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Carnosine protects stimulus-secretion coupling through prevention of protein carbonyl adduction events in cells under metabolic stress

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ABSTRACT

Type 2 diabetes is characterised by failure to control glucose homeostasis, with numerous diabetic complications attributable to the resulting exposure of cells and tissues to chronic elevated concentrations of glucose and fatty acids. This, in part, results from formation of advanced glycation and advanced lipidation end-products that are able to modify protein, lipid, or DNA structure, and disrupt normal cellular function. Herein we used mass spectrometry to identify proteins modified by two such adduction events in serum of individuals with obesity, type 2 diabetes, and gestational diabetes, along with similar analyses of human and mouse skeletal muscle cells and mouse pancreatic islets exposed to glucolipotoxic stress. We also report that carnosine, a histidine containing dipeptide, prevented 65–90% of 4-hydroxynonenal and 3-nitrotyrosine adduction events, and that this in turn preserved mitochondrial function and protected stimulus-secretion coupling in cells exposed to metabolic stress. Carnosine therefore offers significant therapeutic potential against metabolic diseases.

Abbreviations

3-NT	3-nitrotyrosine
4-HNE	4-hydroxynonenal
AGE	advanced glycation end-product
ALE	advanced lipidation end-product
BMI	body mass index
DDA/IDA	data/information-dependent acquisition
ERGIC	endoplasmic reticulum--Golgi intermediate compartment
GD	gestational diabetes
GLT	glucolipotoxic
GLUT4	glucose transporter type 4
HSkM	human skeletal muscle myoblast
IP	immunoprecipitation
NO	nitric oxide
O_2	superoxide
OCR	oxygen consumption rate
$ONOO^-$	peroxynitrite
PANTHER	protein analysis through evolutionary relationships
PBS	phosphate buffered saline
RCS	reactive carbonyl species
ROS	reactive oxygen species
SERCA	sarcoplasmic/endoplasmic reticulum calcium ATPase
T2D	type 2 diabetes

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1. Introduction

Type 2 diabetes (T2D) is a metabolic disorder characterised by chronically elevated blood glucose levels, insulin resistance, and decreasing insulin secretion from the pancreas. Worryingly, the incidence of diabetes continues to grow at an alarming rate, with the projected figure rising from an estimated 451 million adults worldwide in 2017, to 693 million by 2045 [1]. Furthermore, this follows the increased trend in global levels of obesity that have been reported over the last 30 years [2]. Indeed, over 80% of patients with T2D are either overweight or obese, and high BMI has been reported to account for 73.5% of the increase in diabetes mortality rate since 1990 [3]. However, whilst the causal link between obesity and T2D is clear, the underlying mechanisms at the cellular and molecular level remain largely undetermined.

Attempts have been made to slow down the rate of rise in the incidence of obesity and T2D through strategies aimed at reducing sedentary lifestyles and increasing daily exercise. Although there is evidence that this approach can help in the management of T2D and can improve long-term prognosis [4,5], it can be difficult to sustain behaviour change over a long period of time, especially when the input required is not always compatible with a modern lifestyle. In addition, failing health may prevent adherence to extensive exercise programmes in some individuals. In these cases, it behoves us to develop alternative and/or complementary strategies based around adoption of a healthier diet and/or through therapeutic interventions.

Therapeutic strategies to treat T2D have mainly focused on metabolic aspects of the disease. However, there are a limited number of metabolic targets and the efficacy of pharmacological agents aimed at these targets often become less effective over time. This has led to a search for alternative strategies, with one such approach being to try to reduce the level of oxidative stress that results from chronic exposure to high glucose and fatty acid levels in obesity and T2D [6,7]. In this regard, promising data has started to emerge showing a reduction in plasma glucose levels in obese and diabetic individuals who have received regular dietary supplementation with carnosine (β-alanyl-L-histidine) [8–10], a histidine containing dipeptide first discovered in meat extracts by [11]. Carnosine is synthesized by the enzyme carnosine synthase from the combination of the alpha amino acid L-histidine and the beta amino acid β-alanine [12]. It is most abundant in human skeletal muscle [12] and is consumed in the diet through meat and fish [13], although due to the high activity of jejunal mucosa and plasma carnosinases, only trace amounts of intact carnosine are normally present in blood [14].

The majority of research on carnosine has related to its biological role in skeletal muscle, although its role/function in different tissues remains relatively unknown. The high carnosine, and other histidine containing dipeptides, content in many vertebrates, along with the existence of several genes regulating histidine containing dipeptide metabolism, might be suggestive of a relevant homeostatic role. Purported biological functions of carnosine have been suggested to include a physicochemical buffer, a regulator of Ca^{2+} sensitivity and intramuscular Ca^{2+} transients, metallic ion chelation, antioxidant activity and inhibition of by-product formation from advanced glycation and lipoxidation [12,15]. Of direct relevance to the current study, carnosine has been shown to be an effective scavenger of reactive carbonyl species (RCS) in a mouse model of diabetes [16]. Moreover, at the cellular level, carnosine has been shown to increase insulin secretion from pancreatic β-cells and to enhance glucose uptake to skeletal muscle myotubes [17].

Herein, we extend our earlier findings through the identification of specific individual 4-hydroxynonenal (4-HNE) and 3-nitrotyrosine (3-NT) protein adduction events in human serum from patients with obesity, T2D, and gestational diabetes (GD). By so doing, we aimed to identify disease-associated adduction events. Importantly, we have also investigated the extent to which carnosine is able to prevent similar damaging protein modifications in cells central to the control of glucose homeostasis, namely pancreatic islets and skeletal muscle cells, determined the effect on mitochondrial respiration, and performed GLUT4 translocation assays to quantify the impact on stimulus-secretion coupling in these cells.

2. Materials and methods

2.1. Materials

All chemicals or reagents were purchased from Sigma Aldrich (Gillingham, Dorset, UK) and all plasticware from VWR International Ltd (Lutterworth, UK). Antibodies were purchased from Abcam (Cambridge, UK) unless otherwise stated.

2.2. Subjects and sample collection

Following a 10 h overnight fast, venous blood was sampled in the patients detailed below, collected in chilled EDTA-containing tubes with and without aprotinin, aliquoted and frozen at −80°C until assayed. Blood was collected from lean controls (age:24.8 ± (SEM) 2.6yrs; BMI: 21.6 ± (SEM) 0.9 kg/m^2; n = 4), obese non-diabetic (age: 32.8 ± (SEM) 2.7yrs; BMI: 41.6 ± (SEM) 2.0 kg/m^2; n = 6), obese gestational diabetic (age: 33.3 ± (SEM) 2.1yrs; 12-week BMI: 43.3 ± (SEM) 2.2 kg/m^2; n = 6) and obese type 2 diabetic (age: 59.8 ± (SEM) 2.1yrs; BMI: 45.4 ± (SEM) 3.7 kg/m^2; n = 5) non-menopausal, women with informed consent, obtained in accordance with Local Research Ethics Committee (LREC) guidelines and approval. All samples were flash frozen for the *in vitro* studies detailed below. Participants were not on endocrine therapy, steroids, or receiving any anti-hypertensive therapy.

2.3. C2C12 and human skeletal muscle cell culture

Clonetics™ human skeletal muscle myoblasts (HSkM) were purchased from Lonza Bioscience, (Basel, Switzerland). HSkM cells were isolated from the upper arm or leg muscle tissue of a healthy donor (20-year old, Caucasian male, BMI = 21, non-smoker). HSkM cells were incubated in Human Skeletal Muscle Growth Media with supplement pack (PromoCell, Heidelberg, Germany). Mouse C2C12 skeletal myoblasts were maintained in high glucose-DMEM supplemented with 10% (v/v) foetal bovine serum, 10% (v/v) heat inactivated newborn calf serum (Life Technologies, Paisley, UK), and 1% (v/v) penicillin-streptomycin (Life Technologies) in a humidified atmosphere with 5% CO_2 at 37°C. C2C12 cells were switched to Dulbecco's Minimal Eagle's Medium (DMEM) supplemented with 2% (v/v) heat-inactivated horse serum (Life Technologies) for 7 days in order to facilitate myocytic differentiation, whereas HSkM cells were induced to differentiate in DMEM-F12 media. Cells were then incubated for a further 5 days in either control DMEM media (containing 11 mM glucose for C2C12 myotubes; 5 mM glucose for HSkM cells), or GLT media (DMEM with 28 mM glucose, 200 μM oleic acid, 200 μM palmitic acid for C2C12 cells; DMEM with 17 mM glucose, 200 μM oleic acid, 200 μM palmitic acid for HSkM cells), conditions representative of those seen in poorly controlled T2D, and carnosine as indicated.

2.4. Islet isolation

Islets were isolated from male CD1 mice by collagenase injection into the pancreatic duct. As previously detailed [17,18], digested pancreas was washed with MEM-2279 and separated from exocrine tissues by centrifuging through a Histopaque 1.077 g/ml gradient. After washing, islets were picked and incubated at 37 °C in RPMI-1640 (supplemented with 10% [vol/vol] foetal calf serum, 2 mM glutamine and 100U/ml penicillin/0.1 mg/ml streptomycin) for 24h prior to further analysis. All animal procedures were approved by the King's College

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London Ethics Committee and carried out in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986.

2.5. Sample preparation for mass spectrometry

Protein A/G magnetic beads were added to coupling buffer (10 mM sodium phosphate, 150 mM NaCl; pH = 7.2), then 0.02 mM disuccinimidyl suberate and antibody added (anti-3-NT or anti-4-HNE) and the solution gently mixed for 1h at room temperature. Beads were then collected using a magnetic stand, washed twice with immunoprecipitation (IP) wash buffer, then incubated overnight in cell lysate at 4 °C. Beads were washed three times with 50 mM triethylammonium bicarbonate buffer, then incubated for 20 min with 5 mM dithiothreitol at 56 °C with constant shaking. Once cooled down to room temperature, 15 mM iodoacetamide was added and the mixture incubated in the dark for 15min. Afterwards, 0.04 mg/mL proteomics grade trypsin was added and incubated at 37 °C overnight with constant shaking. Beads were then removed using a magnetic stand. Finally, trifluoroacetic acetic acid was added to inactivate the trypsin. The solution was then vacuumed dry and resuspended in 5% (v/v) acetonitrile /0.1% (v/v) formic acid, prior to analysis.

2.6. Mass spectrometry

Samples were analysed by reverse-phase high-performance liquid chromatography-electrospray ionization tandem mass spectrometry analysis (RP-HPLC-ESI-MS) using TripleTOF 6600+ mass spectrometer coupled to Eksigent ekspert nano LC 425 pump system and autosampler (SCIEX, Canada). Data/Information-dependent acquisition (DDA/IDA) was carried out with the following parameters (87 min gradient, DDA/IDA, Top 30 ions, 50 ms accumulation time each, 1.8 s cycle time). Files were processed using ProteinPilot 5.0.2 against either human Swissprot, or mouse Swissprot databases. Number of proteins identified in each adduct IP was used as a proxy for protein adduction (while not being fully quantitative). The mass spectrometry proteomics data of adducted proteins has also been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [19] with the dataset identifier PXD023062.

2.7. Assessment of mitochondrial respiration

One day prior to the assay, a Seahorse sensor cartridge (XFe24 Flux Assay Kit) was hydrated by filling each well with XF calibrant solution and placing in a non-CO_2 incubator at 37 °C overnight. The Seahorse XFe24 Analyzer was also warmed for a minimum of 5 h prior to use to allow it to reach 37 °C. C2C12 cells were seeded at a density of 10,000 cells in a 24-well Agilent Seahorse XF24 Cell Culture microplate, and cultured at 37ºC in 95% air/5% CO_2 atmosphere for 24h. C2C12 cells were then differentiated with DMEM supplemented with 5% horse serum, and after 2 days multinucleated myotubes were treated with control differentiation media ± 10 mM L-carnosine or glucolipotoxic (GLT) media ± 10 mM L-carnosine for 5 days. INS-1 rat pancreatic ß-cells were plated at a density of 100,000 cells/well in a polystyrene 6-well plate and initially treated for 3 days in complete RPMI-1640 media ± 10 mM L-carnosine or GLT media ± 10 mM L-carnosine, and incubated at 37ºC in 95% air/5% CO_2 atmosphere. On the third day, 5000 cells of the treated INS-1 cells were seeded into the Seahorse XF24 cell culture microplate and further exposed to the same treatment for 2 days. On the day of the assay, media was removed, and cells washed twice with Seahorse XF-DMEM medium supplemented with 1 mM pyruvate, 10 mM glucose, and 2 mM glutamine, then incubated at 37 °C for 1h. During this incubation period the bioenergetic modulators oligomycin (1.5 μM), FCCP (2 μM), and rotenone/antimycin-A (0.5 μM) were loaded into the ports of the previously hydrated sensor cartridge, and the cartridge then loaded to the warmed Seahorse XFe24 Analyzer for calibration and equilibration. Once completed, the utility plate was then replaced with the Seahorse plate containing the cells and the Mito Stress test run. Data was then obtained from the Seahorse XF Mito Stress Test Report Generator.

2.8. GLUT4 translocation assay

The GLUT4 translocation assay was conducted following the protocol of [20]; with modifications. Following the desired treatment, cells were serum-starved overnight at 37ºC in DMEM supplemented with 5 mM glucose. Cells were then incubated in glucose-free DMEM ± 100 nM insulin and anti-GLUT4 primary antibody followed by secondary antibody conjugated to AlexaFluor 488 (Abcam, Cambridge, UK). Cells were fixed with 1% paraformaldehyde in PBS and incubated for 20 min at room temperature in the dark. Cells were then transferred to flow cytometer tubes, washed twice with PBS and resuspended in 1% PFA in PBS. Data was acquired using a Beckman Coulter Gallios™ flow cytometer and analysed using Beckman Coulter Kaluza™ software.

2.9. Statistical analysis

Results are expressed as mean ± standard error of the mean (n = 3 or more independent experiments). Parameters were compared either using ANOVA and Tukey *post-hoc* test, or one-tailed student t-test assuming equal variance, with statistical significance determined using an alpha value of 5%. A p value < 0.05 was considered to be statistically significant.

3. Results

3.1. Impact of obesity and diabetes on serum protein adduction

In order to test the validity of our experimental procedures to human obesity and diabetes, we first sought to determine the extent to which serum proteins form adducts with 4-hydroxynonenal and 3-nitrotyrosine as a consequence of metabolic stress. Following a 10 h overnight fast, blood was collected from lean controls, obese non-diabetic, obese GD, and obese T2D non-menopausal women. Serum samples were immunoprecipitated using either 4-HNE or 3-NT primary antibodies, and mass-spectrometry analysis conducted on the recovered proteins.

As can be seen (Table 1), there is 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 are more adducted proteins identified (>60%) in the serum of obese-type 2 diabetic individuals than in the other serum samples investigated (~30%). Protein adduction data were then analysed using PANTHER (protein analysis through evolutionary relationships) software (www.pantherdb.org). This facilitates classification of uploaded protein data from the Uniprot Knowledgebase via a number of available parameters. Fig. 1 shows the relative proportion of biological functions affected by protein adduction from A, 4-HNE and B, 3-NT in each condition.

Consistent with the hypothesis that obesity and diabetes drive metabolic stress [6,7], we found 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, we therefore sought 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, we also determined the extent to which this damage could be prevented by carnosine.

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Table 1
Analysis of serum 4-hydroxynonenal (4HNE) and 3-nitrotyrosine (3NT)-adducted proteins.

Obese BMI = 41.60 ± 4.99; Age = 32.80 ± 6.50	Obese – Type 2 Diabetes BMI = 45.42 ± 8.38; Age = 59.80 ± 4.65	Obese – Gestational Diabetes BMI = 43.31 ± 5.41; Age = 33.33 ± 5.20
4-HNE: Apolipoprotein(a), Extracellular matrix protein 1, Fibrinogen beta chain, Galectin-3-binding protein, Histone H2A type 1–3, Histone H2B type 1–3, Histone H3, Histone H4, Immunoglobulin variables, Myeloperoxidase, Pregnancy-specific beta-1-glycoprotein 3, Putative pregnancy-specific beta-1-glycoprotein 7, Putative transmembrane protein encoded by LINC00477, Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 **3NT:** Afamin, Apolipoproteins A-II, C4b-binding protein beta chain, Coagulation factor XII, Complement component C8 gamma chain, Complement factors (I, H-related protein 5), Dermcidin -Dermokine, Fibrinogen beta chain, Haptoglobin-related protein, Hemoglobin subunit, Histidine-rich glycoprotein, Histone H3, Immunoglobulins (kappa variables, lambda constant/variables, mu heavy chain), Keratin, Leucine-rich alpha-2-glycoprotein, Plasminogen, Polymeric immunoglobulin receptor, Pregnancy zone protein, Putative uncharacterized protein RUSC1-AS1, Vascular endothelial growth factor C, Vitamin K-dependent protein S, Zinc-alpha-2-glycoprotein	**4-HNE:** BPI fold-containing family A member 1, BTB/POZ domain-containing protein 9, Dermcidin, Extracellular matrix protein 1 -Fibrinogen (beta, gamma) chain, Immunoglobulins (heavy and lambda constant 6, heavy/kappa, lambda variables, lambda-like polypeptide 5), Keratin, type I cytoskeletal 9, piRNA biogenesis protein EXD1, Putative transmembrane protein encoded by LINC00477, Statherin, Sulfate anion transporter 1 **3-NT:** Abnormal spindle-like microcephaly-associated protein, Afamin, Alpha-1-antichymotrypsin, Apical junction component 1 homolog, Apolipoproteins (A-II/IV, C-I-III, D-L1), Brefeldin A-inhibited guanine nucleotide-exchange protein 2, Carboxypeptidase B2/N2, Coagulation factor IX /XII/XIII A Cohesin subunit SA-3, Complement component C6/C7/C8 Complement factor I, Dermcidin, DNA polymerase zeta catalytic subunit, E3 ubiquitin-protein ligase MYCBP2, Ecotropic viral integration site 5 protein homolog, Extracellular matrix protein 1, Fanconi-associated nuclease 1, Fibrinogen (beta/gamma chain), Gelsolin, Granulins, Haptoglobin-related protein, HEAT repeat-containing protein 1, Hemoglobin subunit (alpha/beta), Heparin cofactor 2, Histidine-rich glycoprotein, Histone H4, Homeobox protein Mohawk, Hornerin, Immunoglobulin (heavy/kappa/lambda variables), Insulin-like growth factor-binding protein complex acid labile subunit, Inter-α-trypsin inhibitor heavy chain H3, Kallistatin, Keratin, Keratinocyte proline-rich protein , Kinesin-like protein KIF1B, Leucine-rich alpha-2-glycoprotein, Lumican, N-acetylmuramoyl-L-alanine amidase, Phosphatidylinositol-glycan-specific phospholipase D, Pigment epithelium-derived factor, Plasma kallikrein, Plasminogen, Platelet basic protein, Pregnancy zone protein, pre-rRNA processing protein FTSJ3, Putative transmembrane protein encoded by LINC00477, Retinol-binding protein 4, RuvB-like 1, Ryanodine receptor 2	**4-HNE:** Histone H2A (type 1,2,3, X, Z), Immunoglobulin (heavy variables, kappa variables, lambda variables, lambda-like polypeptide 5, Lethal (3)malignant brain tumor-like protein 1, Protein HID1, Putative trypsin-6, Ribonucleoside-diphosphate reductase large subunit, Serine/threonine-protein phosphatase 2A, 65 kDa regulatory subunit A alpha isoform, Trypsin-2, Urea transporter 2 **3-NT:** Alpha-1-acid glycoproteins, Alpha-1-antichymotrypsin Alpha-1-antitrypsin, Alpha-2-macroglobulin, Apolipoproteins (A and B), Complement C1q, Complement C4-A and B, Keratin, Haptoglobin, Haptoglobin-related protein Hemopexin, Immunoglobulin (heavy/kappa/lambda constants, kappa variables, Immunoglobulins (lambda variables, lambda-1 light chain), Immunoglobulins (lambda-like polypeptide 5, mu heavy chain), Inter-alpha-trypsin inhibitor heavy chains (H2 and H4), Vitamin D-binding protein

3.2. Effect of metabolic stress and carnosine on skeletal muscle protein adduction

In order to investigate the effect of metabolic stress on skeletal muscle cells, mouse C2C12 myotubes and a human skeletal muscle (HSkM) cell-line that was developed from isolated muscle tissue of a healthy donor were incubated in parallel in DMEM media (containing 11 mM glucose for C2C12 myotubes; 5 mM glucose for HSkM cells), or DMEM media supplemented to either 28 mM glucose (C2C12 cells) or 17 mM glucose (HSkM cells) and 200 μM palmitic acid and 200 μM oleic acid (GLT media), conditions representative of those seen in poorly controlled type 2 diabetes, and incubated for 5 days ± 10 mM carnosine, a concentration selected based upon the physiological concentration that has previously been reported in skeletal muscle [21,22]. Cells were lysed and then immunoprecipitated against 4-HNE or 3-NT, with mass-spectrometry analysis of the samples then undertaken. Carnosine prevented 90% (4-HNE) and 65% (3-NT) of protein adduction in C2C12 cells, and 80% (4-HNE) and 65% (3-NT) of protein adduction in HSkM cells. These proteins were then classified by molecular function, biological process, and protein class. Data are shown for each of these analyses for C2C12 myotubes (Fig. 2) and HSkM cells (Fig. 3).

3.3. Effect of metabolic stress and carnosine on protein adduction in pancreatic islets

We next sought to determine the extent of protein adduction in pancreatic islets exposed to metabolic stress. Islets were isolated from CD1 mice by collagenase digestion, then incubated in RPMI-1640 media, or RPMI-1640 GLT media, for 5 days ± 10 mM carnosine. Islets were lysed, immunoprecipitated against either 4-HNE or 3-NT and subjected to mass-spectrometry analysis. 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 (Fig. 4).

3.4. Carnosine preserves stimulus-secretion coupling in cells under metabolic stress

Having established the 4-HNE and 3-NT protein adductome that is associated with metabolic stress in skeletal muscle and pancreatic islet cells, it was evident that a large proportion of adducted proteins were involved in metabolic and cellular processes linked to stimulus-secretion coupling, both in terms of insulin-stimulated GLUT4 translocation to the plasma membrane of skeletal muscle, or glucose sensing linked to insulin secretion from pancreatic β-cells. Proteins associated with these processes that were adducted following exposure to glucolipotoxic metabolic stress, but protected from adduction by carnosine, are listed in Table 2.

Given the large number of metabolic and mitochondrial proteins that are subject to adduction, we next analysed mitochondrial function in C2C12 skeletal muscle myotubes and INS-1 pancreatic β-cells. The impact of carnosine scavenging action on mitochondrial function was assessed by Seahorse XF (Agilent Tech., Santa Clara, CA) measuring mitochondrial oxygen consumption rate (OCR). Cells were incubated in either control or GLT media ± 10 mM carnosine for 5 days. Cells displayed robust mitochondrial respiration under control conditions (Fig. 5A, blue traces). However, both C2C12 myotubes and INS-1 pancreatic β-cells displayed a significant reduction in basal mitochondrial respiration when exposed to glucolipotoxicity (Fig. 5A, red traces), suggesting either a dysfunction in total activity or a reduction in the steady state activity. Analysis of the maximal respiratory capacity suggests that GLT leads to a mitochondrial dysfunction. Importantly, carnosine supplementation prevented this damaging GLT action (Fig. 5A, purple traces).

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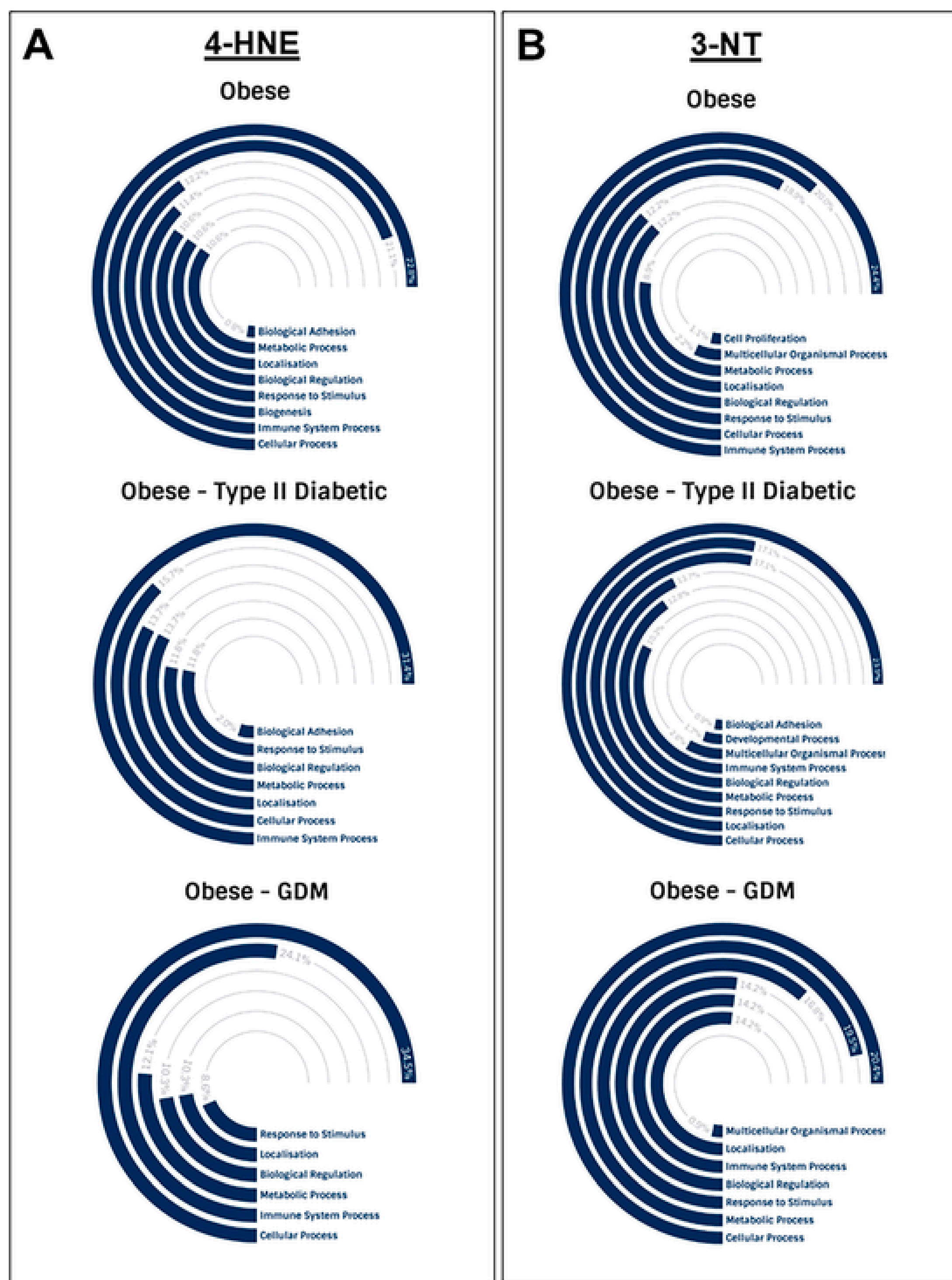

Fig. 1. Biological process classification of proteins identified and associated with (A) 4-hydroxynonenal and (B) 3-nitrotyrosine in Obese, Obese Type 2 Diabetes and Obese-Gestational Diabetes serum samples. Generated using the Panther Classification System.

One of the mitochondrial functions protected by carnosine was ATP generation, which suggests that even in the presence of metabolic stress exocytosis might also be preserved by carnosine. This is the case in pancreatic β-cells, where we previously showed that 10 mM carnosine was able to not only double insulin secretion from both INS-1 β-cells and primary mouse islets, but was also able to prevent GLT-mediated inhibition of insulin secretion [17]. Here, we have now determined the effect of metabolic stress and carnosine on GLUT4 translocation, a process in skeletal muscle that is analogous to exocytosis. However, before doing so we needed to first investigate whether there was any change in GLUT4 protein expression associated with metabolic stress. We observed no significant change in total GLUT4 expression following incubation of C2C12 cells in GLT media (Fig. 5B), data consistent with previously published studies investigating multiple insulin-resistant disease states [23,24]. Given this, we next investigated GLUT4 translocation using flow cytometry. As can be seen (Fig. 5B), under normal conditions there was a significant increase of translocation upon insulin stimulation (100 nM) relative to basal ($p < 0.05$). However, insulin-

stimulated transport of GLUT4 to the cell surface was reduced to 8.9 ± 18.2% of control ($p<0.05$) in cells incubated in GLT media. Importantly, supplementation of 10 mM carnosine to GLT-media resulted in a significant improvement of GLUT4 translocation to 50.0 ± 21.8% ($p<0.05$) of control values. This represents a 5.6-fold increase in insulin-stimulated GLUT-4 transport in the presence of carnosine.

4. 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 [25]. Regulation of the peptides and hormones involved in the pathways control-

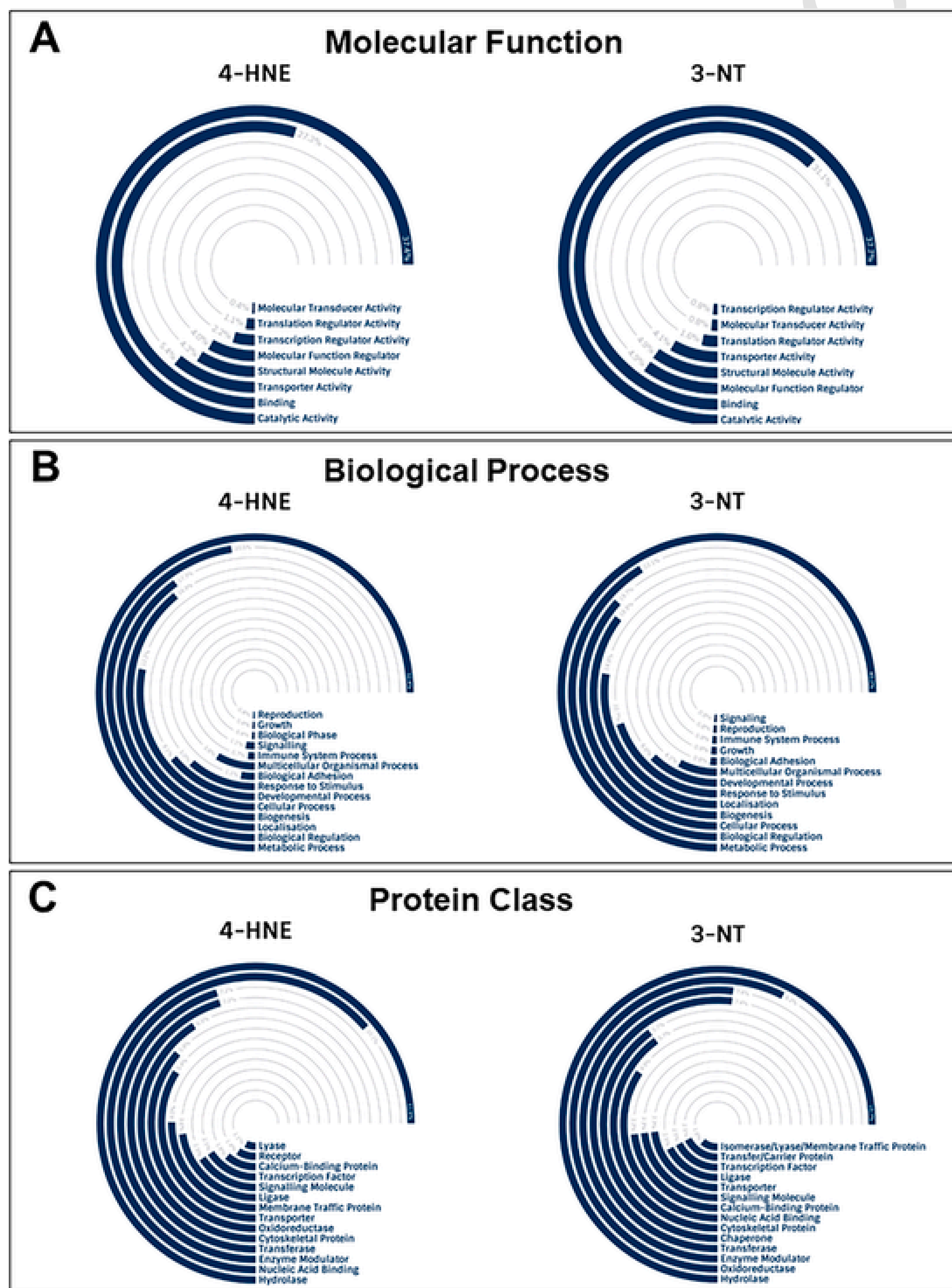

Fig. 2. Molecular function (A), Biological Process (B), and Protein Class (C) of 4-hydroxynonenal (4-HNE) and 3-nitrotyrosine (3-NT) adducted proteins. Proteins that are adducted by 4-HNE or 3-NT in C2C12 myotubes under glucolipotoxic conditions for 5 days, and which are protected from adduction by the presence of carnosine. Generated using the Panther Classification System.

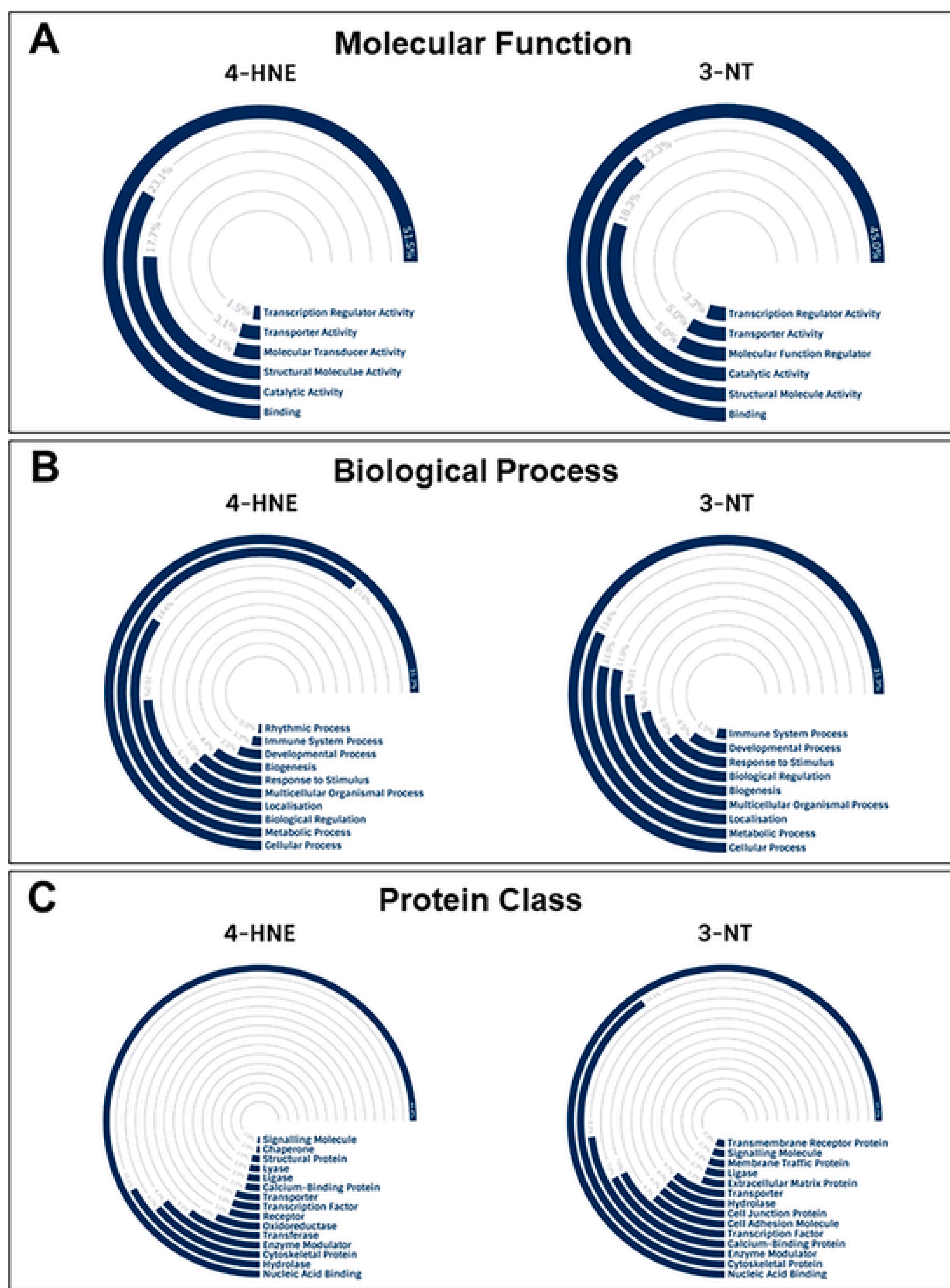

Fig. 3. Molecular function (A), Biological Process (B), and Protein Class (C) of 4-HNE and 3-NT adducted proteins. Proteins that are adducted in 4-HNE or 3-NT in cultured human skeletal myotubes under glucolipotoxic conditions for 5 days, and in which formation is prevented by the presence of carnosine. Generated using the Panther Classification System.

ling glucose homeostasis is, therefore, of paramount importance and failure to maintain this may lead to metabolic disorders, such as T2D [26].

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 [27] and adduction with these end-products can lead to the modification of protein, lipid, or DNA structures, thereby potentially altering their functional capacity and rendering these molecules less efficient or non-functional. As diabetes is characterised by chronic levels of high glucose, this represents a particular problem for these individuals [28]. Surprisingly therefore, few therapeutic strategies exist to treat diabetes that directly target these modifications, with conventional treatments instead seeking to improve glycaemic control through other mechanisms. Crucially, even when diabetes is well controlled, individuals with T2D still have ele-

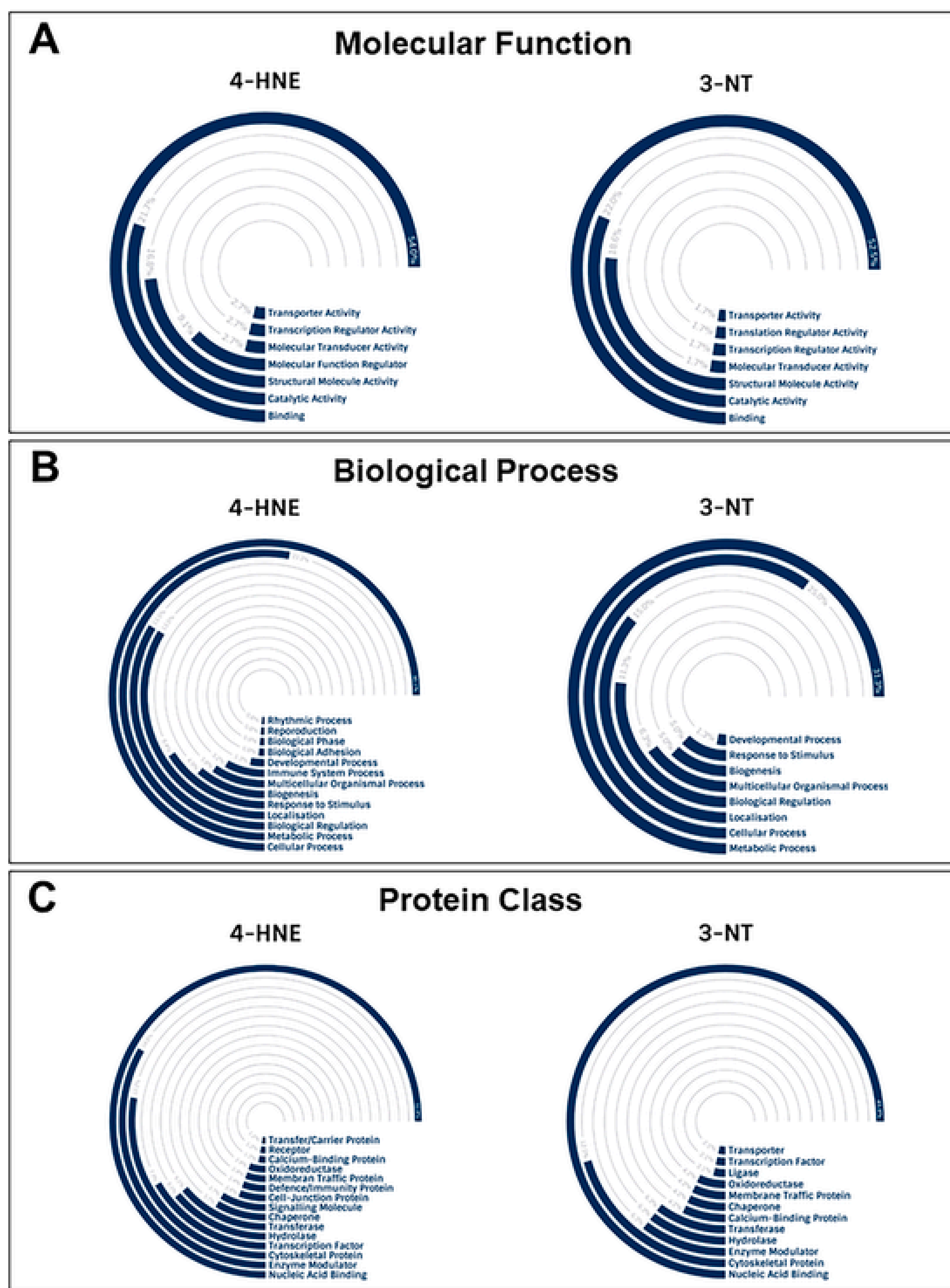

Fig. 4. Molecular function (A), Biological Process (B), and Protein Class (C) of 4-HNE and 3-NT adducted proteins. Proteins that were adducted by 4-HNE or 3-NT in mouse primary islets in GLT conditions for 5 days, and in which formation is prevented by the addition of carnosine. Generated using the Panther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

vated levels of glycated haemoglobin in comparison to non-diabetic healthy individuals (www.diabetes.co.uk). Thus, strategies that can effectively combat the accumulation of reactive species that are associated with glucolipotoxicity could have a unique capability to directly reduce the incidence of diabetes complications, even when T2D is otherwise well managed.

We identified all proteins modified by 4-HNE or 3-NT adduction in serum from lean non-diabetic controls and individuals with obesity, T2D, and GD (data available at http://proteomecentral.proteomexchange.org via the dataset identifier PXD023062.M). In order to better understand how glycation contributes to the pathophysiology of metabolic disease, we then determined specific adduction events seen in individuals with obesity or diabetes, but which were not seen in the control group. From this data we identified 4-HNE and 3-NT adduction of proteins involved in a number of immune and cellular functions.

Table 2
Proteins adducted by 4-HNE and 3-NT in skeletal muscle myotubes (S) and/or primary mouse islets (I) incubated in GLT media for 5 days, but which are protected from adduction by carnosine.

4-HNE	3-NT
Mitochondrial: 60 kDa heat shock protein (**S**), Aconitate hydratase (**S**), Apoptosis-inducing factor 1 (**S**), Aspartate aminotransferase (**I**), ATP synthase β-chain subunit (**S,I**) ATP-citrate synthase (**S**) Electron transfer flavoprotein-ubiquinone oxidoreductase (**S**), Hydroxyacyl-coenzyme A dehydrogenase (**S**), Isoleucyl-tRNA (**S**) Malate dehydrogenase (**S**), Phosphate carrier protein (**S**) Pyruvate carboxylase (**S**), Pyruvate dehydrogenase (**S**), Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 3 (**S**), NADH dehydrogenase (**S**), Stress-70 protein, (**S,I**) Succinate dehydrogenase [ubiquinone] flavoprotein (**S**), Superoxide dismutase (**S**)	**Mitochondrial:** 10 kDa heat shock protein (**S**) Acetyl-CoA acetyltransferase (**I**), Citrate synthase (**S**) Cytochrome *b*-*c*1 complex subunit 1 (**S**) Cytochrome *c* oxidase subunit 7A2 (**S**) Dihydrolipoyl dehydrogenase (**S,I**) Electron transfer flavoprotein subunit alpha (**S**) Enoyl-CoA hydratase (**S**) Isocitrate dehydrogenase [NADP] (**S**) Peroxiredoxin-5 (**S**) Pyruvate carboxylase (**I**) Pyruvate dehydrogenase E1 component (**S**) Stress-70 protein (**I**) Succinyl-CoA ligase (**S**) Superoxide dismutase (**S**) Thioredoxin-dependent peroxide reductase (**S**)
Cytoskeletal and Membrane Trafficking: α-Actinin, actin, and actin-related molecules (**S,I**) ADP-ribosylation factor-like protein 1 (**S**) Catenin delta-1 (**I**) Charged multivesicular body protein 4 (**I**) Coronin-1C (**S**) Cysteine and glycine-rich protein 1 (**I**) Cytoplasmic dynein 1 intermediate chain 2 (**S**) Destrin (**S**) EH domain-containing protein 3 (**S**) ELKS/Rab6-interacting/CAST family member 1 (**S**) Epiplakin (**I**) Ezrin (**S**) F-actin-capping protein subunit alpha-1 (**S**) Gelsolin (**I**) Guanine nucleotide-binding proteins (**I**) Kinesin (**S**) LIM domain and actin-binding protein 1 (**S**) Myosin (**S,I**) Nuclear mitotic apparatus protein 1 (**I**) Plectin (**I**) Profilin-1 (**I**) Protein kinase C and casein kinase substrate in neurons protein 2 (**S**) Ras-related protein Rab-1A (**S**) Ras-related protein Rab-1B (**I**) Ras-related protein Rab-8/Rab 11 (**S**) GTPase activating proteins (**S,I**) Rho GDP-dissociation inhibitor 1 (**I**) Septin-2 (**S**) Spectrin (**I**) Syntaphilin (**I**) Transforming growth factor beta-1-induced transcript 1 protein (**S**) Tropomyosin alpha-1 chain (**I**) Tubulin (**S**) Vacuolar protein sorting (**S**) Vesicle-associated membrane protein (**S**) Vesicle-trafficking protein SEC22 (**I**) Vesicular integral-membrane protein VIP36 (**I**) WD repeat-containing protein 1 (**S**)	**Cytoskeletal and Membrane Trafficking:** Actin and actin-related protein 3 (**S,I**) Coatomer subunit gamma-1 (**S**) EH domain-containing protein 2 (**S**) Endoplasmic reticulum-Golgi intermediate compartment protein 1 (**I**) GTP-binding proteins Sar1A/B (**S**) Macrophage-capping protein (**S**) Moesin (**S**) Myosin light chain 4 (**S**) Myosin regulatory light chain 12B (**I**) Myosin regulatory light polypeptide 9 (**S**) Profilin-1 (**S**) Protein Shroom 3 (**S**) Ras-related protein Rab-13 (**I**) Ras-related protein Rab-5 (**S**) Rho GDP-dissociation inhibitor 1 (**S**) Receptor expression-enhancing protein 5 (**S**) Septin-7 (**S**) Spectrin (**S**) Synaptotagmin-3 (**I**) Synaptic vesicle membrane protein VAT-1 (**S**) Transgelin (**S**) Translationally-controlled tumor protein (**S**) Tropomyosin beta chain (**S**) Tubulin beta-2B chain (**S,I**) Tubulin beta-3 chain (**S**) Unconventional myosin-Ib (**S**) Vacuolar protein sorting-associated protein 35 (**S**) Villin-1 (**I**)

The number of adducted proteins in the serum of obese T2D patients was double that observed in the serum of obese non-diabetics, but when one examines the protein functions affected then a similar pattern emerges. In particular, we see adduction of proteins involved in atherosclerosis and cardiovascular disease, blood clotting, and immune function. In addition, extracellular matrix proteins were also adducted by both 4-HNE and 3-NT in all serum samples (Table 1), which is of interest given that hyperglycaemia-induced alterations of extracellular matrix proteins have been associated with renal dysfunction and compromised cardiac function [29]. By contrast to the other groups studied, there were fewer adduction events in serum of patients with GD, perhaps not unexpectedly given that this is a temporary condition rather than one involving chronic exposure to hyperglycaemia that may have been established for several years. Nevertheless, 3-NT adduction of complement proteins, and extensive immunoglobulin heavy and light chain adduction by both 4-HNE and 3-NT were observed. It is tempting, therefore, to speculate that the compromised immune response to attack from foreign substances and pathogens that is often associated with diabetes could, at least in part, be linked to some of the protein adduction events identified in this study. Future clinical studies will, however, be required in order to determine the specific contribution of these individual protein adduction events to diabetes pathophysiology.

Given the nature and the extent of the protein modifications shown in the serum of patients with obesity and diabetes, we then sought to determine how nutrient excess might affect key cells regulating glucose homeostasis, and in particular skeletal muscle and pancreatic β-cells. As was the case with the patient serum experiments, adduction was determined following IP with either anti-4-HNE or anti-3-NT antibody, and subsequent mass spectrometry peptide analysis. In addition, we were also now able 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 skeletal muscle glucose uptake [17].

In order to determine specific molecular pathways where carnosine supplementation is protective, protein data were uploaded and analysed using PANTHER software. The majority of proteins that were adducted by both 4-HNE and 3-NT, but protected by carnosine, are involved in catalytic and binding activities linked to metabolic processes. In addition, numerous membrane protein trafficking and cytoskeletal proteins were also protected from adduction by carnosine. Together, this is indicative of carnosine having protective actions at multiple points along the stimulus-secretion coupling pathway. The remainder of this manuscript therefore focuses on these aspects of the data.

Defects in GLUT4 glucose transporter translocation occur in insulin resistance, T2D, and metabolic syndrome. In order to facilitate GLUT4 translocation, and subsequent glucose uptake into skeletal muscle, the initial step in stimulus-secretion coupling in these cells is insulin signalling [30,31]. We observed that a number of proteins that either regulate insulin sensitivity, or else have an interaction with components of the insulin signalling pathway, were adducted by 4-HNE. Serine-threonine protein kinases and phosphatases, mitogen-activated protein kinase, and the TBC1 domain family were however protected from adduction by carnosine supplementation.

Mitochondrial dysfunction is known to result from sustained exposure to elevated ROS levels [6], including those typically seen in patients with T2D or obesity [7] as both obesity and high fat diets result in increased production of hydrogen peroxide [32]. 3-NT levels are also known to be significantly higher in small vessels of the skeletal muscles of patients with mitochondrial respiratory chain dysfunction compared with healthy controls [33]. Our data show pyruvate dehydrogenase enzymes, which are important for glucose utilization and in maintaining a supply of acetyl-CoA for the mitochondrial activity [34], 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 adduc-

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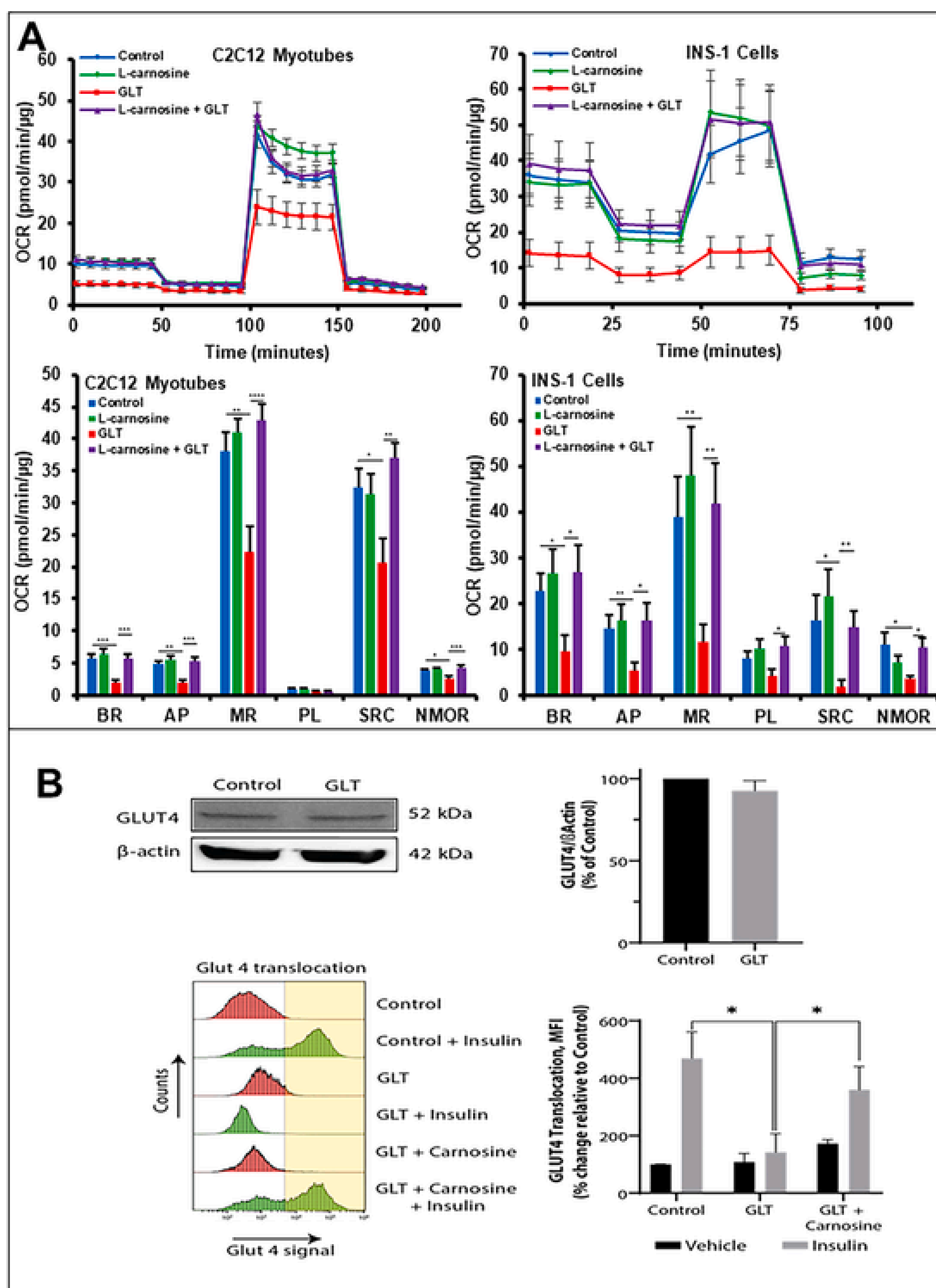

Fig. 5. A) Effect of metabolic stress and carnosine on mitochondrial respiration of skeletal muscle myotubes and pancreatic β-cells. C2C12 myotubes and INS-1 cells were treated with control media, glucolipotoxic (GLT) media, L-carnosine, or GLT supplemented L-carnosine for 5 days. OCR was measured using a Seahorse XFe24 Analyzer. Each data point represents a mean normalised OCR measurement from 3 independent experiments. Abbreviations: Basal Respiration (BR), ATP Production (AP), Proton Leak (PL), Maximal Respiration (MR), Spare Respiration Capacity (SRC), and Non-mitochondrial oxygen respiration (NR). *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. **B) 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 10 mM carnosine for 5 days. Cells were then lysed to extract proteins, and then separated via SDS-PAGE, transferred to nitrocellulose and detected using anti-GLUT4 or anti-actin antibody. Data expressed as mean ± SEM from 3 or more independent experiments, and **Translocation of GLUT4 is impaired in GLT-treated C2C12 muscle myotubes but is enhanced by carnosine supplementation.** Myotubes were incubated in control or GLT media supplemented ± 10 mM 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 min, fixed in 1% PFA for 20 min and data acquisition using flow cytometry. Data are expressed as means ± SEM of 3 independent experiments. *p < 0.05.

tion events by carnosine, included ATP synthase, aconitate hydratase, citrate synthase, cytochrome *b-c*1 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 (Table 2).

Isocitrate dehydrogenase is of particular interest given that it has been considered a promising therapeutic target to counteract T2D and obesity-related metabolic disorders, and purportedly has a role in modulating both insulin sensitivity and substrate metabolism [35]. In addition, cytochrome *b-c*1 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 [36]. 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 (Fig. 5). Peroxiredoxin is another antioxidant enzyme that is modified 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 T2D [37].

Of the adducted mitochondrial enzymes that are protected by carnosine in pancreatic islets, pyruvate carboxylase activity 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 T2D and this had a negative impact on β-cell secretory capacity [38]. Other adducted islet mitochondrial proteins with catalytic activities include acetyl-CoA acetyltransferase, and dihydrolipoyl dehydrogenase. These are key constituent enzymes in multiple essential cellular processes including fatty acid metabolism, pyruvate metabolism, and ketone body degradation.

The transcription factor Nrf2 was also shown to be adducted in islets under glucolipotoxic stress. This is known to be the master regulator of numerous genes encoding antioxidant, detoxifying, and cytoprotective molecules in humans [39]. This is also an emerging target for pharmacological strategies designed to combat oxidative stress in islet transplantation [40]. Therefore, our finding that carnosine is able to protect this molecule from adduction associated with metabolic stress, suggests that carnosine could be of benefit not only to patients with T2D, but could also be utilised to help extend the survival and quality of islets under the oxidative stress associated with transplantation.

We found a number of protein trafficking molecules that carnosine was able to prevent from becoming adducted in primary islets under metabolic stress, indicating that this may be the mechanism by which carnosine enhances insulin secretion in β-cells exposed to glucolipotoxic stress [17]. Insulin is initially synthesized as pre-proinsulin, whereupon it is translocated into the endoplasmic reticulum (ER). Following ER quality control, selective transport of cargo occurs between the ER, the ER-Golgi intermediate compartment (ERGIC), and the Golgi. Interestingly, one of the proteins that we see adducted, but protected by carnosine, is ERGIC protein 1. Vesicular integral-membrane protein 36 is also similarly adducted, and this has also been shown to be involved in ERGIC-mediated cargo transport through the early secretory pathway [41]. This suggests that defective ERGIC transport might, in part, explain why reduced insulin content is found in β-cells under oxidative stress [42], and that carnosine may, therefore, be able to help reverse this defect.

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 [43]. 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 [44] was also shown to be adducted. Coatomer-associated vesicular transport through the early secretory pathway is also dependent upon the small monomeric GTPase, Rab1B [45], a protein that mediates vesicular transport between ER and Golgi [46] 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 [47]. In order to maintain activity, all such GTPases, including Rab1B, therefore require multiple effectors [48]. 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 vesicular transport in non-polarised cells [49], including insulin-stimulated GLUT4 translocation in skeletal muscle cells [50] was also observed to be prevented by the presence of carnosine.

Secretory granules interact with the cytoskeleton in pancreatic β-cells [51] and actin reorganization is required for sustained glucose-stimulated insulin secretion [52]. Interestingly, Rab13 has also been linked with cytoskeletal dynamics through interaction with myosin Va [53] and α-actinin [54]. We see metabolic stress-mediated adduction, and protection by carnosine, of α-actinin1-4 in primary islets. Spectrin interaction with actin also links the plasma membrane to the actin cytoskeleton, and spectrin has been associated with K_{ATP} channel modulation of membrane excitability, which drives insulin secretion [55]. We observed spectrin alpha chain adduction and tubulin beta-2B/3 chain adduction, with tubulin-mediated transport of secretory granules having previously been shown to facilitate sustained insulin secretion through glucose-stimulated anterograde transport of insulin-containing secretory granules to the cell surface [56]. We also show adduction, and protection by carnosine, of villin, a protein important in regulating the exocytosis of insulin secretory granules through controlling the dynamics and size of the F-actin cages or cortical actin cytoskeleton in pancreatic islets [57].

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 [58]. 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 [58–61], with its overexpression increasing insulin secretion [61] and synaptotagmin III-specific antibodies inhibiting exocytosis from permeabilised β-cells [59,60]. 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 disulphide-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-

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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 [62,63]. As carnosine prevents adduction of these molecules, this suggests that carnosine may help reduce ER stress in cells under metabolic stress.

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; [64] 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 [65]. 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 [66]. Carnosine prevents adduction in each of these cases.

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 [67]. The myosins, Rab5 and 11 (thought to regulate endosomal and/or TGN trafficking, as well as GLUT4 recycling; [68,69] were present 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 [70]). Similarly, Rab8, which is implicated in GLUT4 vesicle exocytosis [50,71], and several guanine nucleotide-binding proteins, GTPase activators, and GDP dissociation inhibitors were all adducted by metabolic stress, but protected by carnosine.

There is clearly widespread protein adduction throughout the stimulus-secretion coupling pathway of both 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, we hypothesise that the mitochondrial dysfunction that is seen in insulin resistance [72]. and T2D [73] and is known to result from oxidative stress associated with nutrient excess [6,7,73], is likely the result of protein dysfunction resulting from AGE/ALE adduction. Seahorse data indicates that GLT severely compromises cellular mitochondrial respiration rate in both skeletal muscle myotubes and pancreatic β-cells (Fig. 5), 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 effective substrate metabolism. However, when cell culture media was supplemented with carnosine these defects were prevented, and mitochondria continued to be able to generate ATP. Consequently, we suggest that the protective action of carnosine in preventing damaging adduction of mitochondrial proteins should at the very least delay the onset of mitochondrial dysfunction in T2D.

With cells under metabolic stress largely protected from damaging protein adduction events by the presence of carnosine, we sought to determine whether this would be sufficient to maintain GLUT4 vesicle transport to the cell surface of skeletal muscle cells under metabolic stress. Whilst there was almost complete inhibition of insulin-stimulated GLUT4 translocation following incubation in GLT media, 50% of the control amount of insulin-stimulated translocation was preserved when carnosine was present. Therefore, whilst protection is not complete, this shows that prevention of glucolipotoxic protein adduction through carnosine supplementation results in significant enhancement of C2C12 cellular function. Whilst this work needs to be verified using *in vivo* human skeletal muscle experiments, we nevertheless believe that this data, coupled with our previous work showing increased insulin secretion following carnosine treatment [17], indicates that prevention of AGE/ALE adduction by carnosine scavenging of reactive carbonyl species is effective at preserving normal cellular function in cells and tissues central to the regulation of glucose homeostasis. Given the consistent level of protection from adduction that is afforded by carnosine across the different cell types shown herein, it is possible that significant physiological benefit to diabetic or obese individuals might occur through dietary supplementation with carnosine, or its precursor β-alanine, although well-conducted randomised control trials would be required to confirm this.

5. Conclusions

Data presented here indicates that the majority of 4-HNE and 3-NT adduction events that occur following exposure to glucolipotoxic metabolic stress can be prevented by carnosine supplementation. It is therefore 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. Future clinical studies will however be required to confirm the impact of any single protein adduction event on disease pathophysiology. Carnosine is an effective scavenger of reactive species, however, and by so doing enhances stimulus-secretion coupling in skeletal muscle and pancreatic β-cells, thereby providing a mechanistic basis for findings reporting improved glucose homeostasis and reduced HbA1c levels in patients with type 2 diabetes and obesity.

Author contributions

CL conducted experiments, analysed data, and helped prepare the manuscript.

MPB conducted experiments and analysed data.

KH conducted experiments and analysed data.

DJB contributed to proteomic study design and data analysis.

CC conducted experiments and analysed data.

AKM conducted experiments and analysed data.

GAF conducted experiments and analysed data.

AM collected and analysed data.

AT conducted experiments and analysed data.

LJ collected and analysed data.

SRS conducted experiments and analysed data.

PWC contributed to islet study design and analysed data.

CLD contributed to skeletal muscle study design and analysed data.

PGMcT contributed to patient study design and analysed data.

SLC contributed to mitochondrial study design and analysed data.

CS contributed to carnosine study design and reviewed the manuscript.

MDT directed the study, analysed data, and prepared the manuscript.

Declaration of competing interest

CS has received funding to support a PhD studentship relating to the effects of carnosine on cardiac function from Natural Alternatives International; a company formulating and manufacturing customised nutritional supplements, including CarnoSyn beta-alanine. The same company has also provided CS with supplements for other studies free of charge and has contributed to the payment of open access publication charges for some manuscripts on beta-alanine supplementation. All other authors declare that they have no conflicts of interest with the contents of this article.

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