Role of transglutaminase 2 in A_1 adenosine receptor- and β_2-adrenoceptor-mediated pharmacological pre- and post-conditioning against hypoxia-reoxygenation-induced cell death in H9c2 cells

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Abstract

Pharmacologically-induced pre- and post-conditioning represent attractive therapeutic strategies to reduce ischaemia/reperfusion injury during cardiac surgery and following myocardial infarction. We have previously reported that transglutaminase 2 (TG2) activity is modulated by the A_1 adenosine receptor and β_2-adrenoceptor in H9c2 cardiomyoblasts. The primary aim of this study was to determine the role of TG2 in A_1 adenosine receptor and β_2-adrenoceptor-induced pharmacological pre- and post-conditioning in the H9c2 cells. H9c2 cells were exposed to 8 h hypoxia (1% O_2) followed by 18 h reoxygenation, after which cell viability was assessed by monitoring mitochondrial reduction of MTT, lactate dehydrogenase release and caspase-3 activation. N^6-cyclopentyladenosine (CPA; A_1 adenosine receptor agonist), formoterol (β_2-adrenoceptor agonist) or isoprenaline (non-selective β-adrenoceptor agonist) were added before hypoxia/reoxygenation (pre-conditioning) or at the start of reoxygenation following hypoxia (post-conditioning). Pharmacological pre- and post-conditioning with CPA and isoprenaline significantly reduced hypoxia/reoxygenation-induced cell death. In contrast, formoterol did not elicit protection. Pre-treatment with pertussis toxin ($G_{i/o}$-protein inhibitor), DPCPX (A_1 adenosine receptor antagonist) or TG2 inhibitors (Z-DON and R283) attenuated the A_1 adenosine receptor-induced pharmacological pre- and post-conditioning. Similarly, pertussis toxin, ICI 118,551 (β_2-adrenoceptor antagonist) or TG2 inhibition attenuated the isoprenaline-induced cell survival. Knockdown of TG2 using small interfering RNA (siRNA) attenuated CPA and isoprenaline-induced pharmacological pre- and post-conditioning. Finally, proteomic analysis following isoprenaline treatment identified known (e.g. protein S100-A6) and novel (e.g. adenine phosphoribosyltransferase) protein substrates for TG2. These results have shown that A_1 adenosine receptor and β_2-adrenoceptor-induced protection against simulated hypoxia/reoxygenation occurs in a TG2 and $G_{i/o}$-protein dependent manner in H9c2 cardiomyoblasts.

Key words

A_1 adenosine receptor, β_2-adrenoceptor, cardiomyocytes, transglutaminase 2, pre-conditioning, post-conditioning.

1. Introduction

Ischaemic preconditioning or classical pre-conditioning is a well-characterized phenomenon in which transient exposure to ischaemia protects the myocardium from infarction induced by a subsequent and prolonged ischaemic attack (Murry et al. 1986). Ischaemic post-conditioning is a more recently identified cardioprotective phenomenon, which involves brief periods of ischaemia applied during the start of reperfusion, after a prolonged ischaemic insult (Zhao et al. 2003). The molecular mechanisms underlying ischaemic pre-/post-conditioning have been extensively studied, resulting in a number of membrane-bound receptors, predominantly belonging to the G protein-coupled receptor (GPCR) superfamily, being implicated in cardioprotection including the A_1 adenosine receptor (Sommerschild and Kirkebøen 2000; Headrick et al. 2003; Peart and Headrick 2007; Fretwell and Dickenson 2009; Urmaliya et al. 2009) and the β_2-adrenoceptor (Tong et al. 2005; Gross and Gross, 2006; Salie et al. 2011; Salie et al. 2012). Direct stimulation of the aforementioned receptors or pharmacological activation of signalling pathways associated with them can mimic the cardioprotective effects of ischaemic pre- and post-conditioning (Burley and Baxter 2009; Sanada et al. 2011; Cohen and Downey, 2015; Hausenloy and Yellon, 2016).

The signal transduction mechanisms triggered by the A_1 adenosine receptor and β_2-adrenoceptor include several protein kinases, such as protein kinase C (PKC; Henry et al. 1996), protein kinase B (PKB; Chesley et al. 2000; Germack et al. 2004) and extracellular-signal-regulated kinase 1/2 (ERK1/2; Schmitt and Stork, 2000; Germack and Dickenson, 2005; Shenoy et al. 2006). These pro-survival kinases belong to the Reperfusion Injury Salvage Kinase (RISK) pathway, which when activated promotes cardioprotection

(Hausenloy and Yellon, 2007). It is interesting to note ERK1/2, a prominent member of the RISK pathway, is involved in A_1 adenosine receptor and β_2-adrenoceptor-induced tissue transglutaminase (TG2) activation (Vyas et al. 2016; Vyas et al. 2017).

Transglutaminases (TGs) are a family of calcium (Ca^{2+}) dependent enzymes (TG1-7 and Factor XIIIa) that catalyse the post-translational modification of proteins (Eckert et al., 2014). TG2 is a ubiquitous member of the TG family that possess several enzymic functions that include transamidation, protein disulphide isomerase and protein kinase activity (Gundemir et al. 2012). Although TG2 has been implicated in protection of cardiomyocytes against ischaemia/reperfusion injury by regulating ATP synthesis, at present very little is known regarding its potential role in pre- and post-conditioning (Szondy et al. 2006). We have recently shown that TG2 activity can be modulated by the A_1 adenosine receptor and β_2-adrenoceptor in H9c2 cardiomyoblasts (Vyas et al. 2016; Vyas et al. 2017). Therefore, the primary aim of this study was to determine the role of TG2 in A_1 adenosine receptor and β_2-adrenocepor-induced pre- and post-conditioning against hypoxia/reoxygenation-induced cell death in H9c2 cells. The results obtained indicate for the first time that TG2 plays an anti-apoptotic role in the A_1 adenosine receptor and β_2-adrenocepor-induced pharmacological pre- and post-conditioning in H9c2 cells. It is conceivable that TG2 is a downstream effector of the Reperfusion Injury Salvage Kinase pathway and as such TG2-induced post-translational modification of mitochondrial proteins that are associated with cell death/survival may play a prominent role in cardioprotection.

2. Materials and methods

2.1. Materials

Formoterol, forskolin, ICI 118,551 ((±)-*erythro*-(*S**,*S**)-1-[2,3-(Dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride), PD 98059 (2-(2-Amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one) and propranolol were obtained from Tocris Bioscience (Bristol, UK). DPCPX (1,3-dipropylcyclopentylxanthine), IBMX (3-isobutyl-1-methylxanthine) isoprenaline, MTT (3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), N',N'-dimethylcasein, N^6-cyclopentyladenosine (CPA), pertussis toxin, protease inhibitor cocktail, phosphatase inhibitor cocktail 2 and horseradish peroxidase conjugated ExtrAvidin® (ExtrAvidin®-HRP) were obtained from Sigma-Aldrich Co. Ltd. (Gillingham, Dorset, UK). The TG2 inhibitors Z-DON (Z-ZON-Val-Pro-Leu-OMe; Benzyloxycarbonyl-(6-Diazo-5-oxonorleucinyl)-L-valinyl-L-prolinyl-L-leucinmethylester) and R283 (1,3,dimethyl-2[2-oxo-propyl]thio)imidazole chloride) along with purified standard guinea-pig liver TG2 were obtained from Zedira GmbH (Darmstadt, Germany). Biotin cadaverine (N-(5-aminopentyl)biotinamide) was purchased from Invitrogen UK (Loughborough, UK). CytoTox 96 non-radioactive cytotoxicity assay kit was from Promega (Southampton, UK). DMEM (Dulbecco´s modified Eagle´s medium), foetal bovine serum, trypsin (10X), L-glutamine (200 mM), penicillin (10,000 U/ml)/streptomycin (10,000 μg/ml) were purchased from Lonza, (Castleford, UK). All other chemicals were of analytical grade. Antibodies were obtained from the following suppliers: polyclonal anti-cleaved caspase-3 (Asp175) and monoclonal anti-GAPDH were purchased from New England Biolabs Ltd. (Hitchin, UK); anti-protein S100-A6 antibody was obtained from Abcam (Cambridge, UK); monoclonal anti-transglutaminase 2 (CUB 7402) and monoclonal β-actin from ThermoFisher Scientific (Loughborough, UK).

2.2. Cell culture

Rat embryonic cardiomyoblast-derived H9c2 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). It is important to note that H9c2 cells, derived from embryonic rat heart tissue (Kimes and Brandt, 1976), are increasingly used as an *in vitro* model for studies exploring cardioprotection since they display similar morphological, electrophysiological and biochemical properties to primary cardiac myocytes (Hescheler et al. 1991). Furthermore, recent studies have demonstrated that H9c2 cells are energetically more similar to primary cardiac myocytes than mouse atrial HL-1 cells and thus represent a viable in vitro model to investigate simulated cardiac ischaemia/reperfusion injury (Kuznetsov et al. 2015). In addition to their increasing use in studies exploring cardioprotection, H9c2 cells show hypertrophic responses comparable to primary neonatal cardiac myocytes and therefore also represent a useful cell model system to study heart disease (Watkins et al. 2011). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 10% (v/v) foetal bovine serum and penicillin (100 U/ml)/streptomycin (100 μg/ml). Cells were maintained in a humidified incubator (95% air/5% CO_2 at 37°C) until 70-80% confluent and sub-cultured (1:5 split ratio) using trypsin (0.05% w/v)/EDTA (0.02% w/v).

2.3. cAMP accumulation assay

H9c2 cells (5000 cells/well) were seeded on a white 96 well microtitre plate, with clear-bottomed wells (Fisher Scientific, Loughborough, UK) and cultured for 24 h in fully supplemented DMEM. The medium was removed and the monolayer treated with a range of concentrations of formoterol for 20 min in serum-free DMEM (40 µl/well) in the presence of 20 mM $MgCl_2$ and 500 µM IBMX. Following stimulation, cAMP levels within cells were determined using the cAMP-Glo™ Max Assay kit (Promega UK, Southampton, UK). Briefly, 10 µl of cAMP detection solution were added to all wells and incubated for 20 min at room

temperature. After incubation, Kinase-Glo® reagent (50 µl/well) was added and incubated for 10 min at room temperature, following which luminescence levels across the plate were read using a plate-reading FLUOstar Optima luminometer (BMG Labtech Ltd, Aylesbury, UK). Forskolin (10 µM) was used as a positive control and the luminescence values were converted to cAMP levels using a cAMP standard curve (0-100 nM), according to the manufacturer's instructions.

2.4. Transglutaminase activity assays

Time course profiles and concentration-response curves were obtained for isoprenaline. Following stimulation, cells were rinsed twice with 2.0 ml of chilled PBS, lysed with 500 µl of ice-cold lysis buffer (50 mM Tris-HCl pH 8.0, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v) protease inhibitor cocktail, and 1% (v/v) phosphatase inhibitor cocktail 2 and 3). Cell lysates were clarified by centrifugation at 4°C for 10 min at 14,000 x g prior to being assayed for TG activity, as described below. Protein was determined using the bicinchoninic acid (BCA) protein assay (Smith et al, 1985), using a commercially available kit (Sigma-Aldrich Co. Ltd), with bovine serum albumin (BSA) as the standard.

Biotin-labelled cadaverine incorporation assays were performed according to Slaughter et al. (1992) with the modifications reported by Lilley et al. (1998). Briefly, 96-well microtitre plates were coated overnight at 4° C with 250 µl of N',N'-dimethylcasein (10 mg/ml in 100 mM Tris-HCl, pH 8.0). The plate was washed twice with distilled water, blocked with 250 µl of 3% (w/v) BSA in 100 mM Tris-HCl, pH 8.0 and incubated for 1 h at room temperature. The plate was washed twice before the application of 150 µl of either 6.67 mM calcium chloride or 13.3 mM EDTA (used to check background TG2 activity) assay buffer containing 225 µM biotin-cadaverine (a widely used substrate to monitor TG amine incorporating activity) and 2 mM 2-mercaptoethanol. The reaction was started by the addition of 50 µl of sample, positive control (50 ng/well of guinea-pig liver TG2) or negative control (100 mM

Tris-HCl, pH 8.0). After incubation for 1 h at 37°C, plates were washed as before. Then, 200 µl of 100 mM Tris-HCl pH 8.0 containing 1% (w/v) BSA and ExtrAvidin®-HRP (1:5000 dilution) were added to each well and the plate incubated at 37°C for 45 min; it was then washed as before. The plate was developed with 200 µl of freshly prepared developing buffer (7.5 µg/ml 3, 3´, 5, 5´-tetramethylbenzidine and 0.0005% (v/v) H_2O_2 in 100 mM sodium acetate, pH 6.0) and incubated at room temperature for 15 min. The reaction was terminated by adding 50 µl of 5.0 M sulphuric acid and the absorbance read at 450 nm. One unit of transglutaminase activity was defined as a change in A_{450} of 1.0 per hour. Each experiment was performed in triplicate.

2.5. Hypoxia-reoxygenation-induced cell death

H9c2 cells in glucose-free and serum-free DMEM were exposed to 8 h hypoxia using a hypoxic incubator (5% CO_2/1% O_2 at 37°C), where O_2 was replaced by N_2. Following hypoxic exposure, cells were reoxygenated in DMEM containing 1% foetal bovine serum for 18 h in a humidified incubator (95% air/5% CO_2 at 37°C). For pharmacological pre- and post-conditioning, cells were exposed to either CPA (100 nM; A_1 adenosine receptor agonist; 10 min), formoterol (1 µM; selective β_2-adrenoceptor agonist; 20 min) or isoprenaline (10 µM; non-selective β-adrenoceptor agonist; 20 min) either before hypoxia/reoxygenation (pre-conditioning) or at the start of reoxygenation following 8 h hypoxic exposure (post-conditioning). Where appropriate, cells were treated as follows: for 30 min with DPCPX (1 µM; A_1 adenosine receptor antagonist), ICI 118,551 (1 µM; selective β_2-adrenoceptor receptor antagonist) or propranolol (1 µM; non-selective β-adrenoceptor antagonist); for 1 h with TG2 inhibitors Z-DON (150 µM; Schaertl et al. 2010) or R283 (200 µM; Freund et al. 1994) or for 48 h with 1 µM TG2 siRNA or negative control siRNA before the addition of the appropriate agonist. After the above treatments, the medium containing agonists and inhibitors was removed and replaced with either fresh glucose-free/ serum-free DMEM for

hypoxia or fresh DMEM containing normal glucose and 1% (v/v) foetal bovine serum for the remainder of the reoxygenation period.

2.6. MTT assay

H9c2 cells were plated in 24-well flat bottomed plates (15,000 cells per well) and cultured for 24 h in fully supplemented DMEM prior to hypoxia/reoxygenation. Cell viability was determined by measuring the metabolic reduction of MTT to a coloured formazan product. Cells were incubated for 1 h in 0.5 mg ml^{-1} MTT (added during the last hour of reoxygenation), after which the medium was removed and replaced with 500 μl DMSO. The reduction reaction was measured at 570 nm. Each experiment was performed in quadruplicate.

2.7. Lactate dehydrogenase assay

H9c2 cells were plated in 96-well flat bottomed plates (5,000 cells per well) and cultured for 24 h in fully supplemented DMEM prior to hypoxia/reoxygenation. Following experimentation, the activity of lactate dehydrogenase (LDH) released into the culture medium was detected using the CytoTox 96®non-radioactive cytotoxicity assay (Promega, Southampton, UK). Assays were performed according to the manufacturer's instructions and monitored at 490 nm. Each performed was performed in sextuplicate.

2.8. Western blot analysis of cleaved caspase-3 activity and TG2 expression

Protein extracts (20-30 μg per lane) were separated by SDS-PAGE (12% polyacrylamide gel for caspase-3 and 10% polyacrylamide gel for TG2) using a Bio-Rad Mini-Protean III system. Proteins were transferred to nitrocellulose membranes in a Bio-Rad Trans-Blot system using 25 mM Tris, 192 mM glycine pH 8.3 and 20% (v/v) MeOH). Following transfer, the membranes were washed with Tris-buffered saline (TBS) and blocked for 1 h at room temperature with 3% (w/v) skimmed milk powder in TBS containing 0.1% (v/v) Tween-20.

Blots were then incubated overnight at 4°C in blocking buffer with the following primary antibodies: cleaved caspase-3 (1:500), GAPDH (1:1000), TG2 (1:500) or β-actin (1:1000). The primary antibodies were removed and blots washed three times for 5 min in TBS/Tween 20. Blots were then incubated for 2 h at room temperature with the appropriate secondary antibody (1:1000) coupled to horseradish peroxidase (New England Biolabs (UK) Ltd) in blocking buffer. Following removal of the secondary antibody, blots were extensively washed as above and developed using the Enhanced Chemiluminescence Detection System (Uptima, Interchim, France) and quantified by densitometry using Advanced Image Data Analysis Software (Fuji; version 3.52). The uniform transfer of proteins to the nitrocellulose membrane was routinely monitored by transiently staining the membranes with Ponceau S stain prior to application of the primary antibody.

2.9. Knockdown of endogenous TG2 with siRNA

H9c2 cells were transfected with anti-TG2 (catalogue number: A-092775-15-0005) or negative control siRNA (catalogue number: D-001910-01-05) at 1 μM for 48 h according to the manufacturer's protocol (Dharmacon, Cambridge, UK). Briefly, fully supplemented DMEM cell culture medium was removed and replaced with Accell delivery medium containing either TG2-specific siRNA targeting the open reading frame of rat TG2 mRNA or negative control (non-targeting) siRNA.

2.10. Proteomic analysis of TG2 biotin-X-cadaverine labelled substrate proteins

Cellular proteins acting as substrates for endogenous TG2-catalysed polyamine incorporation reactions were investigated as described by Singh et al. (1995). Cells were labelled with biotin-X-cadaverine (1 mM; 6 h) prior to stimulation with isoprenaline (10 μM; 20 min). Following stimulation with isoprenaline, cells were rinsed twice with 2 ml of chilled PBS and lysed with 500 μl of ice-cold lysis buffer (50 mM Tris-HCl pH 8.0, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v) protease inhibitor cocktail, and 1% (v/v) phosphatase

inhibitor cocktail 2 and 3). Cell lysates were clarified by centrifugation at 4°C for 10 min at 14,000 x g. Biotin-X-cadaverine labelled proteins were enriched using CaptAvidin™-agarose sedimentation beads (Life Technologies, Loughborough, UK) and processed for trypsin digestion (Trypsin, proteomics grade; Sigma-Aldrich, UK).

Samples (~50 µg protein) were reduced and alkylated (1 µl 0.5 M DTT, 56° C for 20 min; 2.7 µl 0.55 M iodoacetamide, room temperature 15 min in the dark), dried in a vacuum concentrator (Eppendorf, UK) and resuspended in 100 µl 50 mM tri-ethyl ammonium bicarbonate (TEAB). Trypsin (2 µg in 2 µl of 1 mM HCl), was added in and incubated overnight at 37° C in a thermomixer. Samples were then evaporated to dryness in a vacuum concentrator and resuspended in 5% (v/v) acetonitrile/0.1% (v/v) formic acid (20 µl) and transferred to a HPLC vial for MS analysis. Samples (3 µl) were injected by autosampler (Eksigent nanoLC 425 LC system) at 5 µl/min directly onto a YMC Triart-C_{18} column (25 cm, 3 µm, 300 µm i.d.) using gradient elution (2-40% Mobile phase B, followed by wash at 80% B and re-equilibration) over either 110 (120 min run time) min (for spectral library construction using data/information dependent acquisition DDA/IDA) or 50 min (60 min run time) for SWATH/DIA (Data Independent Acquisition) analysis (Gillet et al., 2012; Huang et al., 2015). Mobile phases consisted of A: 2% (v/v) acetonitrile, 5% (v/v) DMSO in 0.1% (v/v) formic acid; B: acetonitrile containing 5% (v/v) DMSO in 0.1% (v/v) formic acid.

A spectral library was constructed using the output from ProteinPilot 5 (SCIEX) combining four IDA runs per group (Control, formoterol treated), filtered, and aligned to spiked-in iRT peptides (Biognosys, Switzerland) using PeakView 2.2 (SCIEX) and the SWATH micro app 2.1 plug in. SWATH data extraction, quantitation and fold change analysis were carried out using SCIEX OneOmics cloud processing software (Lambert et al., 2013).

2.11. Validation of biotinylated TG2 substrates

The enriched biotin-X-cadaverine labelled proteins (500 µg) were incubated overnight at 4°C with 2 µg of rabbit anti-protein S100-A6 antibody. Immune complexes were precipitated using Pierce™ Classic Magnetic IP/Co-IP Kit (Loughborough, UK). Precipitated proteins were resolved by SDS-PAGE (15% polyacrylamide gel), transferred to nitrocellulose and probed with ExtrAvidin®-HRP (1:5000 dilution). Reactivity was visualised using the Enhanced Chemiluminescence Detection System (Uptima, Interchim, France) and quantified densitometrically, as described above.

2.12. Data analysis

All graphs and statistics (two-way ANOVA followed by Dunnet's multiple comparison test) were performed using GraphPad Prism® software (GraphPad Software, Inc., USA). Results represent mean ± S.E.M. and p values <0.05 were considered statistically significant.

3. Results

In this study the optimal duration of hypoxia and reoxygenation was determined by monitoring cell viability using MTT reduction, LDH release and caspase-3 activation (data not shown). Based upon these findings, a time course of 8 h of hypoxia followed by 18 h reoxygenation was chosen for further experiments since it produced a significant loss in cell viability as well as significant activation of caspase-3, indicating that apoptosis had occurred (data not shown).

3.1. Role of TG2 in A_1 adenosine receptor-induced pre-conditioning in H9c2 cells

We have previously reported that the A_1 adenosine receptor triggers pharmacological pre-conditioning against hypoxia-induced cell death in H9c2 cells (Fretwell and Dickenson, 2009, Vyas et al. 2016). In this study we have explored the role of TG2 in A_1 adenosine receptor mediated pre-conditioning against hypoxia/reoxygenation-induced cell death. The selective A_1 adenosine receptor agonist CPA and antagonist DPCPX were used to explore A_1 adenosine receptor-mediated pharmacological pre-conditioning. H9c2 cells were pharmacologically pre-conditioned with CPA (100 nM) for 10 min prior to 8 h hypoxia followed by 18 h of reoxygenation. Hypoxia/reoxygenation-induced reduction of MTT and release of LDH was significantly reduced by CPA (Fig. 1). Furthermore, pre-treatment with DPCPX (1 μM) reversed CPA-induced protection (Fig. 1), confirming the involvement of the A_1 adenosine receptor. Since MTT reduction and LDH release do not discriminate between apoptotic and necrotic forms of cell death, caspase 3 activity was monitored by Western blotting using an anti-active caspase 3 antibody, as a marker of apoptotic cell death. Hypoxia/reoxygenation-induced caspase-3 activity was significantly reduced by CPA (Fig. 1). The role of TG2 in A_1 adenosine receptor-induced pre-conditioning was explored using pharmacological inhibitors of TG2 (Z-DON and R283) and knockdown of endogenous TG2 by siRNA. Transfection of H9c2 cells with TG2 siRNA significantly inhibited TG2 protein expression (Fig. 2). Pre-treatment with TG2 inhibitors (either Z-DON (150 μM) or R283 (200 μM)) or transfection of

cells for 48 h with TG2 siRNA reversed CPA-induced protection, confirming the involvement of TG2 in A_1 adenosine receptor-induced pre-conditioning (Fig. 3). Finally, pre-treatment with pertussis toxin (16 h; 100 ng/ml) attenuated CPA-induced pre-conditioning, confirming $G_{i/o}$-protein coupling of the A_1 adenosine receptor (data not shown). Overall these data indicate a prominent role for TG2 in A_1 adenosine receptor-induced pre-conditioning.

3.2. Role of TG2 in A_1 adenosine receptor-induced post-conditioning in H9c2 cells

We have previously reported that the A_1 adenosine receptor triggers pharmacological post-conditioning against hypoxia/reoxygenation-induced LDH release (Fretwell and Dickenson, 2011). In this study, H9c2 cells were pharmacologically post-conditioned with CPA (100 nM) for 10 min at the start of 18 h of reoxygenation. Hypoxia/reoxygenation-induced reduction of MTT, release of LDH and activation of caspase 3 was significantly reduced following the addition of CPA at the onset of reoxygenation (Fig. 4). Furthermore, pre-treatment with DPCPX (1 μM) reversed CPA-induced post-conditioning (Fig. 4). Pharmacological inhibition of TG2 (Z-DON (150 μM) and R283 (200 μM)) and TG2 siRNA reversed CPA-induced protection, indicating the involvement of TG2 in A_1 adenosine receptor-induced post-conditioning (Fig. 5). Finally, pre-treatment with pertussis toxin attenuated CPA-induced post-conditioning (data not shown). Overall these data indicate a prominent role for TG2 in A_1 adenosine receptor-induced post-conditioning.

3.3. Role of TG2 in β_2-adrenoceptor-induced pre-conditioning in H9c2 cells

Our previous studies have indicated functional expression of the β_2-adrenoceptor but not the β_1-adrenoceptor in H9c2 cells (Vyas et al. 2017). Furthermore, the long-acting selective β_2-adrenoceptor agonist formoterol triggers TG2 transamidase activity in H9c2 cells (Vyas et al. 2017). It is also notable that in mouse cardiomyocytes the β_2-adrenoceptor stimulates anti-apoptotic and pro-apoptotic pathways via G_i and G_s-protein coupling, respectively (Zhu et al. 2001). In this study the role of TG2 in β_2-adrenoceptor-induced cell survival was

assessed in H9c2 cells following exposure of cells to simulated hypoxia followed by reoxygenation. Pre-treatment with formoterol (1 μM) had no significant effect on hypoxia/reoxygenation-induced decrease in MTT reduction (Fig. 6A) or release of LDH (Fig. 6B). In marked contrast, pre-treatment with the non-selective β-adrenoceptor agonist isoprenaline (10 μM) significantly attenuated hypoxia/reoxygenation-induced decrease in MTT reduction (Fig. 6C), release of LDH (Fig. 6D) and activation of caspase 3 (Fig. 6E and 6F). Furthermore, treatment with the selective β_2-adrenoceptor antagonist ICI 118,551 (1 μM) and the non-selective β-adrenoceptor receptor antagonist propranolol (1 μM) reversed isoprenaline-induced protection (Fig. 6), suggesting mediation via the β_2-adrenoceptor. Previous studies have shown that isoprenaline-induced cell survival involves G_i-protein coupling (Zhu et al. 2001; Salie et al. 2011). Pertussis toxin (16 h; 100 ng/ml) attenuated isoprenaline-induced pre-conditioning, indicative of $G_{i/o}$-protein involvement (Fig. 7). Finally, pharmacological inhibition of TG2 (R283 and Z-DON) and TG2 siRNA attenuated isoprenaline-induced cell survival (Fig. 8). Overall, these data indicate that isoprenaline-induced β_2-adrenoceptor activation triggers pharmacological pre-conditioning via a $G_{i/o}$-protein and TG2 dependent pathway.

As expected, isoprenaline triggered transient (peaking at 20 min; Fig. 9A) and concentration dependent (Fig. 9B; EC_{50} = 980 nM; $p[EC_{50}] = 6.13 \pm 0.15$; n=4) increases in TG2 catalysed biotin-cadaverine incorporation. The antagonists ICI 118,551 and propranolol blocked isoprenaline-induced TG2 activation (Fig. 9C). Responses to isoprenaline were inhibited following pre-treatment of cells with the TG2 inhibitors Z-DON and R283 (Fig. 9D). TG2 responses to isoprenaline were also blocked by pertussis toxin, indicating a role for $G_{i/o}$-proteins (Fig. 9E). We have recently shown that formoterol-induced TG2 activation involves ERK1/2, a component of the Reperfusion Injury Salvage Kinase pathway (Vyas et al. 2017). In this study, treatment with PD 98059 (MEK1/2 inhibitor; 50 μM) blocked isoprenaline-induced TG2 activation suggesting ERK1/2 involvement (Fig. 9F). Finally, it is notable that isoprenaline triggers only a modest increase in cAMP (Fig. 10; EC_{50} = 24 nM; $p[EC_{50}]$ = 7.63

± 0.07; n=4). However, following treatment of cells for 16 h with pertussis toxin (100 ng/ml), isoprenaline triggered a robust cAMP response (EC_{50} = 2.3 nM; p[EC_{50}] = 8.68 ± 0.09; n=4). Overall, these data suggest that isoprenaline-induced activation of the β_2-adrenoceptor in H9c2 cells signals predominantly via $G_{i/o}$-proteins.

3.4. Role of TG2 in β_2-adrenoceptor-induced post-conditioning in H9c2 cells

H9c2 cells were pharmacologically post-conditioned with isoprenaline (10 μM) for 20 min at the start of 18 h of reoxygenation. Hypoxia/reoxygenation-induced reduction of MTT, release of LDH and activation of caspase 3 was significantly reduced following the addition of isoprenaline at the onset of reoxygenation (Fig. 11). Pre-treatment with either ICI 118,551 (1 μM) or propranolol (1 μM) reversed isoprenaline-induced post-conditioning (Fig. 11). Furthermore, pertussis toxin attenuated isoprenaline-induced post-conditioning indicative of $G_{i/o}$-protein involvement (Fig. 12). Finally, pharmacological inhibition of TG2 (Z-DON (150 μM) and R283 (200 μM)) and TG2 siRNA reversed isoprenaline-induced protection, indicating the involvement of TG2 in β_2-adrenoceptor-induced post-conditioning (Fig. 13). Overall these data indicate a prominent role for TG2 in β_2-adrenoceptor-induced post-conditioning.

3.5. Identification of biotin-X-cadaverine labelled protein substrates

We have previously identified TG2 substrates following stimulation of the β_2-adrenoceptor with formoterol (Vyas et al. 2017). In this study, we identified TG2 substrates following stimulation with isoprenaline (10 μM for 20 min). Initially, cell extracts from biotin-X-cadaverine labelled cells (1 mM for 6h) were enriched using CaptAvidin™-agarose sedimentation beads. To identify the proteins captured and eluted from CaptAvidin™-agarose beads, eluates were analysed by SWATH-MS (Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra; Huang et al. 2015). This technique allows quantification of mass spectrometry data and the results presented are shown as

isoprenaline-induced fold-changes in proteins eluted from CaptAvidin™-agarose compared to control unstimulated cells. These experiments identified numerous proteins, including several novel TG2 substrates, whose profiles either increased or decreased following isoprenaline treatment (Table 1). Further work is needed to determine whether these changes represent altered transamidation and/or interactions with TG2 substrate binding partners.

Protein S100-A6 was chosen for validation via immunoprecipitation, SDS-PAGE and Western blot analysis since we recently identified this protein as a TG2 substrate that is transamidated following β_2-adrenoceptor activation with formoterol (Vyas et al. 2017). Enhanced incorporation of the biotinylated amine into protein S100-A6 was shown using ExtrAvidin®-HRP following treatment with isoprenaline (Fig. 14). Furthermore, isoprenaline-induced increases in biotinylated protein S100-A6 were reversed by the TG2 inhibitors Z-DON (150 μM) and R283 (200 μM).

4. Discussion

At present there is very little information regarding the physiological role(s) of TG2 in the heart. Studies employing TG2 knockout mice have revealed a role for TG2 in protecting against ischaemia/reperfusion injury via the regulation of mitochondrial respiratory function (Szondy et al. 2006). In marked contrast, TG2 triggers apoptosis in rat neonatal cardiomyocytes exposed to H_2O_2-induced oxidative stress (Song et al. 2011). These conflicting reports suggest the role of TG2 in cardiac myocyte cell survival/apoptosis is unresolved and may depend upon the model employed and the mode of stress used to induce cell death. Our recent studies have shown that the A_1 adenosine receptor and β_2-adrenoceptor stimulate TG2 transamidase activity in the rat embryonic cardiomyoblast-derived cell line H9c2 (Vyas et al. 2016; Vyas et al. 2017). Furthermore, we have demonstrated that TG2 plays a role in A_1 adenosine receptor-mediated cytoprotection against hypoxia-induced cell death in H9c2 cells (Vyas et al. 2016). Given the conflicting role of TG2 in cardiomyocyte cell survival/apoptosis we have extended our previous findings to investigate further the role of TG2 in A_1 adenosine receptor and β_2-adrenoceptor-induced pre- and post-conditioning against hypoxia/reoxygenation-induced cell death in H9c2 cells. The data presented clearly show for the first time that pharmacological inhibition and siRNA knockdown of TG2 attenuates the A_1 adenosine receptor and β_2-adrenoceptor-mediated pre- and post-conditioning in H9c2 cells exposed to simulated hypoxia/reoxygenation. The role of TG2 in protecting against hypoxia/reoxygenation-induced cell death is in agreement with Szondy et al. (2006). In contrast, TG2 appears to promote cardiomyocyte cell death when using H_2O_2-induced oxidative stress as the model for ischaemia/reperfusion injury (Song et al. 2011).

4.1. A_1-adenosine receptor-induced cell survival

The role of the A_1 adenosine receptor in ischaemic pre-conditioning and associated signalling pathways have been widely studied in many model systems (Peart and Headrick,

2007; McIntosh and Lasley, 2012; Headrick et al. 2013). Components of A_1 adenosine receptor-mediated cardioprotection include PKC (Henry et al. 1996), ERK1/2 (Germack and Dickenson, 2005) and the large-conductance Ca^{2+}-activated K^+ channel (Fretwell and Dickenson, 2009). In this study we have shown a role for TG2 in mediating A_1 adenosine receptor-induced pre-conditioning.

The role of the A_1 adenosine receptor in ischaemic post-conditioning is controversial (Burley and Baxter, 2009). Evidence for a role include studies showing that the infarct limiting protection of ischaemic post-conditioning was absent in A_1 adenosine receptor knockout mice (Xi et al. 2008). Likewise, the A_1 adenosine receptor antagonist DPCPX blocked ischaemic post-conditioning in normal and hyper-cholesterolemic rabbits (Donato et al. 2007). Cell-based studies using rat ventricular myocytes have also demonstrated that pharmacological post-conditioning with adenosine is blocked by DPCPX and involves the mitochondrial K_{ATP} channel (Lu et al. 2006). Finally, our own work has revealed A_1 adenosine receptor-induced pharmacological post-conditioning in H9c2 cells (Fretwell and Dickenson 2011). In contrast, several reports suggest that the A_1 adenosine receptor is not involved in ischaemic post-conditioning (Kin et al. 2005; Philip et al. 2006). The reasons for these differences may reflect differences in experimental protocol and model systems used. In this study we have shown for the first time that TG2 is involved in the anti-apoptotic effect of A_1 adenosine receptor-induced post-conditioning.

4.2. β_2-adrenoceptor-induced cell survival

In the current study, formoterol had no significant effect on hypoxia/reoxygenation-induced decrease in MTT reduction or release of LDH. These observations contrast with previous studies reporting formoterol-induced cardioprotection in isolated rat hearts (Salie et al. 2011). The reason(s) for the discrepancy are unclear but may reflect the use of different models (e.g. cell line versus isolated heart) and end points (cell viability assays versus infarct size) employed. In marked contrast, isoprenaline, which we have shown in

the present work to signal predominantly via G_i-protein, significantly attenuated hypoxia/re-oxygenation-induced decrease in MTT reduction, release of LDH and caspase 3 activation via a TG2-dependent pathway. This is in agreement with previous studies showing that isoprenaline-induced cell survival involved G_i-protein coupling (Zhu et al. 2001; Salie et al. 2011). It is important to note that in H9c2 cells formoterol induces a robust cAMP response with no apparent $G_{i/o}$-protein coupling (Vyas et al. 2017). However, at present it is not clear why formoterol does not elicit increased cell survival despite triggering TG2 activation (Vyas et al. 2017). Possible explanations for this include; (i) only G_i-protein-mediated TG2 stimulation is cytoprotective, possibly via the selective activation of alternative enzymic functions of TG2 (e.g. protein kinase) not activated via G_s-dependent pathways, or (ii) opposing effects of robust formoterol-induced cAMP signalling on cell death which counteract TG2-induced cell survival. Indeed, the β_2-adrenoceptor triggers a pro-apoptotic signal in cardiomyocytes via G_s-protein dependent signalling (Zhu et al. 2001). Overall these data highlight the need to carefully consider the signalling profiles (G_s versus G_i) when selecting β_2-adrenoceptor agonists for use in studies investigating cardioprotection.

Finally, whilst previous studies have demonstrated β_2-adrenoceptor-induced cardioprotection via pharmacological pre-conditioning in mouse and rat heart (Tong et al. 2005; Bernstein et al. 2011; Salie et al. 2001), very little is known regarding β_2-adrenoceptor-induced post-conditioning. However, it is notable that the β_2-adrenoceptor plays a role in post-conditioning mediated by the anaesthetic desflurane in rabbit heart (Lange et al. 2009). Hence, the data presented in this study demonstrate β_2-adrenoceptor-induced post-conditioning in a cell based model.

4.3. Role of TG2 in cell survival

TG2 appears to have a dual role in cell survival/apoptosis with its exact function being dependent upon many factors including cell type, cellular location, and mode of stress (Fésüs and Szondy, 2005; Tatsukawa et al. 2016). Although the A_1 adenosine receptor and

β_2-adrenoceptor stimulate TG2 transamidase activity in H9c2 cells (Vyas et al. 2016; Vyas et al. 2017) it is not known at present if the protective roles of TG2 observed in this study are dependent upon its transamidase activity. Whilst the TG2 inhibitors Z-DON and R283 blocked A_1 adenosine receptor and β_2-adrenoceptor-induced TG2 transamidase activity (Vyas et al. 2016; Vyas et al. 2017) it is conceivable that the protective effects of TG2 are mediated via the protein disulphide isomerase and/or protein kinase functions of TG2. It is not known if Z-DON and R283 inhibit the protein disulphide isomerase and/or protein kinase activity of TG2. Alternatively, the protective effects of TG2 may relate to the non-enzymatic functions of TG2 (e.g. adapter protein and cell signalling regulator), although this would seem unlikely given the effects of Z-DON and R283 on A_1 adenosine receptor and β_2-adrenoceptor-induced cell survival.

Whilst A_1 adenosine receptor and isoprenaline-induced TG2 transamidase activity is dependent upon $G_{i/o}$-protein coupling, at present the precise signal transduction mechanism(s) linking $G_{i/o}$-protein and TG2 activation is unclear. However, it would seem unlikely that stimulation of TG2 involves direct activation via $G_{i}\alpha$ and/or $G\beta\gamma$ subunits. Our previous studies have shown that TG2 is phosphorylated in response to A_1 adenosine receptor and β_2-adrenoceptor activation via a pathway involving ERK1/2 (Vyas et al. 2016; Vyas et al. 2017). However, at present it is not known if A_1 adenosine receptor and β_2-adrenoceptor-induced TG2 activity is dependent upon TG2 phosphorylation. Finally, since the A_1 adenosine receptor and isoprenaline trigger ERK1/2 activation in H9c2 cells via a $G_{i/o}$-protein dependent pathway (data not shown) it is likely that TG2 activation proceeds via this route.

Cell death induced by hypoxia/reoxygenation is mainly mediated by mitochondrial dysfunction and opening of the mitochondrial permeability transition pore (Assaly et al. 2012). Hence it is likely that the cell survival role of TG2 observed in this study involves modulation of protein(s) associated with the intrinsic mitochondrial apoptotic pathway. For example, recent studies have shown that TG2 inhibits apoptosis via the down-regulation of

Bax expression and subsequent inhibition of caspase 3 and 9 (Cho et al. 2010). Furthermore, we have recently identified hexokinase 1 (HK1) and voltage-dependent anion-selective channel protein 1 (VDAC1) as TG2 protein substrates following A_1 adenosine receptor activation (Vyas et al. 2016). This is notable since hexokinases influence mitochondrial permeability transition pore opening and subsequent ischaemia/reperfusion injury via their interaction with VDAC1 (Calmettes et al. 2015). In this study, SWATH™-MS analysis identified 11 proteins not previously identified as TG2 protein substrates (e.g. adenine phosphoribosyltransferase) and 20 known substrates (Table 1) in response to isoprenaline stimulation. It is beyond the scope of the present discussion to describe the biological functions/roles of all of these proteins but it is interesting to note that induction of protein S100-A6 expression by tumour necrosis factor-α attenuates apoptosis in cardiac myocytes, suggesting a role for this TG2 substrate in cardioprotection (Tsoporis et al. 2008). Furthermore, five of the proteins, whose profile increased following isoprenaline stimulation, are involved with metabolism. Of these, it is notable that malate dehydrogenase is the primary enzyme in the malate-aspartate shuttle and recent studies have shown that inhibition of this important mitochondrial process is cardioprotective (Støttrup et al. 2010).

Further interrogation of the SWATH data identified a large number of proteins that displayed a pronounced decrease following treatment with isoprenaline, indicative of altered levels of transamidation of specific substrates and/or proteins interacting with them. It is notable that TG2 can catalyse simultaneous transamidation and deamidation of heat shock protein 20 and thus it is conceivable that β_2-adrenoceptor-induced activation also promotes deamidation of TG2 substrates (Boros et al. 2006). Overall, these data have identified a large number of proteins whose elution profile from CaptAvidin™-agarose beads changes markedly in isoprenaline treated cells. Future work will explore the role of such TG2-mediated modifications in the cellular functions and signalling pathways mediated by the β_2-adrenoceptor.

Further studies are also required to assess the temporal effects of hypoxia/reoxygenation on the cellular localization and transamidase activity of TG2. Whilst previous studies have identified mitochondrial TG2 substrates including the Bcl-2 family member Bax (Rodolfo et al. 2004) and adenine nucleotide translocator 1 (Malorni et al. 2009), it would be of interest to identify additional TG2 protein substrates that undergo cross-linking and/or incorporation of small molecule mono- and polyamines during hypoxia/reoxygenation in cardiomyocytes in order to clarify the mechanism(s) of TG2-mediated cell survival. In summary, we have shown for the first time that TG2 is involved in the A_1 adenosine receptor and β_2-adrenoceptor-induced pharmacological pre- and post-conditioning. Furthermore, since A_1 adenosine receptor and β_2-adrenoceptor-induced TG2 activation is ERK1/2 dependent it is conceivable that TG2 is a downstream effector of the Reperfusion Injury Salvage Kinase pathway. Future experiments will endeavour to determine how TG2 protects against hypoxia/reoxygenation-induced cell death in H9c2 cardiomyoblasts.

Conflict of Interest:

The authors state no conflict of interest.

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Figure legends

Figure 1. A_1 adenosine receptor-induced pharmacological pre-conditioning against hypoxia/reoxygenation-induced cell death. Where indicated, H9c2 cells were pre-treated for 30 min with DPCPX (1 μM) before the addition of CPA (100 nM) for 10 min prior to 8 h hypoxia (1% O_2) followed by 18 h reoxygenation (H/R) or normoxia (NX). Cell viability was assessed by measuring the metabolic reduction of MTT by cellular dehydrogenases (A) and release of LDH into the culture medium (B). Caspase-3 activation was assessed via Western blotting using anti-activated caspase-3 antibody (C) and quantified (D). Data are expressed as a percentage of normoxia control cell values (=100%) and represent the mean ± S.E.M. from four independent experiments. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001, (a) versus normoxia control, (b) versus H/R control, (c) versus CPA alone in the presence of H/R.

Figure 2. Knockdown of endogenous TG2 by siRNA. H9c2 cells (6-well plate; 45,000 cells/well) were transfected for 48 h with 1 μM TG2 siRNA or negative control siRNA using Accell delivery medium. Control cells were cultured for 48 h in fully supplemented DMEM. (A) Levels of TG2 were subsequently assessed by Western blotting. Levels of β-actin are included for comparison. (B) Quantified data expressed as the ratio of TG2 to β-actin and represent the mean ± S.E.M. of three independent experiments. **P<0.01 versus Accell delivery medium control.

Figure 3. The role of TG2 in A_1 adenosine receptor induced pharmacological pre-conditioning against hypoxia/reoxygenation-induced cell death. Where indicated, cells were pre-treated with TG2 inhibitors (Z-DON (150 μM) or R283 (200 μM)) for 1 h or for 48 h with 1 μM TG2 siRNA or negative control siRNA before the addition of CPA (100 nM) for 10 min prior to 8 h hypoxia (1% O_2) followed by 18 h reoxygenation (H/R) or normoxia (NX). Cell

viability was assessed by measuring the metabolic reduction of MTT by cellular dehydrogenases (A,E) and release of LDH into the culture medium (B,F). Caspase-3 activation was assessed via Western blotting using anti-activated caspase-3 antibody (C) and quantified (D). Data are expressed as a percentage of normoxia control cell values (=100%) and represent the mean ± S.E.M. from four independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$, (a) versus normoxia control, (b) versus H/R control, (c) versus CPA alone in the presence of H/R.

Figure 4. A_1 adenosine receptor-induced pharmacological post-conditioning against hypoxia/reoxygenation-induced cell death. Following 8 h hypoxia cells were treated with the CPA (100 nM) for 10 min at the start of reoxygenation. Where appropriate, cells were pre-treated with DPCPX (1 μM) for 30 min prior to stimulation with CPA. Cell viability was assessed by measuring the metabolic reduction of MTT by cellular dehydrogenases (A) and release of LDH into the culture medium (B). Caspase-3 activation was assessed via Western blotting using anti-activated caspase-3 antibody (C) and quantified (D). Data are expressed as percentage of normoxic control (=100%) and represent the mean ± S.E.M. from four independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$, and ****$P<0.0001$, (a) versus normoxia control response, (b) versus H/R control, (c) versus CPA alone in the presence of H/R.

Figure 5. The role of TG2 in A_1 adenosine receptor-induced pharmacological post-conditioning against hypoxia/reoxygenation-induced cell death. Following 8 h hypoxia cells were treated with the CPA (100 nM) for 10 min at the start of reoxygenation. Where indicated, cells were pre-treated with TG2 inhibitors (Z-DON (150 μM) or R283 (200 μM)) for 1 h or for 48 h with 1 μM TG2 siRNA or negative control siRNA prior to stimulation with CPA. Cell viability was assessed by measuring the metabolic reduction of MTT by cellular dehydrogenases (A,E) and release of LDH into the culture medium (B,F). Caspase-3

activation was assessed via Western blotting using anti-activated caspase-3 antibody (C) and quantified (D). Data are expressed as percentage of normoxic control (=100%) and represent the mean ± S.E.M. from four independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$, and ****$P<0.0001$, (a) versus normoxia control response, (b) versus H/R control, (c) versus CPA alone in the presence of H/R.

Figure 6. The effect of pharmacological pre-conditioning with formoterol and isoprenaline on hypoxia/reoxygenation-induced cell death. Where indicated, H9c2 cells were pre-treated for 30 min with ICI 118,551 (1 μM) or propranolol (1 μM) before the addition of formoterol (1 μM) or isoprenaline (10 μM) for 20 min prior to 8 h hypoxia (1% O_2) followed by 18 h reoxygenation (H/R) or normoxia (NX). Cell viability was assessed by measuring the metabolic reduction of MTT by cellular dehydrogenases (A,C) and release of LDH into the culture medium (B,D). Caspase-3 activation was assessed via Western blotting using anti-activated caspase-3 antibody (E) and quantified (F). Data are expressed as a percentage of normoxia control cell values (=100%) and represent the mean ± S.E.M. from four independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$, (a) versus normoxia control, (b) versus H/R control, (c) versus isoprenaline alone in the presence of H/R.

Figure 7. The role of $G_{i/o}$-proteins in isoprenaline-induced pharmacological pre-conditioning against hypoxia/reoxygenation-induced cell death. Where indicated, cells were pre-treated for 16 h with pertussis toxin (PTX; 100 ng/ml) before the addition of isoprenaline (10 μM) for 20 min prior to 8 h hypoxia (1% O_2) followed by 18 h reoxygenation (H/R) or normoxia (NX). Cell viability was assessed by measuring the metabolic reduction of MTT by cellular dehydrogenases (A) and release of LDH into the culture medium (B). Caspase-3 activation was assessed via Western blotting using anti-activated caspase-3 antibody (C) and quantified (D). Data are expressed as a percentage of normoxia control cell values (=100%)

and represent the mean ± S.E.M. from four independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$, (a) versus normoxia control, (b) versus H/R control, (c) versus isoprenaline alone in the presence of H/R.

Figure 8. The role of TG2 in isoprenaline-induced pharmacological pre-conditioning against hypoxia/reoxygenation-induced cell death. Where indicated, cells were pre-treated with TG2 inhibitors (Z-DON (150 μM) or R283 (200 μM)) for 1 h or for 48 h with 1 μM TG2 siRNA or negative control siRNA before the addition of isoprenaline (10 μM) for 20 min prior to 8 h hypoxia (1% O_2) followed by 18 h reoxygenation (H/R) or normoxia (NX). Cell viability was assessed by measuring the metabolic reduction of MTT by cellular dehydrogenases (A,E) and release of LDH into the culture medium (B,F). Caspase-3 activation was assessed via Western blotting using anti-activated caspase-3 antibody (C) and quantified (D). Data are expressed as percentage of normoxic control (=100%) and represent the mean ± S.E.M. from four independent experiments. **$P<0.01$, ***$P<0.001$, and ****$P<0.0001$, (a) versus normoxia control response, (b) versus H/R control, (c) versus isoprenaline alone in the presence of H/R.

Figure 9. Effect of isoprenaline on TG2 transamidase activity in H9c2 cells. (A) Cells were stimulated with isoprenaline (10 μM) for the indicated time periods. (B) Concentration-response curve for isoprenaline in cells treated with agonist for 20 min. (C) Cells were pre-treated for 30 min with the antagonists ICI 118,551 (1 μM) and propranolol (1 μM) or (D) for 1 h with the TG2 inhibitors Z-DON (150 μM) and R283 (200 μM) prior to stimulation with isoprenaline (ISO; 10 μM; 20 min). (E) Where indicated H9c2 cells were pre-treated for 16 h with 100 ng/ml pertussis toxin (PTX) prior to 20 min stimulation with 10 μM isoprenaline. (F) Cells were pre-treated with PD 98059 (50 μM) for 30 min prior to 20 min stimulation with 10 μM isoprenaline. Cell lysates were subjected to the biotin-cadaverine incorporation assay and data points represent the mean ± S.E.M. for TG2 specific activity from four

independent experiments. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001, (a) *versus* control response and (b) versus isoprenaline alone.

Figure 10. Isoprenaline-induced cAMP accumulation in H9c2 cells. Where indicated, cells were pre-treated for 16 h with 100 ng/ml pertussis toxin (PTX) prior to stimulation for 20 min with the indicated concentrations of isoprenaline. Levels of cAMP were determined as described in Materials and Methods. Data are presented as levels of cAMP in nM. The results represent the mean ± S.E.M. of four experiments each performed in triplicate. *P<0.05, **P<0.01, and ****P<0.0001 versus control.

Figure 11. The effect of pharmacological post-conditioning with isoprenaline on hypoxia/reoxygenation-induced cell death. Following 8 h hypoxia cells were treated with the isoprenaline (10 μM) for 20 min at the start of reoxygenation. Where indicated, H9c2 cells were pre-treated for 30 min with ICI 118,551 (1 μM) or propranolol (1 μM) prior to stimulation with isoprenaline. Cell viability was assessed by measuring the metabolic reduction of MTT by cellular dehydrogenases (A) and release of LDH into the culture medium (B). Caspase-3 activation was assessed via Western blotting using anti-activated caspase-3 antibody (C) and quantified (D). Data are expressed as a percentage of normoxia control cell values (=100%) and represent the mean ± S.E.M. from four independent experiments. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001, (a) versus normoxia control, (b) versus H/R control, (c) versus isoprenaline alone in the presence of H/R.

Figure 12. The role of $G_i/_o$-proteins in isoprenaline-induced pharmacological post-conditioning against hypoxia/reoxygenation-induced cell death. Following 8 h hypoxia cells were treated with the isoprenaline (10 μM) for 20 min at the start of reoxygenation. Where indicated, cells were pre-treated for 16 h with pertussis toxin (PTX; 100 ng/ml) prior to stimulation with isoprenaline. Cell viability was assessed by measuring the metabolic

reduction of MTT by cellular dehydrogenases (A) and release of LDH into the culture medium (B). Caspase-3 activation was assessed via Western blotting using anti-activated caspase-3 antibody (C) and quantified (D). Data are expressed as a percentage of normoxia control cell values (=100%) and represent the mean ± S.E.M. from four independent experiments. *P<0.05, ***P<0.001 and ****P<0.0001, (a) versus normoxia control, (b) versus H/R control, (c) versus isoprenaline alone in the presence of H/R.

Figure 13. The role of TG2 in isoprenaline-induced pharmacological post-conditioning against hypoxia/reoxygenation-induced cell death. Following 8 h hypoxia cells were treated with the isoprenaline (10 μM) for 20 min at the start of reoxygenation. Where indicated, cells were pre-treated with TG2 inhibitors (Z-DON (150 μM) or R283 (200 μM)) for 1 h or for 48 h with 1 μM TG2 siRNA or negative control siRNA prior to stimulation with isoprenaline. Cell viability was assessed by measuring the metabolic reduction of MTT by cellular dehydrogenases (A,E) and release of LDH into the culture medium (B,F). Caspase-3 activation was assessed via Western blotting using anti-activated caspase-3 antibody (C) and quantified (D). Data are expressed as percentage of normoxic control (=100%) and represent the mean ± S.E.M. from four independent experiments. *P<*0.05*, **P<*0.01*, ***P<*0.001*, and ****P<*0.0001*, (a) versus normoxia control response, (b) versus H/R control, (c) versus isoprenaline alone in the presence of H/R.

Figure 14. Detection of biotin-X-cadaverine into protein S100-A6 following isoprenaline treatment of H9c2 cells. (A) Cells were incubated with 1 mM biotin-X-cadaverine for 6 h after which they were treated with isoprenaline (10 μM) for 20 min. Where indicated, cells were pre-treated with TG2 inhibitors Z-DON (150 μM) or R283 (200 μM) for 1 h prior to stimulation with isoprenaline. Biotin-X-cadaverine-labelled proteins were enriched using CaptAvidin™ agarose sedimentation beads and eluted proteins subjected to immunoprecipitation using anti-protein S100-A6 antibody. Immunoprecipitated proteins

were subjected to SDS-PAGE, transferred to nitrocellulose membrane and detected using ExtrAvidin®-HRP. Immunoprecipitated samples were also analysed on separate blots using anti-protein S100-A6 antibody to confirm equal protein loading. (B) Quantified data are expressed as the percentage of biotinylated protein S100-A6 in control cells (= 100%) and represent the mean ± S.E.M. of three independent experiments. *$P<0.05$ and **$P<0.01$, (a) versus control response, (b) versus 10 μM isoprenaline alone.

Table 1. Identification of proteins showing increased or decreased levels in eluates from CaptAvidin™-agarose columns following isoprenaline-induced β_2-adrenoceptor activation.

Protein Name	Uniprot Accession	Uniprot Name	Absolute Fold Change*
Isoprenaline-induced increases			
[b]Adenine phosphoribosyltransferase	P36972	APT_RAT	2.61
[a]*Protein S100-A6*	P05964	S10A6_RAT	2.46
[e]*Collagen alpha-1(III) chain*	P13941	CO3A1_RAT	2.27
[g]*Annexin A5*	P14668	ANXA5_RAT	2.15
[h]Protein disulfide-isomerase A5	Q5I0H9	PDIA5_RAT	2.02
[a]*Protein SET*	Q63945	SET_RAT	2.01
[b]*Malate dehydrogenase, mitochondrial*	P04636	MDHM_RAT	1.96
[e]*Collagen alpha-1(I) chain*	P02454	CO1A1_RAT	1.91
[b]Acetyl-CoA carboxylase 1	P11497	ACACA_RAT	1.81
[b]*L-lactate dehydrogenase A chain*	P04642	LDHA_RAT	1.75
[g]Caspase recruitment domain-containing protein 9	Q9EPY0	CARD9_RAT	1.74
[b]Aldehyde dehydrogenase, mitochondrial	P11884	ALDH2_RAT	1.73
[d]*Ribonuclease inhibitor*	P29315	RINI_RAT	1.71
Isoprenaline-induced decreases			
[d]40S ribosomal protein S24	P62850	RS24_RAT	-2.88
[d]*Transcription intermediary factor 1-beta*	O08629	TIF1B_RAT	-2.87
[f]PDZ and LIM domain protein 5	Q62920	PDLI5_RAT	-2.60
[d]*Cysteine and glycine-rich protein 1*	P47875	CSRP1_RAT	-2.47
[g]*Lactadherin*	P70490	MFGM_RAT	-2.24
[e]*Dynactin subunit 2*	Q6AYH5	DCTN2_RAT	-1.90
[d]Elongation factor 1-delta	Q68FR9	EF1D_RAT	-1.88
[a]*Transforming growth factor beta-1-induced transcript 1 protein*	Q99PD6	TGFI1_RAT	-1.82
[e]*Dynactin subunit 1*	P28023	DCTN1_RAT	-1.80
[a]*Integrin beta-1*	P49134	ITB1_RAT	-1.79
[c]*Vimentin*	P31000	VIME_RAT	-1.78
[d]*Eukaryotic translation initiation factor 5*	Q07205	IF5_RAT	-1.68
[e]Cytoplasmic dynein 1 heavy chain 1	P38650	DYHC1_RAT	-1.57
[h]*Heat shock protein HSP 90-alpha*	P82995	HS90A_RAT	-1.56
[c]Tubulin beta-4B chain	Q6P9T8	TBB4B_RAT	-1.52
[b]*Fatty acid synthase*	P12785	FAS_RAT	-1.42
[f]*14-3-3 protein theta*	P68255	1433T_RAT	-1.42
[f]14-3-3 protein zeta/delta	P63102	1433Z_RAT	-1.38

H9c2 cells were pre-incubated with biotin-X-cadaverine prior to treatment with isoprenaline (10 μM) and biotin-cadaverine labelled proteins were captured and analysed by SWATH MS. *Absolute fold changes in isoprenaline-treated samples versus control (n=4) were calculated using SCIEX OneOmics with parameters MLR weight > 0.15, confidence >70%, algorithms used described by Lambert *et* al., (2013). Known TG2 targets appearing in the TG2

substrate database (Csósz et al., 2009) or identified by Yu et al. (2015), Almami et al. (2014), Vyas et al. (2016, 2017) are indicated in *italics*. Proteins are grouped according to their functions and/or cellular function as follows: [a]cell signalling; [b]metabolism; [c]cytoskeletal; [d]transcription/translation; [e]vesicular trafficking/extracellular matrix constituent; [f]structural/scaffolding protein; [g]apoptosis; [h]protein folding

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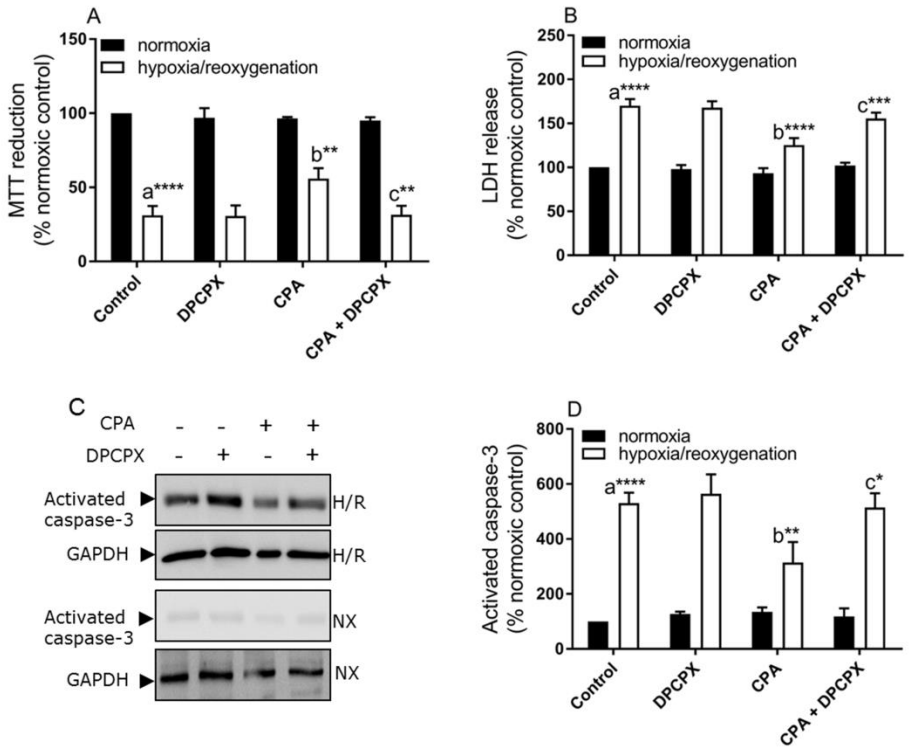
Figure 1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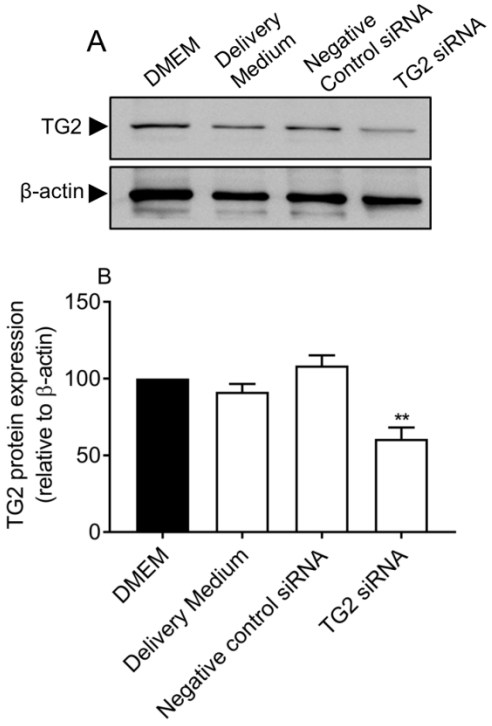
hypoxia/reoxygenation
MTT reduction
(% normoxic control)
150
100
50
0
a****
b**
c**
Control
DPCPX
CPA
CPA + DPCPX
B
normoxia
hypoxia/reoxygenation
LDH release
(% normoxic control)
250
200
150
100
50
0
a****
b****
c***
Control
DPCPX
CPA
CPA + DPCPX
C
CPA - - + +
DPCPX - + - +
Activated caspase-3
H/R
GAPDH
H/R
Activated caspase-3
NX
GAPDH
NX
D
normoxia
hypoxia/reoxygenation
Activated caspase-3
(% normoxic control)
800
600
400
200
0
a****
b**
c*
Control
DPCPX
CPA
CPA + DPCPX

Figure 2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Delivery Medium
Negative Control siRNA
TG2 siRNA
TG2
β-actin
B
TG2 protein expression (relative to β-actin)
150
100
50
0
**
DMEM
Delivery Medium
Negative control siRNA
TG2 siRNA

Figure 3

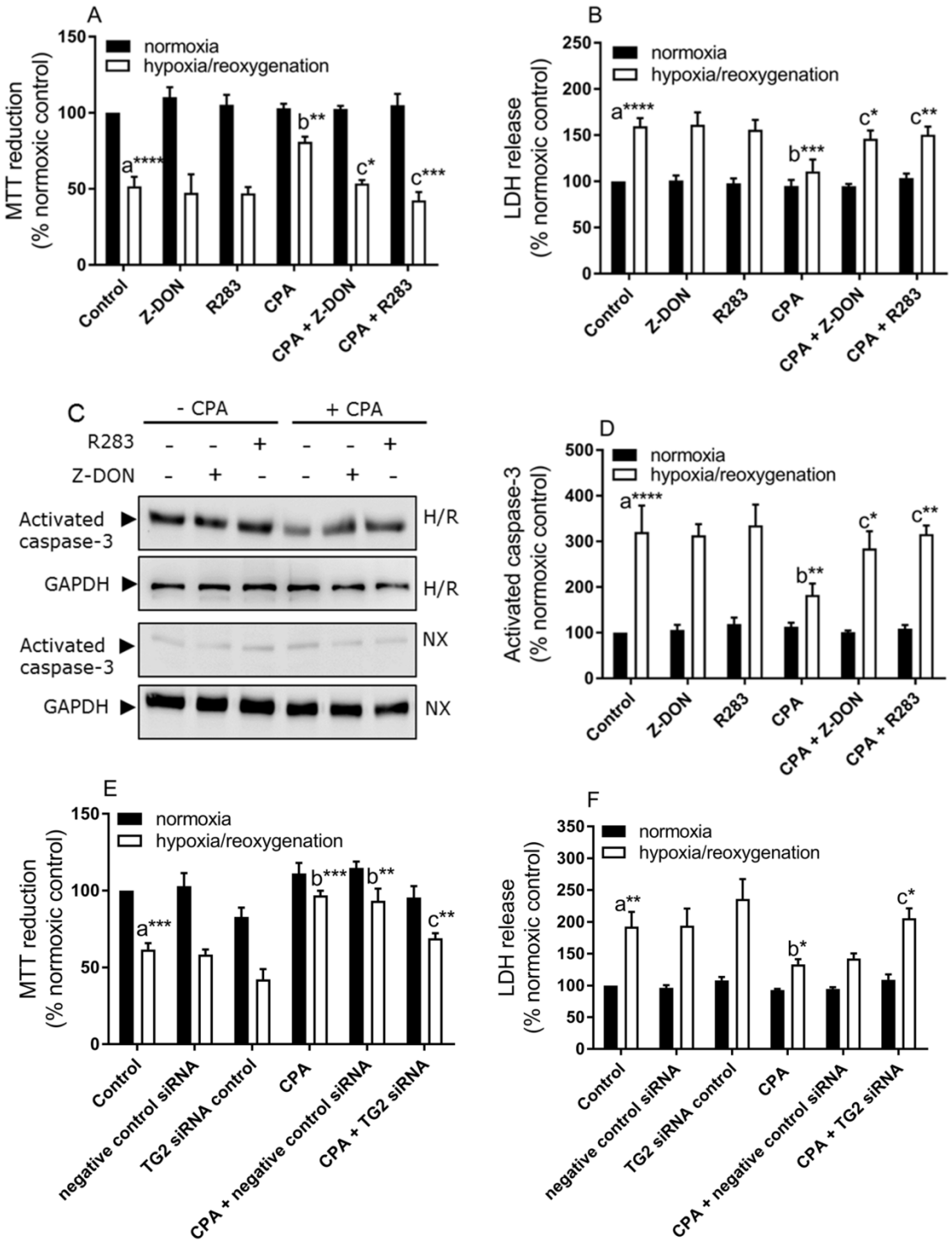
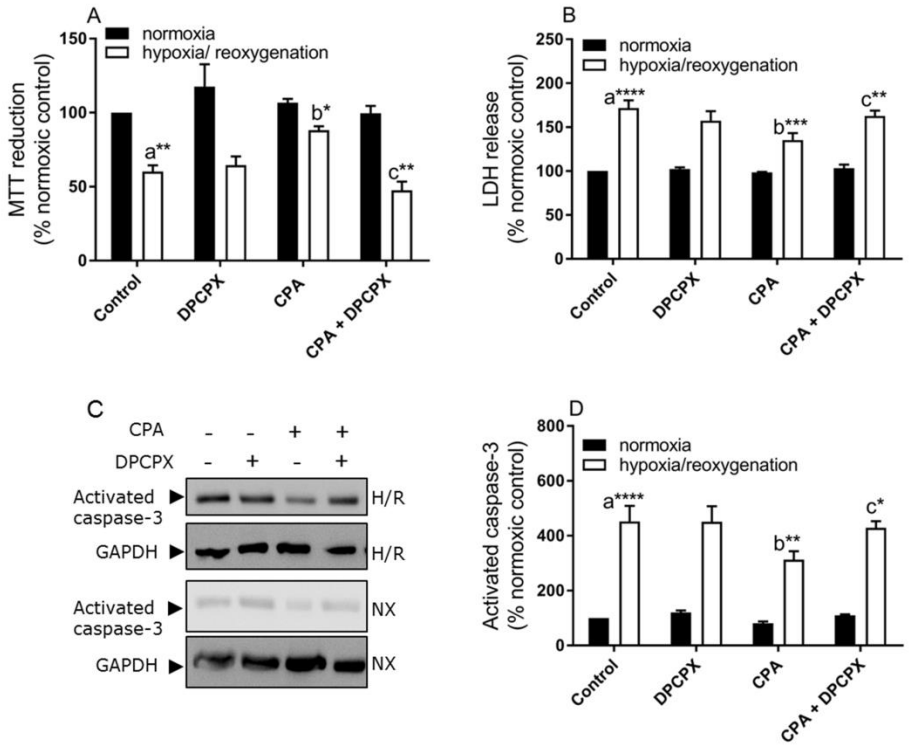

A
normoxia
hypoxia/reoxygenation
MTT reduction
(% normoxic control)
150
100
50
0
a****
b**
c*
c***
Control
Z-DON
R283
CPA
CPA + Z-DON
CPA + R283
B
normoxia
hypoxia/reoxygenation
LDH release
(% normoxic control)
250
200
150
100
50
0
a****
b***
c*
c**
Control
Z-DON
R283
CPA
CPA + Z-DON
CPA + R283
C
- CPA
+ CPA
R283
Z-DON
Activated caspase-3
GAPDH
Activated caspase-3
GAPDH
H/R
H/R
NX
NX
D
normoxia
hypoxia/reoxygenation
Activated caspase-3
(% normoxic control)
500
400
300
200
100
0
a****
b**
c*
c**
Control
Z-DON
R283
CPA
CPA + Z-DON
CPA + R283
E
normoxia
hypoxia/reoxygenation
MTT reduction
(% normoxic control)
150
100
50
0
a***
b***
b**
c**
Control
negative control siRNA
TG2 siRNA control
CPA
CPA + negative control siRNA
CPA + TG2 siRNA
F
normoxia
hypoxia/reoxygenation
LDH release
(% normoxic control)
350
300
250
200
150
100
50
0
a**
b*
c*
Control
negative control siRNA
TG2 siRNA control
CPA
CPA + negative control siRNA
CPA + TG2 siRNA

Figure 4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normoxia
hypoxia/ reoxygenation
MTT reduction
(% normoxic control)
150
100
50
0
a**
b*
c**
Control
DPCPX
CPA
CPA + DPCPX
B
normoxia
hypoxia/reoxygenation
LDH release
(% normoxic control)
250
200
150
100
50
0
a****
b***
c**
Control
DPCPX
CPA
CPA + DPCPX
C
CPA - - + +
DPCPX - + - +
Activated caspase-3
H/R
GAPDH
H/R
Activated caspase-3
NX
GAPDH
NX
D
normoxia
hypoxia/reoxygenation
Activated caspase-3
(% normoxic control)
800
600
400
200
0
a****
b**
c*
Control
DPCPX
CPA
CPA + DPCPX

Figure 5

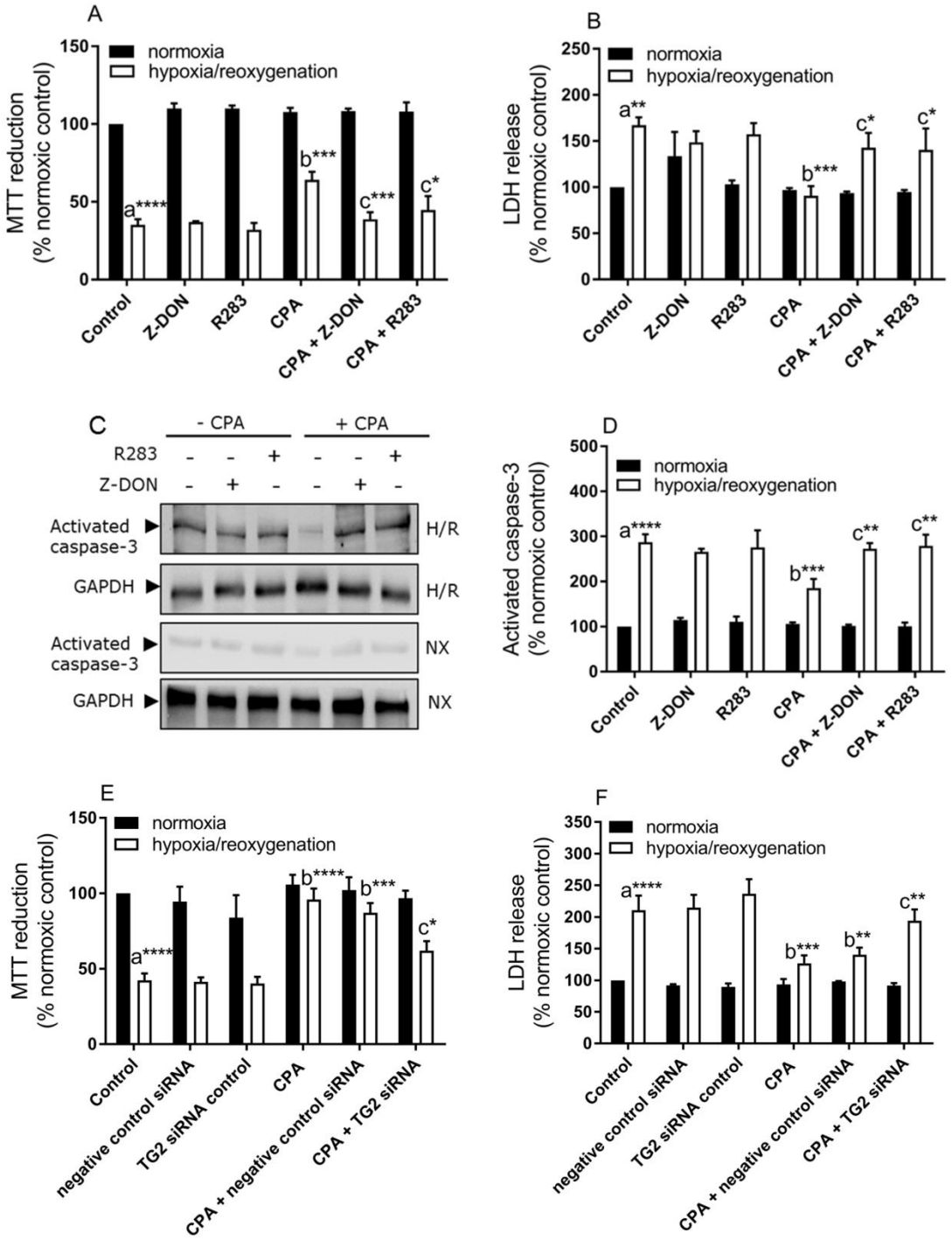

A
normoxia
hypoxia/reoxygenation
MTT reduction (% normoxic control)
150
100
50
0
a****
b***
c***
c*
Control
Z-DON
R283
CPA
CPA + Z-DON
CPA + R283
B
normoxia
hypoxia/reoxygenation
LDH release (% normoxic control)
250
200
150
100
50
0
a**
b***
c*
c*
Control
Z-DON
R283
CPA
CPA + Z-DON
CPA + R283
C
- CPA
+ CPA
R283 - - + - - +
Z-DON - + - - + -
Activated caspase-3
H/R
GAPDH
H/R
Activated caspase-3
NX
GAPDH
NX
D
normoxia
hypoxia/reoxygenation
Activated caspase-3 (% normoxic control)
500
400
300
200
100
0
a****
b***
c**
c**
Control
Z-DON
R283
CPA
CPA + Z-DON
CPA + R283
E
normoxia
hypoxia/reoxygenation
MTT reduction (% normoxic control)
150
100
50
0
a****
b****
b***
c*
Control
negative control siRNA
TG2 siRNA control
CPA
CPA + negative control siRNA
CPA + TG2 siRNA
F
normoxia
hypoxia/reoxygenation
LDH release (% normoxic control)
350
300
250
200
150
100
50
0
a****
b***
b**
c**
Control
negative control siRNA
TG2 siRNA control
CPA
CPA + negative control siRNA
CPA + TG2 siRNA

Figure 6

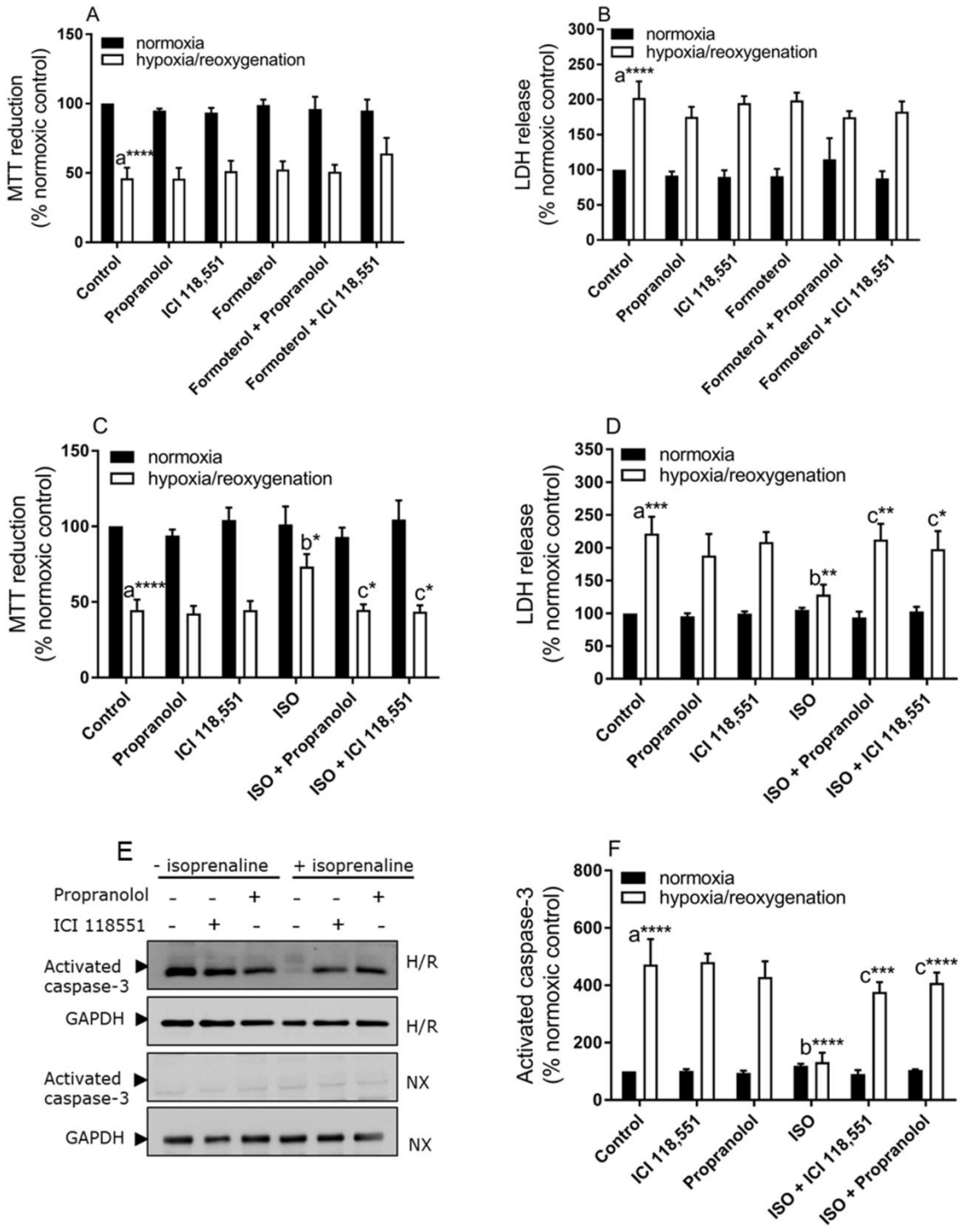

A
normoxia
hypoxia/reoxygenation
MTT reduction (% normoxic control)
a****
Control
Propranolol
ICI 118,551
Formoterol
Formoterol + Propranolol
Formoterol + ICI 118,551
B
normoxia
hypoxia/reoxygenation
LDH release (% normoxic control)
a****
Control
Propranolol
ICI 118,551
Formoterol
Formoterol + Propranolol
Formoterol + ICI 118,551
C
normoxia
hypoxia/reoxygenation
MTT reduction (% normoxic control)
a****
b*
c*
c*
Control
Propranolol
ICI 118,551
ISO
ISO + Propranolol
ISO + ICI 118,551
D
normoxia
hypoxia/reoxygenation
LDH release (% normoxic control)
a***
b**
c**
c*
Control
Propranolol
ICI 118,551
ISO
ISO + Propranolol
ISO + ICI 118,551
E
- isoprenaline
+ isoprenaline
Propranolol
ICI 118551
Activated caspase-3
H/R
GAPDH
H/R
Activated caspase-3
NX
GAPDH
NX
F
normoxia
hypoxia/reoxygenation
Activated caspase-3 (% normoxic control)
a****
b****
c***
c****
Control
ICI 118,551
Propranolol
ISO
ISO + ICI 118,551
ISO + Propranolol

Figure 7

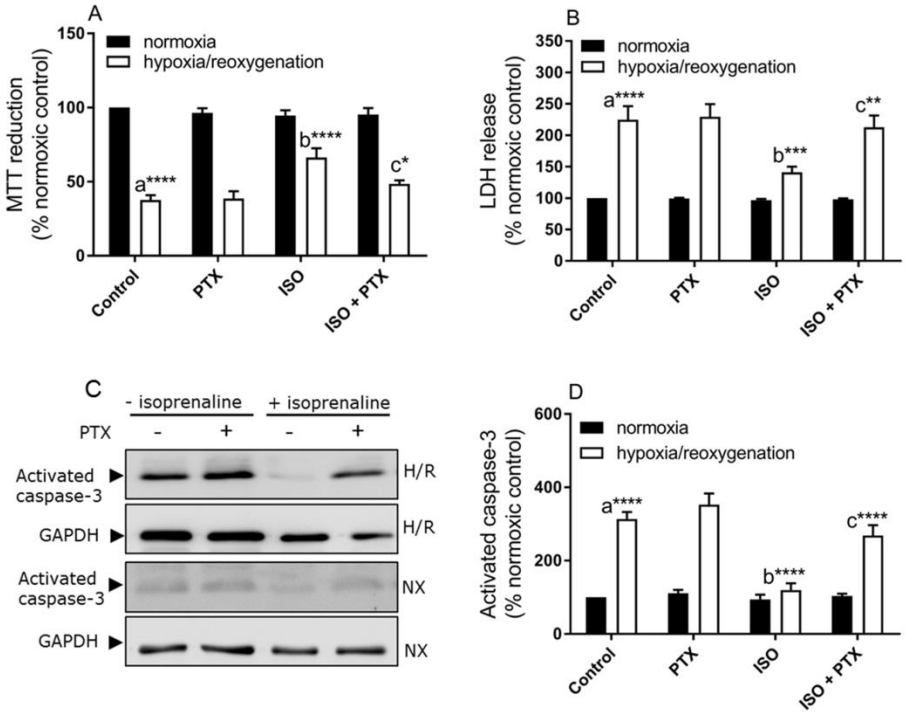

A
normoxia
hypoxia/reoxygenation
MTT reduction
(% normoxic control)
150
100
50
0
a****
b****
c*
Control
PTX
ISO
ISO + PTX
B
normoxia
hypoxia/reoxygenation
LDH release
(% normoxic control)
350
300
250
200
150
100
50
0
a****
b***
c**
Control
PTX
ISO
ISO + PTX
C
- isoprenaline
+ isoprenaline
PTX
- + - +
Activated caspase-3
GAPDH
Activated caspase-3
GAPDH
H/R
H/R
NX
NX
D
normoxia
hypoxia/reoxygenation
Activated caspase-3
(% normoxic control)
600
400
200
0
a****
b****
c****
Control
PTX
ISO
ISO + PTX

Figure 8

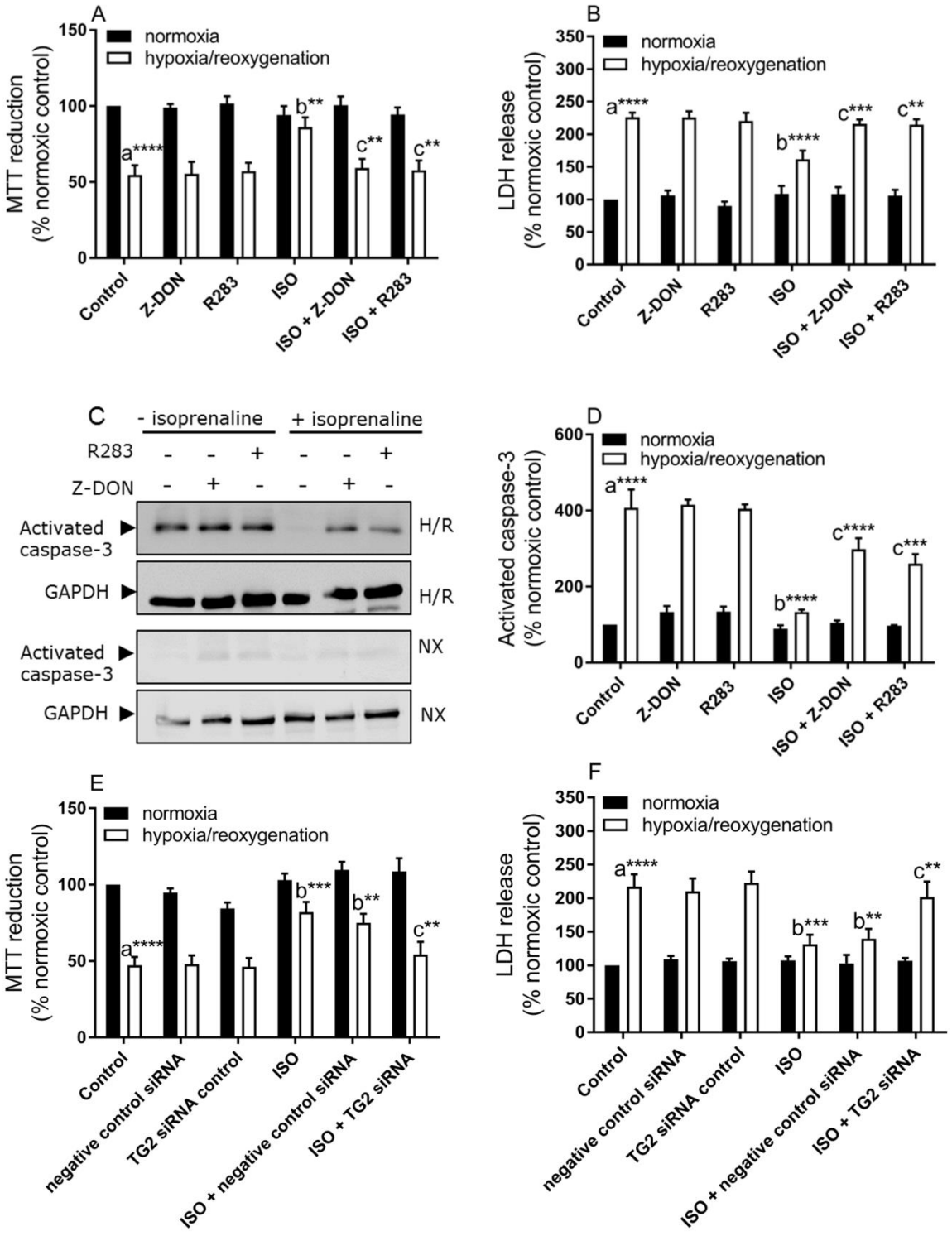

A
normoxia
hypoxia/reoxygenation
MTT reduction (% normoxic control)
150
100
50
0
a****
b**
c**
c**
Control
Z-DON
R283
ISO
ISO + Z-DON
ISO + R283
B
normoxia
hypoxia/reoxygenation
LDH release (% normoxic control)
350
300
250
200
150
100
50
0
a****
b****
c***
c**
Control
Z-DON
R283
ISO
ISO + Z-DON
ISO + R283
C
- isoprenaline
+ isoprenaline
R283
Z-DON
Activated caspase-3
GAPDH
H/R
H/R
Activated caspase-3
GAPDH
NX
NX
D
normoxia
hypoxia/reoxygenation
Activated caspase-3 (% normoxic control)
600
400
200
0
a****
b****
c****
c***
Control
Z-DON
R283
ISO
ISO + Z-DON
ISO + R283
E
normoxia
hypoxia/reoxygenation
MTT reduction (% normoxic control)
150
100
50
0
a****
b***
b**
c**
Control
negative control siRNA
TG2 siRNA control
ISO
ISO + negative control siRNA
ISO + TG2 siRNA
F
normoxia
hypoxia/reoxygenation
LDH release (% normoxic control)
350
300
250
200
150
100
50
0
a****
b***
b**
c**
Control
negative control siRNA
TG2 siRNA control
ISO
ISO + negative control siRNA
ISO + TG2 siRNA

Figure 9

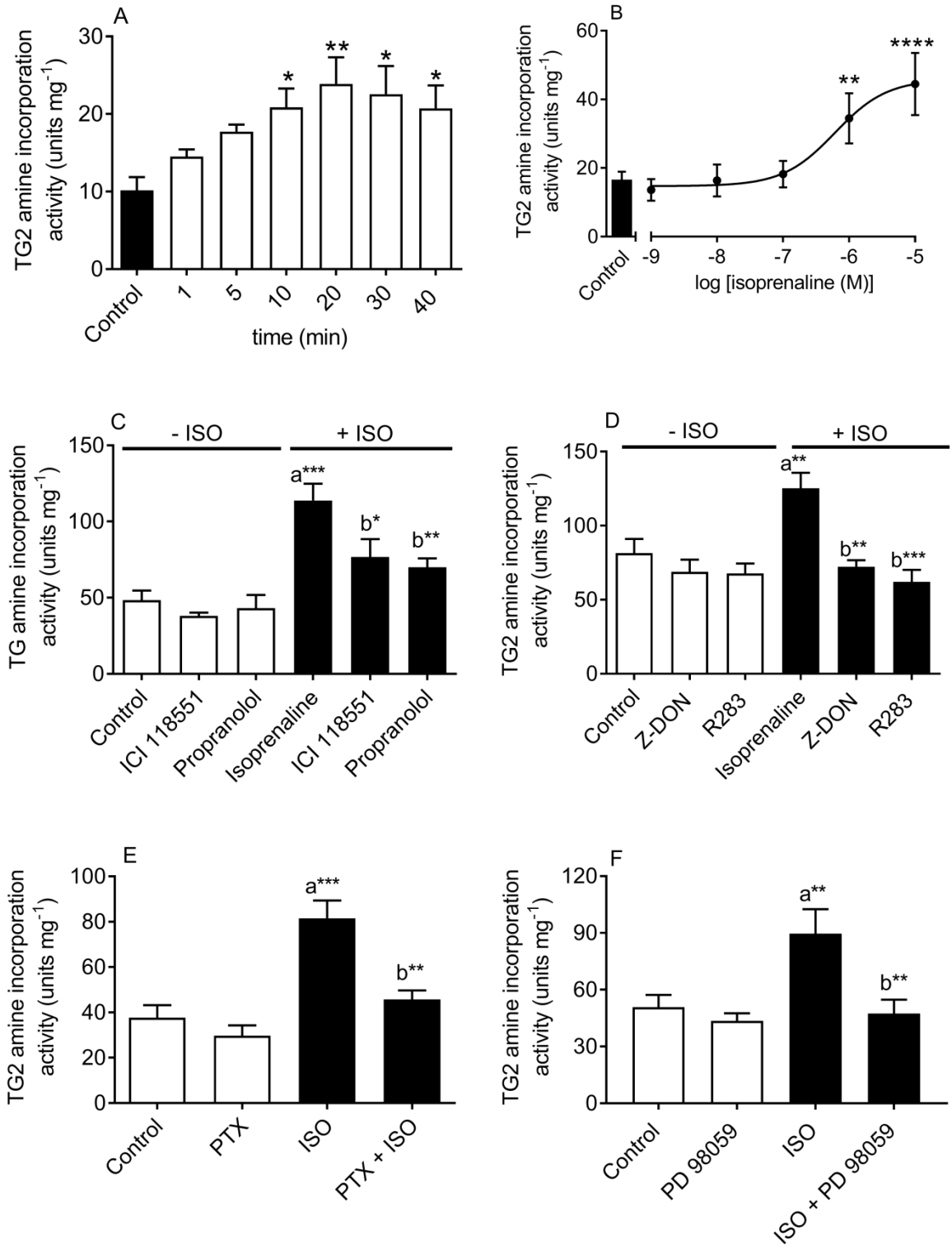

A
TG2 amine incorporation activity (units mg-1)
30
20
10
0
*
**
*
*
Control
1
5
10
20
30
40
time (min)
B
TG2 amine incorporation activity (units mg-1)
60
40
20
0
**
****
Control
-9
-8
-7
-6
-5
log [isoprenaline (M)]
C
TG amine incorporation activity (units mg-1)
- ISO
+ ISO
150
100
50
0
a***
b*
b**
Control
ICI 118551
Propranolol
Isoprenaline
ICI 118551
Propranolol
D
TG2 amine incorporation activity (units mg-1)
- ISO
+ ISO
150
100
50
0
a**
b**
b***
Control
Z-DON
R283
Isoprenaline
Z-DON
R283
E
TG2 amine incorporation activity (units mg-1)
100
80
60
40
20
0
a***
b**
Control
PTX
ISO
PTX + ISO
F
TG2 amine incorporation activity (units mg-1)
120
90
60
30
0
a**
b**
Control
PD 98059
ISO
ISO + PD 98059

Figure 10

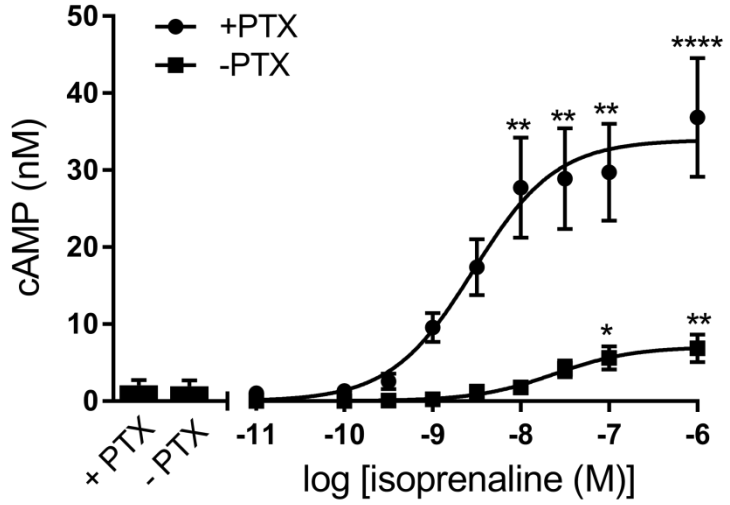

+PTX
-PTX
cAMP (nM)
50
40
30
20
10
0
****
**
**
**
*
**
+PTX
-PTX
-11
-10
-9
-8
-7
-6
log [isoprenaline (M)]

Figure 11

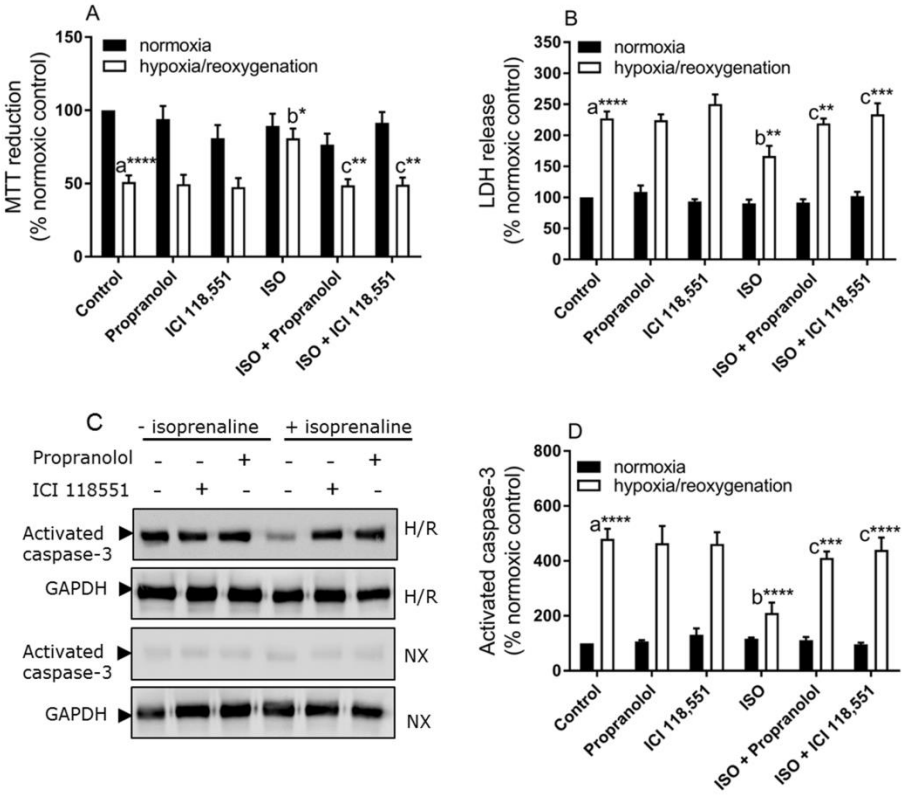

A
normoxia
hypoxia/reoxygenation
MTT reduction (% normoxic control)
150
100
50
0
a****
b*
c**
c**
Control
Propranolol
ICI 118,551
ISO
ISO + Propranolol
ISO + ICI 118,551
B
normoxia
hypoxia/reoxygenation
LDH release (% normoxic control)
350
300
250
200
150
100
50
0
a****
b**
c**
c***
Control
Propranolol
ICI 118,551
ISO
ISO + Propranolol
ISO + ICI 118,551
C
- isoprenaline
+ isoprenaline
Propranolol - - + - - +
ICI 118551 - + - - + -
Activated caspase-3
H/R
GAPDH
H/R
Activated caspase-3
NX
GAPDH
NX
D
normoxia
hypoxia/reoxygenation
Activated caspase-3 (% normoxic control)
800
600
400
200
0
a****
b****
c***
c****
Control
Propranolol
ICI 118,551
ISO
ISO + Propranolol
ISO + ICI 118,551

Figure 12

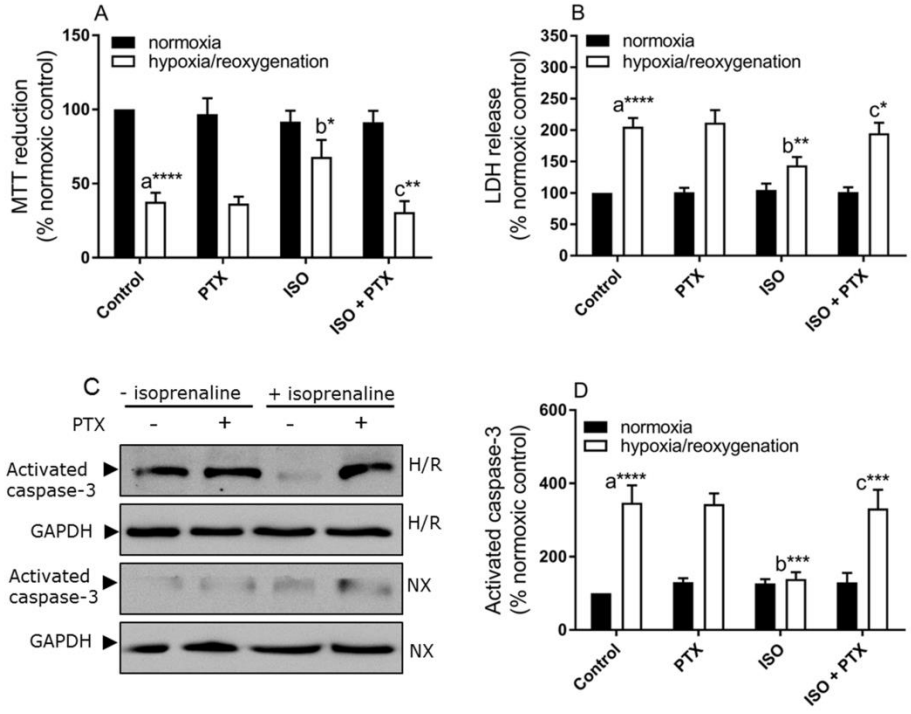

A
normoxia
hypoxia/reoxygenation
MTT reduction
(% normoxic control)
150
100
50
0
a****
b*
c**
Control
PTX
ISO
ISO + PTX
B
normoxia
hypoxia/reoxygenation
LDH release
(% normoxic control)
350
300
250
200
150
100
50
0
a****
b**
c*
Control
PTX
ISO
ISO + PTX
C
- isoprenaline
+ isoprenaline
PTX
-
+
-
+
Activated caspase-3
H/R
GAPDH
H/R
Activated caspase-3
NX
GAPDH
NX
D
normoxia
hypoxia/reoxygenation
Activated caspase-3
(% normoxic control)
600
400
200
0
a****
b***
c***
Control
PTX
ISO
ISO + PTX

Figure 13

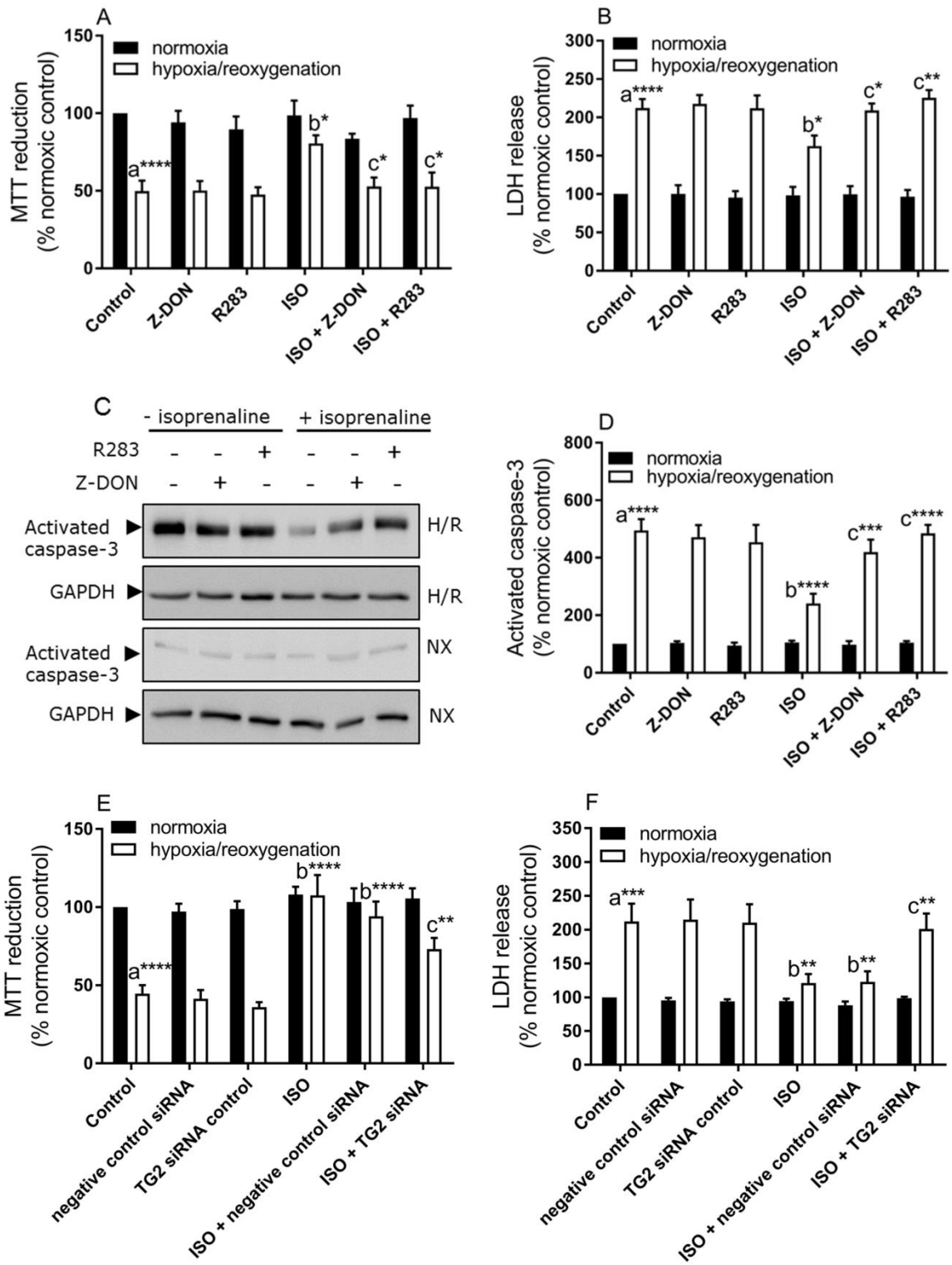
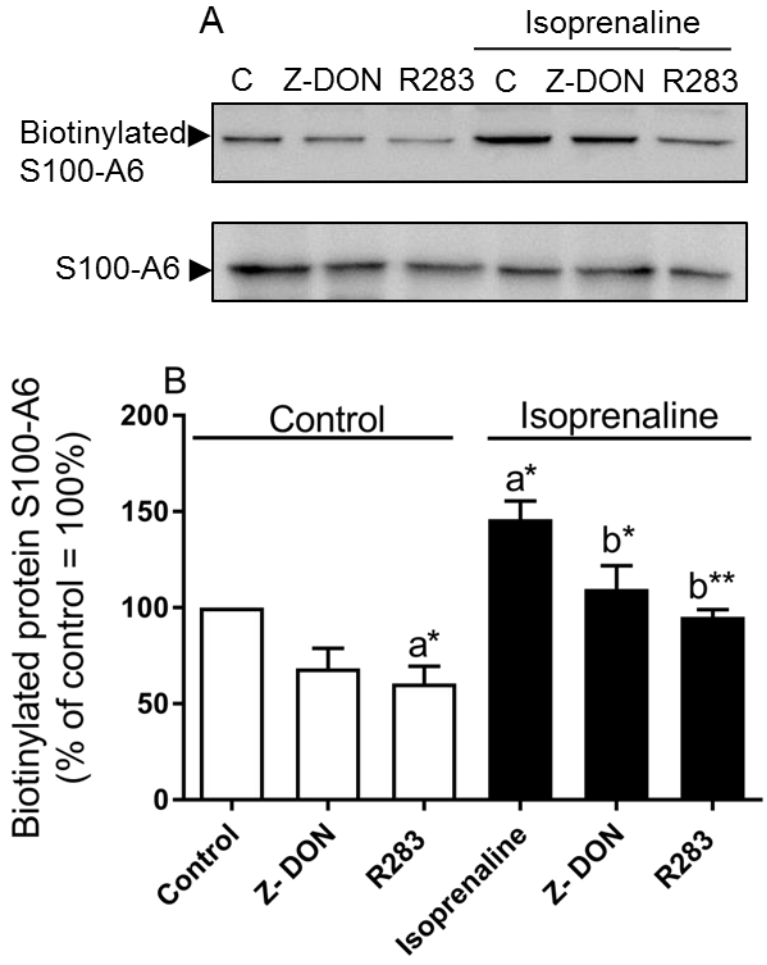

A
MTT reduction (% normoxic control)
normoxia
hypoxia/reoxygenation
Control
Z-DON
R283
ISO
ISO + Z-DON
ISO + R283
B
LDH release (% normoxic control)
C
- isoprenaline
+ isoprenaline
R283
Z-DON
Activated caspase-3
GAPDH
H/R
NX
D
Activated caspase-3 (% normoxic control)
E
negative control siRNA
TG2 siRNA control
ISO + negative control siRNA
ISO + TG2 siRNA
F

Figure 14


A
Isoprenaline
C
Z-DON
R283
C
Z-DON
R283
Biotinylated S100-A6
S100-A6
B
Control
Isoprenaline
Biotinylated protein S100-A6 (% of control = 100%)
200
150
100
50
0
a*
b*
b**
a*
Control
Z- DON
R283
Isoprenaline
Z- DON
R283