Muscle Cells

Tables 4.6 and 4.7, indicate those proteins that were adducted in diabetic GLT conditions, but not in healthy control conditions. In all cases, proteins shown in green are those where adduct formation was prevented by carnosine supplementation. By contrast, carnosine supplementation did not prevent adduction of those proteins indicated in black. Data are generated from three independent experiments.

Again, carnosine showed to have protected protein adduction (80% for 4-HNE and 65% for 3-NT) in HSkM cells.

Table 4.6. 4-HNE protein adduction in human skeletal muscle cells incubated in GLT media for 5 days.

• 40S ribosomal proteins (S12,S15,S27,S29,S30)	• Elongation factor Tu, mitochondrial
• 60 kDa heat shock protein, mitochondrial	• Enhancer of rudimentary homolog
• 60S acidic ribosomal protein P0	• Eukaryotic initiation factor 4A-III
• 60S ribosomal proteins (L9,L10,L13,L24, L27,L35,L37)	• FACT complex subunit SSRP1
• Aconitate hydratase, mitochondrial	• F-actin-capping protein subunit alpha-1
• Actin-related protein 2/3 complex subunit 5	• G protein-activated inward rectifier potassium channel 4
• Actin-related protein 3	• Glucagon
• ADP-ribosylation factor 1	• Guanine nucleotide-binding protein G
• Alpha-actinin-4	• Heterochromatin protein 1-binding protein 3
• Alpha-crystallin B chain	• Heterogeneous nuclear ribonucleoproteins
• Annexin A5	• Histones (H1,H2A,H2B,H3)
• Apoptotic chromatin condensation inducer in the nucleus	• HLA class I histocompatibility antigen, Cw-5 alpha chain
• ATP synthase subunit beta, mitochondrial	• Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial
• ATP synthase subunit	• Hypoxia up-regulated protein 1
• ATP-citrate synthase	• Insulin
• ATP-dependent RNA helicase A	• Keratin, type I (cuticular and cytoskeletal)
• ATP-dependent RNA helicase DDX1	• Keratin, type II(cuticular and cytoskeletal)
• ATP-dependent RNA helicase DDX3Y	• Keratin-associated proteins
• Calreticulin	• Lamin-B1/B2
• Coagulation factor X	• LIM domain and actin-binding protein 1
• Collagen alpha-1(VI) chain	• Malate dehydrogenase, mitochondrial
• Collagen alpha-2(VI) chain	• Matrin-3
• Collagen alpha-3(VI) chain	• Metallothionein-1X
• Core histone macro-H2A.1	• Methyl-CpG-binding protein 2

• Core histone macro-H2A.2	• Myosin-14
• Coronin-1C	• NADH-cytochrome b5 reductase 3
• Creatine kinase B-type	• Neuroendocrine convertase 2
• Destrin	• NHP2-like protein 1
• Electron transfer flavoprotein subunit alpha, mitochondrial	• Nucleolar protein 10/58
• Elongation factor 1-alpha 1	• Nucleophosmin
• rRNA 2'-O-methytransferase fibrillarin	• Peptidyl-prolyl cis-trans isomerase B
• Sarcoplasmic/endoplasmic reticulum calcium ATPase 3	• Phosphoglycerate kinase 1
• Serine/threonine-protein phosphatase PP1-beta catalytic subunit	• Poly(rC)-binding protein 1 / 2
• Signal recognition particle 14 kDa protein	• Polypyrimidine tract-binding protein 3
• Small nuclear ribonucleoprotein Sm D1 / D3	• Pre-mRNA-processing-splicing factor 8
• Somatostatin	• Probable ATP-dependent RNA helicase DDX5
• Splicing factor 3B subunit 3	• Protein disulfide-isomerase, A3/A6
• Splicing factor U2AF 35 kDa subunit	• Putative RNA-binding protein Luc7-like 2
• Stress-70 protein, mitochondrial	• Pyruvate carboxylase, mitochondrial
• Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	• Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial
• Superoxide dismutase [Cu-Zn]	• Ras-related protein Rab-1A, 3A,8A
• SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	• Receptor of activated protein C kinase 1
• THO complex subunit 4	• RNA-binding motif protein, X chromosome
• Transformer-2 protein homolog beta	• Tricarboxylate transport protein, mitochondrial
• Tubulin alpha-1A chain	• Vinculin
• Tubulin beta-2A chain	• WD repeat-containing protein 1
• Ubiquitin-40S ribosomal protein S27a	• YY1-associated protein 1
• 14-3-3 protein epsilon	• Corneodesmosin
• 40S ribosomal protein S11,S13	• Desmoglein-1
• 60S ribosomal protein L11,L19	• eIF-2-alpha kinase GCN2
• Alpha-enolase	• Elongation factor 2
• Apolipoprotein M	• Endoplasmin
• ATP synthase subunit alpha, mitochondrial	• Fructose-bisphosphate aldolase A
• Calmodulin-like protein 5	• Galectin-1
• Clathrin heavy chain 1	• Glutathione S-transferase P
• Clusterin	• Heat shock protein HSP 90-alpha
• Cofilin-1	• Heterogeneous nuclear ribonucleoprotein
• Immunoglobulin gamma-1 heavy chain	• Histone H1.5
• Junction plakoglobin	• Histone H2A.J
• Keratin, type II cytoskeletal 6C	• Peroxiredoxin-1
• L-lactate dehydrogenase A chain	• Plakophilin-1
• Protein S100-A11, A8, A9	• Skin-specific protein 32

Table 4.7. 3-NT protein adduction in human skeletal muscle cells incubated in GLT media for 5 days.

• 40S ribosomal protein (S11, S23, S4, S5, S8, S9)	• Keratin, type II cytoskeletal 6C
• 60S ribosomal protein L14, L22, L7	• Large neutral amino acids transporter small subunit 4
• Actin, cytoplasmic 2	• Leucine-rich repeat-containing protein 59
• Alpha-actinin-4	• LIM and SH3 domain protein 1
• ATP-binding cassette sub-family A member 8	• Long-chain-fatty-acid--CoA ligase ACSBG2
• Calcium-activated chloride channel regulator family member 3	• Matrix metalloproteinase-28
• Calmodulin-lysine N-methyltransferase	• Matrix-remodeling-associated protein 5
• Clusterin	• Moesin
• Desmocollin-1	• Myosin light chain 4
• EH domain-containing protein 2	• Myosin regulatory light polypeptide 9
• Endoplasmic reticulum chaperone BiP	• Myosin-3
• Exportin-T	• Nebulin
• Fc receptor-like protein 2	• PC4 and SFRS1-interacting protein
• Four and a half LIM domains protein 1	• Polycystin-1
• Gamma-interferon-inducible protein 16	• Profilin-1
• GTP-binding nuclear protein Ran	• Protein Shroom3
• Heat shock 70 kDa protein 1-like	• Putative uncharacterized protein C5orf17
• Heterogeneous nuclear ribonucleoproteins C1/C2	• Rho-related GTP-binding protein RhoC
• Histone H1, H2B, H3	• Trypsin-1
• Immunoglobulin mu heavy chain	• Tubulin beta-2B chain
• Spectrin alpha chain, non-erythrocytic 1	• Unconventional myosin-Ib
• Tetratricopeptide repeat protein 28	• UTP--glucose-1-phosphate uridylyltransferase
• Tropomyosin beta chain	• Versican core protein
• Zinc finger protein 587B	• Immunoglobulin lambda-1 light chain
• 40S ribosomal protein S15, S2, S7	• Inter-alpha-trypsin inhibitor heavy chain H4
• 60S ribosomal protein L6, L8, L11, L19, L35, L36	• KAT8 regulatory NSL complex subunit 1
• Actin, alpha cardiac muscle 1	• Myotubularin-related protein 3
• Alpha-1-antitrypsin	• Nestin
• Alpha-actinin-1	• Neuroblast differentiation-associated protein AHNAK
• Caveolae-associated protein 1	• Peptidyl-prolyl cis-trans isomerase B
• Coiled-coil domain-containing protein 80	• Plasminogen activator inhibitor 1
• Collagen alpha-1(I) chain	• Prelamin-A/C
• Fibrillin-1	• Protein S100-A8
• Galectin-1	• Skin-specific protein 32
• Heat shock cognate 71 kDa protein	• Spermatogenesis-associated protein 31D1
	• Unconventional myosin-Ic

4.2.5 PANTHER Classification System of 4-HNE and 3-NT Associated Proteins in GLT-exposed Human Skeletal Muscle Cells

Using the same classification analysis as above, the following pie charts depict the molecular functions, the biological processes and what protein class the proteins listed in Tables 4.6 and Table 4.7 are involved in.

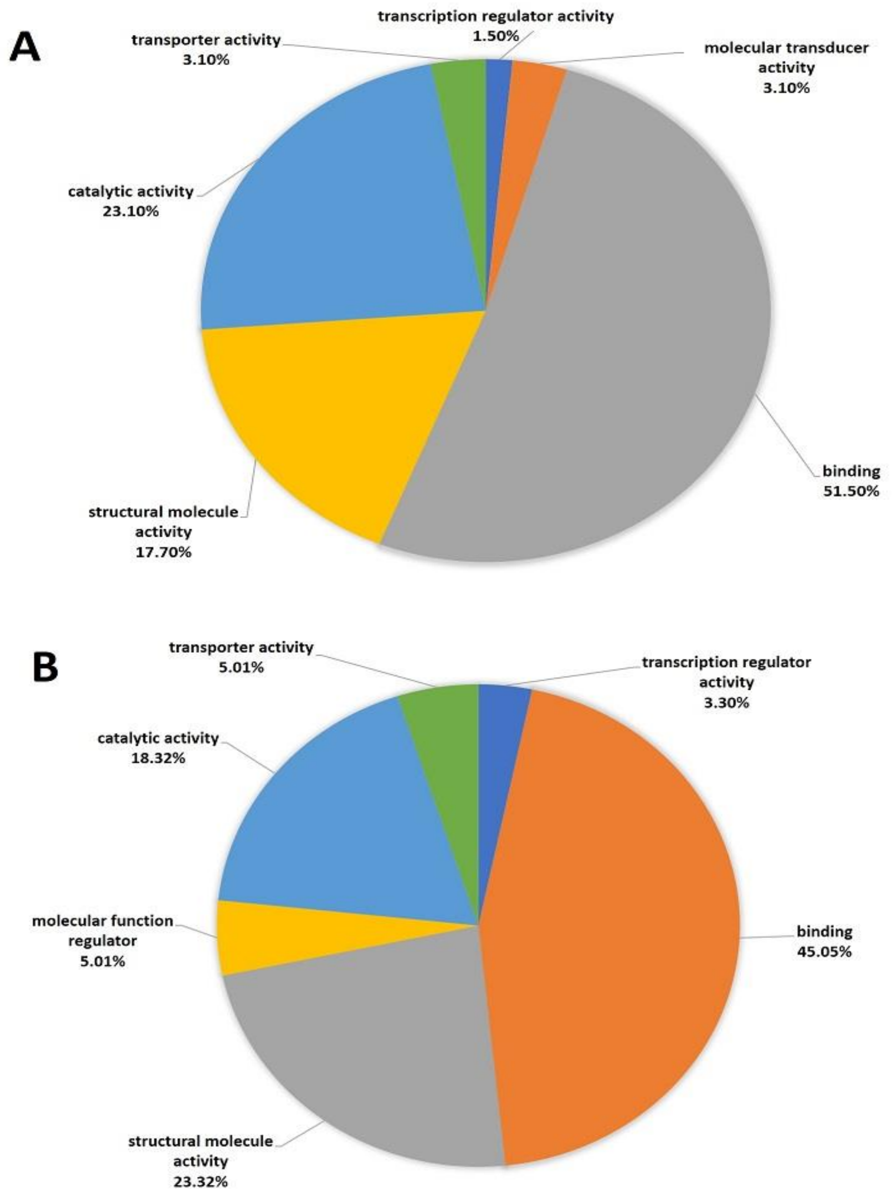


Figure 4.6 Molecular function of 4-HNE (A) and 3-NT (B) adducted proteins. Proteins that are adducted in 4-HNE or 3-NT in cultured human skeletal myotubes under glucolipotoxic conditions for 5 days and in which formation is prevented by the addition of carnosine. Generated using the Panther Classification System.

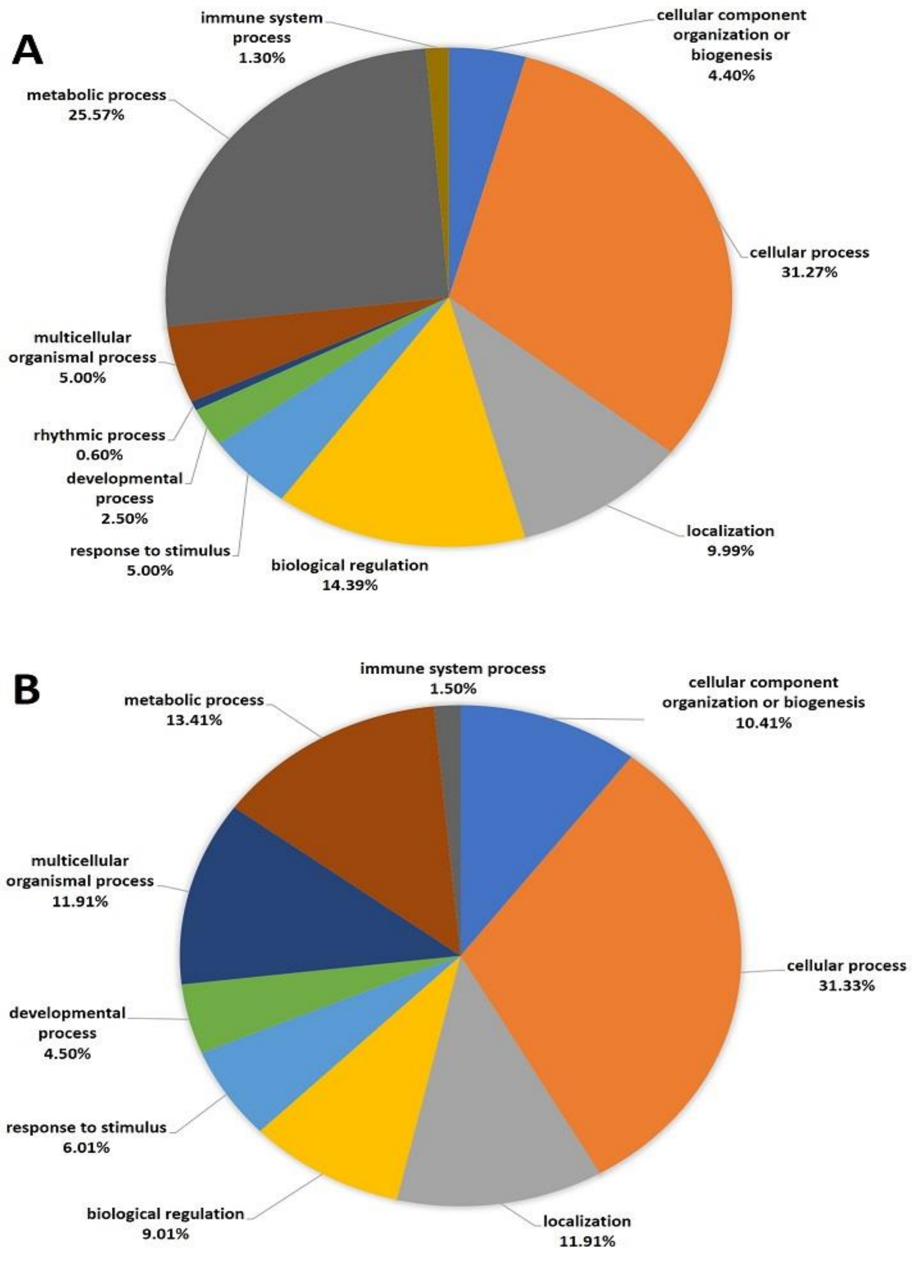


Figure 4.7 Biological process of 4-HNE (A) and 3-NT (B) adducted proteins. The biological process of associated proteins that formed adducts with 4-HNE or 3-NT in human skeletal myotubes treated with GLT for 5 days and in which formation is prevented by the addition of carnosine. Generated using the Panther Classification System.

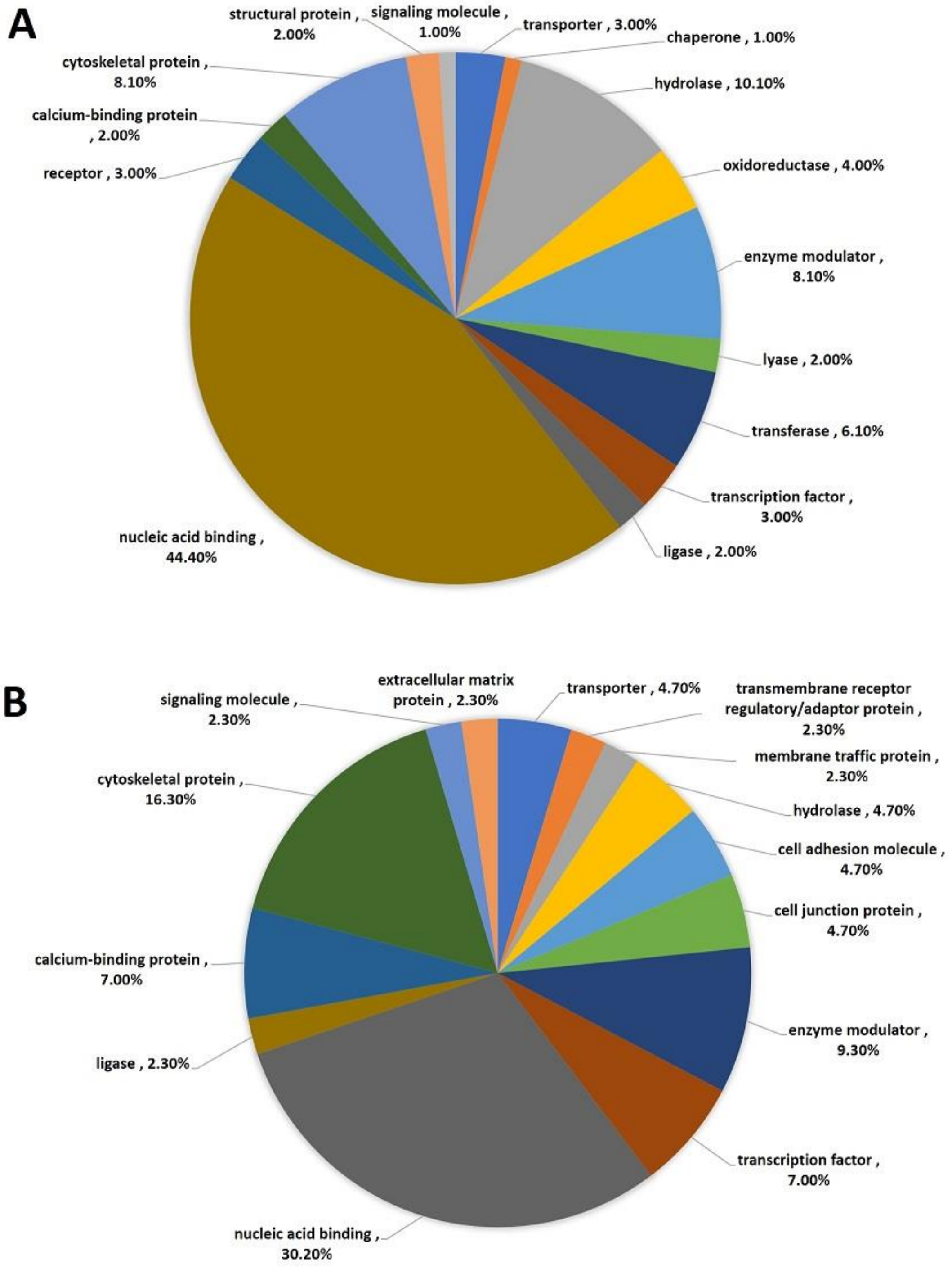


Figure 4.8 Protein class of 4-HNE (A) and 3-NT (B) adducted proteins. The protein class of associated proteins that formed adducts with 4-HNE or 3-NT in human skeletal myotubes incubated in GLT for 5 days and in which formation is prevented by the addition of carnosine. Generated using the Panther Classification System.

4.2.6 4-HNE and 3-NT Protein Adduction in Mouse Primary Islets

In addition to skeletal muscle, the pancreas also plays a central role in the control of glucose homeostasis. INS-1 β-cells cultured for 5 days in GLT media have elevated levels of 3-nitrotyrosine and 4-hydroxynonenal (Cripps et al., 2017). However, tissue culture cells are not always fully representative of animal physiology, so in the current study it utilised mouse primary islets that were kindly isolated and cultured by Dr. Paul Caton (King's College London). Isolated islets were incubated either in control RPMI-1640 media, or RPMI- GLT media ± 10mM carnosine for 5 days. Cell lysates were frozen, then shipped on dry ice to the Turner group for processing and analysis, and as detailed above for skeletal muscle cells. In all cases, proteins shown in green are those where adduct formation was prevented by carnosine supplementation. By contrast, carnosine supplementation did not prevent adduction of those proteins shown in black. Data are generated from three independent experiments.

In line with the findings from skeletal muscle cells, 88% (4-HNE) and 75% (3-NT) of protein adduction in primary islets was prevented by carnosine supplementation. PANTHER analysis of proteins protected from adduction by carnosine was also undertaken in order to determine their respective molecular function, biological process, and protein class.

Table 4.8. 4-HNE protein adduction in primary mouse islets incubated in GLT media for 5 days.

• 14-3-3 protein gamma	• Gelsolin
• 40S ribosomal protein S27-like	• Glutathione peroxidase 3
• 60 kDa SS-A/Ro ribonucleoprotein	• Glutathione S-transferase Mu 7
• 60S ribosomal protein L38	• Guanine nucleotide-binding protein G
• Actin, alpha cardiac muscle 1	• Guanine nucleotide-binding protein subunit alpha-13
• Actin-related protein 3	• Heat shock 70 kDa protein 1-like
• Activated RNA polymerase II transcriptional coactivator p15	• Heterochromatin protein 1-binding protein 3
• ADP/ATP translocase 1	• Heterogeneous nuclear ribonucleoprotein F
• Alpha-1-antitrypsin 1-6	• Histone acetyltransferase KAT7
• Alpha-2-macroglobulin-P	• Histone deacetylase complex subunit SAP18
• Alpha-actinin-1	• Histone H2A and H2B
• Annexin A4	• Histone H2B type 2-E
• Aspartate aminotransferase, mitochondrial	• Ig heavy chain V region 1-72
• ATP synthase subunit d, mitochondrial	• Ig heavy chain V region 345
• Carboxylesterase 1F	• Ig kappa chain V-V region MOPC 21
• Catenin delta-1	• Inter-alpha-trypsin inhibitor heavy chain H1
• Centromere protein V	• Keratin, type I and II cuticular
• Ceruloplasmin	• Keratin, type I cytoskeletal 15
• Charged multivesicular body protein 4c	• Macrophage migration inhibitory factor
• Chromatin target of PRMT1 protein	• Matrin-3
• Chromobox protein homolog 3	• Methyl-CpG-binding protein 2
• Complement factor B	• NHP2-like protein 1
• Core histone macro-H2A.2	• Nuclear mitotic apparatus protein 1
• Cullin-associated NEDD8-dissociated protein 1	• Nucleolar protein 56/58
• Cysteine and glycine-rich protein 1	• Nucleophosmin
• Cytoskeleton-associated protein 4	• Nucleoside diphosphate kinase A
• Dedicator of cytokinesis protein 11	• Parathymosin
• Dihydropyrimidinase-related protein 2	• Phosphate carrier protein, mitochondrial
• DNA topoisomerase 2-beta	• Plasminogen
• DnaJ homolog subfamily C member 3	• Plectin
• Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	• Polypyrimidine tract-binding protein 1
• E3 ubiquitin-protein ligase RNF181	• Pre-mRNA-processing factor 19/38A
• Enhancer of rudimentary homolog	• Pre-mRNA-splicing factor 38A
• Epiplakin	• Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15
• Eukaryotic initiation factor 4A-III	• Tetratricopeptide repeat protein 30B
• Filamin-B	• Thrombospondin-1
• Frizzled-2	• Transcription initiation factor TFIID subunit 1
• Pre-mRNA-splicing factor CWC22 homolog	• Transmembrane channel-like protein 5
• Profilin-1	• Tropomyosin alpha-1 chain

• Protein AMBP	• U5 small nuclear ribonucleoprotein 200 kDa helicase
• Protein FAM135A	• Unconventional myosin-Ic/Va
• Putative ATP-dependent RNA helicase Pl10	• Vesicle-trafficking protein SEC22b
• Ras GTPase-activating-like protein IQGAP2	• Vesicular integral-membrane protein VIP36
• Ras-related protein Rab-1B/8B	• Sorbin and SH3 domain-containing protein 2
• Ras-related protein Rab-8B	• Spectrin alpha chain, non-erythrocytic 1
• Regulator of G-protein signaling 22	• Splicing factor 3B subunit 1,3&4
• Rho GDP-dissociation inhibitor 1	• Structural maintenance of chromosomes protein 1A
• Ribonuclease inhibitor	• Syntaphilin
• RNA-binding motif protein, X chromosome	• Serine protease HTRA1
• RNA-binding protein FUS	• Serine/arginine repetitive matrix protein 2
• RNA-binding protein with serine-rich domain 1	• Serine/arginine-rich splicing factor 9/10
• rRNA 2'-O-methyltransferase fibrillarin	• Serine/threonine-protein phosphatase PP1-beta catalytic subunit
• Scaffold attachment factor B2	• Neutral alpha-glucosidase AB
• 60S ribosomal protein L10	• Phosphoglycerate mutase 1
• ATP synthase subunit O, mitochondrial	• Poly(rC)-binding protein 1
• Heterogeneous nuclear ribonucleoprotein H2	• Protein transport protein Sec31A
• Heterogeneous nuclear ribonucleoproteins C1/C2	• Secretogranin-1
• Keratin, type I cytoskeletal 28	• Signal recognition particle subunit SRP68
• Keratin, type II cytoskeletal 75	• Small nuclear ribonucleoprotein Sm D1
• N-acetylglucosamine-6-sulfatase	• Sodium channel protein type 11 subunit alpha
• Tubulin beta-2A chain	•

Table 4.9. 3-NT protein adduction in primary mouse islets incubated in GLT media for 5 days.

• 40S ribosomal protein S10/SA	• Dihydrolipoyl dehydrogenase, mitochondrial
• 60S ribosomal protein L24/L6	• ELAV-like protein 1
• Acetyl-CoA acetyltransferase, mitochondrial	• Elongation factor 1-beta
• Actin-related protein 3	• Elongation factor 1-gamma
• Alpha-actinin-1/4	• Endoplasmic reticulum-Golgi intermediate compartment protein 1
• Aly/REF export factor 2	• EPM2A-interacting protein 1
• ATP-dependent RNA helicase A/DDX39A	• Eukaryotic initiation factor 4A-III
• Clusterin	• Glutathione S-transferase P 2
• Coatomer subunit gamma-1	• Prolyl 4-hydroxylase subunit alpha-2
• Complement factor B	• Protein turtle homolog B
• Delta-1-pyrroline-5-carboxylate synthase	• Pyruvate carboxylase, mitochondrial
• Mas-related G-protein coupled receptor member X2	• Ras-related protein Rab-13
• Heterogeneous nuclear ribonucleoprotein H,L,M,C1/C2	• RNA binding motif protein
• Keratin, type I cuticular Ha5	• RNA-binding protein 44

• Keratin, type II cytoskeletal 8	• Small nuclear ribonucleoprotein E
• Methyltransferase-like protein 25	• Small nuclear ribonucleoprotein-associated protein B/N
• Myosin regulatory light chain 12B	• Spliceosome RNA helicase Ddx39b
• Myosin-14	• Splicing factor 3B subunit 3
• Myosin-9	• Stress-70 protein, mitochondrial
• Neuronal migration protein doublecortin	• Synaptotagmin-3
• NHP2-like protein 1	• T-complex protein 1 subunit beta
• Non-POU domain-containing octamer-binding protein	• THO complex subunit 4
• Nuclear factor erythroid 2-related factor 2	• Transforming protein RhoA
• Nuclear receptor coactivator 7	• Transmembrane protein 82
• Nucleobindin-2	• Tubulin beta-2B/3 chain
• Nucleophosmin	• U5 small nuclear ribonucleoprotein 200 kDa helicase
• Poly(rC)-binding protein 1	• Villin-1
• Poly(rC)-binding protein 2	• Vimentin
• Prelamin-A/C	• Voltage-dependent anion-selective channel protein 2
• Pre-mRNA-splicing factor 38A	• Keratin, type II cytoskeletal 7
• 60S ribosomal protein L36a/L7a	• Keratin, type I cytoskeletal 15/18
• ADP/ATP translocase 4	• Phosphoglycerate kinase 2
• ATP synthase protein 8	• Phosphoglycerate mutase 1
• Calmodulin-like protein 3	• Protein ERGIC-53
• Cofilin-1/2	• Protein S100-A11
• Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	• Ras-related protein Rab-15
• DnaJ homolog subfamily B member 11	• Stromelysin-1/2
• Endoplasmic reticulum resident protein 44	• Toll-like receptor 11
• Glial fibrillary acidic protein	• Triosephosphate isomerase
• Heterogeneous nuclear ribonucleoprotein	•

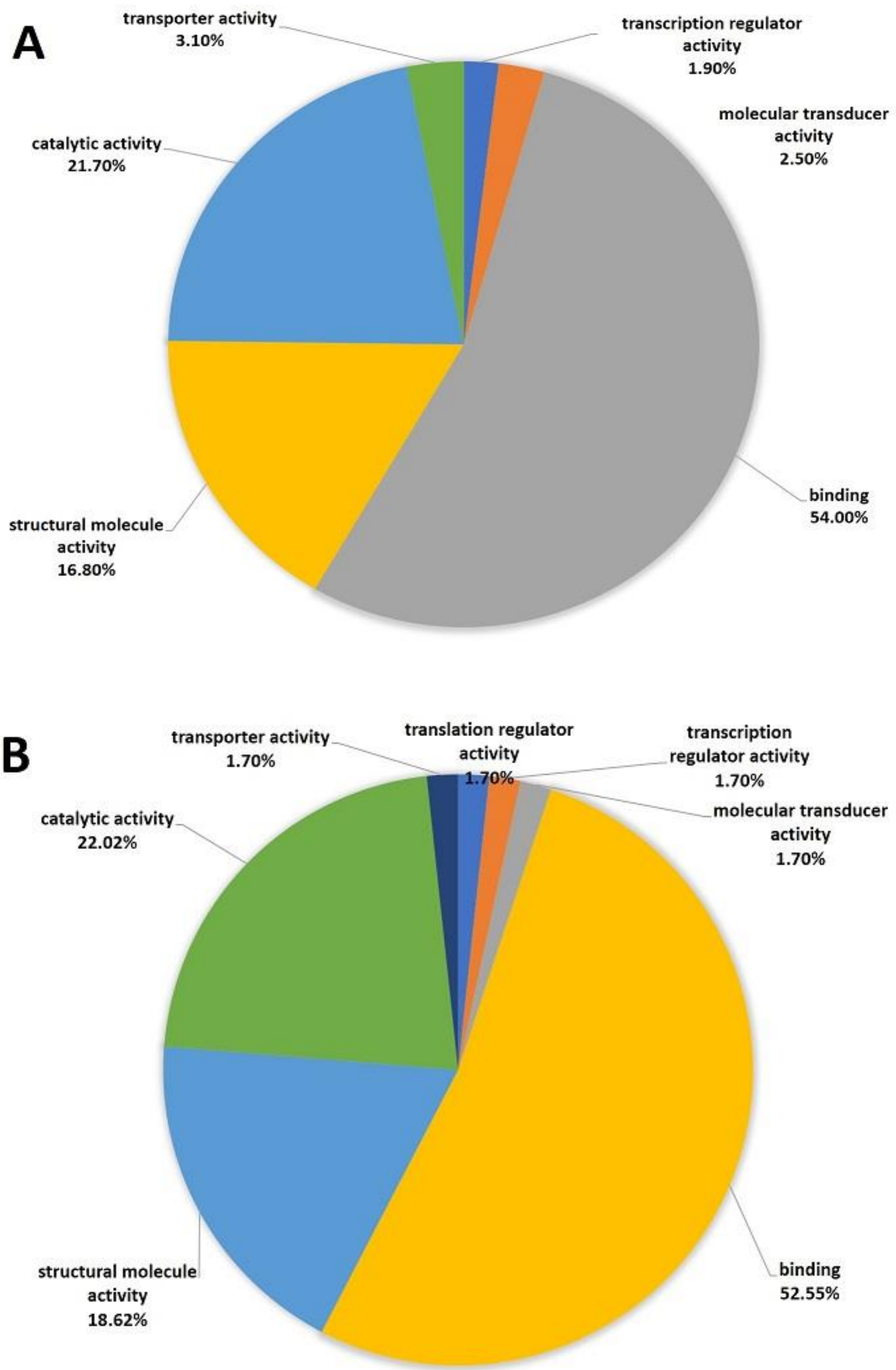


Figure 4.9. Molecular function of 4-HNE (A) and 3-NT (B) adducted proteins. Proteins that were adducted by 4-HNE or 3-NT in mouse primary islets in GLT conditions for 5 days and in which formation is prevented by the addition of carnosine. Generated using the Panther Classification System.

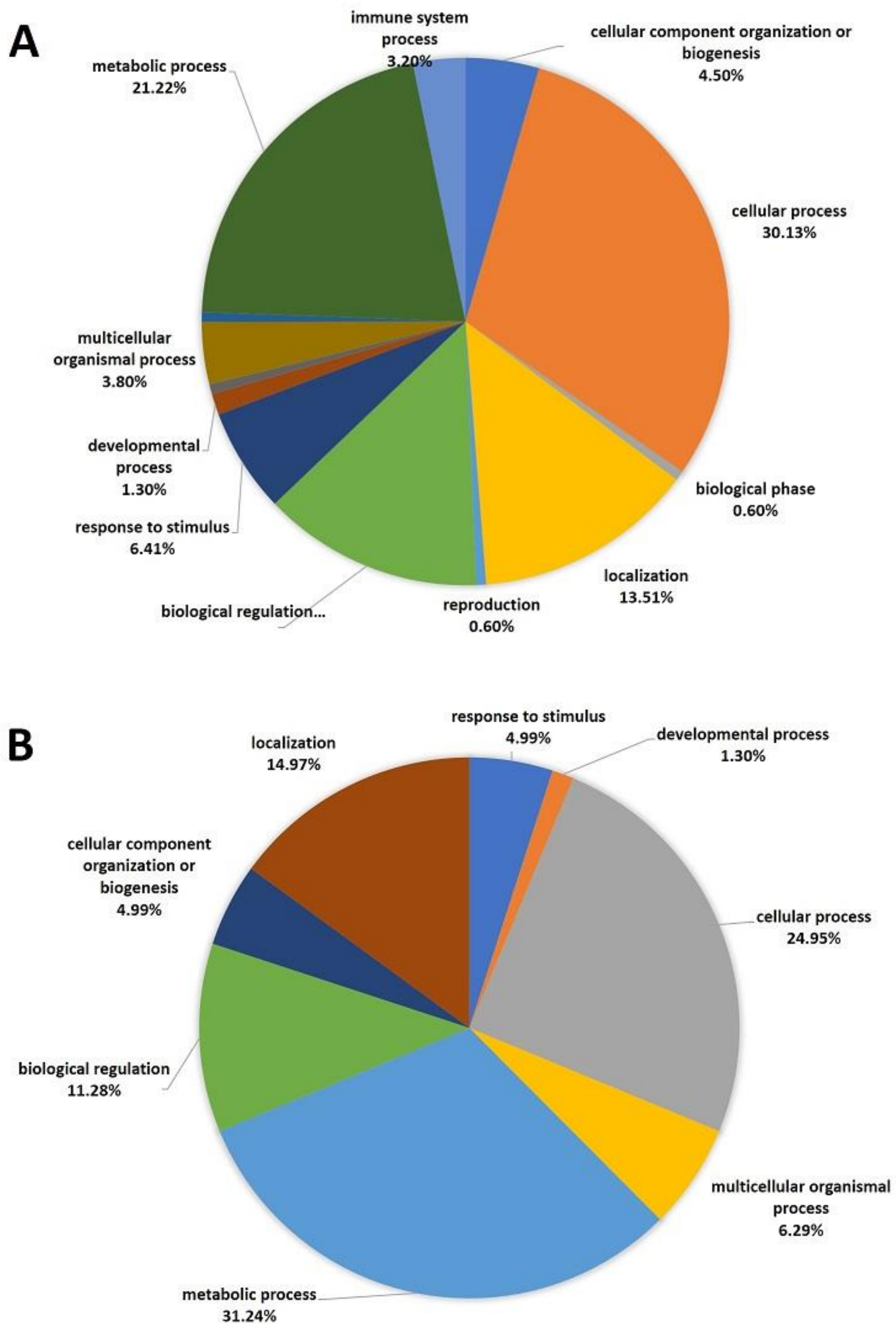


Figure 4.10 Biological process of 4-HNE (A) and 3-NT (B) adducted proteins. Proteins that were adducted by 4-HNE or 3-NT in mouse primary islets in GLT conditions for 5 days and in which formation is prevented by the addition of carnosine. Generated using the Panther Classification System.

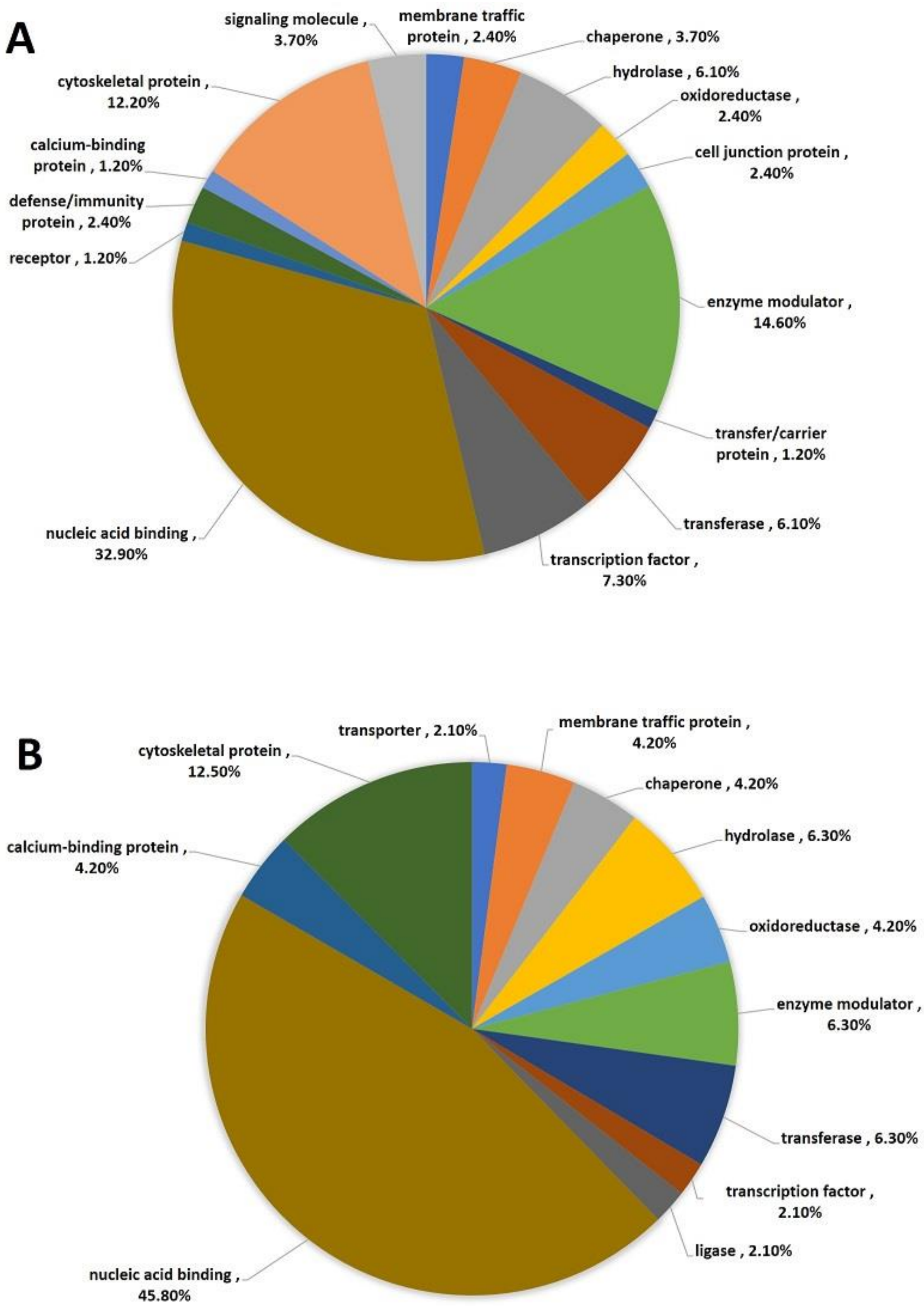


Figure 4.11 Protein class of 4-HNE (A) and 3-NT (B) adducted proteins. Protein class of associated proteins forming adducts with 4-HNE or 3-NT in mouse primary islets incubated in GLT for 5 days and in which formation is prevented by the addition of carnosine. Generated using the Panther Classification System.

Data indicated above showed that both 4-HNE and 3-NT are elevated in skeletal muscle cells incubated in media supplemented with high-glucose and high fatty acid (glucolipotoxicity), as compared to controls. Similar findings were also shown in rat pancreatic β-cells incubated in GLT media. The results obtained from these experiments indicated that 4-HNE and 3-NT are important biomarkers implicated in the impaired functions of these tissues essential to glucose homeostasis. Importantly, however, supplementation of carnosine protected against generation of those cell-damaging species.

The PANTHER classification system (Figure 4.3-4.5) showed that for instance in GLT-treated C2C12 skeletal muscle cells, the majority of the proteins that are adducted by both 4-HNE and 3-NT are involved in catalytic and binding activities and are linked to metabolic processes. In the case of the human skeletal muscle cell-line, Figures 4.6-4.8 showed that the majority of proteins that are adducted by both 3-NT and 4-HNE are also linked to metabolic processes. Using mouse primary islets, most proteins adducted by both 4-HNE and 3-NT (Figures 4.9-4.11) are also linked to metabolic and binding activities.

Having established the 4-HNE and 3-NT protein adductome that is associated with metabolic stress in skeletal muscle and pancreas, it was evident that there are adducted proteins which were either involved in metabolic and cellular processes linked to stimulus-secretion coupling, both in terms of insulin-stimulated GLUT4 translocation to the skeletal muscle plasma membrane, or glucose sensing linked to insulin secretion from pancreatic β-cells. Some of these proteins associated with these processes that were adducted following exposure to glucolipotoxic metabolic stress, but protected from adduction by carnosine, are detailed in Discussion section.

4.2.7 Effect of Carnosine Against GLT-Mediated Mitochondrial Dysfunction

Given the extent of GLT-mediated protein adduction shown in metabolic and mitochondrial proteins, the next step was then to investigate how this might affect mitochondrial function. This was accomplished by employing a Seahorse Mito Stress Test kit (Agilent Tech., USA), and determining the impact of carnosine scavenging action on mitochondrial function. This test measures the mitochondrial oxygen consumption rate of live cells to assess basal, maximal and ATP-linked respiration using three compounds that will be introduced serially in an analyser (Seahorse XFe24). These compounds modulate cellular respiration and specifically target components of the electron transport chain. For specific experiments, myotubes (C2C12, mouse primary muscle cells, and human skeletal muscle cell-line) were treated in either control or GLT media ± 10mM carnosine for 5 days, then analysed according to the protocol detailed in Chapter 2.

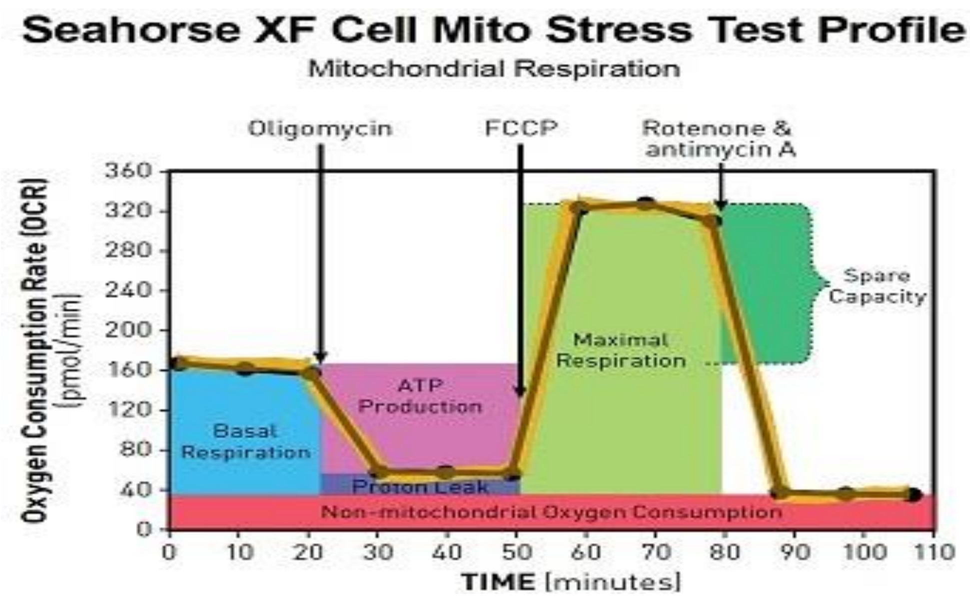


Figure 4.12. Schematic representation of mitochondrial stress test indicating key parameters of mitochondrial function. (Adapted from Agilent Technology, USA)

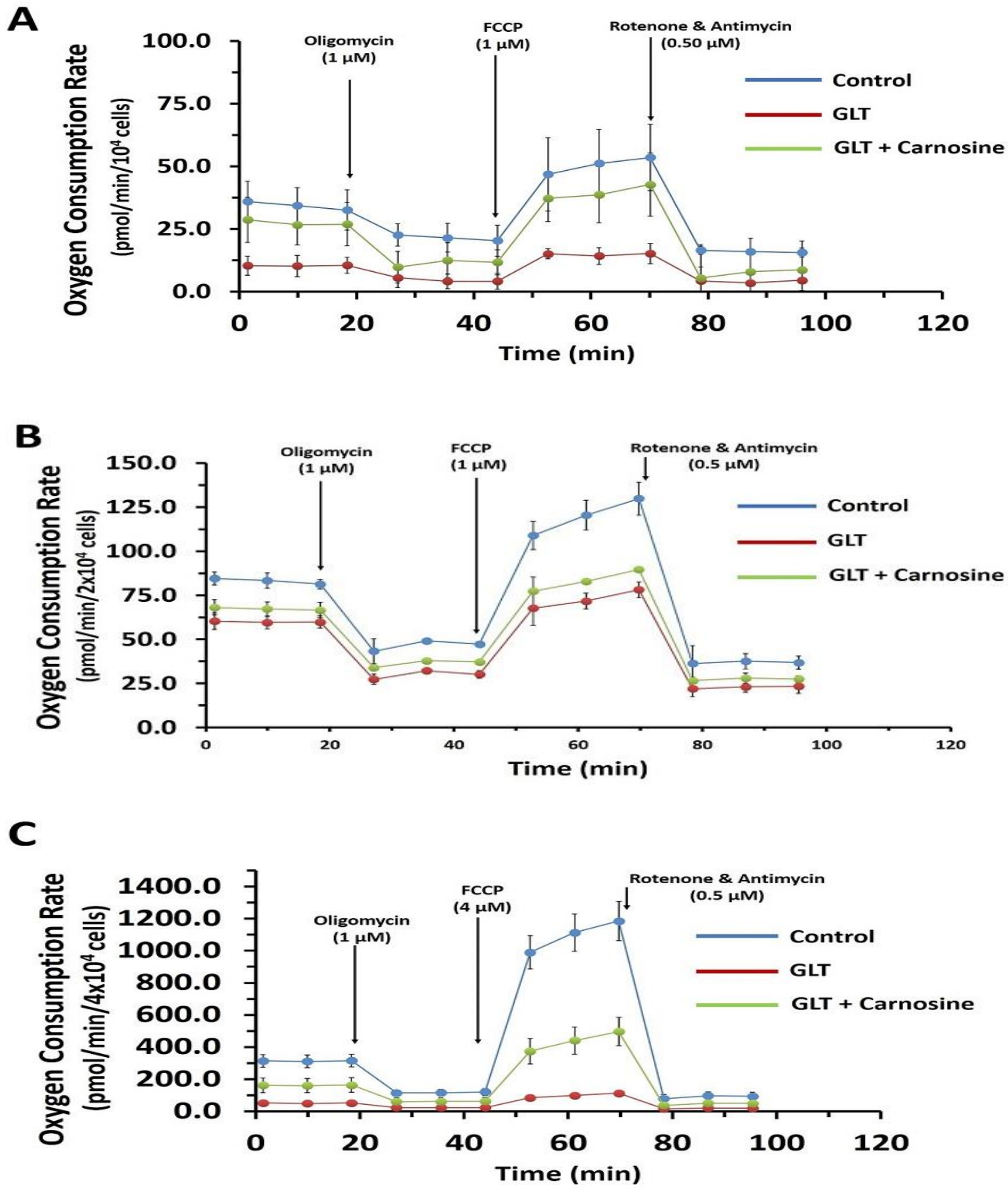


Figure 4.13. The effect of carnosine on mitochondrial respiration of glucolipotoxicity-treated skeletal muscle cells from (A) C2C12, (B) primary mouse, and (C) human skeletal muscle cell-line. Previously treated muscle cells either in Control or GLT±10mM carnosine were seeded at the indicated number of cells in Seahorse XFe24 microplates using supplemented Seahorse XF DMEM and incubated for 24 hrs (last day treatment) and OCR measured from XFe24 Analyser. Each data point represents an OCR measurement, and Figures are representative images from 3-4 independent experiments.

Figure 4.13 indicates that all cells displayed a significant reduction in basal mitochondrial activity when exposed to the GLT treatment suggesting either a reduction in the steady state activity or a dysfunction in total activity. The cells displayed robust mitochondrial respiration under control conditions (blue traces). However, ATP production and mitochondrial respiration were strongly inhibited in these cells that had been incubated in GLT media (red traces). In all cases however, carnosine supplementation (green traces) had a protective action against GLT-mediated dysfunction, albeit this was not fully preventative in restoring respiratory rates to those of healthy control cells.

4.3 Discussion

The regulation of blood glucose level is essential for the human body in order to ensure that energy requirements of vital organs are met. This is achieved by a highly complex network of signalling events involving hormone and neuropeptide signalling and crosstalk involving the brain, pancreas, liver, intestine, adipose and skeletal muscle tissues (Han et al., 2016, Roder et al., 2016). Regulation of the peptides and hormones involved in the pathways controlling glucose homeostasis is therefore of paramount importance, and failure to maintain this may lead to metabolic disorders such as type 2 diabetes (Gonzalez et al, 2018).

One way in which the aforementioned pathways can become disrupted is through the formation of advanced glycation (AGE) and advanced lipidation (ALE) end products. Non-enzymatic reactions with glucose were first reported over a century ago (Maillard, 1912), and adduction with these end-products potentially leads to the modification of protein, lipid, or DNA structure. This then alters their functional capacity, typically rendering these molecules less efficient or non-functional, and consequently this therefore represents a particular problem for individuals with diabetes (Vlassara et al,1994). Despite this knowledge however, there are currently few therapeutic strategies to treat diabetes that directly target these modifications, with conventional therapies instead seeking to improve glycaemic control through other mechanisms. Crucially, even when diabetes is well controlled individuals with T2DM still have elevated levels of glycated haemoglobin in comparison to non-diabetic healthy individuals. Therefore, strategies that can effectively combat reactive species associated with glucolipotoxicity would potentially have a unique capability to directly reduce the incidence of diabetes complications, even when T2DM is otherwise well managed.

In order to better understand the relationship between glucolipotoxic metabolic stress and both obesity and diabetes, this PhD study sought to identify all proteins modified by 4-HNE or 3-NT adduction in serum from individuals with obesity, T2DM and gestational diabetes mellitus (GDM). Serum from obese non-diabetic patients contains both 4-HNE and 3-NT adducts of proteins involved in several immune and cellular functions, and their regulation. Among these are apolipoproteins, proteins which once adducted would likely result in defective lipid transport, uptake and clearance. For example, defects that result in apolipoprotein A-II deficiency, one of the proteins that we found to be adducted, have been reported to result hypercholesterolemia (Al-Allaf et al, 2015). Adductions of this nature are therefore highly likely to contribute to the development of atherosclerosis and coronary heart disease in people with obesity.

Fibrinogen was another protein adducted by both 4-HNE and 3-NT in the serum of obese non-diabetics. Reduced and/or dysfunctional fibrinogen occurs in various congenital and acquired human fibrinogen-related disorders and can result in liver and kidney disease (de Moerloose et al., 2013). These disorders represent a group of conditions in which individuals may present with severe episodes of pathological bleeding and thrombosis. We also showed 3-NT adduction of coagulation factor XII, which is involved in blood clotting, and plasminogen, the precursor of plasmin, which is responsible for fibrin clot degradation. Similarly, there was 3-NT adduction of vitamin K-dependent protein S, a protein which, when present in the blood at reduced levels, results in increased risk of thrombosis (Beauchamp et al., 2004). As obesity has previously been shown to increase the propensity to thrombosis, the leading cause of death in the Western World, through pathways associated with inflammation, oxidative stress, dyslipidaemia, insulin resistance and the coagulation cascade (Darvall et al., 2007), we suggest that metabolic stress-driven adduction may be a unifying mechanistic basis for these pathologies, and that carnosine may be able to reverse this.

Obese non-diabetic serum contained 3-NT adducts to a few members of the complement system, which enhances the ability of antibodies and phagocytic cells to attack pathogens and clear microbes and damaged cells from the bloodstream. This is part of a wider pattern of 4-HNE and 3-NT adduction of immunoglobulin heavy and light chain regions. Together these events would impair the body's adaptive immune response, thereby leaving these individuals immunocompromised, and hence susceptible to attack from foreign substances and pathogens, including viral infections such as Covid-19.

Histone H2A, H2B, H3 and H4 are all adducted by 4-HNE in the serum of obese non-diabetic individuals. In addition, histone H3 is also adduced by 3-NT. As histones are the chief protein component of chromatin, this has important implications for DNA packing and access to factors regulating gene expression, be that either directly as part of the transcriptional machinery or indirectly (epigenetics) through allowing access to molecules that induce further chemical modification of histone or DNA structure, such as acetylation and methylation.

The number of adducted proteins observed in obese T2DM patient serum was double than that observed in obese non-diabetic serum, but a similar pattern nevertheless emerges when one examines the specific protein functions. In particular, adduction of proteins involved in atherosclerosis and cardiovascular disease, blood clotting, and immune function were also observed. In addition, another group of proteins also shown to be adducted by both 4-HNE and 3-NT in all serum samples are the extracellular matrix proteins, with hyperglycaemia-induced alterations of extracellular matrix proteins importantly have been being shown to be associated with renal dysfunction and compromised cardiac function (Law et al., 2012).

By contrast to the other groups studied, there were fewer adduction events in serum of patients with GDM, perhaps that this is a temporary condition rather than one involving chronic exposure to hyperglycaemia. Nevertheless, 4-HNE adduction of multiple core histone H2A molecules, 3-NT adduction of complement proteins, and extensive immunoglobulin heavy and light chain adduction by both 4-HNE and 3-NT were observed. As it has been hypothesised that epigenetic mechanisms contribute to the effect of GDM on offspring adiposity and type 2 diabetes (Elliot et al., 2019), AGE/ALE adduction of histones could provide a possible modifiable mechanism to help reduce type 2 diabetes in the next generation, with carnosine and possibly other reactive species scavengers potential tools to achieve this goal.

Given the nature and the extent of the potentially damaging protein modifications shown in the serum of patients with obesity and diabetes, this PhD work also aimed to determine how nutrient overload from the combined effects of hyperglycaemia and hyperlipidaemia might affect the two main cell types regulating glucose homeostasis, namely skeletal muscle and pancreatic β-cells. Similarly with serum experiments, adduction was determined following immunoprecipitation with either anti-4-HNE or anti-3-NT antibody, and subsequent mass spectrometry peptide analysis. In addition, as live cells were used in the analysis at this point, it would then now possible to determine how specific individual protein adduction events might be influenced by the presence of carnosine, a histidine-containing dipeptide that we have previously shown to enhance both insulin secretion and glucose uptake (Cripps et al, 2017).

PANTHER software was used to determine specific cellular and molecular pathways most influenced by carnosine supplementation. With regard to C2C12 myotubes, HSkM cells, and primary islets, the majority of proteins that were adducted by both 4-HNE and 3-NT are involved in catalytic and binding activities and are linked to metabolic processes. Further analysis by protein class also revealed that several metabolic enzyme classes are protected from glucolipotoxic damage by carnosine. As numerous membrane protein trafficking and cytoskeletal proteins were also protected from adduction by carnosine, this is indicative of carnosine having protective actions along the stimulus-secretion coupling pathway. It is also worth noting that in HSkM cells and primary islets, nucleic acid binding was the single main protein class of molecule protected, suggesting that carnosine might also be able to prevent some of the dysregulation in gene expression that is associated with metabolic stress, either directly through preserving the function of transcription factors and associated transcriptional and translational machinery, or indirectly through histone structure and epigenetic mechanisms.

Glucose is the primary energy source for most cells in the body, making it an essential nutrient. However, it cannot freely cross the plasma membrane and thus requires a carrier-mediated system in order to promote cellular glucose entry. Glucose transport into most tissues is achieved by the action of glucose transporters, which transport glucose by facilitative diffusion down concentration gradients. In the brain and pancreas this is an ongoing constitutive process that is primarily facilitated by GLUT1 and GLUT2 transporters, thereby ensuring that these cells can constantly sense their extracellular environment and rapidly adapt to change. By contrast, in skeletal muscle in the basal (unstimulated) state, glucose transport is very low, with >90% of the glucose transporters internally sequestered in intercellular compartments, such as endosomes, the trans-Golgi network and GLUT4-specialised vesicles (Martin et al., 1996). After consuming a meal, however, insulin is released from pancreatic β-cells, and glucose uptake is rapidly stimulated via GLUT4 transporters that translocate from their intracellular storage pool to the cell surface, whereupon the vesicles carrying these transporters fuse with the plasma membrane by a process of exocytosis (Rea and James, 1997). Once integrated into the plasma membrane, they are then able to effect glucose transport into the cell. Upon termination of the insulin stimulus, glucose transporters are recycled back from the plasma membrane to the intracellular pool, ready for the next insulin stimulus (Wardzala et al., 1978).

Defects in the translocation process occur in insulin resistance, T2DM, and metabolic syndrome. In order to facilitate GLUT4 translocation and glucose uptake into skeletal muscle, the initial step in stimulus-secretion coupling in these cells is insulin signalling (Czech, 1995; Summers et al., 1999). We showed that a number of proteins that either regulate insulin sensitivity or else have an interaction with components of the insulin signalling pathway are adducted by 4-HNE. Encouragingly, however, proteins including serine-threonine protein kinases and phosphatases, mitogen-activated protein kinase, and the TBC1 domain family could be protected by carnosine supplementation.

Mitochondrial dysfunction has been shown to result from sustained exposure to elevated ROS levels (Evans et al, 2002), such as that typically seen in patients with

T2DM or obesity, with both obesity and high fat diets resulting in increased production of hydrogen peroxide (Anderson et al., 2009). 3-NT levels are significantly higher in the small vessels of the skeletal muscles of patients with mitochondrial respiratory chain dysfunction compared with healthy controls (Vatemmi et al., 2011). This led to the further identification of proteins that have been affected by tyrosine nitration – proteins that mostly have important roles in energy metabolism and mainly localise in the mitochondria, including aconitate hydratase, ATP synthase β-chain subunit, pyruvate dehydrogenase E1 component and succinate dehydrogenase. These modifications would explain the observed functional changes of the mitochondria.

Pyruvate dehydrogenase enzymes, which are important for glucose utilization and in maintaining a supply of acetyl-CoA for the mitochondrial activity (Lee, 2014), were adducted in C2C12 myotubes and HSkM cells under metabolic stress, but not in the presence of carnosine. Other mitochondrial enzymes adducted following exposure to metabolic stress, but protected from these adduction events by carnosine, included ATP synthase, aconitate hydratase, citrate synthase, cytochrome b-c1 complex subunit 1, dihydrolipoyl dehydrogenase, electron transfer flavoprotein subunit, enoyl-CoA hydratase, isocitrate dehydrogenase, malate dehydrogenase, peroxiredoxin, pyruvate carboxylase, stress-70 protein, succinate dehydrogenase [ubiquinone] flavoprotein subunit, superoxide dismutase, and thioredoxin-dependent peroxide reductase. Of these, isocitrate dehydrogenase is of particular interest given that it has been considered a promising therapeutic target to counteract T2DM and obesity-related metabolic disorders, and purportedly has a role in modulating both insulin sensitivity and substrate metabolism (Lee et al., 2016). In addition, cytochrome b-c1 complex subunit 1 is an integral part of the mitochondrial respiratory chain that catalyses the oxidation of ubihydroquinone and the reduction of cytochrome c, which contribute to ATP synthesis. As such, defects in key regions such as catalytic sites could result in mitochondrial myopathy that could, in turn, enhance superoxide production (Crofts, 2004). This situation would also be further exacerbated by adduction of the antioxidant enzyme, superoxide dismutase, which is consistent with our findings showing changes in the maximal OCR. Peroxiredoxin is another antioxidant enzyme that is targeted by 3-NT adduction, and peroxiredoxin-knockout mice have previously been shown to have impaired insulin signalling and reduced muscle glucose uptake that is associated with overt hyperglycaemia in T2DM (Pacifici et al., 2014).

Fewer mitochondrial enzymes were adducted in pancreatic islets, but of those observed, the activity of pyruvate carboxylase has a direct role in pancreatic β-cell adaptation to insulin resistance. Indeed, reduction of its activity has previously been reported in animal models of T2DM and this had a negative impact on β-cell secretory capacity (Xu et al, 2008). Other islet mitochondrial proteins with catalytic activities include acetyl-CoA acetyltransferase, and dihydrolipoyl dehydrogenase.

As a result of increased glucose uptake by pancreatic β-cell GLUT transporters, such as that seen following digestion of food, the resulting glucose metabolism leads to an elevated ATP/ADP ratio that causes K_{ATP} channels to close. This in turn results in β-cell membrane depolarisation and opening of L_c-type Ca^{2+} channels that drive Ca^{2+}-dependent exocytosis of insulin-containing secretory granules (Hou et al, 2009). Crucially, a number of protein trafficking molecules that carnosine was able to prevent from becoming adducted in primary islets under metabolic stress were observed, indicating that this may be the mechanism by which casrnosine enhances insulin secretion in β-cells exposed to glucolipotoxic stress (Cripps et al, 2017). Interestingly, one of the proteins that we see adducted, but protected by carnosine, is endoplasmic reticulum-Golgi intermediate compartment protein 1 (ERGIC). Vesicular integral-membrane protein 36 is also similarly adducted, and this has been shown to be involved in ERGIC-mediated cargo transport through the early secretory pathway (Dahm et al., 2001). This suggests that defective ERGIC transport might be one reason why reduced insulin content is found in β-cells under oxidative stress (Kajimoto and Kaneto, 2004), with our data suggesting that carnosine may, therefore, be able to reverse this defect.

Oxidative damage will also be further impacted by our observed adduction of the transcription factor Nrf2 in islets under glucolipotoxic stress, as this is known to be the master regulator of numerous genes encoding antioxidant, detoxifying, and cytoprotective molecules in humans (Masuda et al., 2015). This is also an emerging target for pharmacological strategies designed to combat oxidative stress in islet transplantation (Jarrin-Lopex et al., 2020). Therefore, our finding that carnosine is able to protect this molecule from the damage associated with metabolic stress, suggests that carnosine could be of benefit not only to patients with T2DM, but could also be utilised to help extend survival and quality of islets under the oxidative stress associated with transplantation.

Ras-related proteins recruit effectors and help in controlling tethering and docking of secretory vesicles and also play a role in stimulating insulin secretion (Xiong et al., 2017). There are also binding proteins that are essential for tight control of insulin secretion and in improving its morphology and function which are also adducted by 3-NT. For instance, clusterin, which induces and promotes cytodifferentiation of endocrine islet cells (Kim et al., 2006). Another protein class adducted by 4-HNE is a family of molecules called G-protein coupled receptors (GPCRs). These are known to be involved in islet cell signaling and in regulating insulin secretion, and thus could potentially become a new target for T2DM treatment (Layden et al., 2010; Cripps et al, 2020).

Coatomer is also an essential constituent component of vesicle transport through the early secretory pathway, and coatomer subunit gamma-1 has an important role to play in COP-I coat formation (Wegmann et al., 2004). This is also a protein that was adducted in islets, but protected from adduction by carnosine supplementation in the current study. Additionally, SEC22 (a protein that interacts with COP-II coatomer during ER to Golgi transport; Springer and Schekman, 1998) adduction was also shown. Coatomer-associated vesicular transport through the early secretory pathway is also dependent upon the small monomeric GTPase, Rab1B (Peter et al, 1993), a protein that mediates vesicular transport between ER and Golgi (Pluttner et al, 1991) and which was adducted in islets but protected by carnosine in the current study. Small monomeric GTPases act as molecular switches, alternating between the active GTP-bound form and the inactive GDP-bound form, a process facilitated by guanine nucleotide exchange factors and GTPase-activating proteins (Li and Marlin, 2015). In order to maintain activity, all such GTPases, including Rab1B, therefore require multiple effectors (Martinez et al, 2016). Crucially, we showed that a number of guanine nucleotide-binding proteins, GTPase activators, and GDP dissociation inhibitors were adducted in the presence of metabolic stress, but were protected by carnosine. Adduction of Rab13, a GTPase that is associated with tight junctions in polarized cells, and with cytoplasmic vesicular transport in non-polarised cells (Zahraoui et al, 1994) was also shown and is likely associated with exocytosis, as has been reported for insulin-stimulated GLUT4 translocation in skeletal muscle cells (Sun et al, 2010).

The final stage in stimulus-secretion coupling in β-cells is Ca^{2+}-dependent exocytosis. Whilst the composition of the core machinery of the SNARE-mediated fusion event is generally accepted, identification of the Ca^{2+}-sensors linked to exocytosis in these cells is more controversial (Aganna et al, 2006). In particular, a number of studies have advocated roles for different members of the synaptotagmin family in insulin secretion. Multiple studies have, however, shown that synaptotagmin III is part of the functional protein complex regulating beta-cell exocytosis (Mizuta et al, 1997; Brown et al, 2000; Gao et al, 2000; Aganna et al, 2006), with its overexpression increasing insulin secretion (Gao et al, 2000) and synaptotagmin III-specific antibodies inhibiting exocytosis from permeabilised β-cells (Mizuta et al, 1997, Brown et al, 2000). We find this protein to be adducted in islets incubated in GLT media, but to be protected from adduction by carnosine supplementation.

There are numerous stimulus-secretion coupling protein adduction parallels between our data for islets and skeletal muscle. For instance, there are several adducted proteins that function in the early secretory pathway. The ER performs molecular chaperone activities, as well as participating in protein glycosylation. Quality control is mediated by proteins that include protein disulfide-isomerase, the related family member endoplasmic reticulum resident protein 44, and the ER chaperone protein BiP, all of which were adducted in HSkM cells, but protected by carnosine in the current study. Other proteins with catalytic activities that were adducted in GLT-treated skeletal muscle cells include guanine-nucleotide binding proteins, creatine kinase B type, Ras-related proteins, protein disulphide isomerase, and sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA). Impaired function of SERCA in muscle affects its quality and quantity and triggers ER stress, which would also promote the progression of insulin resistance in obesity and diabetes (Kang et al., 2016; Qaisar et al., 2018).

ER cargo transport is also likely to be affected by the HSkM myotube adduction of Rab1A, and the C2C12 myotube adduction of the GTP-binding protein Sar1, as well as VAT-1 (a soluble protein involved in multiple vesicular transport steps; Watanabe et al, 2020) adduction shown herein. Further adduction comes via vesicle-associated membrane protein-associated protein B, a protein that functions as an adaptor protein to recruit target proteins to the ER and to execute various cellular functions, including lipid transport, membrane traffic, and ER stress (Lev et al, 2008). We also see adduction in both skeletal muscle cell types of ADP-ribosylation factor 1, a protein associated with coatomer function in intra-Golgi trafficking in skeletal muscle cells (Nori et al, 2004).

The cytoskeleton is also a feature of skeletal muscle adduction, with α-actinin, actin, and actin-related molecules each being adducted both in HSkM cells and C2C12 myotubes. As was shown in pancreatic islets, we also see adduction, and carnosine protection, of tubulin, spectrin, and other actin-interacting proteins. GLUT4 exocytosis, and subsequent recycling, is known to involve the actin cytoskeleton, myosin motors, and several Rab GTPases, as well as the exocytotic machinery itself (Stöckli et al, 2011). The myosins, Rab5 and 11 (thought to regulate endosomal and/or TGN trafficking, as well as GLUT4 recycling; Kessler et al, 2000; Huang et al, 2001) were included in our skeletal muscle adduction and carnosine protection dataset, as were vacuolar protein sorting-associated proteins 18 and 35 (thought to direct endosomal membrane remodelling and scission; Schöneberg et al, 2017). In addition, we showed that Rab8, which is implicated in GLUT4 vesicle exocytosis (Ishikura et al, 2007; Sun et al, 2010), and several guanine nucleotide-binding proteins, GTPase activators, and GDP dissociation inhibitors were all adducted by metabolic stress, but protected by carnosine.

Seahorse OCR values indicated that GLT compromised cellular mitochondrial respiration rate, resulting in a diminished ability to meet endogenous ATP demand, to drive synthesis of ATP, and to maintain mitochondrial membrane potential, all of which are vital for an effective and normal substrate metabolism. Furthermore, the data also suggest the possible negative effects that GLT-mediated OS or damaging

4-HNE and 3-NT adduction have upon key molecules related to insulin signaling or insulin secretion. There is clearly widespread protein adduction throughout the stimulus-secretion coupling pathway of pancreatic islets and skeletal muscle following exposure to metabolic stress. As carnosine is able to prevent 65-90% of the respective 3-NT and 4-HNE adduction events measured, this PhD work hypothesised that the mitochondrial dysfunction that is seen in insulin resistance (Kelley and Mandarino, 2000) and T2DM (Rovira-Llopis et al., 2017) and is known to result from oxidative stress associated with nutrient excess (Evans et al, 2002; Rovira-Llopis et al, 2017), is likely the result of protein dysfunction resulting from AGE/ALE adduction, and that the protective action of carnosine in preventing/reducing damaging adduction of mitochondrial proteins could, however, offer important therapeutic benefits and, should at the very least, delay the onset of mitochondrial dysfunction in T2DM.

Adduction data presented herein indicates target molecules that could be utilised for early detection and/or therapeutic interventions for those who have, or are at risk of developing, T2DM. The data also indicates the potential use of carnosine as a tool to prevent these damaging adductions. However, despite the promising role of carnosine as a supplement, its action would likely require regular administration at high dosage, due to the presence of tissue and serum carnosinase enzymes that catalyse carnosine turnover. One possible strategy to offset this limitation would be to design or synthesise carnosinase inhibitors and carnosine mimetics that are stable against the hydrolytic action of carnosinases. This forms the premise for work presented in Chapter 5.

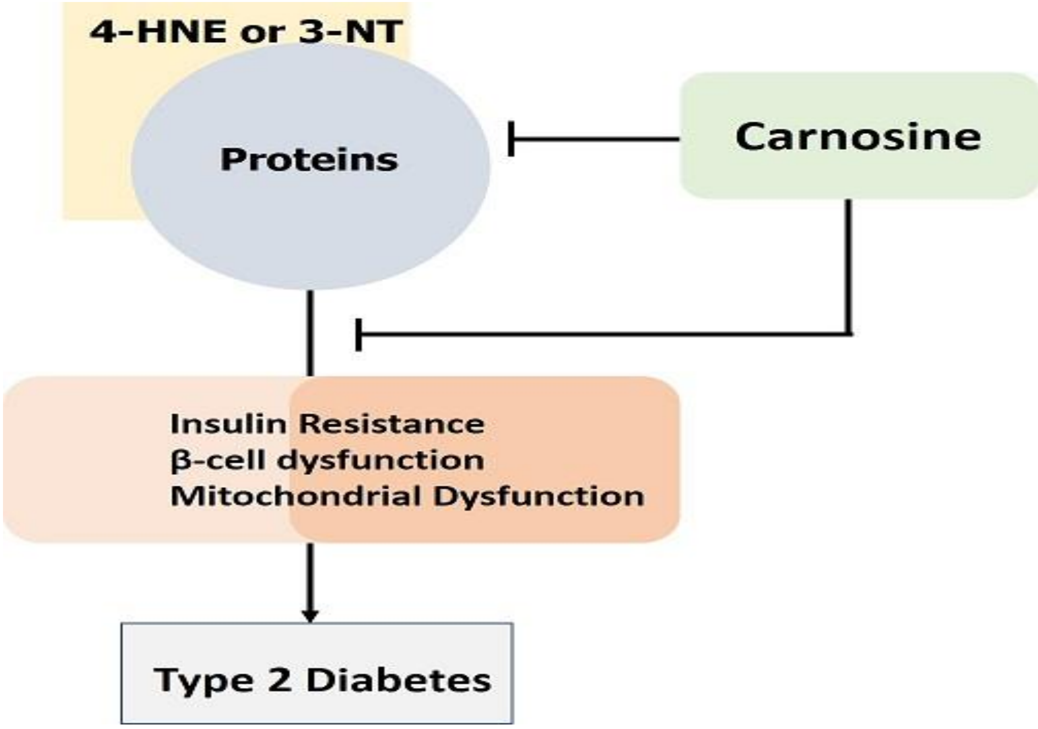


Figure 4.14; A schematic representation of how carnosine in this Ph.D. work could potentially protect cells from impaired insulin signaling or diminished insulin production and preserve mitochondrial function via quenching 3-NT and 4-HNE thereby inhibiting them to crosslink and form adducts with key proteins and enzymes affecting their functionalities and activities.

Chapter 5

Evaluation of Carnosine-Derivative Molecules and β-Alanine Supplementation as Potential Candidates for the Treatment of Type 2 Diabetes

5.1 Introduction

The overproduction of reactive species and/or a decrease in the body's antioxidant defences are associated with a number of chronic diseases, including diabetes, and the elevated incidence of type 2 diabetes has inspired the search for new drug candidates. One emerging strategy relates to the identification of compounds with antioxidant profiles, either from a natural or synthetic source. Another promising research direction is to design molecules, as either derivative of bioactive naturally-occurring substances or as novel compounds that could exert therapeutic actions. The Turner laboratory reported a natural dipeptide, carnosine, to be a promising agent for regulating glucose homeostasis through enhancement of both insulin secretion by the pancreas, and glucose uptake to skeletal muscle cells (Cripps et al., 2017).

Despite carnosine's promising potential as a therapeutic agent for oxidative-based diseases, its effectiveness is limited in humans due to the presence of serum and tissue carnosinase enzymes that catalyse the hydrolysis of carnosine to its amino acid components (Vistoli et al., 2012; Teufel et al., 2003). Upon oral ingestion, carnosine can be absorbed in the small intestine via PEPT1 transporters (oligopeptide transporter 1), with approximately 14% being transported intact (Gardner et al., 1991). Carnosine is then either hydrolysed by carnosinase-2 (CN2) in intestinal cells, or else further transported to the circulation where it is potentially susceptible to hydrolysis by carnosinase-1 (CN1) before it reaches any peripheral tissues to render its beneficial action (Quinn et al., 1992; Boldyrev et al., 2013). Given this limitation of carnosine, one possible solution to counteract this rapid hydrolysis is regular administration of high doses of exogenous carnosine in order to increase the available circulating level of carnosine in the system that is then able to elicit a biological effect.

Although reported to be safe during long-term administration due to the excess that could be cleaved by carnosinase, this mode, however, could nevertheless exert negative effects in rare cases such as carnosinemia (deficient of carnosinase), which is believed to have been associated with certain neurological disorders (Willi et al., 1997, Boldyrev, 2009). An alternative route to achieve the desired beneficial action is therefore desirable, and this forms the basis for the latter part of this section.

Investigations of the physiological roles of carnosine have mainly been directed towards skeletal muscle, as this is the tissue with the highest carnosine concentration. Initial findings indicated an anti-fatigue effect that is believed to be due to its pH-buffering capacity, or its proton sequestering effect during acidosis-associated muscular contractile fatigue. This might, therefore, be the reason for its high abundance in skeletal muscle (Smith, 1938; Sahlin, 1980; Allen and Westerblad, 2008). The level of carnosine in tissues is also controlled by the balance between carnosine synthase and carnosinase enzymes (Pegova et al., 2000). Consistent with this hypothesis, a study by Baguet et al.,(2010) showed that increased carnosine content in muscle due to nutritional supplementation of β-alanine attenuated the degree of acidosis in the blood during high-intensity exercise. Similarly, increased total muscle buffering capacity was observed after significant increase of carnosine content in skeletal muscle due to ingestion of β-alanine for 2-4 weeks, and this has the potential to elicit improvements in physical performance during high-intensity exercise (Sale et al., 2009; Hill et al., 2006). Supplementation of β-alanine does not only contribute to increasing the carnosine concentration but more importantly, rather than L-histidine, its availability is the rate-limiting factor for the synthesis of carnosine (Dunnet and Harris, 2009) through the reaction catalysed by carnosine synthase (CARNS). Given this available evidence on the effects of β-alanine supplementation on muscle carnosine synthesis, it follows that this strategy could potentially also exert beneficial actions upon glucose homeostasis in T2DM.

Carnosine is the best substrate for the human serum carnosinase, with a rate of 50-200 fold higher as compared to other histidine-containing dipeptides (HCDs) (Peters et al., 2011). Importantly, a study involving diabetic mice and humans showed that under diabetic conditions, increased carnosinase-1 activity might be due to post-translational modification of this gene through carbonylation and s-nitrosylation reactions that are influenced by the reactive metabolite methylglyoxal (MG) (Peters et al., 2015). Furthermore, reactive oxygen and nitrogen species could also increase carnosinase activity (Peters et al., 2011 and Peters et al., 2018). This being the case, in diabetic patients where tissue damage is driven by glucolipotoxicity, it might be possible to offset high serum or tissue carnosinase activity by pharmacologically inhibiting these hydrolysing enzymes in order to maximise the potential benefits of oral carnosine supplementation.

This chapter aims to evaluate whether supplementation of β-alanine could have a similar beneficial effect to that of carnosine scavenging of glucolipotoxic intracellular reactive species, and if so, to determine whether this would enhance glucose uptake or improve insulin sensitivity in GLT-induced insulin-resistant skeletal muscle cells. Secondly, this chapter also aims to identify potential drug candidates that are carnosinase inhibitors designed through *in silico* screening and docking techniques, or bespoke synthesis of hydrolysis-resistant carnosine mimetics – achieved through collaboration with Dr. Christopher Garner and Prof. John Wallis (Chemistry, NTU). Thereafter these compounds will be screened for their ability to potentiate and sustain the action of endogenous carnosine, or to augment its known biological action, and importantly to improve its limitation as a therapeutic agent. Amongst the screened compounds, selected molecules will then be utilised for further *in vivo* studies which are an integral part of the future direction of this PhD work – through collaboration with Dr. Paul Caton of Kings College London. Either or both strategies from this work, if successful, will provide essential steps to create future therapeutic perspectives in oxidative-based diseases through a rational design of potent and selective quenchers of reactive species deleterious to the normal functions of cells and tissues.

5.2 Results

5.2.1 Identification of Carnosine Synthase Expression in C2C12 Myotubes

Carnosine is synthesized from β-alanine and L-histidine by an ATP-dependent synthase known as ATP-grasp domain-containing protein 1 (Drozak et al., 2010). Preliminary data from the Turner laboratory group has shown it to be expressed at a low level (data not shown, unpublished) in INS-1 β-cells, thereby indicating that β-cells are likely capable of synthesising carnosine. Consistent with this, supplementation of β-cells with the rate-limiting β-alanine had a positive impact upon insulin secretion. I therefore sought to determine whether glucolipotoxicity would affect the level of this enzyme in C2C12 muscle cells.

In order to accomplish this, C2C12 myotubes were cultured in DMEM media with and without GLT treatment for 5 days, after which cell lysates were generated in RIPA buffer and the resulting protein samples separated using SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted using Ab against ATPGD-1 / carnosine synthase (Santa Cruz, USA). As shown in Figure 5.1, the enzyme is expressed in skeletal muscle cells, where two isoforms were detected - namely ATPGD1 isoform 1 at 88 kDa, and isoform 2 at about 37 kDa. It was also observed that there was no significant difference in the expression level of the enzyme in C2C12 cells cultured in

GLT media. However, this warrants further investigation in animal models of diabetes, and thus forms an additional goal for future research.

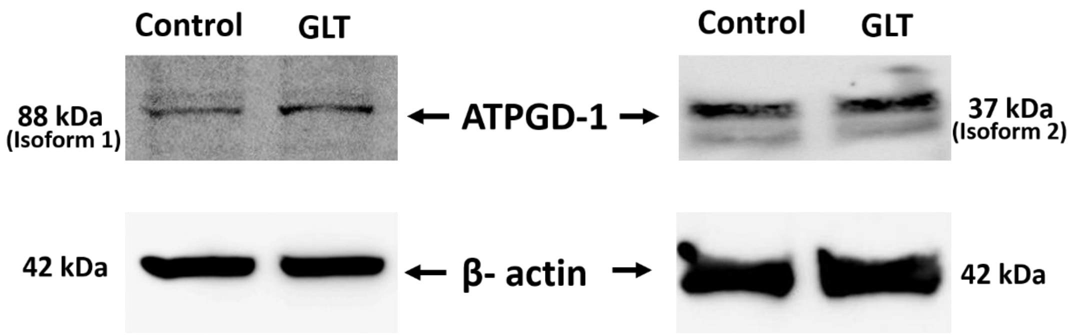


Figure 5.1; ATP-grasp domain-containing protein 1 is expressed in muscle cells. C2C12 myotubes were incubated in control or GLT media supplemented for 5 days. Cells were then lysed to extract proteins and then separated via SDS-PAGE, transferred to nitrocellulose and detected using anti-ATPGD-1 or anti-actin antibody. Data expressed as mean ± SEM from 3 independent experiments. *$p < 0.05$.

5.2.2 Effect of β-Alanine Supplementation on Scavenging of Glucolipotoxic Intracellular Reactive Species in C2C12 Myotubes

Skeletal muscle is able to take up β-alanine via a specific β-amino acid transport protein (Miyamoto et al., 1990), and this could potentially contribute to the increase in skeletal muscle carnosine concentration following supplementation of this amino acid that has been reported in several studies (Hill et al., 2007; Saunders et al., 2017). The present study thus sought to determine whether β-alanine supplementation could also exert scavenging action towards reactive species, similar to that seen with carnosine in my previous experiments. In order to answer this, C2C12 myotubes were treated with control or GLT media for 5 days ± 10 mM β-alanine. Intracellular reactive species were estimated based on fluorescence intensity using DCFDA dye.

Figure 5.2 shows that GLT-exposed C2C12 cells had a higher level of reactive species, with an increase of 87.25 ± 15.33 % compared to control. Whilst β-alanine supplementation had no significant effect in the control condition, it significantly lowered the increase of reactive species in GLT-treated cells. Although scavenging of reactive species is fast, β-alanine was supplemented for 5 days in order to allow for synthesis of carnosine to take place, albeit intracellular carnosine was not formally measured in this particular experiment. Data (not shown) generated by a colleague in the Turner group working with INS-1 β-cells, did not, however, show any significant effect upon scavenging of GLT-mediated reactive species. This might, however, have been due to the β-alanine incubation time being only one hour, and so treating this for longer might still elicit an effect.

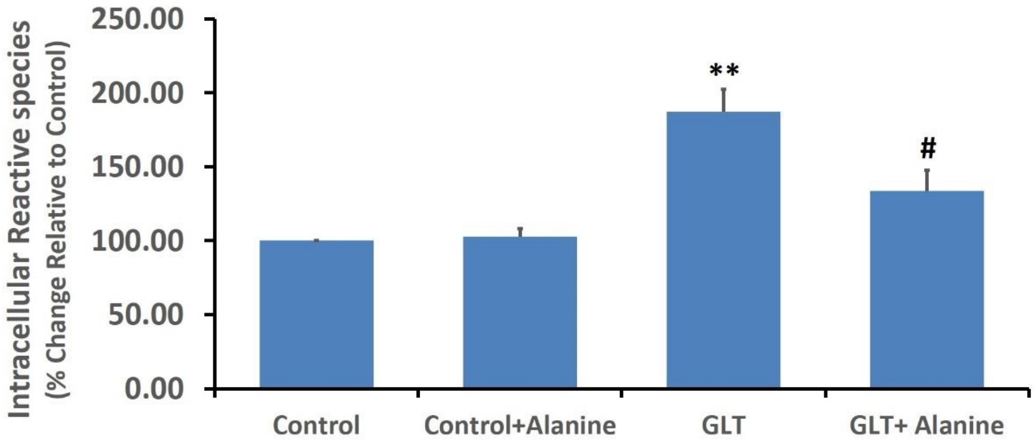


Figure 5.2; **β-alanine scavenging reactive species in C2C12 skeletal muscle cells.** C2C12 myotubes were cultured in control or GLT media for 5 days ± 10mM β-alanine. A 20μM DCFDA in KREBS buffer was then added to cells for 1h and reactive species detected via fluorescence with excitation and emission of 495nm and 530nm. Reactive species are expressed as a percentage change in comparison to control from 3 independent experiments ± SEM. (***p*<0.005 vs Control, $^{\#}p$<0.05 vs GLT; Tukey's test)

5.2.3 Effect of β-Alanine Supplementation on Glucose-Uptake in Glucolipotoxicity Treated C2C12 Myotubes

As supplementation of β-alanine significantly reduced the level of intracellular reactive species generated in glucolipotoxicity-treated muscle cells, then this could potentially elicit similar effects to those shown with carnosine to enhance glucose uptake in the GLT-induced model of insulin-resistant muscle cells. In order to evaluate this, differentiated C2C12 muscle cells were incubated in either control or GLT media for 5 days in the presence or absence of 10mM β-alanine. Glucose uptake was then determined using a luminescence-based assay.

As shown in Figure 5.3, GLT-treated cells (insulin-stimulated) have significantly lower glucose-uptake (69.92 ± 11.36) compared to healthy control with insulin stimulation. However, the observed increase in glucose uptake upon supplementation of β-alanine was found to be statistically not significant. The observed enhancement could be attributed either to the level of carnosine that could have endogenously formed, or to β-alanine itself. Further, the addition of β-alanine to GLT media neither elicited toxicity to the cells, nor significantly affected cell number, an observation that was made using Calcein-AM fluorescence-based cell viability assay and depicted in Figure 5.4.

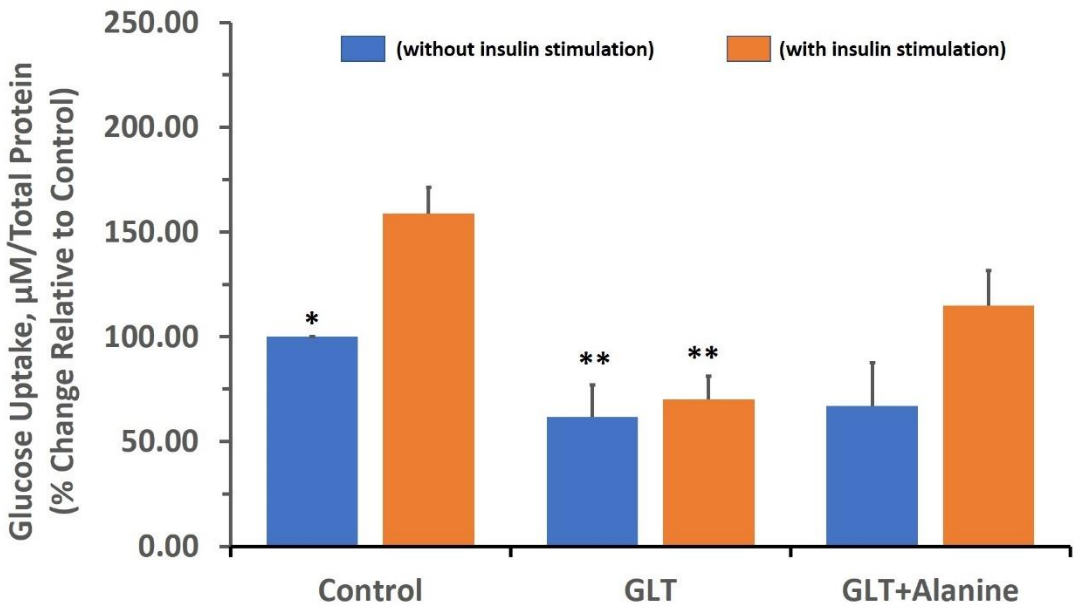


Figure 5.3; Glucose-uptake assay of β-alanine-supplemented GLT-exposed C2C12 muscle cells. C2C12 myotubes were cultured in DMEM media, or DMEM GLT media for 5 days ± 10mM β-alanine. Cells were serum-starved overnight in DMEM supplemented with 5 mM glucose, then incubated for 1 h in glucose-free DMEM +/−100nM insulin. Medium was replaced with PBS + 0.125 mM 2-deoxy glucose (2-DG). Glucose uptake reactions were conducted for 30 min. 2DG6P was detected using a luminometer. Data are expressed as means ± SEM of 3 independent experiments. (*$p < 0.05$,**$p < 0.005$; Tukey's test)

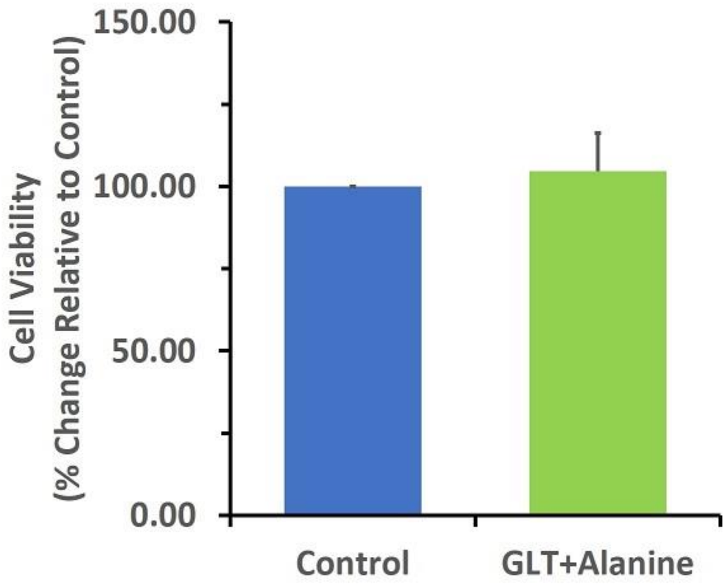


Figure 5.4; β-alanine supplementation does not affect cell viability of C2C12 Myotubes. C2C12 myotubes were cultured in control or GLT media for 5 days. After 1h incubation with 5μM solution of Calcein AM, fluorescence intensity was measured using excitation and emission of 490nm and 520nm. Results shown are expressed as percentage change compared to control from 4 independent experiments ± SEM. ($p > 0.05$; t-test)

5.2.4 CNDP-2 / Tissue Carnosinase Expression in Healthy / Control, and Glucolipotoxicity-Treated C2C12 Myotubes

Increased expression of carnosinase, and thus its activity, has been reported to be associated with increased risk of diabetic nephropathy and altered kidney function in type 2 diabetes (Ahluwalia et al., 2011). A study by Chiu et al., 2014, also indicated that cytosolic non-specific dipeptidase or carnosine dipeptidase -2 (CNDP2) expression was observed to be significantly elevated in both male and female hypertensive mice, wherein there was a reduced carnosine level and decreased protection against oxidative stress.

In order to assess whether or not the level of CNDP2 changed in GLT-exposed muscle cells, C2C12 myotubes were treated in either control or GLT media for 5 days. The cells were then lysed and the proteins separated by SDS-PAGE, before being subjected to immunoblot analysis using an anti-CNDP2 primary antibody. Figure 5.5, showed that under GLT conditions CNDP2 is significantly upregulated relative to control, with an increase of 88.86 ± 19.42%.

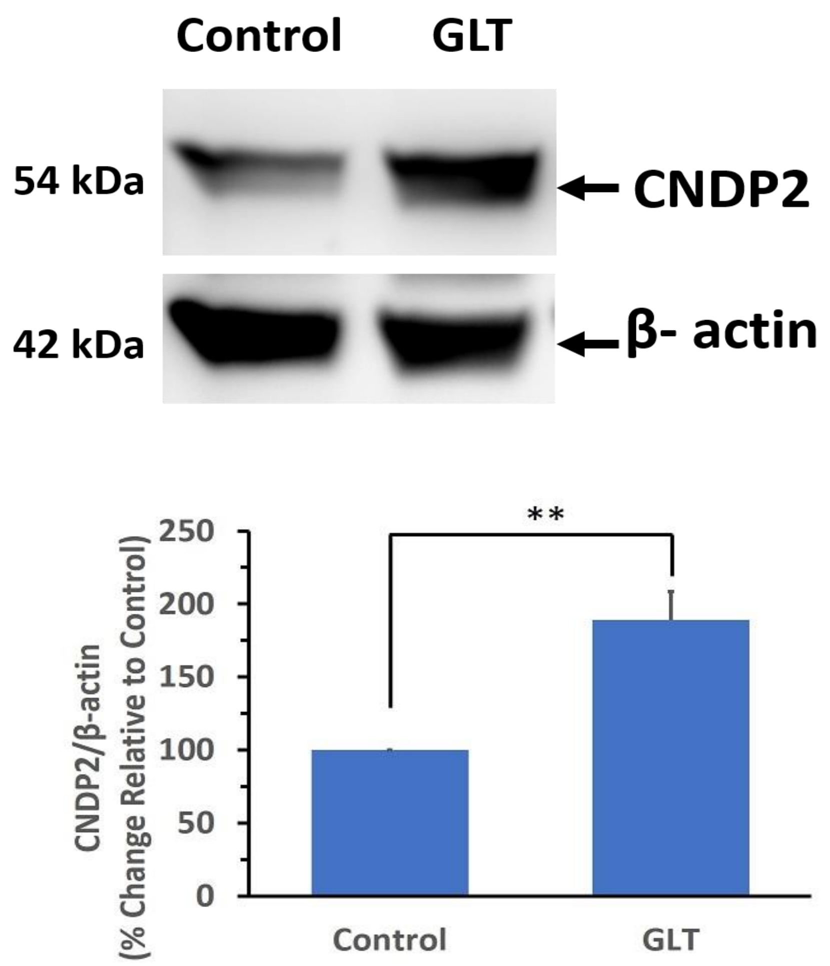


Figure 5.5; Expression of CNDP2 is upregulated in GLT-exposed C2C12 myotubes. C2C12 myotubes were incubated in control or GLT media supplemented for 5 days. Cells were then lysed to extract proteins and then separated via SDS-PAGE, transferred to nitrocellulose and detected using anti-CNDP2 or anti-actin antibody. Data expressed as mean ± SEM from 4 independent experiments. (***p* < 0.005; t-test).

Since the potential beneficial action of carnosine could be hampered by the activity of serum and tissue carnosinase, resulting in its rapid degradation, I therefore next sought to identify molecules that could inhibit the activity of tissue CNDP2. Molecules were assessed using *in silico* computational analysis of predictive catalytic cleft spacial interactions in order to predict those most likely to be stable against hydrolysis and/or CNDP2 inhibitors. If effective, this strategy could sustain the action of endogenous carnosine.

Using carnosine as the template, a library of over 50,000, compounds available from Maybridge (www.maybridge.com) were virtually screened to initially identify hit compounds that are similar in shape with carnosine. After assessing the stereochemical and physical quality of the molecules, the selected hit compounds (top 500) were docked with the binding site of carnosinase-2 (CN2), particularly in the region involved in hydrolysis, using the GOLD software (https://www.ccdc.cam.ac.uk/solutions/csd-discovery/components/gold) and the Tanimoto combo scoring function, which quantitatively measures and ranks the similarity between carnosine and Maybridge molecules. The top 50 hits were then chosen for further screening and were re-docked to the active site of CN1 and CN2 with increased search efficiency in order to determine which of these had the most similar shape to carnosine. Bestatin, an analogue of carnosine, was used as an internal control to validate and identify any malfunction and error in the method. From this measurement, 14 of these molecules met the said criteria and these are labelled as M4, M8, M14, M17, M21, M28, M36, M37, M38, M43, M44, M47, M48, M49.

5.2.5 Effect of Carnosinase Inhibitors and Carnosine Mimetics on Cell Viability

In order to investigate the effect of the potential drug candidates on cell number and viability, fluorescence-based cell viability testing was conducted using calcein-AM dye. This was initially performed using C2C12 muscle cells (Figures 5.6, 5.7 and 5.8), showing that the five analogues initially selected did not cause any significant toxicity. Consequently, the present study then went on to determine their scavenging activity in GLT-treated muscle cells. Figure 5.8 shows some representative images of C2C12 myotubes that were treated for 5 days with the indicated compounds, as well as a separate compound that was toxic to the cells (M44). The reason for M44 toxicity remains unknown, but might be better explained by a future comprehensive structure-function analysis.

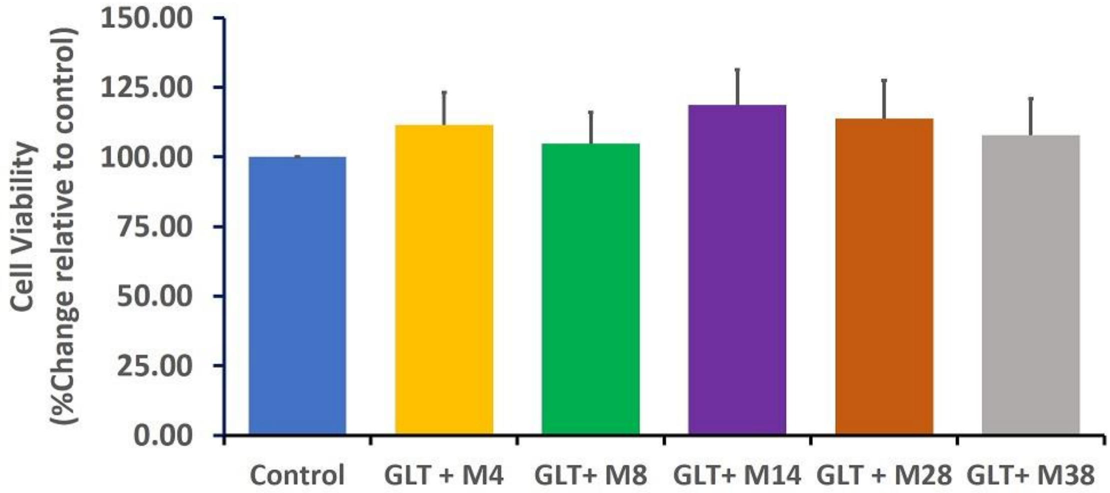


Figure 5.6. Selected carnosine-derivative molecules did not affect C2C12 cell viability. C2C12 myotubes were cultured in control or GLT media + compound for 5 days. After 1h incubation with 5µM solution of Calcein AM, fluorescence intensity was measured using excitation and emission of 490nm and 520nm. Results shown are expressed as a percentage change compared to control from 3 independent experiments ± SEM. ($p>0.05$; ANOVA)

Similarly, different carnosine esters (sythesised through a condensation reaction between alcohol and carnosine in the presence of trimethylsilyl chloride) were also tested whether these compounds would have significant effect on cell viability of GLT-treated cells. Introducing an ester group is designed to make these derivatives more stable against hydrolysis by carnosinase, whilst retaining biological actions similar to that of carnosine. Indeed, this may potentially improve the nucleophilic character of the histidine moiety, thereby making it more reactive towards electrophilic toxic aldehydes; resulting in it becoming a more preferential site for protein glycation due to the proximity of imidazole and carboxylate. The synthesis of these carnosine esters was conducted in the Chemistry Department of NTU under the supervision of Dr. Christopher Garner. Methyl, ethyl, and isopropyl were allowed to react for about 15 minutes with trimethylsilyl chloride, before L-carnosine was added, after which the resulting solution was allowed to reflux at 95°C overnight to obtain alkyl carnosine esters labelled as CE1, CE2, and CE3. The purity of the compounds was ensured and validated using magnetic resonance spectroscopy. Figure 5.7 showed that each of 100μM carnosine methyl ester (CE1), carnosine ethyl ester (CE2), and carnosine isopropyl ester (CE3) in GLT-treated cells did not significantly affect cell viability as compared to healthy control.

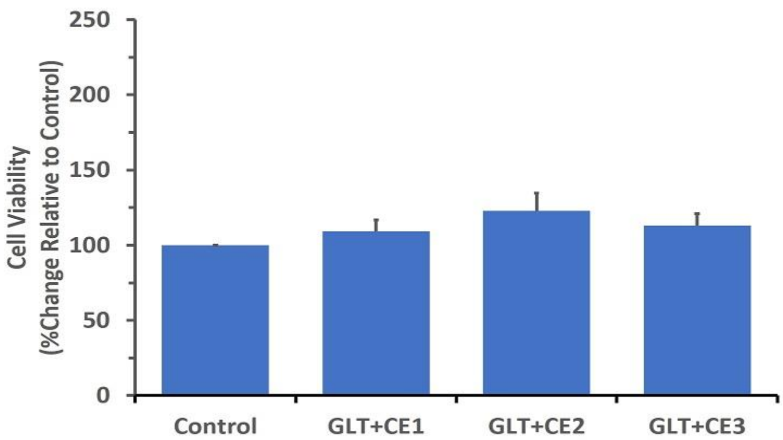


Figure 5.7. The effect of three carnosine esters on C2C12 cell viability. C2C12 myotubes were cultured in control or GLT media + compound for 5 days. After 1h incubation with 5μM solution of Calcein AM, fluorescence intensity was measured using excitation and emission of 490nm and 520nm. Results shown are expressed as a percentage change compared to control from 3 independent experiments ± SEM. ($p>0.05$; ANOVA)

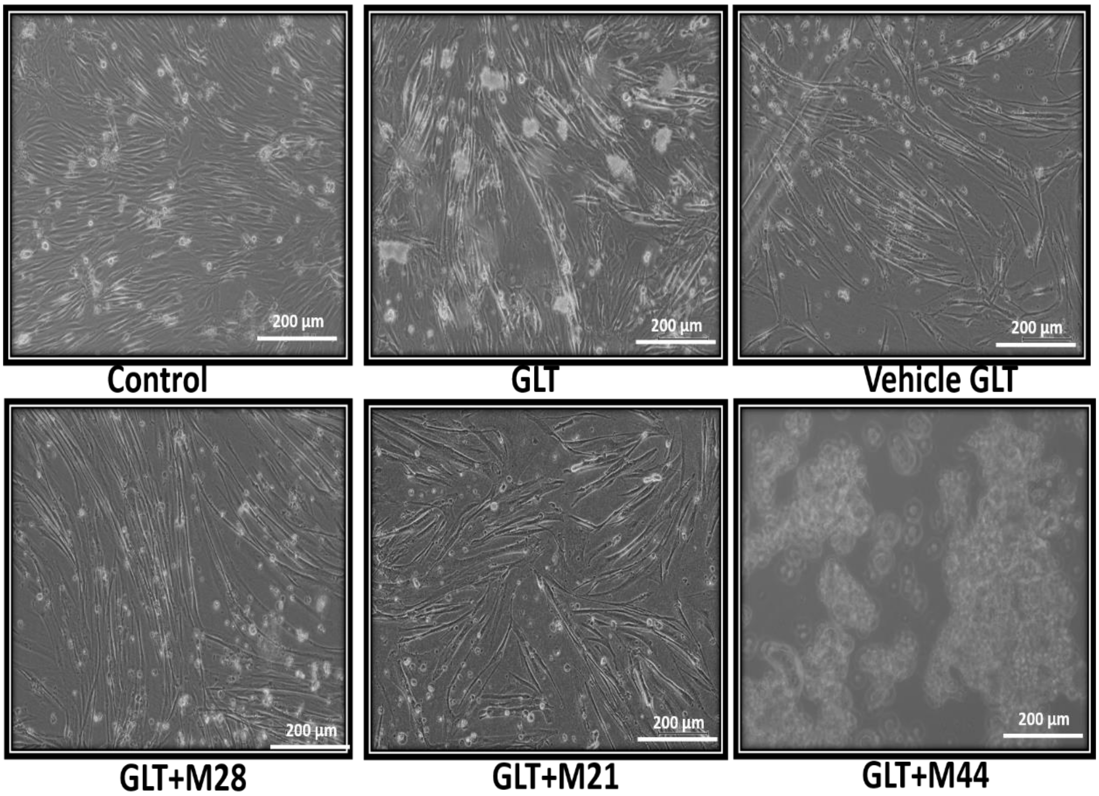


Figure 5.8; Representative images of C2C12 myotubes treated with different selected Maybridge molecules using the Olympus CKX53 microscope.

The potential toxicity of carnosine-derived molecules was also determined on a human skeletal muscle cell-line. As indicated in Figure 5.9, 5-day of treatment with 100µM each of M4, M8, M14, M28 and M38, and in addition with three different carnosine mimetics (100 µM each of CE1, CE2, and CE3 or carnosine esters) in GLT-treated human myotubes were not significantly different to the healthy control cells, suggesting that the treatments did not have cytotoxic effects to the human muscle cell-line. Nevertheless, this dosing might have to change when administered in animal models during *in vivo* validation moving forward. In this collaborative *in vivo* work, three molecules (M8, M28, and CE3) were initially chosen for further analysis, based upon scavenging and glucose uptake data (Figures 5.12-15). Figure 5.10 shows example images of human myotubes treated for 5 days in GLT with the indicated molecules.

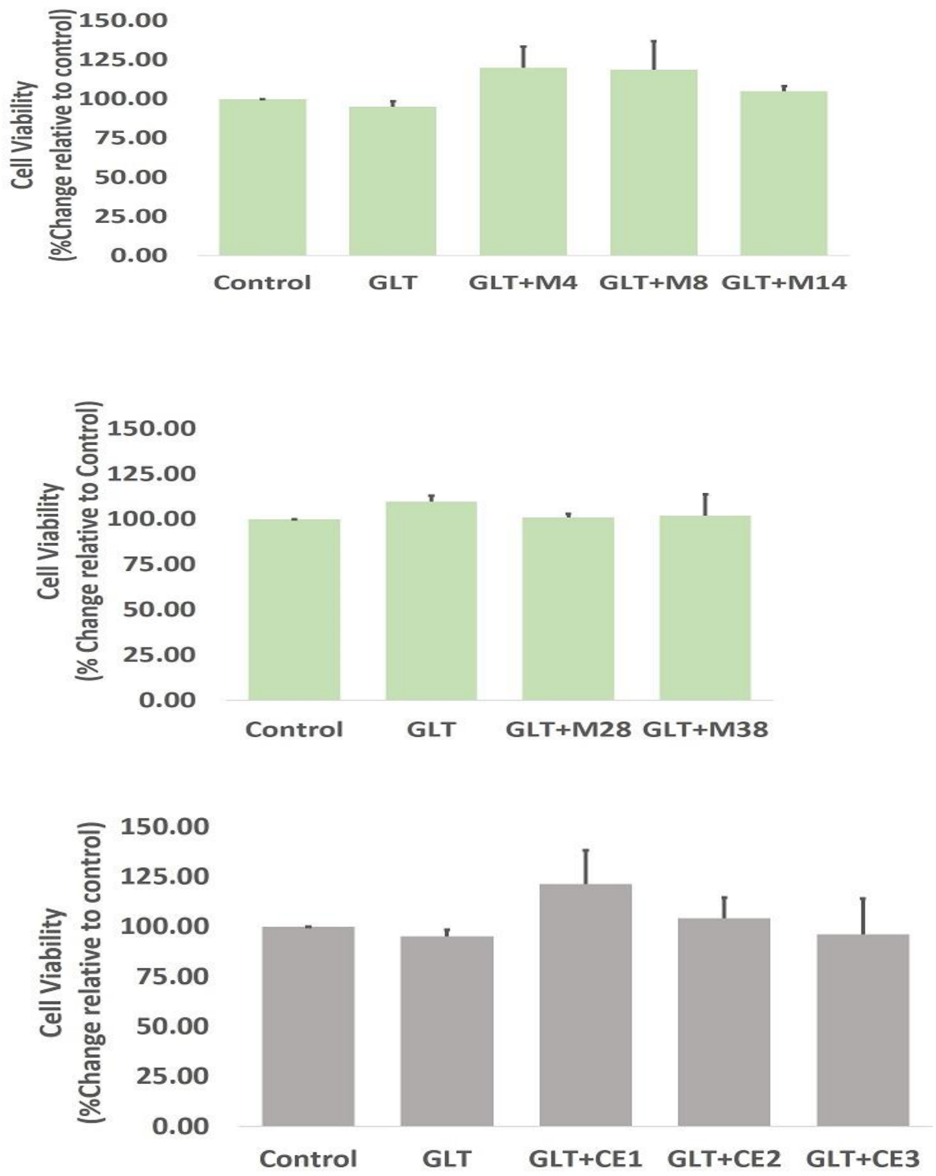


Figure 5.9; Treatment of the proposed carnosinase inhibitors and isopropyl carnosine ester on GLT-exposed cells did not affect cell viability. HSkM myotubes were cultured in control or GLT media for 5 days. After 1h incubation with 5μM solution of Calcein AM, fluorescence intensity was measured using excitation and emission of 490nm and 520nm. Results shown are expressed as a percentage change compared to control from 3 or more independent experiments ± SEM. (p>0.05; ANOVA)

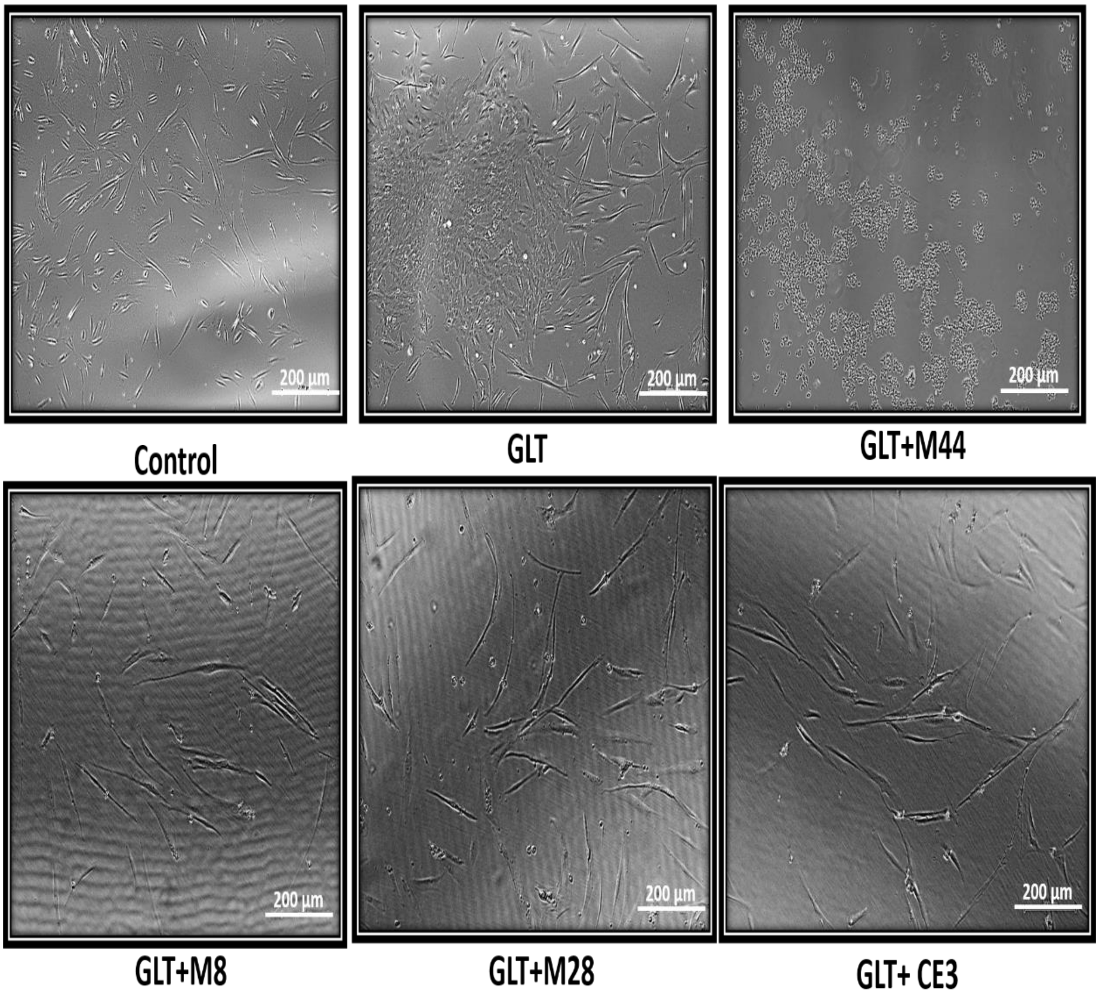


Figure 5.10; Human skeletal myotubes with 5-day treatment of some selected molecules using the Olympus CKX53 microscope.

5.2.6 Scavenging Activity Assay on Skeletal Myotubes Treated with Potential Carnosinase Inhibitors and Carnosine Mimetics

Promising drug candidates were next investigated for biological action, through scavenging, insulin secretion and glucose-uptake assays. Based upon the preliminary glucose uptake data (Figure 5.11), five molecules, namely M4, M8, M14, M28, and M38, were initially selected and further screened for scavenging activity using the DCFDA fluorescent dye. Following this, glucose uptake was determined. These molecules were also then evaluated for other functional activity relevant to glucose homeostasis, such as insulin secretion assay (performed and reported by Michael Cripps in the Turner laboratory).

A stock solution of these molecules was prepared in 100% sterile ethanol and further diluted with sterile KREBS to yield a solution containing 10mM of each of the compounds. A 100µM solution (1:100, compound to experimental media) was chosen as the treatment concentration for all the screening conducted in this chapter work, as 100µM is optimal to elicit significant scavenging effect, whilst for many compounds does not affect cell viability. Although carnosine is optimally effective at a concentration of 10mM, a much lower concentration of optimal activity was desired in the use of synthetic compounds.

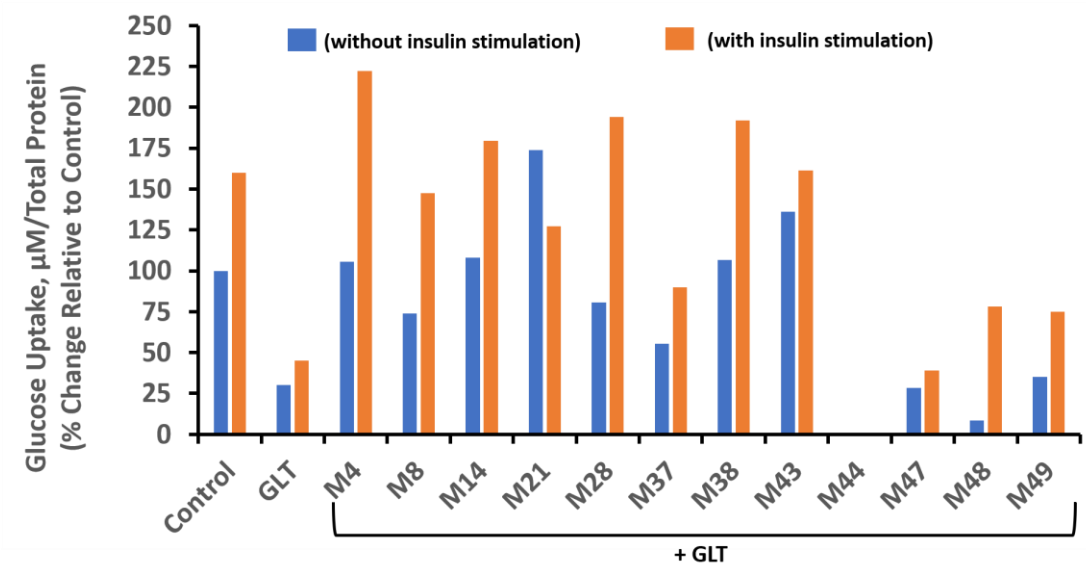


Figure 5.11. Initial glucose-uptake assay of some carnosine-shaped molecules. C2C12 myotubes were cultured in DMEM media, or DMEM GLT media for 5 days ± 100µM of the indicated labelled molecules. Cells were serum-starved overnight in DMEM supplemented with 5 mM glucose, then incubated for 1 h in glucose-free DMEM +/−100nM insulin. Medium was replaced with PBS + 0.125 mM 2-deoxy glucose (2-DG). Glucose uptake reactions were conducted for 30 min. 2DG6P was detected using a luminometer (n=1).

The following figures depict the scavenging potential of five molecules (M4, M8, M14, M28, M38) believed to inhibit carnosinase-2. The molecular weight of these molecules ranges from 188 g/mol to 213 g/mol not far from that of carnosine 226 g/mol.

There was no significant difference in reactive species levels between the control condition and the control treated with the selected molecules (Figure 5.12). As GLT resulted in a significant increase in reactive species (215.55±9.58%) compared to control, it is interesting to note that supplementation of GLT-exposed cells with 100μM of these five molecules (M4, M8, M14, M28, and M38) for 5 days resulted in a significant reduction of intracellular reactive species when compared to the GLT condition.

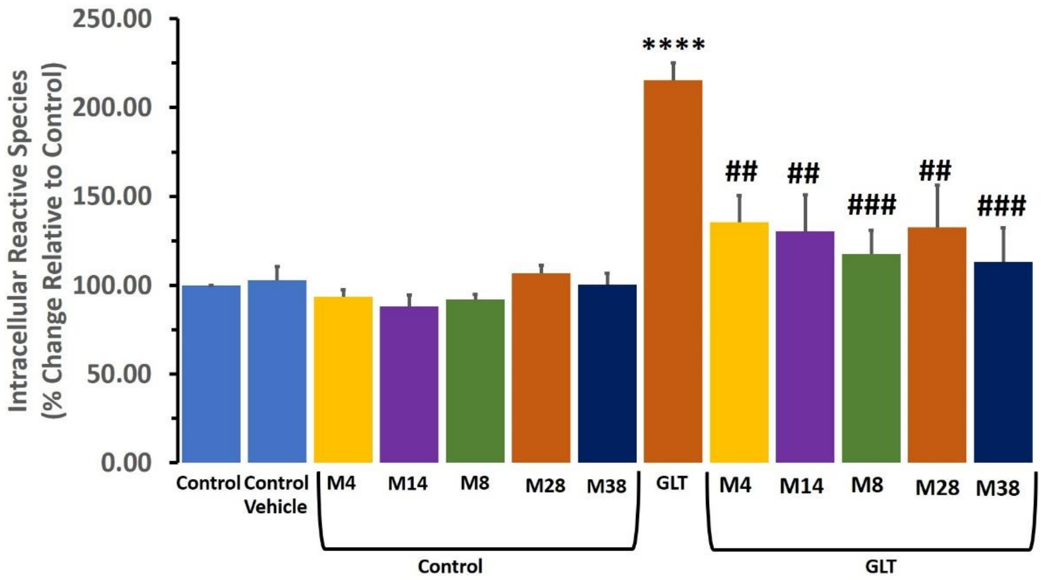
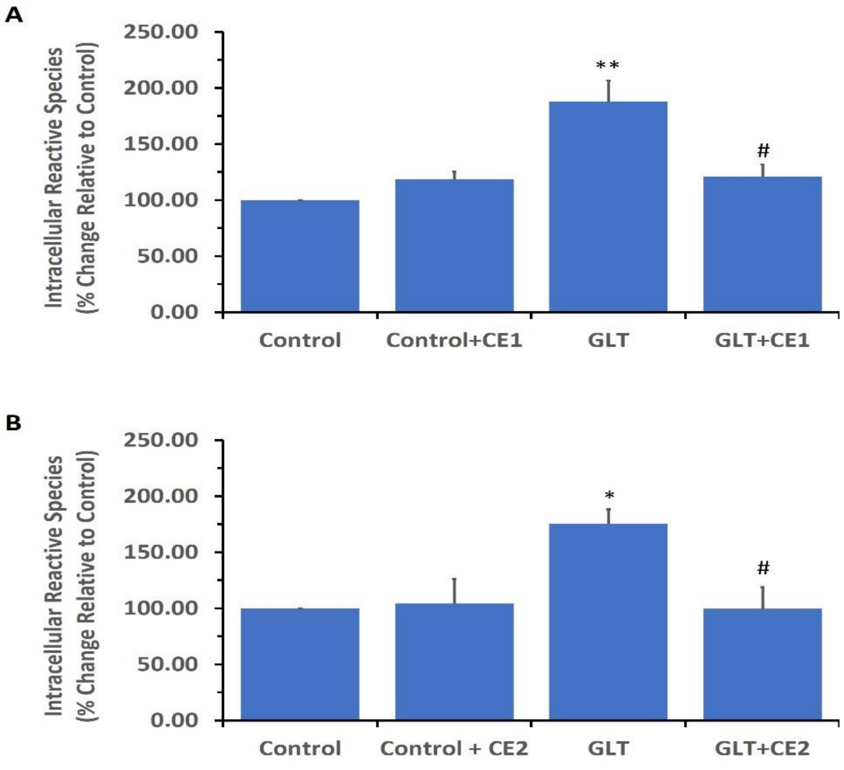


Figure 5.12; Scavenging assay of selected carnosinase inhibitors. C2C12 myotubes were cultured in control or GLT media for 5 days ± 100μM of indicated labelled molecules. A 20μM DCFDA in KREBS buffer was loaded for 1h and reactive species detected via fluorescence with excitation and emission of 495nm and 530nm. [Control Vehicle – 1% ethanol] Reactive species are expressed as a percentage change in comparison to control from 4 independent experiments ± SEM. (****p<0.0001 vs Control, $^{\#\#}$p<0.005, $^{\#\#\#}$p<0.0005 vs GLT; Tukey's test)

In conjunction with carnosine analogs that are putative carnosinase inhibitors that have already been investigated using scavenging and glucose-uptake assays, this PhD work also evaluated the carnosine-like properties of three ester derivatives of carnosine.

As shown in Figure 5.13, all ester derivatives of carnosine significantly scavenged or reversed the increased intracellular reactive stress generated following incubation in GLT media, with both ethyl and isopropyl carnosine esters having the highest scavenging potential. Specifically, GLT media containing 175.50 ± 12.83% and 184.64 ± 19.11 reactive species (relative to control) was reduced to 99.38 ± 19.35% and 100.66 ± 17.37% following incubation with CE2 and CE3.



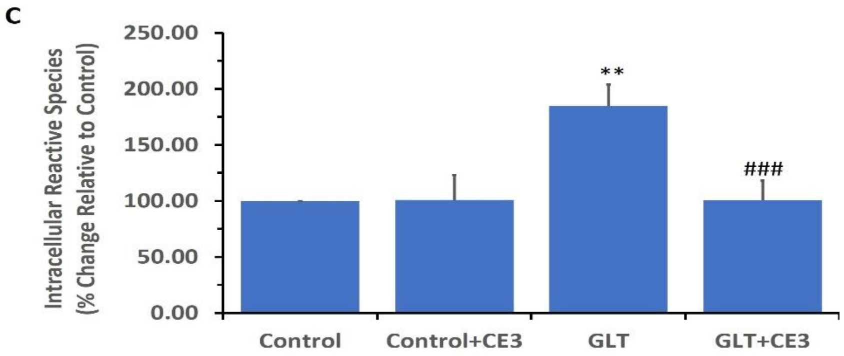


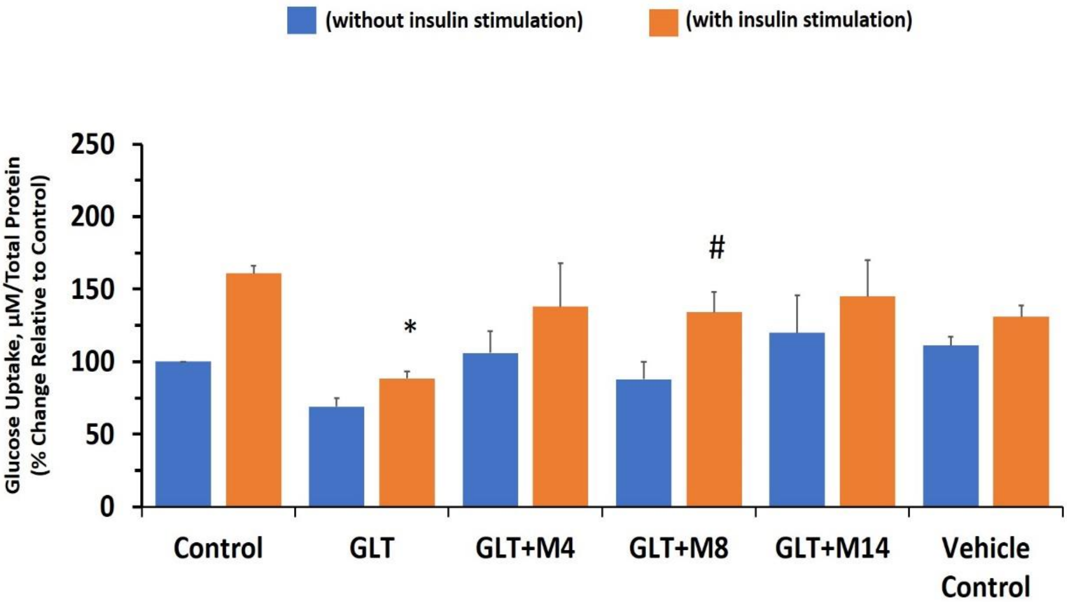
Figure 5.13; Three carnosine esters have shown scavenging action against glucolipotoxicity mediated generation of reactive species. (A) methyl carnosine ester (B) ethyl carnosine ester (C) isopropyl carnosine ester. C2C12 myotubes were cultured in control or GLT media for 5 days. Corresponding media were then replaced and supplemented ± 100μM of different alkyl carnosine esters for 1h. A 20μM DCFDA in KREBS buffer was loaded for 1h and reactive species detected via fluorescence with excitation and emission of 495nm and 530nm. Reactive species are expressed as a percentage change in comparison to control from 4 independent experiments ± SEM. (*p<0.05, **p<0.005 vs Control, $^{\#}$p<0.05, $^{\#\#\#}$p<0.001 vs GLT; Tukey's test)

5.2.7 The Effect of Potential Carnosinase Inhibitors and Carnosine Mimetics on Glucose-Uptake in Glucolipotoxicity-Treated Skeletal Myotubes

Previous observations made following carnosine and β-alanine supplementation (as discussed in Chapter 3 and above) demonstrated that when cells are incubated in the presence of these compounds there is beneficial impact on glucose homeostasis, both through improved insulin secretion and glucose uptake. As such, carnosine-based *in silico* synthetic molecules could provide an important new direction in the development of novel treatment strategies beneficial for those either at risk or who are patients with type 2 diabetes.

In order to find out what influence the selected molecules had upon glucose uptake, differentiated C2C12 cells were treated with either control media or GLT ± 100μM of carnosine ester or carnosinase inhibitor for 5 days. Following this treatment, they were then serum-starved overnight and insulin-stimulated for an hour before glucose uptake was measured using a luminescence-based assay.

The improvement in glucose-uptake shown in Figures 5.14 and 5.15, could be attributed to the positive effect seen in the scavenging data of selected Maybridge molecules (M4, M8, M14, M28, and M38) and the isopropyl carnosine ester (CE3) on cells incubated under glucolipotoxic conditions. In all cases, stimulated GLT-exposed cells had significantly lower uptake of glucose ($p < 0.005$) as compared to control-stimulated cells. 5-days of treatment of these molecules (with the exception of M4) resulted in increased glucose uptake to GLT-treated cells ($p < 0.05$, GLT-stimulated vs GLT-stimulated + carnosine analogue). Since CE2 and CE1 were not effective (Figure 5.15) in improving glucose uptake under GLT conditions, only CE3 was chosen for further evaluation with either a human skeletal muscle cell-line or for future *in vivo* work.



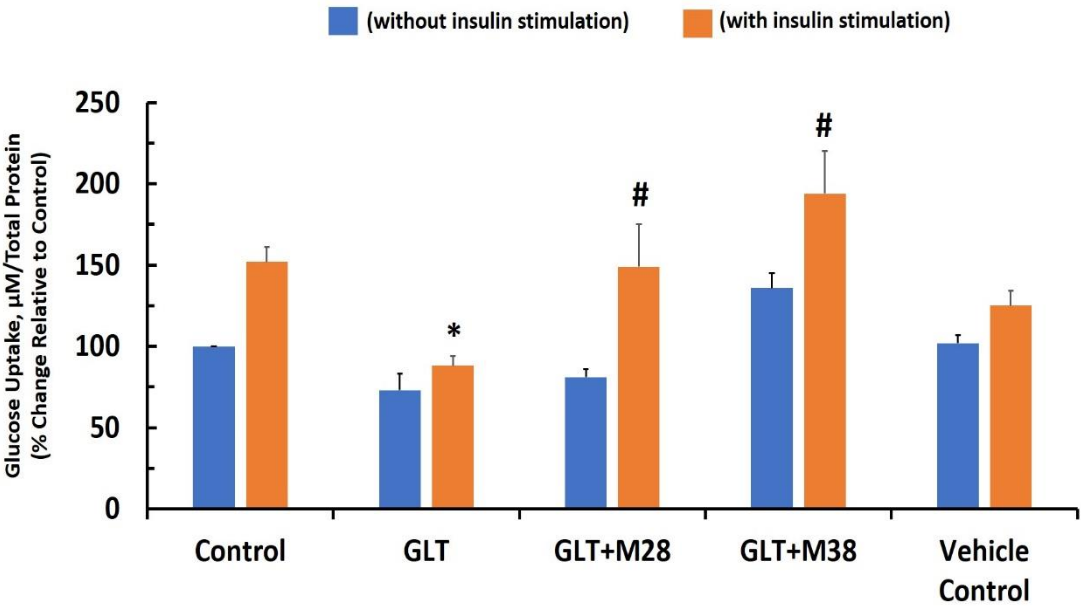


Figure 5.14; Glucose-uptake measurements of GLT-exposed cells treated with selected carnosine-shaped molecules. C2C12 myotubes were cultured in DMEM media, or DMEM GLT media for 5 days ± 100µM of indicated compound. Cells were serum-starved overnight in DMEM supplemented with 5 mM glucose, then incubated for 1 h in glucose-free DMEM +/−100nM insulin. Medium was replaced with PBS + 0.125 mM 2-deoxy glucose (2-DG). Glucose uptake reactions were conducted for 30 min. 2DG6P was detected using a luminometer. Data are expressed as means ± SEM of 3 independent experiments. (*p<0.05 vs Insulin-stimulated Control, $^{\#}p$<0.05 vs Insulin-stimulated GLT; Tukey's test).

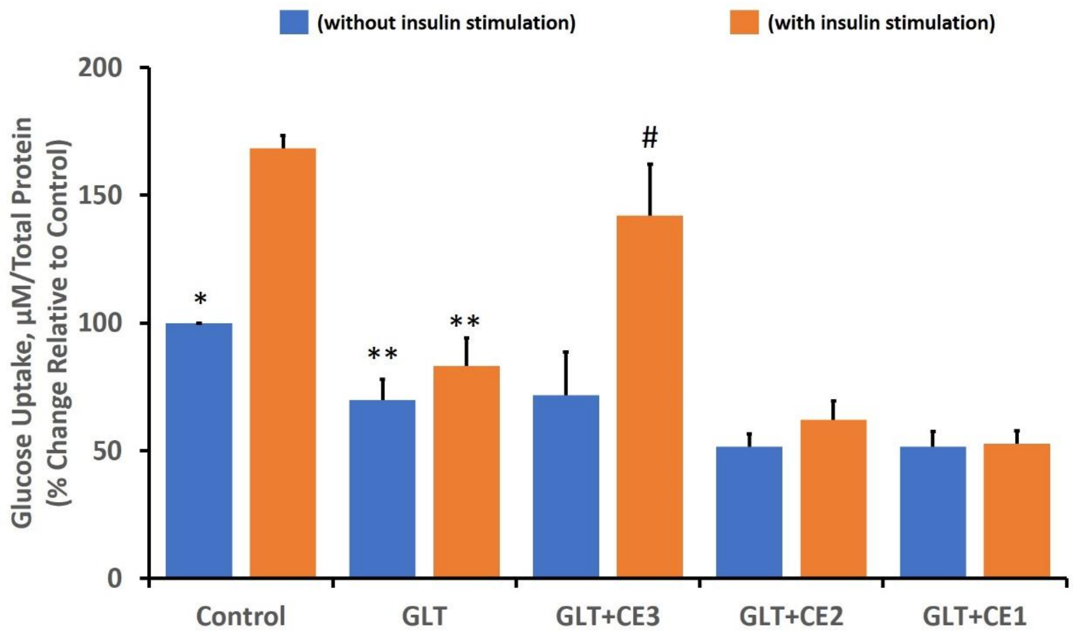


Figure 5.15; Isopropyl carnosine ester enhances glucose-uptake of GLT-induced insulin resistant C2C12 muscle cells. C2C12 myotubes were cultured in DMEM media, or DMEM GLT media for 5 days ± 100µM of isopropyl carnosine ester (CE3). Cells were serum-starved overnight in DMEM supplemented with 5 mM glucose, then incubated for 1 h in glucose-free DMEM +/−100nM insulin. Medium was replaced with PBS + 0.125 mM 2-deoxy glucose (2-DG). Glucose uptake reactions were conducted for 30 min. 2DG6P was detected using a luminometer. Data are expressed as means ± SEM of 3 independent experiments. (***p*<0.01 vs Insulin-stimulated Control, $^{\#}p$<0.05 vs Insulin-stimulated GLT; Tukey's test).

Following the *in silico* analysis and subsequent *in vitro* experiments detailed above, three leading candidate molecules (M8, M28, and CE3) were selected for further evaluation, This *in vivo* study was conducted by a collaborator at King's College London (Dr. Paul Caton), and employed glucose tolerance test (GTT) primarily to assess beneficial effect(s) of these drug candidates on a high-fat fed mouse model of type 2 diabetes. Whilst this *in vivo* work was underway, glucose-uptake assays were conducted using human skeletal muscle cells that were differentiated and treated either in control or GLT media ± 100µM each of M8, M28 and CE3 for 5 days, and similarly M21 as a negative control. Figure 5.16 shows that all compounds, except

M21 and M28, significantly increased glucose uptake in the GLT model of insulin-resistance using myotubes from a human skeletal muscle cell line.

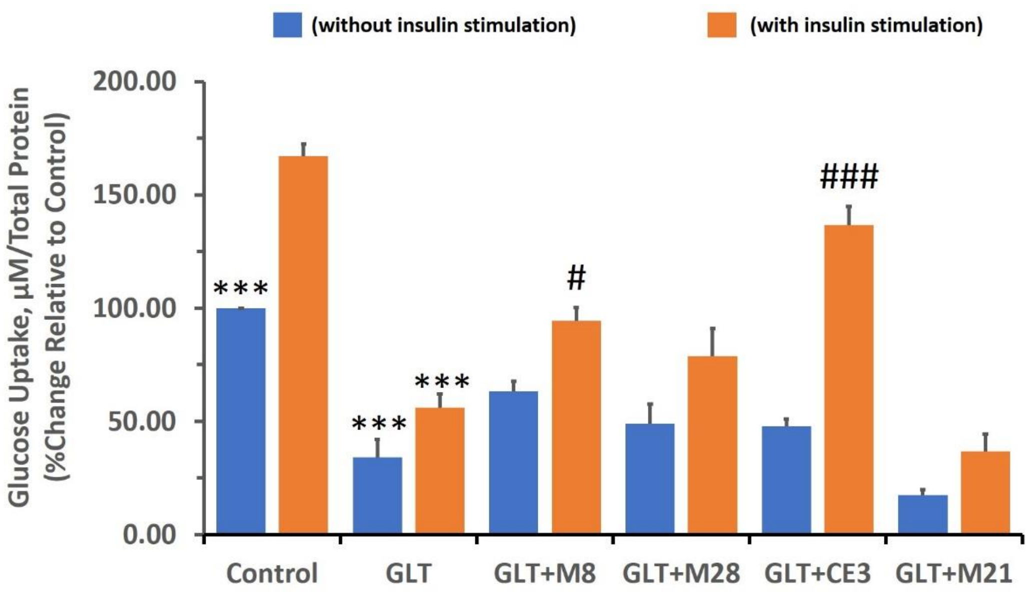


Figure 5.16; Glucose-uptake Assay of selected carnosine-shaped and carnosine mimetic molecules on human skeletal muscle cell-line. Human skeletal myotubes (HSkM) were cultured in DMEM-F12 media, or DMEM-F12 GLT media (17mM glucose, 200 µM Palmitic acid, 200 µM Oleic acid) for 5 days ± 100µM of indicated compound. Cells were serum-starved overnight in DMEM supplemented with 5 mM glucose, then incubated for 1 h in glucose-free DMEM +/− 1µM insulin. Medium was replaced with PBS + 0.150 mM 2-deoxy glucose (2-DG). Glucose uptake reactions were conducted for 30 min. 2DG6P was detected using a luminometer. Data are expressed as means ± SEM of 3 independent experiments. ($^{***}p<0.001$ vs Insulin-stimulated Control, $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ vs Insulin-stimulated GLT; Tukey's test).

5.2.8 Effect of β-Alanine Supplementation, Carnosinase Inhibitors, and Carnosine Mimetics on Insulin Secretion in Glucolipotoxicity-Treated INS-1 β-cells

The expression of carnosine synthase has been shown in INS-1 β-cells by colleagues (K. Hannah and M. Cripps doctoral theses) in the Turner group. This suggests that the entry of β-alanine could increase the active pool of intracellular carnosine in these cells. In order to investigate this hypothesis, the effect of β-alanine supplementation was evaluated in INS-1 cells via scavenging and insulin secretion assays conducted by M. Cripps. The results (data available from http://irep.ntu.ac.uk/id/eprint/36698/) indicated a significant reduction in glucolipotoxicity-mediated reactive species following the 5-day treatment with β-alanine, which consequently elicited an increased secretagogue-stimulated insulin secretion. Importantly, a reversal of glucolipotoxic inhibition of insulin secretion was also shown.

Incubation of INS-1 β-cells with putative carnosinase inhibitors was toxic in certain instances, e.g. M44, but for the most part these compounds (M4, M8, M28, and M14) showed no significant effect on cell viability. They had varied scavenging actions, however, and showed no significant increase in insulin secretion (M. Cripps thesis). Initial screening of the carnosine mimetic, isopropyl carnosine ester (CE3), on INS-1 β-cells (performed with N.N. Dilla in the Turner group) showed that 5-day treatment of these pancreatic cells with CE3 did not affect cell viability and was not toxic to these cells. Importantly the addition of this molecule significantly reduced the increase of GLT-mediated intracellular reactive species.

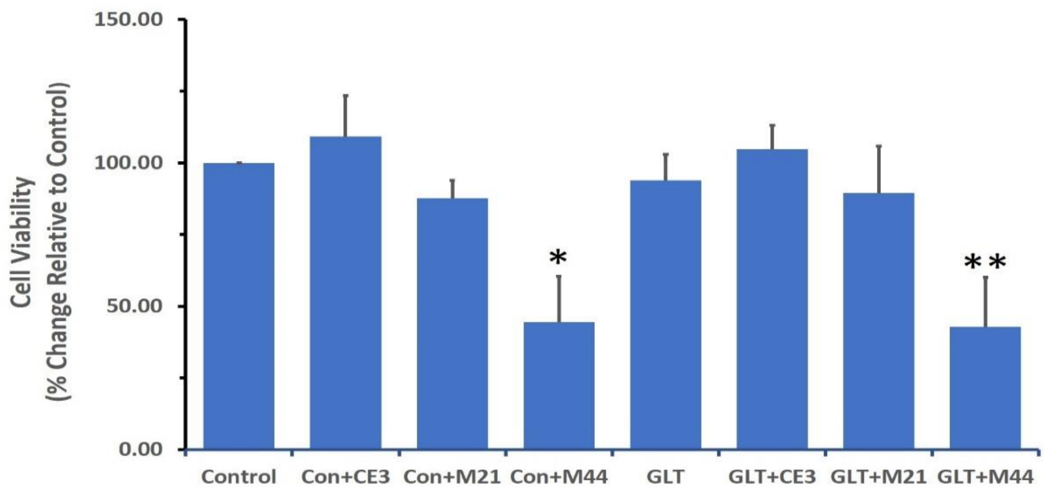


Figure 5.17; The effect of some carnosine-shaped molecules (M44 and M21) and carnosine mimetic (CE3) on INS-1 β-cells. INS-1 β-cells were cultured in control or GLT media for 5 days. After 1h incubation with 5μM solution of Calcein AM, fluorescence intensity was measured using excitation and emission of

490nm and 520nm. Results shown are expressed as a percentage change compared to control from 3 or more independent experiments ± SEM. * $p < 0.05$, ** $p<0.01$ vs Healthy control; Dunnett's test)

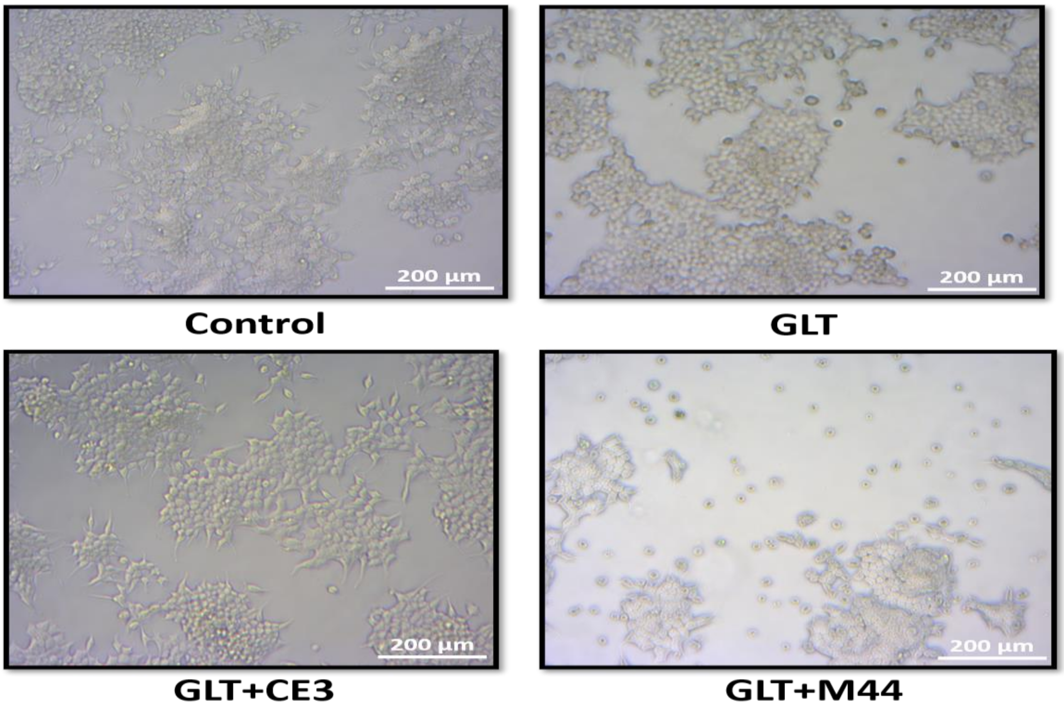


Figure 5.18; Example of INS-1 images treated for 5-days with the indicated compound. [*Images obtained by N.N. Dilla, MSc student in Turner group*]

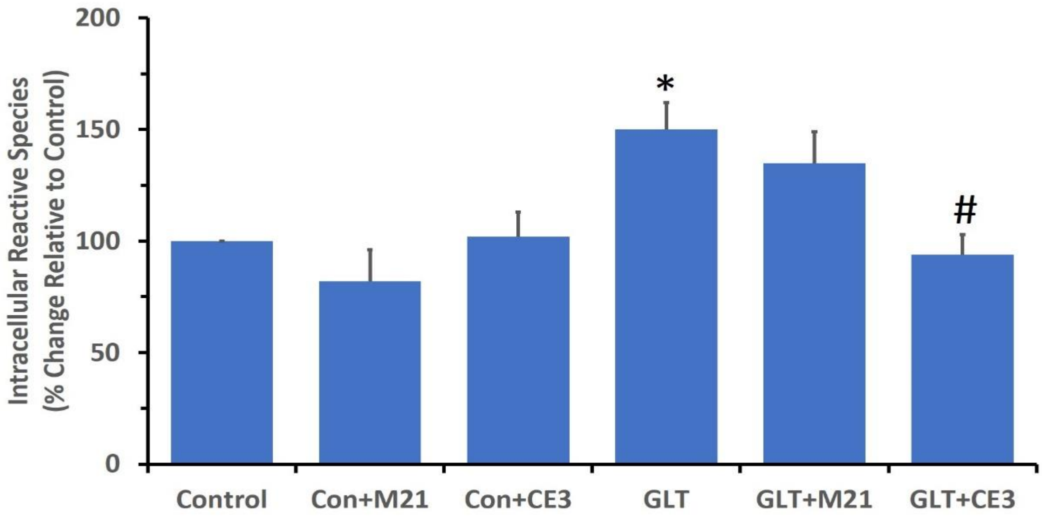


Figure 5.19; Carnosine isopropyl ester is effective as a scavenger of GLT-mediated intracellular reactive species generated in GLT-exposed pancreatic β-cells. INS-1 pancreatic beta cells were cultured in control or GLT media ± 100µM of different alkyl carnosine esters. A 20µM DCFDA in KREBS buffer

was loaded for 1h and reactive species detected via fluorescence with excitation and emission of 495nm and 530nm. Reactive species are expressed as a percentage change in comparison to control from 3 independent experiments ± SEM. (* $p<0.05$ vs Control, $^{\#}p<0.05$ vs GLT).

5.2.9 Evaluation of Carnosinase-2 Activity on Selected Carnosinase Inhibitors and Carnosine Mimetics

Following the *in-silico* screening of carnosine-shaped molecules for likely stability against carnosinase hydrolysis, their theoretical inhibitory actions were next tested using commercially available recombinant human CNDP2 (rhCNDP2) in conjunction with a fluorescence-based assay using carnosine as substrate. The fluorescence intensity produced was then used to assess the inhibitory action of each molecule based upon the complex formed between the histidine component from the cleaved carnosine substrate and the derivatization reagent ortho-pthaldialdehyde (o-PA) used in the assay (protocol detailed in Chapter 2). Similarly, to assess the hydrolysis of carnosine ester derivatives, the same approach was employed by making it as the substrate and allowing it to react with rhCNDP2. The lower the flourescence intensity, the lower the amount of histidine that was available to form the fluorescent complex, and thus the lower the amount of carnosine ester that was hydrolysed to release the histidine moiety.

As indicated in Figure 5.20, M4 ($p < 0.05$), M14, M28 and M8 ($p < 0.005$) significantly inhibited the enzyme and protected carnosine from cleavage when compared to control (rhCNDP2 + carnosine only), whilst M38 ($p = 0.2007$) and M49 ($p= 0.2410$) showed no significant inhibition of carnosinase 2 cleavage of carnosine. In the case of the carnosine mimetics / carnosine esters, comparing to carnosine there were negligible fluorescence intensity values observed, indicating that these carnosine analogs are highly effective at preserving carnosine integrity.

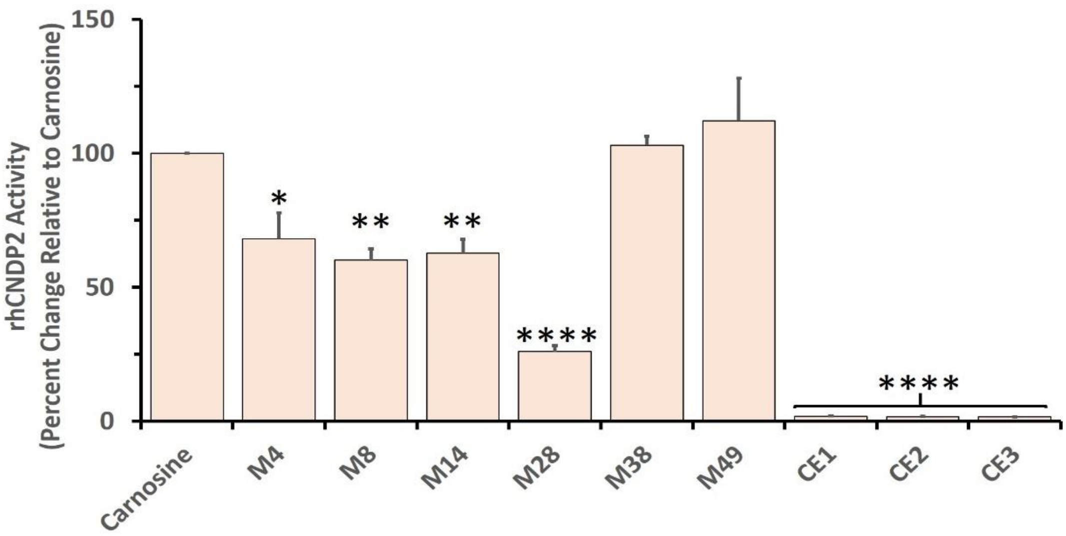


Figure 5.20; Estimation of the inhibitory effects of selected carnosinase inhibitors and hydrolytic property of the carnosine esters. Each substrate (e.g., CE3) was allowed to react at room temperature with rhCNDP2 for 1hr (prior to this is a 30-min reaction period in the presence of inhibitor e.g M8). After the reaction was stopped with trichloroacetic acid, o-pthaldialdehyde detection reagent was added and incubated for 30 mins to obtain a fluorescent complex measured at 360 nm (excitation) and 460 (emission). Data reported as average values ± SEM of n=3. (* $p < 0.05$, **** $p < 0.0001$ vs Carnosine Control; Dunnett's test).

5.3 Discussion

Supplementation of β-alanine has been found to have a positive influence on the carnosine concentration in skeletal muscle (Hill et al., 2006), and studies by Bakardjiev and Dunnet indicated that β-alanine, and not histidine, limits the synthesis of carnosine. Carnosine synthase also known as ATPGD1 is an enzyme that is required for the endogenous synthesis of carnosine, and has been found to be mainly expressed in skeletal muscle and in the brain (Miyaji et al., 2012; Drozak, et al., 2010). Similarly, the data presented here indicated the expression of the enzyme ATPGD1 in C2C12 mouse muscle cells, although in the duration of this experiment GLT had no significant effect on its expression level.

The experimental data obtained herein indicate that 5-days of supplementation with β-alanine in GLT exposed C2C12 muscle cells significantly attenuated the increase of GLT-mediated intracellular reactive species. This effect resulted to an increased glucose-uptake although not statistically significant. However, this does not mean β-alanine would have no effect nor conclusive to state that it is biologically unimportant in glucose homeostasis. Most likely the condition or number of experiments conducted here might not be more enough to reveal its real effect. On the other hand, carnosine synthase is present in INS-1 pancreatic cells (Hanna, 2018) suggesting that carnosine synthesis is possible in these cells. Consistent with this hypothesis, 5-days of treatment of β-alanine reduced the GLT-generated reactive species levels in these cells, resulting in improved insulin secretion (Cripps, 2019). Taken together, our data from skeletal muscle and pancreatic β-cell experiments suggest that taking β-alanine as a dietary supplement might offer therapeutic benefit to patients with diabetes.

Carnosine as a natural constituent in excitable tissues possesses diverse biological effects and the level of carnosine in tissues is regulated by several enzymes, particularly those involved in its hydrolysis (e.g., tissue carnosinase (CNDP2) and serum carnosinase (CNDP1). The overexpression or high activity of carnosinase, specifically CNDP1, has been linked to diabetic nephropathy that is common in patients with type 2 diabetes (Janssen et al., 2005; Albrecht et al., 2017). The activity of tissue carnosine-degrading enzyme (CNDP2) also regulates the role of imidazole-containing dipeptides in tissues like brain and skeletal muscles. Together with CNDP1, when both expression or activity is increased these enzymes might lead to decreased concentrations of endogenous carnosine and other imidazole-containing dipeptides and, therefore, this could potentially limit the action of available natural antioxidant or defence mechanisms for cells under oxidative stress. Collectively, the interplay between related carnosine transporters (stated in Chapter 1) and related enzymes (e.g. CNDP1 and CNDP2) and nutritional stimuli (e.g. β-alanine supplementation), might have important roles in regulating muscle carnosine homeostasis.

The concentration of carnosine in skeletal muscle has previously been shown to be relatively stable (Baguet et al., 2009), and the pH in muscle is also not optimal for degradation to take place (Teufel et al., 2003). This has led some to question, although not established, that there may be an absence of carnosine-degrading enzyme in mammalian skeletal muscle. However, the data presented earlier in this PhD work disproved this observation, as it was shown that at least in differentiated C2C12 muscle cells, CNDP2 was expressed through western blot analysis. Importantly, data herein also showed that glucolipotoxicity potentially reduces cellular carnosine levels, albeit both observations warrant further validation using more relevant physiological models.

In type 2 diabetic patients, the level of carnosine content in muscle was observed to be significantly reduced compared to healthy controls (Gualano et al., 2012), and as part of the ageing process, carnosine level is believed to decline over time (Everaert et al., 2011). This might therefore be attributed to the decreased ability to synthesise carnosine with increasing age, plus human conditions such as diabetes generate increased levels of reactive species and thus accelerated ageing, which could lead to a decreased production and increased destruction of carnosine (Boldyrev et al., 2001; Bellia et al., 2009; Everaert et al., 2011).

Carnosine degrading enzymes may therefore be considered as a risk factor in type 2 diabetes and its associated complications, and they pose a challenge to any anti-diabetic treatment / prevention strategy involving carnosine. One of the objectives of this PhD programme of work was to address this issue through the identification and synthesis of carnosine-related molecules that could have the same beneficial action upon glucose homeostasis, but importantly are resistant towards hydrolysis. Initial data obtained in the Turner group has identified a number of promising candidates for use as anti-diabetic agents, from which three molecules (M8, M28, and CE3) were chosen to be further evaluated in subsequent *in vivo* studies. However, once the most effective compound is identified, further comprehensive dose-response experiments will need to be conducted in order to establish a suitable concentration for physiological application.

The carnosine-shaped molecules studied here are relatively small in size and possess some aromaticity due to the presence of either the 6-membered ring (benzene or pyridine) or 5-membered ring (furan, azole, imidazole) in their structures, with mainly amino functional groups in their side alkyl chain. Just like carnosine, these functional groups are nucleophilic which might be useful for sequestering reactive species. However, this needs to be further validated via structure-activity relationship analysis or prediction of activity spectra for substances (PASS) approach - by comparing the structure of new compound with structure of well-known biological active substrate existing in the database, which at this stage is beyond the scope of this PhD project. Both of these functional groups are relevant in the sequestering action towards reactive and deleterious aldehydes, like 4-HNE, via Schiff base formation and intramolecular Michael addition reaction mechanism. The orientation of these molecules towards the active site of carnosinase-2 is similar to that of carnosine, where the aromatic group occupies one pocket of the enzyme's active site and the carboxyl-alkyl chains map to the other functional site. Also, when these molecules were docked in carnosinase-1 almost similar desired binding scores were observed, indicating that these molecules also fit with this enzyme with a similar extent of interaction involved, and, as such, are likely to be resistant against hydrolysis from either carnosinase.

Carnosine and its analogue bestatin - a known inhibitor of carnosinase, were allowed to interact with CN2 computationally in order to establish interaction conditions, molecular conformations, functional groups or bonding type present for substrate recognition. From the Maybridge database, this step then allowed to identify carnosine analogs that are resistant to hydrolysis. Due to intellectual property considerations and a pending patent application, sadly the exact chemical structural details of the chosen molecules must remain confidential at this time. This *in silico* work was kindly undertaken by the Garner group of NTU Chemistry Department.

The 5-day treatment of selected carnosine-related molecules on both mouse and human myotubes under glucolipotoxic conditions successfully attenuated the increased formation of intracellular reactive species, and consequently improved glucose uptake. Whilst some compounds did not affect pancreatic β-cell viability, an ineffective response was seen on insulin secretion following incubation with these carnosinase inhibitors. One likely reason for the different responses shown between skeletal muscle and pancreatic β-cells is that the carnosine concentration in skeletal muscle is very high, whereas there are no data available to indicate the concentration in β-cells. Importantly, preliminary data obtained by a colleague in Turner group has in fact determined that there is a very low concentration of carnosine in INS-1 β-cells. This is consistent with the observation of Robertson and Hammon (2007) that islets are one of the least protected tissues in terms of an inherent antioxidant defence system. This makes these cells more susceptible to damage under oxidative stress, such as prolonged exposure to glucolipotoxicity. However, whilst carnosinase inhibitors are likely to prove of little direct benefit in enhancing insulin secretion, Cripps et al. (2017) have demonstrated that carnosine can significantly increase insulin secretion. In contrast to carnosinase inhibitors, therefore, it seems likely that biologically active carnosine mimetics, which are also resistant to hydrolysis, will eventually prove to be highly effective insulin secretagogues.

In conclusion, data obtained from functional assays relevant to glucose homeostasis showed that selected carnosinase inhibitors are effective agents to increase skeletal muscle glucose uptake, and hence are likely to be effective at combating insulin resistance. Carnosine mimetics are also effective in this capacity, and have the added benefit of being likely to increase insulin secretion from pancreatic β-cells as well. Together they are promising drug candidates that could potentially offer novel therapeutic strategies for the prevention and treatment of type 2 diabetes.

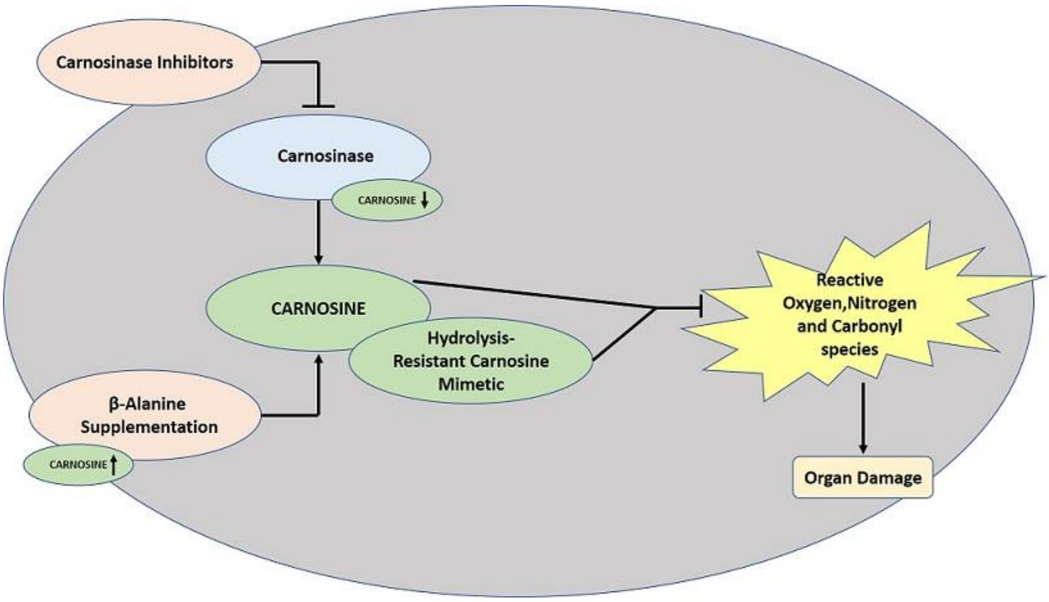


Figure 5.21; A schematic representation of how carnosinase inhibitors, carnosine mimetics, or β-alanine could be utilised as potential treatment strategies for oxidative-stress driven diseases like type 2 diabetes.

5.4 Future Work

The increasing and improved data on protein 3D structures has contributed to the success of structure-based approaches in the development of several experimental and theoretical techniques for the rational design of protein ligands, and thus through the application of combinatorial chemistry provides a potentially rapid identification of potential new drug candidates. With this advancement, the Turner laboratory, in conjunction with the Garner Synthetic Chemistry research group at NTU, are engaged in the ongoing search, design, and evaluation of anti-diabetic agents based on carnosine-carnosinase interactions. The use of chromatographic techniques like high-performance liquid chromatography (HPLC) can also provide understanding as to the likely effectiveness of carnosinase inhibitors by quantifying the concentration of carnosine within different cell types, as well as in the presence or absence of metabolic stress or therapeutic treatments.

Evaluation of additional molecules identified to elicit improved insulin secretion and glucose uptake in cells exposed to glucolipotoxicity will also be further evaluated *in vivo* using high-fat-fed mice – through an ongoing collaboration that has been established between the Turner and Caton group in King's College London. Ultimately, it is planned that NTU and the Philippines' MSU-IIT will maintain collaborative work by moving forward the initial data obtained in this project to future clinical trials and food supplementation studies aimed to be initiated upon the conclusion of this PhD study – a research initiative that will be most likely supported by the Philippine government research agency which has contributed much to the success of this entire PhD project. By so doing it is hoped that we may be able to develop a new class of therapeutic agents with ever increasing efficacy and effectiveness.

Chapter 6:

General Discussion and Conclusions

6.1 General Discussion

Diabetes mellitus is an endocrinological and/or metabolic disorder characterised by persistently high blood glucose levels stemming from inadequate or defective pancreatic insulin secretion, and impaired insulin-directed transport or utilisation of glucose by target cells, such as skeletal muscles. The diagnosis of diabetes is based upon an oral glucose tolerance test and/or measurement of glycated haemoglobin (World Health Organisation, 2011, Section 2). The clinical diagnosis of diabetes is typically prompted by several associated symptoms, which may include excessive thirst and frequent urination, intermittent infections, unexplained reduction of weight, glycosuria, and in severe instances, drowsiness and coma (Alberti and Zimmet, 1998).

There are three major types of diabetes and each of these has different causes and risk factors. Among these types, type 2 diabetes mellitus (T2DM) is the most common and accounts for about 95% of diabetes cases (Dardano et al., 2014). The prevalence of T2DM has been growing at an alarming rate worldwide; it is predicted that in 2045 there will be over 1 billion people living with or at high risk of diabetes. Around 80% of these patients will be in low-middle income countries and most of them will be 45-64 years old (Wild et al, 2004, International Diabetes Federation, 2017; Harvard T.H. School of Public Health, 2016).

Genetic and environmental factors, poor diet (low in fibre, high in fat, salt, sugar) and a sedentary lifestyle are some of the factors believed to promote the development of T2DM (Fletcher et al., 2002; Stumvol et al., 2005). Also, while there is a clear association between the ageing of the population and greater prevalence of T2DM, the increasing incidence of obesity has resulted in a dramatic rise of T2DM among children, teenagers and adolescents, which also increases their risk of health complications in later life (Pulgaron et al., 2014; Silverstein et al, 2001). T2DM often develops over a period of years, and therefore symptoms can also develop gradually. Diabetic individuals are more susceptible to several forms of acute and chronic complications, which could lead to serious damage to both small and large blood vessels. These complications may include some of the macrovascular diseases like hypertension, hyperlipidemia, heart attacks, coronary artery disease, strokes, cerebral vascular disease, and peripheral vascular disease, and microvascular diseases namely, retinopathy, nephropathy, and neuropathy (Forbes, 2013, Wu et al, 2014 and Evans, 2015).

A 2016 study indicated that the global cost of diabetes has reached 825 billion US dollars per year (Diabetes UK, 2019; Harvard School of Public Health, 2017). The alarming prevalence of T2DM led to the development of several therapeutic approaches for hyperglycaemia, these are in the form of either oral or injectable drugs, mainly to reduce and maintain patients' blood glucose concentrations and prevent patients from developing further complications. However, due to the complex nature of the pathophysiology of T2DM and the varied responses of patients towards these drugs, over time they lose their effectiveness. Therefore, it is vital to constantly identify and develop novel targets and treatment strategies to combat this challenging health problem.

The aim of this PhD thesis was to investigate the biological action and therapeutic potential of carnosine and related molecules for T2DM treatment through targeted action to improve insulin resistance in skeletal muscles. In this study, a glucolipotoxicity cellular model of T2DM was employed. In order to induce insulin resistance, skeletal muscle cells (C2C12 and primary mouse muscle cells) were cultured in DMEM, or ready to use skeletal muscle growth media (human skeletal muscle cell-line) supplemented to a final concentration of either 28mM glucose (animal muscle cells) or 17mM glucose (human muscle cells), along with 200μM palmitic acid and 200μM oleic acid for 5 days to mimic diabetic extracellular glucolipotoxic conditions or GLT (high glucose and high fatty acids). The choice of these fatty acids and their concentrations were selected as they are the most abundant fatty acids in the diet and in the serum. The Turner Group initially published data demonstrating that glucolipotoxicity decreased insulin secretion (Marshall et al, 2007) and subsequent studies also showed that GLT can initiate pancreatic beta-cell death (Baganati et al., 2016). This PhD work has contributed to the existing international state of the art on this topic by confirming that glucolipotoxic conditions did negatively affect insulin secretion and glucose uptake (Cripps et al., 2017).

The work presented in Chapter 3 showed that carnosine supplementation in C2C12, human skeletal myoblasts, and primary muscle cells could protect these cells against the oxidative stress driven by reactive species that are generated following chronic exposure to high glucose and free fatty acid concentrations, and thereby determined that this could enhance insulin-dependent glucose uptake. Oxidative stress (OS) has been considered as the common denominator for the pathogenesis of several diseases including cancer, diabetes, obesity and neurodegenerative disorders (Son, 2012; Devi, 2015; Reuter, 2010). In particular, OS is associated with the pathogenesis of the two most relevant hallmarks of T2DM, namely insulin resistance and β-cell dysfunction (Pitocco et al., 2013; Poitout et al., 2008). Oxidative stress ensues when the oxidant production in the living system exceeds that of the cell's antioxidant machinery, in other words, a disease results when there is a imbalance in the redox system of the cell (Henriksen et al., 2011).

Oxidants are either generated intentionally, or as by-products which can be in the form of reactive oxygen species and reactive nitrogen species. My experiments showed that GLT-treated cells have increased 3-nitrotyrosine (3-NT), a molecule that has been detected in many human pathologies. Importantly, 3-NT is a useful marker for the presence of a strong oxidant or reactive nitrogen species called peroxynitrite, which is also a product from the reaction of common reactive species nitric oxide and superoxide (Ischiropoulos and Al-Mehdi, 1995; Ischiropoulos et al., 1992). Another reactive nitrogen-derived species that has been shown to be modulated by GLT is the inducible nitrogen oxide synthase (iNOS), which is an instigator of inflammation, insulin resistance, and is increased in the skeletal muscles of type 2 diabetic patients (Ceriello et al., 2002; Tannous et al., 1995; Torres et al, 2004).

In addition to the RNS and ROS above, another reactive species that could easily react with the nucleophilic sites of proteins such as Lys, His, and Cys, and DNA thereby causing cellular dysfunction are the reactive carbonyl species (RCS). Proteins are the target of RCS, and through a series of oxidative and nonoxidative reactions yield the irreversible advanced glycation end products (AGEs), which are shown to be amplified and accumulated in diabetes because of hyperglycaemia (Uchida, 2003). Proteins are also modified by lipids through lipid peroxidation and produce advanced lipid peroxidation end products (ALEs). Possible mechanisms by which AGEs and ALEs modify proteins, and thereby impair their functions, involve the induction of signal transduction (e.g. receptor activation inducing inflammatory cascades) , which would potentially lead to cellular damage and the mediation of the functional disorganisation of their target molecules as a consequence of conformational changes or catalytic distortion (Coughlan et al., 2009; Bierhaus et al., 2009). One particular reactive aldehyde quantified in this work is 4-hydroxynonenal or 4-HNE.

4-HNE is the most intensively investigated and quantitatively most important product of lipid peroxidation due to its high cytotoxic role inhibiting gene expression (Ayala et al., 2014). It enhances the development and progression of several pathological states, including diabetes, and GLT-treated cells had increased levels of these species. This was consistent with the negative impact of glucolipotoxicity on insulin secretion.

Thus, given the implication of oxidative stress in the onset of type 2 diabetes, it is possible that antioxidant strategies would be effective in the prevention or treatment of diabetes. These are molecules or compounds that could scavenge, quench or even neutralise excess reactive species, thereby preventing cellular damage and preserving function.

In order to address this, the Turner group have shown that a natural dipeptide, carnosine, has the ability to offset the negative effects that reactive species have upon beta cells and skeletal muscle cells, and by so doing increased insulin secretion and glucose uptake in cells exposed to glucolipotoxic conditions. The antioxidant and scavenging action of carnosine towards radicals or reactive species can be attributed in many ways. For example, it is a good buffer not only for protons but also for regulating the level of mixed-valence metal ions (copper, cobalt, manganese, iron, and cadmium) that take an active part in many metabolic processes activating free-radical processes. Another one is its anti-glycating or anti-crosslinking properties that could block oxidative damage of biomolecules (Prokopieva et al., 2015). In the case of RNS and ROS, carnosine could form a charge-transfer complex with free-radicals converting them to a stable or unreactive molecule (Boldyrev et al., 2013), and with RCS like 4-HNE, carnosine could trap this cytotoxic aldehyde through a sacrificial mechanism in lieu of the target substrate by acting like the preferable site of addition by HNE (Liu, Xu and Sayre, 2003).

Data shown in Chapter 4 detailed proteins that both interacted and formed adducts with 4-HNE and 3-NT in skeletal muscle and pancreatic islet cells under metabolic stress due to exposure to glucolipotoxic conditions for 5-days. In order to generate these data, cell lysates were immunoprecipitated against 4-HNE and 3-NT primary antibodies prior to analysis using high-resolution mass spectrometry (SCIEX TripleTOF 6600). The proteins identified were classified according to molecular function, biological process, and protein class using the program PANTHER (Protein Analysis Through Evolutionary Relationship). Initial data obtained using the C2C12 cell-line revealed that the majority of 4-HNE and 3-NT associated or adducted proteins are linked to metabolic processes and involved in catalytic activity. Importantly, about 65% and 95% of this adduction, respectively, were prevented by carnosine supplementation. Interestingly in this list, there are particularly mitochondrial enzymes (e.g., pyruvate dehydrogenase [acetyl-transferase] kinase isozyme 3, pyruvate dehydrogenase protein X component, isoleucine-tRNA ligase, ATP citrate synthase, and citrate synthase) that are promising therapeutic targets for patients with metabolic syndrome and are also important in glucose utilisation and in maintaining a steady supply of ATP in the cell (Lee, 2014; Zhou et al., 2019). Also, included in this list are mitochondrial processing peptidase, apoptosis-inducing factor, glycogen synthase and magnesium transport proteins that have been implicated in mitochondrial dysfunction and mitochondrial-related diseases (Gakh, Cavadini and Isaya, 2002; Padrao et al., 2012; Bano and Prehn, 2018). Mutations or dysregulated activities of some of these enzymes are also believed to have a causal link in severe muscle mitochondrial dysfunction, leading to atrophy and neurodegeneration.

This glucolipotoxicity-related mitochondrial impairment in skeletal muscle could potentially compromise protein quality control and normal skeletal muscle function in the mitochondria. The data obtained in this study (Chapter 4) indicated that there are also catalytic enzymes adducted by both 4-HNE and 3-NT relevant in regulating insulin sensitivity and action, and related signalling pathways. In a human skeletal muscle cell-line, the extent of adduction by 4-HNE and 3-NT was also determined and data showed that the majority of proteins that are adducted by both 3-NT and 4-HNE are also involved in binding, catalytic and structural molecule activities. In terms of biological process, the classification system in PANTHER showed that just like what has been shown from C2C12, these proteins generally participate in metabolic and cellular processes.

In terms of 4-HNE adduction, more than 80% of adducted mitochondrial proteins (e.g., ATP synthase, aconitate hydratase, ATP-citrate synthase, electron transfer flavoprotein subunit, malate dehydrogenase, pyruvate carboxylase, stress-70 protein, succinate dehydrogenase [ubiquinone] flavoprotein subunit, and superoxide dismutase with catalytic activities) are prevented by carnosine. The data for this adduction experiment also indicated that other proteins thought to be adducted by 4-HNE and 3-NT in GLT-treated human skeletal muscle cells are guanine-nucleotide binding proteins, creatine kinase B type, Ras-related proteins, protein disulphide isomerase, and sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA). Impaired function of SERCA in muscle affects its quality and quantity and triggers ER stress, which could also promote the progression of insulin resistance in obesity and diabetes (Kang et al., 2016; Qaisar et al., 2018)

Dr Katie Hannah (of the Turner group) conducted similar IP-MS analysis on INS-1 pancreatic β-cells, which have been cultured in either control or GLT media ± carnosine to obtain a preliminary profile of proteins adducted by the reactive carbonyl (4-HNE) and nitrogen (3-NT) species adduction. This has been furthered by working with mouse primary islets (isolated at Kings College London by Dr Paul Caton, working with Home Office and local Ethics Committee approval) similarly treated for 5 days with either RPMI control or GLT ± 10mM carnosine. Interestingly, based on this list, more than 80% of protein-HNE and protein-3NT adduction in GLT conditions was also prevented by the addition of 10mM carnosine. Data for this showed that proteins with binding molecular functions involved in metabolic biological process are the ones that are mostly adducted by both 4-HNE and 3-NT. The mitochondrial enzymes, such as acetyl-CoA acetyltransferase, dihydrolipoyl dehydrogenase, pyruvate carboxylase, and stress-70 protein, are important in normal mitochondrial activity in pancreatic β-cell metabolism and energy production, and when these are modified by adduction for instance studies showed that this could possibly lead to a progressive failure to potentiate insulin secretion (Kowluru et al., 2006; Gorrepati et al., 2018; Kim et al., 2006; Mziaut et al., 2016). Also, in the list are proteins that are important in islet signalling, and are effectors in controlling, tethering and docking of secretory vesicles to help regulate insulin secretion.

The concept of protein adduction mediated by carbonylation and nitrosylation may not be confined to the glucose homeostasis-relevant tissues described above. This PhD work also included the identification of proteins adducted by 3-NT and 4-HNE in human serum samples from obese, type 2 diabetic, and gestational diabetic individuals. Results for this are not only helpful to construct a detailed road map of how obesity and diabetes contribute to the pathology of HNE and 3-NT adduction, but they might also be important in aiding early disease detection (possibly through blood test), allowing the implementation of therapeutic interventions earlier in the disease process to prevent the manifestation of damaging and irreversible diabetes complications. Analysis of the serum proteins adducted by both 3-NT and 4-HNE identified molecules involved in adaptive immunity. This implies that oxidative stress-driven adductions of important serum molecules may contribute to impairment of the immune defences of obese and diabetic individuals, which could influence the progression of diabetic complications.

Interestingly, extracellular matrix proteins (ECM) in these serum samples were also associated with both 4-HNE and 3-NT, and, as such, they might modify ECM protein structures and change cellular interactions. Law et al., 2012 showed that persistent elevation of blood glucose and oxidative stress could induce ECM alterations that are indicative of several diabetic complications, including renal dysfunction and cardiac dysfunction.

Another group of proteins called apolipoproteins are observed in this work to be most likely adducted by 3-NT in all serum samples used in this study. The oxidative stress and disrupted antioxidant defences that occur in diabetes is associated with perturbed lipid metabolism. This high index of oxidation modifies plasma apolipoproteins, as reflected by the observed increase in oxidised forms of these proteins, and, as a consequence, cardiovascular complications can arise due to the poorly mediated efflux of cholesterol in the circulation (Azizkhanian et al., 2016).

Several types of histones were also associated with 4-HNE in the serum of obese and gestational diabetes individuals, suggesting that they might be modified by this reactive alkenal. The potential modification of histones by 4-HNE might serve as a common denominator between obesity and diabetes, but could also suggest that the offspring of mothers with gestational diabetes are predisposed to obesity and type 2 diabetes, as this inherited genetic variant has been shown in observational studies (Michalczyk et al., 2016; Kasinska et al., 2016).

Immunoprecipitation and mass spectrometric analysis of skeletal muscles and pancreatic islet cells (Chapter 4) showed that some mitochondrial proteins are adducted with both 4-HNE and 3-NT, and this finding might explain how oxidative stress-driven mitochondrial dysfunction is linked with glucolipotoxicity-induced insulin resistance and β-cell dysfunction, thereby contributing to the pathogenesis of type 2 diabetes. Further evidence of this is provided by the profile of mitochondrial stress shown Seahorse XFe24 data, where key parameters of mitochondrial function, such as basal, maximal and ATP-linked respiration, were evaluated. The effect of glucolipotoxicity treatment on the skeletal muscle cells, as reflected by a decreased oxygen consumption rate (OCR) compared to control, could be indicative of mitochondrial dysfunction. The change shown in GLT-treated cells is suggestive of compromised mitochondrial respiration rate, which could result in a diminished ability for effective and normal substrate metabolism. The addition of 10mM carnosine improved mitochondrial respiration, particularly in mouse C2C12 and human skeletal muscle cell lines, which might be attributed to its scavenging or quenching effect towards reactive species and/or its by-products (4-HNE and 3-NT), thereby preventing key biomolecules like proteins and enzymes from damaging adducts formation.

Carnosine synthase (ATPGD1) is an enzyme that is required for the endogenous synthesis of carnosine and is mainly expressed in skeletal muscles and in the brain (Miyaji et al., 2012; Drozak, et al., 2010). The data presented in Chapter 5 indicated that although ATPGD1 was detected in C2C12 mouse muscle cells, there was no significant difference in its expression between healthy control and GLT-treated cells. β-alanine supplementation increases the carnosine content of skeletal muscles, with β-alanine being the limitation to the synthesis of carnosine (Harris et al., 2006). After β-alanine has been transported into skeletal muscles by the proton-assisted amino acid transporter 1 (PAT1) and taurine transporter (TauT) it combines with L-histidine to form carnosine through the enzyme carnosine synthase. Work conducted in this thesis indicated that 5-day supplementation of β-alanine in GLT exposed C2C12 muscle cells resulted in a significant decrease in GLT-mediated intracellular reactive species, and enhancement of glucose-uptake, as compared to healthy control. Carnosine synthase was also detected in INS-1 pancreatic β-cells (study conducted by colleagues Cripps and Hanna). Interestingly, a 5-day treatment of β-alanine also lowered the GLT-generated reactive species in these cells and importantly also prevented GLT-impaired insulin secretion.

The promising potential of carnosine as a therapeutic agent for oxidative-based diseases is limited in humans due to the presence of serum (CNDP1) and tissue carnosinase (CNDP2), which catalyse the hydrolysis of carnosine to its amino acid components (Vistoli et al., 2012; Teufel et al., 2003). In this work, the level of enzyme CNDP2 was evaluated and data showed that, under GLT conditions, CNDP2 is significantly upregulated relative to control. Upregulation of carnosinase, and thus its activity, has been reported to be associated with increased risk of diabetic nephropathy and altered kidney function in type 2 diabetes (Ahluwalia et al., 2011). Chiu et al. (2014) has shown that CNDP2 expression is significantly elevated in both male and female hypertensive mice. Consequently, the carnosine-carnosinase equilibrium may be disturbed across a broad spectrum of cardiometabolomic diseases.

The design and synthesis of carnosine-related molecules was an integral component to this project. Importantly, these molecules have been designed to be stable against hydrolysis by carnosinases. The data obtained from the evaluation of these molecules showed some promising candidates for use as potential anti-diabetic agents. From these, three (M8, M28 and CE3) were initially chosen to be evaluated *in vivo* for anti-diabetes action in a high fed mouse model of diabetes. The carnosine-related molecules used in this work were relatively small in size with an aromatic ring and amino functional group in their structures. Both of these functional groups are relevant in the sequestering action towards reactive species and deleterious aldehydes like 4-HNE. The 5-day treatment with these selected carnosine-related molecules in both mouse and human myotubes under glucolipotoxic conditions significantly lowered the increased formation of intracellular reactive species, and consequently improved glucose uptake.

Some of these molecules were also tested in GLT-treated pancreatic β-cells for 5-days (work conducted by a colleague M. Cripps). However, those that did not affect cell viability showed varied responses or were ineffective at enhancing the insulin secretion. One reason for the different responses observed between skeletal muscles and pancreatic β-cells might be due to the level of endogenous carnosine, as that is known to be high in the former (Suzuki et al., 2002) while so far, there is no published data or known value to indicate this in the latter. There was, however, a very low concentration of carnosine detected in INS-1 β-cells based on preliminary data obtained by a colleague in Turner group, which might explain why the carnosinase inhibitors were ineffective in β-cells. This observation is also consistent with what was reported by Robertson and Hammon (2007), indicating that the pancreas is one of the least protected tissues in terms of inherent antioxidant defence system, and thereby making the β-cells more susceptible to damage under oxidative stress. It is then tempting to speculate that carnosine mimetics such as carnosine esters might be better for the pancreas, although this warrants further work to prove.

6.2 Conclusions

This PhD thesis has demonstrated that when skeletal muscle cells were cultured in glucolipotoxic (GLT) media, levels of cellular reactive oxygen and nitrogen species significantly increased. GLT mediated the increased generation of two biomarkers that are implicated in several pathologies, including diabetes, namely 4-HNE and 3-NT. The increase of these cellular reactive species in GLT-exposed muscle cells was negatively correlated with their ability to uptake glucose. The negative impact of glucolipotoxicity was not only demonstrated with insulin resistance, but also had a negative impact on insulin secretion and elevated quantities of the aforementioned reactive species (shown by a related project in the Turner group). Carnosine supplementation was able to scavenge or quench those glucolipotoxicity-mediated free radicals and reactive species, thereby resulting in an improved insulin sensitivity through increased glucose uptake in skeletal muscle cells. Similarly, carnosine also inhibited the GLT-driven generation of reactive species in β-cells and increased insulin secretion. Therefore, the scavenging action and the use of carnosine as a blocking agent against those deleterious species could offer potential treatment and therapeutic perspectives for T2DM patients with dual beneficial actions on glucose homeostasis.

The immunoprecipitation-mass spectrometry tandem experiment allowed us to identify possible proteins that are associated or adducted by 4-HNE and 3-NT species in muscle cells and pancreatic islets under GLT conditions. Using the PANTHER classification system, it was shown that the majority of these proteins are involved in catalytic and binding activities that relate to metabolic processes, some of which are relevant to the biosynthesis and action of insulin. Treatment of cells under GLT-induced metabolic stress with 10mM carnosine prevented the majority of adduct formation, indicating that the protective action of carnosine in this context was effective, thereby preserving mitochondrial and other cellular functions. In addition to skeletal muscle and pancreatic islets, this work also identified different classes of proteins that are mostly adducted by 4-HNE and 3-NT from obese, type 2 diabetes and gestational diabetes serum samples. Analysis showed that some of these are critical for immune responses, such as the immunoglobulins, whilts others include apolipoproteins and histones. This approach not only allowed us to evaluate the extent of carnosine's protective action towards protein damage by 4-HNE and 3-NT adduction, but also identified a class of proteins that could serve as a tool for early detection. They may also be potential targets for therapeutic intervention in the treatment or prevention of diabetes and its complications.

Supplementation of the rate-limiting constituent of carnosine synthesis, β-alanine, to GLT-treated myotubes enhanced glucose uptake and reduced glucolipotoxic-driven generation of reactive species, suggesting that this amino acid could potentially offer protective actions, most probably by sustaining or increasing the intracellular pool of carnosine. Likewise, screening of carnosinase inhibitors or carnosine mimetics, which were designed through computational and bespoke organic synthetic chemistry approaches, showed that some are able to reduce glucolipotoxic-mediated formation of cellular reactive species and consequently improved glucose uptake. Therefore, carnosine and synthetic carnosine-derived molecules are potentially promising drug candidates that could offer novel therapeutic strategies for the prevention and treatment of type 2 diabetes.

Finally, this work proposed that future studies in this area should be extended to focus upon carnosine actions on the immune system. The fact that several immune system proteins adducted in the serum of obese and diabetic individuals were seen in this work may, at least in part, help to explain why individuals from these groups are more susceptible to infection than the general population. Given that data presented in here indicated that the majority of adduction resulting from glucolipotixic metabolic stress can be prevented by carnosine supplementation of cells and tissues as far as this PhD work is concerned, it is tempting to speculate that carnosine might prove equally as effective at preserving serum immune function as it did in preserving stimulus-secretion coupling in cells that mediate insulin secretion and glucose uptake. This is especially pertinent at the present time, given that immunocompromised individuals are particularly susceptible to Covid-19, and that ~30% of all UK hospital deaths attributed to Covid-19 in April 2020 were reported to be from individuals with diabetes and obesity. Carnosine could, therefore, potentially improve the prognosis of patients with diabetes and obesity not only by enhancing stimulus-secretion coupling in skeletal muscle and pancreatic β-cells and thereby reducing HbA1c levels, but also by preserving immune function.

Chapter 7

Limitations of the Study and Future Directions

This study utilised only one method in assessing cell viability. As the Calcein-AM may not always be an ideal or sole measure of cell viability, other test and method such as apoptosis assay and flow cytometry would provide stronger evidence to indicate that the GLT response was not an artefact of either GLT-driven cell death or cell proliferation. In addition, the present study did not include a positive or negative control to check the working condition of the reagent employed in the assay.

The list of proteins identified and indicated in Chapter 4 is based on the use of one immunoaffinity enrichment-based technique (magnetic bead immunoprecipitation), and so there could possibly GLT adduction – carnosine protection events that might be outside the level of detection by this method. Other enrichment strategies or enrichment kits for the mass spectrometry-based identification of specifically targeted class of proteins would be more advantageous complementary tools for greater protein identification. Protein classification was solely based on PANTHER software which might have some limitations too. In addition, patients' ethnicity as indicated in Table 4.1 were not specified since additional information are unavailable and thus were only presented as Asian, White and Black.

The current study, in conjunction with that of others in the Turner group, has identified that carnosine is effective in scavenging reactive species in different glucolipotoxicity-based cellular models of type 2 diabetes, thereby preventing protein damage through deleterious adduction by oxidative biomarkers (4-HNE and 3-NT) and preserving mitochondrial function of cells under metabolic stress. As such, future studies are likely to include similar adduction experiments and obtain mitochondrial stress test profiles in these cellular models for the leading candidate carnosine-related molecule(s) in order to characterise their biological actions.

Initial adduction data obtained from this work, and future analysis using additional and/or other clinical samples for adipose tissue and human primary adipocytes combined, might also provide information related to potential anti-inflammatory actions of carnosine directed against adipocytokine release (both types of samples were initially processed but due to technical and facility-related problems, this needed to be resampled and re-processed). If so, this could help us to better understand how obesity and diabetes contribute to the pathology of HNE and 3-NT adduction. In turn, this could provide novel therapeutic intervention strategies for type 2 diabetes and its complications.

The choice of carnosine-derived molecules presented in Chapter 5 was initially based on computational data and criteria (geometrical). Thereafter, the selection of molecules which have been further studied both *in vitro* and *in vivo* were then based on their bioactivities (ROS scavenging or glucose uptake assays). Dose-dependence experiments in all carnosine-derived molecules were not conducted at the beginning and would only be examined after selecting top 3 best compounds for future work in vivo. Such experiment (dose-response) would have been necessary to define the most effective compound. Particularly, assays where carnosine-derived molecules are involved, vehicle control in healthy condition was only included during the initial ROS scavenging assay (e.g. Figure 5.12) and in one glucose uptake assay (Figure 5.14), and there was no inclusion of a vehicle control in GLT condition. It was only assumed that the solvent used for the preparation of molecules had no effect on other similar assays both in control and GLT based on prior cell viability experiments.

Based on the preliminary scavenging data and glucose uptake experiments of more than 10 carnosine-related molecules that have been evaluated in the present study, three candidate molecules (designated as M8, M28 and CE3) have emerged from this work as potential therapeutic agents. These were initially chosen to be evaluated for anti-diabetic effects *in vivo* using high fat fed mice as a representative animal model of diabetes that mirrors the dyslipidaemia and hyperglycaemia typically seen in obese humans with T2DM. The graph below indicates a representative glucose tolerance test conducted after 8-weeks of high fat diet. This part of work has been conducted by our collaborator Dr. Paul Caton of King's College London, and all animal procedures are approved by the King's College London Ethics Committee and are carried out in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986.

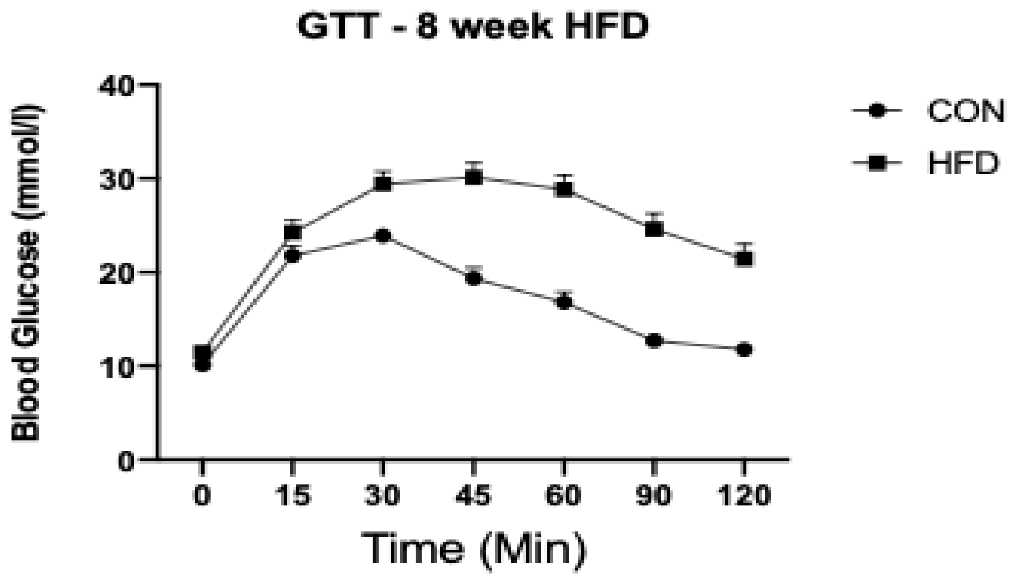


Figure 7.1. **Glucose tolerance test data from 8-weeks high fat fed mice**.
(*Conducted by Dr. Paul Caton, King's College London*)

Preliminary data with the analogues is encouraging, but full analysis is still ongoing at the time of this submission. If this *in vivo* study is as successful as early data indicates, then this will form the basis for future pharmacokinetic studies wherein absorption, distribution, metabolism, excretion and toxicity data will be generated in multiple animal species. It should also then be possible to extend these analyses to predict likely human dose tolerance as a prelude to conducting a small-scale clinical trial. If successful, that could lead to wider roll-out for the benefit of a significant number of those with type 2 diabetes.

In parallel to this work, the Turner Laboratory and the Garner Synthetic Chemistry research groups of NTU continue to design and evaluate further potential anti-diabetic candidates based upon carnosine-carnosinase interactions and/or other diabetes-relevant biological targets. This structure-based screening for more molecules will also utilise chemical libraries other than Maybridge. In addition, carnosine-related molecules investigated in this PhD work and some others that can be developed by Turner and Garner group in the future can also be further investigated using concentrations other than what had been used in this work.

Sedentary lifestyle is a factor known to contribute to the development of insulin resistance and T2DM. Regular physical exercise, by contrast, is believed to help in the management and delayed onset of this disease (Lumb, 2014), and therefore considered a possible solution to improve metabolic health of patients with T2DM. Skeletal muscle glucose uptake depends upon both GLUT4 translocation and expression, a process that can also be regulated by exercise. This contraction-mediated pathway is an insulin-independent mechanism of glucose disposal into skeletal muscles. Therefore, one possible future direction would be to identify the molecular mechanisms regulating the effect of exercise in improving glucose uptake in skeletal muscles, and to determine the impact this might have upon mitochondrial function, and its influence on metabolic genes.

It would also be interesting to determine the effects there might be when exercise is combined with carnosine supplementation. Similarly, does exercise alone have any influence on the concentration of endogenous carnosine in muscles, or on its release to the circulation? *In vitro* studies for this purpose could involve the application of stimuli to the cells such as electrical stimulation and mechanical loading to mimic acute or chronic exercise, followed by functional analysis including glucose-uptake measurement or by quantitative analysis of carnosine by HPLC or mass spectrometry.

In summary, the findings from this PhD show that carnosine enhances insulin secretion from the pancreas and increases glucose uptake into skeletal muscle. This has major implications for diabetes prevention and for the treatment of diabetes and associated complications. Future work the author would like to embark upon is to commence translation of this basic research into strategies that could have significant benefit to people (particularly in the Philippines) who are living with diabetes. Preliminary clinical trials on carnosine have begun to support the mechanistic studies. The benefit of carnosine appears greatest in obese individuals, again supporting our previously published work that showed carnosine could prevent damage to skeletal muscle and insulin producing cells exposed to high sugar and fat. The pilot carnosine studies to date have, however, been conducted in Western Europe, Australia, or North America, where individuals have a different genetic make-up to individuals in the Philippines. By contrast, there have been no such studies conducted in countries in the Western Pacific region and, as such, the extent to which carnosine might benefit individuals in this region is currently unknown. If the above strategies and plans are successful, planned collaborative work with NTU (Turner Laboratory) will allow future work to embed development of these novel therapeutic tools (in the Philippines) and thereby offer a significant future benefit to individuals at high risk of T2DM.

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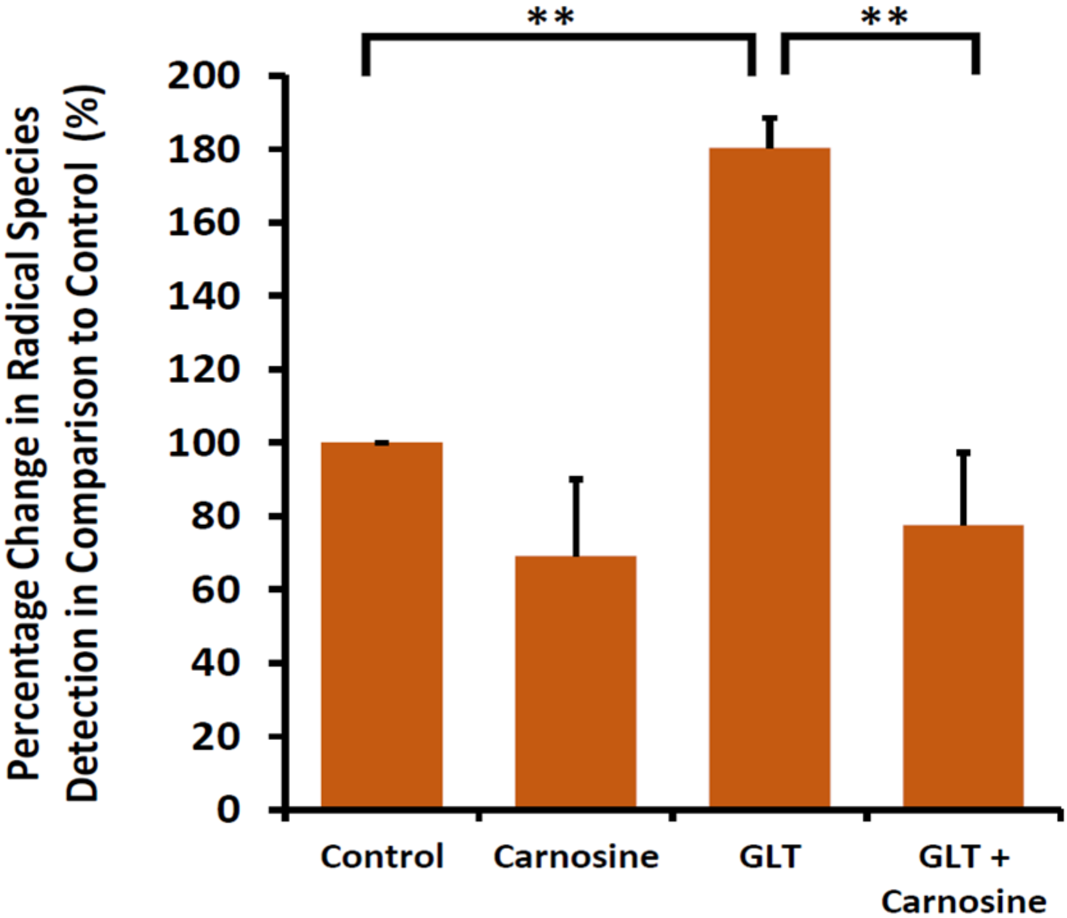
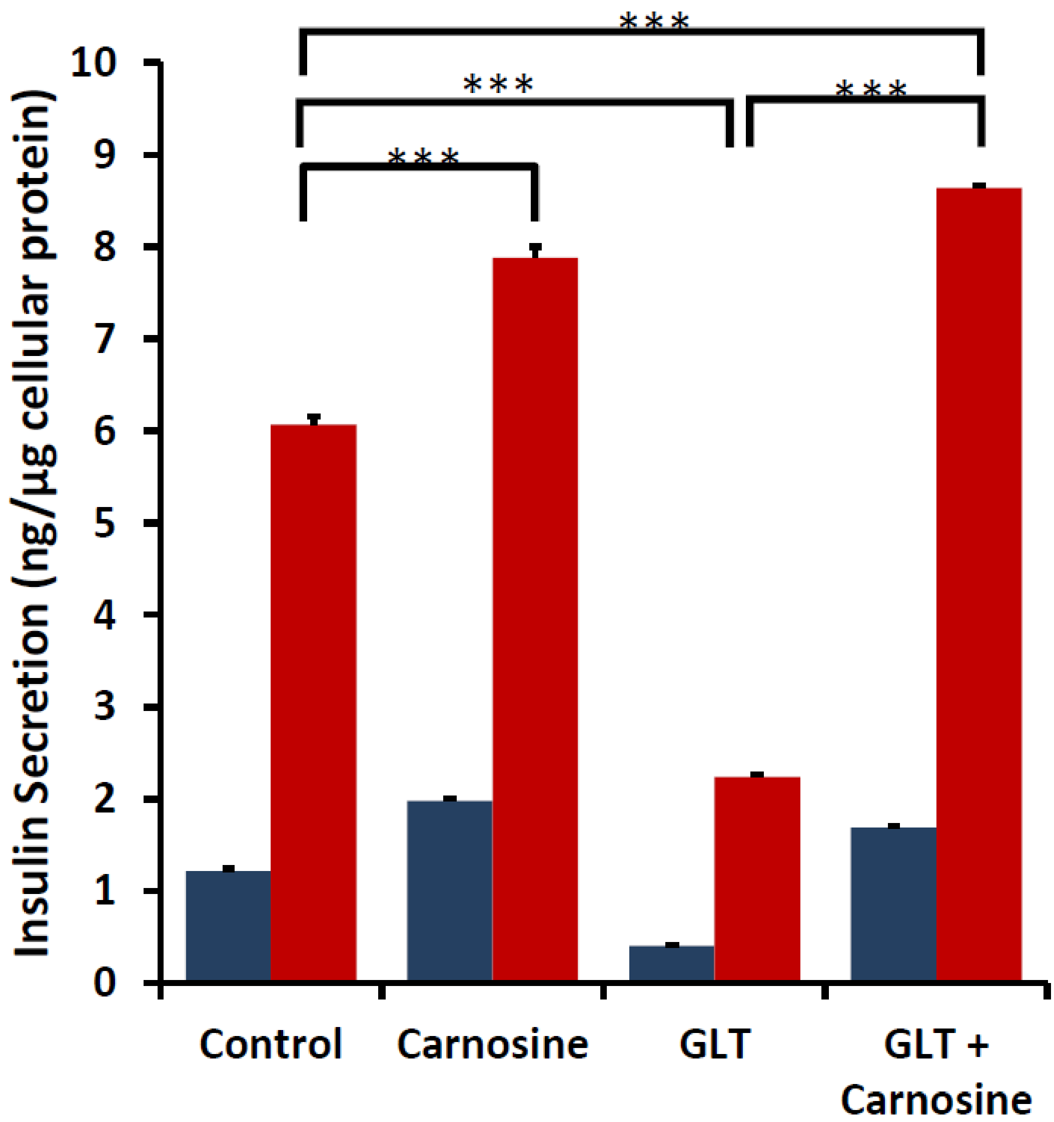
Appendices

Appendix 1



Carnosine is an effective scavenger of glucolipotoxic reactive species in β-cells. INS-1 cells were cultured in RPMI-1640 media or GLT media for 5 days before incubation with media supplemented ± 10mM carnosine for 1h. 20μM DCFDA was loaded in KREBS buffer for 1h and reactive species detected via fluorescence with excitation and emission of 495nm and 530nm. Reactive species are expressed as a percentage change in comparison to control from 4 independent experiments ± SEM. **p<0.005 (Obtained from Michael Cripps).

Appendix 2



Reversal of GLT-inhibited insulin secretion by carnosine in INS-1 cells. INS-1 cells were cultured in control or GLT media for 5 days. 2 days prior to stimulation, cells were incubated with or without experimental conditioned media supplemented with 10mM carnosine. Insulin secretion was determined by ELISA following incubation ± secretagogue cocktail for 2h [(-) blue, (+) red] with data normalised to protein content. Data are expressed as mean ± SEM from 5 independent experiments. ***$p<0.0005$ (conducted by Michael Cripps)