Caspase inhibition via A3 adenosine receptors: A new cardioprotective mechanism against myocardial infarction

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Caspase inhibition via A_3 adenosine receptors: a new cardioprotective mechanism against myocardial infarction

Running Title: 2-CL-IB-MECA mediated cardioprotection

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Abstract

Purpose.2-CL-IB-MECA, (A_3 adenosine receptor agonist)(A_3AR) mediated cardioprotection is well documented although the intracellular signalling pathways associated remain unclear. Here we demonstrate a role of the pro-survival signalling pathways MEK1/2-ERK1/2 and PI3K/AKT and their effect on modifying Caspase-3 activityin A_3AR mediated cardioprotection.

Methods. Isolated perfused rat hearts or primary adult rat cardiac myocytes were subjected to ischaemia/hypoxia and reperfusion/reoxygenation, respectively. 2-CL-IB-MECA (1nM) was administered at the onset of reperfusion/reoxygenation in the presence and absence of either the PI3K inhibitor Wortmannin (5nM) or MEK1/2 inhibitor UO126 (10μM). Heart tissues were harvested for assessment ofp-ERK1/2$_{(Thr202/Tyr204)}$ or p-AKT$_{(ser473)}$ status or underwent infarct size assessment. Cardiac myocytes underwent flow-cytometric analysis for apoptosis, necrosis, cleaved-caspase 3 / p-BAD (ser112) activity post-reoxygenation.

Results. 2-CL-IB-MECA significantly reduced infarct size compared to non-treated controls, where co-administration with either of the kinase inhibitors abolished the infarct sparing effects. Administration of 2-CL-IB-MECA at reperfusion significantly upregulated the status of p-ERK1/2 and p-AKT compared to time matched controls in a UO126 and Wortmannin sensitive manner respectively. 2-CL-IB-MECA when administered throughout reoxygenation significantly reduced apoptosis, necrosis, cleaved-caspase 3 activity and increased p-BAD (ser112) and p-BAD (ser136) activity in myocytes subjected to hypoxia/reoxygenation injury. The cytoprotective effect was abolished by co-administration with the kinase inhibitors Wortmannin and/or UO126.

Conclusions. We have described the molecular mechanisms associated with A_3AR mediated cardioprotection indicating a role for the pro-survival signalling pathways that decrease caspase-3 activity. These observations provide novel insight into the pharmacological effects of A_3ARs in ameliorating myocardial ischaemia/reperfusion injury.

Keywords: A3 Adenosine receptor, Reperfusion injury, MEK1/2, PI3K, Apoptosis, Caspase 3, BAD.

Introduction

Adenosine is a purine nucleoside that is highlyabundantthroughout body where it plays a critical role in cellular function. Previous studies have shown adenosine, a metabolite of adenosine triphosphate (ATP), to accumulate within the interstitial fluid within the ischaemic myocardium(1). Increased adenosine has profound effects in the cardiovascular system including vasodilation,hypotension and bradycardia. Adenosine exerts its physiological responses via four purinergic G-protein coupled adenosine receptors designated A_1, A_{2a}, A_{2b} and A_3(2).

Research over the past decade has implicated the putative role of adenosine in mediating an innate role in protecting the myocardium from the deleterious consequences of ischaemia-reperfusion (I/R) injury (3-6).A_1, A_{2a}, A_{2b} and A_3 have been shown to exhibit cardioprotective properties, although controversy remains regarding the precise timing of activation of these receptors required to induce cardioprotection, as well as their involvement in ischaemic preconditioning and postconditioning(7-11).The ability of adenosine and adenosine analogues to mediate cardioprotection is of clinical relevance as a possible adjunctive therapy to treat patients with myocardial infarction or during cardiac surgery to limit the consequences of reperfusion injury(12).

Maddock *et al*. have previously shown activation of A_3AR at the onset of reperfusion to protect the myocardium from post ischaemic injury via an anti-apoptotic/necrotic mechanism that was abolished in the presence of A_3AR antagonist MRS 1191(8).A_3 adenosine receptor-mediatedcardioprotection has also been reported to limit myocardial I/R injury in a number of animal models including therat (10), rabbit(13), guinea pig (14), mice (7, 15)and dog (16).Adenosine receptors have previously been shown to be functionally expressed in a number of organs including the myocardium (17, 18).The protection afforded by A_3AR agonists can be directly attributed to A_3ARs, as in A_3AR gene knock-out mice subjected to myocardial ischaemia reperfusion injury, administration of the A_3AR agonist failed to induce cardioprotection(10).

A_3AR agonists have been reported to mediate protection from I/R injury in a number of organs including the feline lung (19-21) and rat brain(22, 23). A_3AR agonists have also been shown to promote cell survival in HL-60 leukaemia and U-937 lymphoma cells exposed to UV light by means of restricting apoptosis(24). Surprisingly, there is limited data available that indicates the mechanisms responsible for this protection in the intact heart, despite some information obtained in cellular models.

Recent studies have shown that mitogen activated protein kinases MEK1/2 - extracellular regulated kinase (ERK1/2) and phosphatidylinositol 3 kinase (PI3K) to become activated by a range of stimuli like growth factors and hormones following receptor stimulation (25-28). Mitogen activated protein kinases (MAPKs) play a role in cell survival, growth and differentiation and apoptosis (29). In this context, the MEK1/2-ERK1/2-BADand

PI3K-AKTcell survival pathwayshave been shown in numerous studies to regulate cardioprotection upon receptor stimulation where it is referred to as the reperfusion injury salvage kinase pathway (RISK) (30).

Studies by Germackand Dickenson showed direct stimulation of A_3ARs in neonatal rat cardiomyocytes to be directly linked to the serine/threonine kinase ERK1/2 and AKT in a dose and time dependent manner in normoxic conditions (31, 32). These further showed that preconditioning with the A_3AR agonist 2-CL-IB-MECA protected neonatal myocytes from hypoxia/reoxygenation injury in neonatalmyocytes. Interestingly, a number of cardioprotective agents including insulin, bradykinin and erythropoietin have been shown to have infarct sparing effectswhen administered alone either before or during reperfusion via upregulation at AKT and ERK pro-survival kinases(30).

The aim of this study was to evaluate the putative role of PI3K and MEK1/2 signalling pathways in A_3AR receptor mediated cardioprotection, asprevious studies have shown A_3ARs to activate PI3K and MEK1/2 in normoxic conditions. Therefore, it was feasible that the cardioprotection exerted by 2-Cl-IB-MECA when administered at reperfusion may involve potential recruitment of the PI3K and MEK1/2 MAPK signalling pathways.

Infarct sizes were measured after 2-Cl-IB-MECA (1nM) was administered andthe mechanisms responsible for 2-CL-IB-MECAdependent cardioprotection in the isolated perfused heart were assessed using the MEK1/2 inhibitor UO126(10μM)and the PI3K inhibitor Wortmannin (5nM).Tissues were also harvested at various time points post reperfusion do determine the phosphorylation status of $AKT_{(Ser473)}$, $ERK_{(Thr202/Tyr204)}$ and $BAD_{(Ser112)}$. Furthermore, we investigated the effect of the A_3AR agonist 2-CL-IB-MECA in the presence and absence of U0126 (10μM) or Wortmannin(5nM)when administered at reoxygenation on apoptosis, necrosis and cleaved-caspase 3 activity in adult rat cardiomyocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation.

Materials and Methods

Chemicals:A_3ARagonist 1-[2-Chloro-6-[[(3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-1-deoxy-*N*-methyl-*b*-D-ribofuranuronamide (2-CL-IB-MECA), 1,4-Diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (UO126), (1S,6bR,9aS,11R,11bR) 11-(Acetyloxy)-1,6b,7,8,9a,10,11,11b-octahydro-1-(methoxymethyl)-9a,11b-dimethyl-3H-furo[4,3,2-de]indeno[4,5,-h]-2-h]-2-benzopyran-3,6,9-trione (Wortmannin) were purchased from Tocris Cookson (Bristol.UK). Total ERK,AKT,p-ERK1/2 (Thr^{202} /Tyr^{204})p-AKT (Ser^{473}), Cleaved-Caspase-3 (Asp^{175}) (5A1), p-BAD(Ser^{112}) and p-BAD (Ser^{136}) Rabbit mAb antibodies were purchased from New England Biolabs (Hertfordshire, UK). Alexa Fluor 488 F(ab')2 fragment of goat anti-rabbit IgG (H+L)was purchased

from Invitrogen (Paisley, UK). β-actinantibody was purchased from Abcam (Cambridge, UK). Drugs were dissolved in DMSO and stored at -20°C.

Animals

Male Sprague-Dawley rats were used in all experiments (350-400g b/w). Experiments were conducted in accordance with the Guidelines on the operation of Animals (Scientific Procedures) Act 1986. Animals were obtained from Charles River (Margate, UK).

Isolated perfused heart preparation.

Animals were sacrificed by cerebral dislocation. Hearts were rapidly excised and placed into ice-cold Krebs Heinsleit buffer (KH). Hearts were quickly mounted onto the Langendorff system and retrogradely perfused with modified Krebs Henseleit (KH) bicarbonate buffer containing (in mmol) NaCl 118.5, $NaHCO_3$ 25, KCL 4.8, $MgSO_4$ 1.2, KH_2PO4 1.2, $CaCl_2$ 1.7, and glucose 11.KH buffer was gassed using 95% O_2 and 5% CO_2 and maintained at 37°C.

The left atrium was trimmed away and a latex isovolumic balloon was carefully introduced into the left ventricle and inflated up to 5-10mmHg. Functional recording was via a physiological pressure transducer connected to a bridge amp and a Powerlab (AD Instruments Ltd. Chalgrove, UK). Left ventricular pressure (LVDP), heart rate (HR) and coronary flow (CF) were measured at regular intervals. To induce regional ischaemia a surgical needle was inserted under the left main coronary artery and the ends of the thread were passed through a tube to form a snare. Tightening of the snare induced regional ischaemia and releasing the threadinitiated reperfusion. At the end of reperfusion, the snare was tightened to re-occlude the coronary artery branch.

A solution of 0.25% Evans blue in saline was then infused slowly via the aorta to delineate the non risk zone of the myocardium, which stained dark blue. The hearts were weighed and frozen at -20°C. Frozen hearts were then sliced into 2mm thick transverse sections and incubated in triphenyl-tetrazolium chloride solution (1% in phosphate buffer) at 37°C for 10-12minutes and fixed in 10% Formalin for at least 4 hours.

In the risk zone the viable issue stained red and infarct tissue appeared pale. The risk zone and infarct areas were traced onto acetate sheets. Using computerised planimetry (Summasketch II, Summagraphics) the percentage of infarct tissue within the volume of the myocardium at risk was calculated (I/R%).

Experimental Groups.

Isolated hearts were split into two groups. Group 1 hearts underwent a protocol for infarct size assessment as shown in Fig 1a. Group 2 hearts underwent protocols for western blotting as summarised in Fig 1b.

Protocol 1: All hearts were allowed to stabilise for 20min prior to being subjected to 35min regional ischaemia followed by 120min reperfusion. Hearts were randomly assigned to the following groups 1min before reperfusion. a) hearts perfused with KH buffer alone with no drugs(control); b) hearts perfused with 2-CL-IB-MECA (1nM) throughout the duration of reperfusion, previously shown to be cardioprotective by Maddock et al. c) hearts perfused with 2-CL-IB-MECA (1nM) in the presence and absence of UO126 (10μM) (highly specific MEK1/2 inhibitor) or Wortmannin (5nM)(selective irreversible inhibitor of PI3K) throughout the reperfusion period.

Protocol 2: Hearts were allowed to stabilise for 20min after which they were randomly assigned to one of the following groups a) perfused for 60min b) 35min ischaemia with 5,10, or 20min reperfusion in the presence and absence of 2-CL-IB-MECA (1nM) c) 35min ischaemia followed by 10min reperfusion with 2-CL-IB-MECA in the presence and absence of U0126 (10μM) or Wortmannin (5nM)d) 35 min ischaemia followed administered at the onset 10 min reperfusion in the presence and absence of UO126 (10 μM) or Wortmannin (5nM).

Protein Extraction

At the end of each experiment tissues were isolated from the risk zone and snap frozen in liquid nitrogen. Tissues from the ischaemic zone were homogenised in suspension buffer containing (in mM) 0.1 NaCl, 10 Tris, 1 EDTA (pH8), 2 sodium pyrophosphate, 2 NaF, 2 β-glycerophosphate, 0.1mg/ml 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and protease inhibitor cocktail mixture (Roche, UK) at 4°C using the IKA-Ultra-Turrax homogeniser. Samples were then centrifuged for 5min at 11,000 rpm and stored at -80°C until further analysis. Samples were assayed for protein content using the bicinchoninic acid (BCA) protein assay reagent kit (Pierce,Northumberland,UK).

Western blot analysis

Protein samples were mixed with an equal volume of sample buffer containing 100mM Tris (pH6.8), 200mM DTT, 2%SDS, 0.2% Bromophenol blue, 20% glycerol and heated to 95°C for 10min and stored at -20°C. Samples were defrosted on ice and 60μg of protein were separated by SDS-PAGE (12.5% acrylamide gel) using a Bio-Rad mini protean II system.

Proteins were transferred to polyvinylidenedifluoride (PVDF) membrane using the Bio-Rad mini Trans blot system. After transfer the membranes were washed with Ambo Life Tris buffered saline (TBS) and blocked for 1 hour using TBS, 5%w/v non fat milk, 0.1% Tween-20. Blots were incubated overnight at 4°C with the primary antibody to phosphor-AKT(Ser^{473}) phospho-ERK1 / ERK2 (Thr^{202}/Tyr^{204})at a dilution of 1:1000 in TBS, 5%w/v bovine serum albumin fraction V, 0.1% Tween-20. Blots were washed and incubated with anti rabbit

IgGantibody conjugated to horseradishperoxidase at a concentration of 1:2000 at room temperature for 1 hour. Blots were further washed and were incubated for a few seconds in enhanced chemiluminescence substrates (AMBO Lumifast ECL Kit).

Blots were exposed to Hyperfilm ECL (Amersham, Buckinghamshire, UK) and developed using Kodak developing/fixing solution (Sigma, Poole. UK). Equal loading was confirmed by developing the blots for either total AKT, ERK1/2, antibody.The transfer efficiency was assessed by Ponceau S Stainingprior to primary antibody application. Films were scanned and the band densitometry was assessed using the NIH Image J (v1.33) software.

Rat cardiac myocytemodel

Adult rat cardiomyocytes were isolated by conventional enzymatic dissociation. Briefly, Male Sprague Dawley rats (350-500g body weight) were sacrificed by cerebral dislocation. Hearts were quickly excised and mounted on a modified Langendorff apparatus. Hearts were perfused with modified Krebs Heinsleit bicarbonate buffer (NaCl 116mM, $NaHCO_3$ 25.0mM, KCl 5.4mM, $MgSO_4$ .7.H_2O 0.4mM, $CaCl_2$ 1.7mM, glucose 10mM, taurine 20mM, pyruvate 5mM and Na_2HPO_4.12H_2O 0.9mM). The buffer was oxygenated with 95% O_2 and 5% CO_2 (BOC Gases) and heated to 37°C (pH 7.4).

Hearts were perfused for 5 minutes with Krebs buffer containing calcium followed by 5 minutes of perfusion with calcium free Krebs Heinsleit buffer. Upon perfusion with calcium free buffer the heart ceased to contract. The hearts underwent a final 5 minute perfusion cycle with modified Krebs Heinsleit digestion buffer containing BSA 0.5%, Worthingtons Type II Collagenase 0.075%, $CaCl_2$ 4.4μM pH 7.4 with NaOH. During perfusion with collagenase the effluent was collected and reused.

After perfusion with collagenase the heart was removed and the atria were cut away.The ventricles were teased apart and incubated with fresh digestion buffer for 10 minutes on an orbital shaker. The digestion buffer was aspirated and was passed through a nylon mesh into a sterile falcon tube and centrifuged at 400 rpm for 2 minutes.

The supernatant was removed using a sterile pipette and the pellet was redistributed in 25ml freshly prepared restoration buffer (NaCl 116mM, $NaHCO_3$ 25.0mM, KCl 5.4mM, $MgSO_4$ .7.H_2O 0.4mM, glucose 10mM, taurine 20mM, pyruvate 5mM Na_2HPO_4.12H_2O 0.9mM, 1% BSA and 1% Pen-Strep). The calcium concentration was gradually brought back to 1.25mM.Myocyte viability was assessed using trypan blue and were discarded if the viability was below 70% or myocytes were incubated in suspension bufferfor 24 hours before being used in experiments.

Experimental Protocol.

Cardiac myocytes were centrifuged at 500rpm for 5 min and buffer was replaced with hypoxic buffer (12 mM KCL, 0.49mM $MgCl_2$, 0.9 mM $CaCl_2$, 4 mM HEPES, 10 mMdeoxyglucose and 20 mMlactate). Myocytes were incubated in a pre-heated (37°C) hypoxic chamber for 6 hours where the O_2 was replaced with 5% CO_2 balanced in Argon (BOC Gases). After hypoxia, cells were removed from the chamber and centrifuged at 500 rpm for 5 minutes. The supernatant was removed and replaced with restoration buffer.The A_3 agonist 2-Cl-IB-MECA (1nM) and the MEK1/2 inhibitor UO126 (10μM) or PI3K inhibitor Wortmannin (5nM) were placed into the appropriate wells in the 24 well plate. Cells were also kept for the normoxic group and unstained group.

Cells were reoxygenated in the incubator for 18 hours before undergoing fluorescence-activated cell sorting (FACS) analysis for assessment of cellular apoptosis , necrosis, cleaved-caspase3 and p-BAD (Ser^{112}) and p-BAD (Ser^{136}) activity.

Assessment of apoptosis and necrosis by FACS Analysis

The Vybrant Apoptosis Assay Kit #10 (Invitrogen, Paisley,UK) provides a three-colour fluorescence assay that distinguishes live, apoptotic, and late apoptotic cells from one another. Cells were incubated with fluorochromes according to the manufacturer's instructions. Briefly, cells were pelleted by centrifugation and resuspended in Annexin V buffer. The appropriate volumes of fluorescent probes were added to the cells and incubated at 37°C for 15 minutes. The samples were analysed on the FL-2 and FL-4 channels on the BD FACS Calibur® Flow cytometer and setup to count 10,000 events. Quantitative assessment of live, apoptotic and necrotic cell populations was made using the Cell Quest software.

Assessment of p-BAD (Ser^{112}) and p-BAD (Ser^{136}) and cleaved-caspase 3 activity by FACS Analysis

To determine the activity of p-BAD (Ser^{112}), p-BAD (Ser^{136}) and cleaved-caspase 3 in adult rat myocytes at the end of reoxygenation cells were centrifuged at 1200rpm for 2min(33). The supernatant was aspirated and the pellet was resuspended in 250μl phosphate buffered saline andfixed by the addition of 250μl of 6% formaldehyde. The cells were incubated for 10min at 37°C and placed on ice for 1 min.

The eppendorffs were centrifuged at 1200 rpm for 2min after which the supernatant was aspirated and the cells were permeabilised by resuspending in ice-cold methanol (90%). Cells were kept on ice for 30min and centrifuged at 1200 rpm for 2min. The supernatant was removed and the cells were with incubation buffer (0.5% BSA in PBS stored @ 4°C). The eppendorffs were centrifuged at 1200 rpm for 2min followed by the removal of the supernatant. The cells were blocked by the addition of 100μl of incubation buffer for 10 minutes at room temperature. The cleaved-Caspase 3_{ASP175} (5A1) rabbit monoclonal primary antibody or p-BAD (ser112) (New

England Biolabs, Hitchin, UK) was added to the blocking buffer to give a final concentration of 1:100 and incubated at room temperature for 60 minutes.

Theeppendorffs were centrifuged again at 1200 rpm for 2 minutes followed by the removal of supernatant and washed twice in incubation buffer. The cells were resuspended in 200µl of incubation buffer containing the Alexa Fluor® 488 secondary antibody (Invitrogen®) to give a final concentration of 1:1000. The samples were covered in foil and incubated for 30 minutes at room temperature. The cells were centrifuged at 1200 rpm for 2 min and washed in incubation buffer. Finally, the cells were resuspended in 500µl of phosphate buffered saline and analysed on the BD FACS Calibur® flow cytometer on the FL-1 channel. Histograms were plotted for each of the groups showing the mean fluorescence for 10,000 counts.

Statistical Analysis

All values are expressed as mean ±SEM. Infarct size, cell death and band densities were tested for group differences using one way analysis of variance (ANOVA) with Fishers post hoc tests. A P-value of $P<0.05$ was considered statistically significant. Left ventricular developed pressure, heart rate and coronary flow were assessed for statistical difference using a Two way ANOVA for repeated measures.

Exclusion Criteria

In total 5 hearts were excluded from the study where heart rate or left ventricular developed pressure failed to recover during the stabilisation phase.

Results

Homogeneity among the experimental groups

The results from 68successful experiments were included. Heart rate, coronary flow and left ventricular developed pressure were not significantly different during stabilisation, ischaemia and reperfusion in all groups (data not included).

Protection afforded by the A_3AR agonist 2-CL-IB-MECA when administered at reperfusion is mediated via MEK1/2 activity

Isolated perfused rat hearts underwent 35 minutes of ischaemia followed by 120min of reperfusion where the A_3 agonist 2-CL-IB-MECA (1nM) was administered throughout the reperfusion period. Our studies showed that the administration of the highly specific A_3AR agonist 2-Cl-IB-MECA (1nM) throughout the reperfusion period significantly attenuated infarct development (% of the risk area) (I/R %) from 65 ± 1.7% in control hearts to 32

±4% in 2-CL-IB-MECA treated hearts, a reduction of 51% (P<0.01, Fig.2). The cardioprotective effect of 2-CL-IB-MECA has previously been demonstrated by Maddock et al.(8).

To determine whether the protection afforded by the A_3AR agonist 2-Cl-IB-MECA (1 nM) when administered at reperfusion was mediated via MEK1/2 activity, we used the MEK1/2 inhibitor UO126 (10 μM). Alone, UO126 had no significant effect on infarct size compared to control (55 ± 8% vs. 65 ± 1.5% control P>0.05, Fig.2). Co-administration of UO126 in the presence of 2-CL-IB-MECA significantly abrogated the protection afforded by 2-CL-IB-MECA (59 ± 9% vs. 32 ± 4% 2-CL-IB-MECA P<0.05, Fig.2). These data suggest that MEK1/2 dependent signalling pathways are involved in A_3AR mediated cardioprotection when administered at reperfusion.

Protection afforded by the A_3AR agonist 2-CL-IB-MECA at reperfusion is mediated via PI3K activity

To determine whether the protection afforded by the A_3AR agonist 2-Cl-IB-MECA (1 nM) when administered at reperfusion was mediated by enhanced PI3K activity, we used the PI3K inhibitor Wortmannin (5nM). Alone, Wortmannin had no significant effect on infarct size compared to control (65 ± 8% vs. 65 ± 2% respectively, P>0.05, Fig.3).

Co-administration of Wortmannin with 2-CL-IB-MECA significantly abrogated the protection afforded by 2-CL-IB-MECA (68 ± 12% vs. 32 ± 4% P<0.05; respectively, Fig.3). These data suggest that PI3K dependent signalling pathways are also involved in A_3AR mediated cardioprotection when administered at reperfusion.

The role of MEK1/2 – ERK1/2 MAPK pathway in A_3 adenosine receptor mediated cardioprotection in adult rat cardiomyocytes subjected to hypoxia/ reoxygenation injury

As shown in Figure 4 and 6, isolated adult rat cardiac myocytes underwent 6 hours of hypoxia followed by 18 hours of reoxygenation resulting in a 3.2 fold increase in the number of apoptotic cells (41.8 ± 5.4% vs. 12.9 ± 1.3% normoxic group, P<0.001), and a 3.1 fold increase in the number of necrotic cells (33.2 ± 2 vs. 10.7 ±2.1% normoxic group, P<0.001, Fig 4).

To ascertain the role of A_3ARs in limiting the deleterious consequences of reoxygenation injury the A_3AR agonist 2-Cl-IB-MECA (1 nM) was administered throughout the reoxygenation period. Administration of the A_3 agonist 2-Cl-IB-MECA (1 nM) significantly decreased the percentage of apoptotic myocytes (26 ± 6% vs. 41.8 ± 5.4 % Hyp/Reox group, P<0.001, Fig 4), and necrotic myocytes (16 ± 4 vs. 33.2 ± 2 Hyp/Reox group, P<0.001, Fig 4).

Administration of the A_3 adenosine receptor agonist 2-Cl-IB-MECA (1 nM) at reoxygenation significantly decreased the number of apoptotic and necrotic myocytes compared to non-treated groups. To determine which

mechanisms are involved in 2-CL-IB-MECA mediated cardioprotection we assessed the role of the MEK1/2 – ERK1/2 cell survival pathway using the MEK1/2 inhibitor UO126 (10 μM). Cardiac myocytes were incubated with the MEK1/2 inhibitor UO126 (10 μM) in the presence and absence of the A_3 agonist 2-CL-IB-MECA (1 nM).

Co-administration of the A_3 agonist 2-CL-IB-MECA (1 nM) with the MEK 1/2 inhibitor UO126 (10 μM) significantly abolished the anti-apoptotic (38 ± 4 % vs. 26 ± 6 % 2-CL-IB-MECA, P<0.001) and anti-necrotic potential of 2-Cl-IB-MECA (25 ± 3 vs. 16 ± 4 2-CL-IB-MECA, P<0.001, Fig 4). UO126 alone had no significant effect of cellular apoptosis when compared to the hypoxic reoxygenated group (42 ± 6 % vs. 42 ± 5 % Hyp/Reox, P>0.05, Fig 4). UO126 when administered at reoxygenation was seen to significantly decrease the number of cells dying by cellular necrosis when compared the hypoxic reoxygenated group (23 ± 3 % vs. 32.2 % vs. Hyp/Reox, P<0.05, Fig 4).

The role of PI3K survival pathway in A_3 adenosine receptor mediated cardioprotection in adult rat cardiomyocytes subjected to hypoxia/ reoxygenation injury.

Administration of the A_3 adenosine receptor agonist 2-Cl-IB-MECA (1 nM) at reoxygenation significantly decreased the number of apoptotic and necrotic myocytes compared to non-treated groups. To determine which cellular mechanisms are involved in 2-CL-IB-MECA mediated cytoprotection we assessed the role of the PI3K cell survival pathway using the PI3K inhibitor Wortmannin (5 nM).

Administration of the A_3 agonist 2-CL-IB-MECA (1 nM) in the presence of the PI3K inhibitor Wortmannin (5 nM) significantly abolished the anti-apoptotic effect of 2-CL-IB-MECA (1 nM) compared to when administered alone throughout reoxygenation (37 ± 6 % 2-CL-IB-MECA + Wortmannin vs. 26 ± 6 % 2-CL-IB-MECA, P<0.05, Fig 5).

Administration of 2-CL-IB-MECA (1 nM) in the presence of the PI3K inhibitor Wortmannin (5 nM) also significantly abolished the anti-necrotic effect of 2-CL-IB-MECA (1 nM) when administered alone throughout reoxygenation (28 ± 3 % 2-CL-IB-MECA + Wortmannin vs. 16 ± 4 % 2-CL-IB-MECA, P<0.05, Fig 5).

Wortmannin (5 nM) alone had no significant effect of cellular apoptosis when compared to the Hyp/Reox group (36 ± 5% Wortmannin vs. 42 ± 5 % Hyp/Reox, P>0.05) Fig 5. Interestingly, Wortmannin (5 nM) when administered alone throughout reoxygenation was seen to have a significant effect on reducing cellular necrosis when compared to the Hyp/Reox group (22 ± 3% Wortmannin vs. 32 ± 2 % Hyp/Reox P<0.001, Fig 5.

Time course of ERK 1/2 phosphorylation in non treated and 2-CL-IB-MECA ischaemic reperfused rat hearts

In order to investigate the role of MAPKs ERK1/2 inA_3AR mediated cardioprotection 2-CL-IB-MECA (1 nM) was administered at reperfusion and the heart tissues were harvested at different time intervals in both treated and non-treated groups and assessed by western blot analysis for total and Phospho-ERK1/2.

Administration of the A_3 agonist 2-Cl-IB-MECA (1nM) at reperfusion significantly upregulated the phosphorylation of ERK1/2 after 10 minutes of reperfusion ($P<0.05$; Fig 6) compared to non-treated time matched controls. This upregulation of ERK 1/2 phosphorylation by 2-CL-IB-MECA (1nM) after 10 minutes of reperfusion was abrogated by the co-administration of the MEK1/2 inhibitor UO126 (10μM) ($P<0.01$; Fig 6). Administration of UO126 (10μM) for 10 minutes of reperfusion had no significant effect of p-ERK expression compared to time matched controls ($P>0.05$; Fig 6). Administration of Wortmannin alone or in the presence of 2-CL-IB-MECA had no significant effect p-ERK levels.

Time course of AKT $_{(ser\ 473)}$ phosphorylation in non-treated and 2-CL-IB-MECA treated ischaemic reperfused rat hearts.

In order to determine the role of the PI3K/AKT pro-survival pathway in A_3AR mediated cardioprotection we assessed the phosphorylation status of p-AKT $_{(Ser\ 473)}$, at various time intervals post reperfusion in the presence and absence of the A_3 agonist 2-CL-IB-MECA (1 nM).

Administration of 2-CL-IB-MECA (1nM) for 10 minutes of reperfusion significantly upregulated the phosphorylation of AKT $_{(ser473)}$ compared totime matched controls ($P<0.01$vs. time matched controls, Fig 7).2-CL-IB-MECA dependent increase in p-AKT at 10 minutes of reperfusion was significantly reversed in the presence of the PI3K Inhibitor Wortmannin ($P<0.001$;Fig 7).Administration of Wortmannin (5nM) for 10 minutes of reperfusion significantly decreased p-AKT expression compared to time matched controls ($P<0.001$; Fig 7). Administration of UO126 alone had no significant effect p-AKT compared to time matched control ($P>0.05$; Fig 7). Administration of UO126 + 2CL-IB-MECA significantly increased p-AKT compared to time matched control ($P<0.01$; Fig 7).

Role of 2-CL-IB-MECA when administered at reoxygenation on p-BAD (ser112)and p-BAD (ser136) activity in isolated rat cardiomyocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation

Recruitment of pro-survival pathways has been shown to inhibit by phosphorylation downstream pro-apoptotic protein such as BAD. BADis involved in initiating apoptosis, but when phosphorylated at Ser112 and Ser136 BAD is inactivated and apoptosisisreduced. In order to investigate whether the cardioprotection observed by the administration of 2-CL-IB-MECA involved phosphorylation of p-BAD (Ser^{112}) and p-BAD (Ser^{136}),cardiac myocytes were subjected to hypoxia and reoxygenation injury where 2-CL-IB-MECA (1nM) was administered at reoxygenation in the presence and absence of the kinase inhibitors.

Administration of the A_3 agonist 2-Cl-IB-MECA (1 nM) at reoxygenation significantly up regulated the phosphorylation of BAD_{ser112} and BAD $_{ser136}$compared to the Hyp/Reox group ($P<0.05$) (Fig 8). Co-administration of 2-CL-IB-MECA (1nM) with UO126 (10uM) or Wortmannin (5nM) significantly abolished 2-CL-IB-MECA (1nM) mediated upregulation of p-BAD(ser112) and p-BAD (Ser136) respectively ($P<0.05$) (Fig 8). The kinase inhibitors Wortmannin or UO126 alone had no significant effect on p-BAD(ser112) and p-BAD (Ser136)compared to the Hyp/Reox group (Fig 8).

Role of 2-CL-IB-MECA when administered at reoxygenation on cleaved-caspase 3 activity in isolated rat cardiomyocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation

Isolated adult rat cardiomyocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation resulted in a 2.5 fold increase in cleaved-caspase 3 activity compared to the normoxic group $P<0.001$ (Fig 9). Administration of the A_3 agonist 2-Cl-IB-MECA (1nM) at reoxygenation significantly decreased Hyp/Reox dependent increases in cleaved-caspase 3 activity ($P<0.001$,Fig 9). The data strongly suggests that the cardioprotective effects of 2-CL-IB-MECA are via anti-apoptotic mechanisms.

Role ofMEK1/2-ERK1 and PI3K 2 cell survival pathway in cleaved-caspase 3 activity upon administration of 2-CL-IB-MECA (1nM) at reoxygenation in isolated myocytes subjected to hypoxia/reoxygenation injury.

Administration of 2-Cl-IB-MECA (1nM) at reoxygenation significantly reduced cleaved-caspase 3 activity compared to non-treated isolated adult rat cardiomyocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation ($P<0.001$, Fig 9). To determine whether the decrease in cleaved - caspase 3 activity involved the MEK1/2 – ERK1/2 cell survival pathway we used the MEK1/2 inhibitor UO126 (10μM) or PI3K inhibitor Wortmannin (5nM) in the presence and absence of 2-C-IB-MECA (1nM).

Administration of the MEK1/2 inhibitor UO126 with the A_3 agonist 2-CL-IB-MECA (1nM) at reoxygenation failed to block the decrease in cleaved-caspase 3 activityafforded when 2-CL-IB-MECA was administered alone at reoxygenation ($P>0.05$, Fig 9). UO126 when administered alone had no significant effect on cleaved-caspase 3 activity compared to the Hyp/Reox group ($P>0.05$, Fig 9).

Activation of A_3ARs throughout reoxygenation significantly decreased cleaved-caspase 3 activitywhich was significantly abolished in the presence of the PI3K inhibitor Wortmannin (5 nM) ($P<0.05$,Fig 10). Administration of Wortmannin (5nM) throughout reoxygenation alone had no significant effect of cleaved-caspase 3 activity compared to the non-treated Hyp/Reox($P>0.05$,Fig 10).

Discussion

The findings from the current study demonstrate that activation of adenosine A_3 receptors at reperfusion protects the isolated perfused rat heart from ischaemia/reperfusion injury and cardiac myocytes from hypoxia/reoxygenation injury via recruitment of the survival kinases PI3K and MEK1/2.

Our data are in agreement with those of Maddock and others whichhave previously shown that activation of A_3AR at reperfusion significantly attenuated infarct development when administered during reperfusion(7, 8, 10, 13, 14). The selective A_3AR antagonist I-ABOPX has previously been co-administered with the A_3AR agonist 2-CL-IB-MECA confirming that the protection was directly attributed to A_3ARs and not other adenosine receptor subtypes (14). 2-CL-IB-MECA dependent cerebral protection has also been described in the gerbil model of ischaemia / reperfusion injury leading to neuronal protection and decreased microglial infiltration(23). 2-CL-IB-MECA has been shown to protect against simulated models of ischaemia reperfusion injury in the brain (34) and feline lung (19, 21).

Our results confirm those of Maddock and colleagues showing 2-CL-IB-MECA (1nM) attenuated apoptosis and necrosis cell death in cardiac myocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation(8). Indeed, activation of A_3ARs has been shown to protect rat basophilic leukaemia mast cells exposed to UV light reducing cellular apoptosis by 50% (24).

MAPKs play an essential role in myocardial ischaemia reperfusion injury (35, 36). Adenosine A_3 receptors have been shown to activate MAPKs ERK1/2 in Chinese hamster ovary cells exhibiting the human A_3 adenosine receptor (37, 38). Our data revealed that the infarct sparing effects associated with 2-CL-IB-MECA was via recruitment of the reperfusion injury salvage kinase MEK1/2 – ERK 1/2 cell survival pathway in the isolated perfused rat heart. These results are in accordance with other studies showing that activation of A_3ARs at reperfusion can limit the deleterious consequences of ischaemia reperfusion injury in a number of organs including the heart (8, 13-15, 39), lung (19, 21) and brain (23). Our studies show that co-administration of 2-CL-IB-MECA in the presence of the MEK1/2 inhibitor UO126 abolished A_3 agonist dependent cardioprotection in the isolated heart. In concurrence with other cardioprotective agents, previous studies have shown cardioprotection with erythropoietin or adipocytokinevisfatin protects against ischaemic injury via activation of MEK1/2, where inhibition with UO126 an inhibitor of MEK1/2 abolished protection(40, 41).

Our data showed that the administration of 2-CL-IB-MECA at reoxygenation significantly decreased the number of apoptotic and necrotic cells compared to non-treated cells. Interestingly, the MEK1/2 inhibitor when administered together with 2-CL-IB-MECA abolished 2-CL-IB-MECA-dependent anti apoptotic/necrotic effects. Our results are in accordance with numerous studies that have shown activation of A_3ARs to protect

myocytes from hypoxia reoxygenation injury (32, 42). They further showed that the cytoprotection was abolished in the presence of the A_3AR antagonist MRS 1191 implicating a direct role for A_3ARs.Matot and colleagues have shown the A_3 agonist 2-CL-IB-MECA when administered at reperfusion attenuated ischaemia reperfusion injury in the in vivo feline lung via anti apoptotic/necrotic signalling pathways (19). They further showed that the A_3 antagonist MRS 1191 abolished this protective effect. A number of groups have shown activation of A_3ARs to protect cells from ischaemic injury (23, 24). This is the first study to show that activation of adenosine A_3receptors at reperfusion/reoxygenation to activate the MEK1/2-ERK1/2-BAD cell survival pathways in isolated hearts and cardiomyocytes.

Our results support those of Germack and Dickenson who showed preconditioning with adenosine protected myocytes from hypoxia reoxygenation injury measured by decreased caspase 3 releases and lactate dehydrogenase release(32). They further showed this cardioprotection was associated with recruitment of MEK1/2-ERK1/2 signalling pathways where inhibition of MEK1 with PD98059 completely abolished adenosine-dependent decreases in LDH and caspase 3.

Our data showed that 2-Cl-IB-MECA when administered at reperfusion significantly upregulated ERK1/2 phosphorylation at the time points assessed compared to time matched controls in a UO126 sensitive manner. Our results confirm those of Dickenson and colleagues who showed recruitment of the MEK1/2 – ERK1/2 cell survival pathway in neonatal myocytes preconditioned with 2-CL-IB-MECA (32). Importantly, Matot and colleagues showed that the administration of the A_3 agonist IB-MECA or the newly synthesised A_3 agonist MRS3558 at the onset of reperfusion significantly protected the feline ischaemic lung from reperfusion injury(19). They further showed upregulation of ERK1/2 phosphorylation with the A_3 agonists where ERK1/2 phosphorylation was abolished in the presence of the A_3 antagonist MRS 1191(19).

Our findings showed 2-CL-IB-MECA mediated infarct sparing effects were abolished in the presence of the PI3K inhibitor Wortmannin in the isolated perfused heart. 2-CL-IB-MECA mediated protection involved recruitment of PI3K /AKT pathway, where the PI3K inhibitor Wortmannin abolished 2-CL-IB-MECA mediated protection. Similarly, Park and colleagues have shown that the protection afforded by the administration of 2-CL-IB-MECA (1μM) at reperfusion involves recruitment of the PI3K/AKT survival pathway as inhibition of this pathway with the PI3K inhibitor Wortmannin abolished 2-CL-IB-MECA mediated protection in the isolated perfused rat heart (43). Our findings may be explained as 2-CL-IB-MECA has been shown to activate AKT in a dose dependent manner, where 2-CL-IB-MECA increases AKT phosphorylation (31).We show for the first time that the anti-apoptotic and anti-necrotic effects of 2-CL-IB-MECA were dependent on PI3K signalling where the

cytoprotective effects were reversed in the presence of the PI3K inhibitor Wortmannin in cardiac myocytes subjected to hypoxia reoxygenation injury.

Recruitment of the MEK1/2-ERK1/2 and PI3K/AKT pathways has been shown to recruit downstream effector proteins including the pro-apoptotic protein BAD(41, 44, 45). BAD remains in the cytosol when phosphorylated on either of its phosphorylation sites $BAD_{ser\ 112}$ and $BAD_{ser\ 136}$ bound to 14-3-3. In its phosphorylated state BADs pro-apoptotic potential is diminished and upon dephosphorylation BAD translocates to mitochondria where it leads to the release of cytochrome c from the mitochondria initiating the apoptosis(46). Our results indicate that activation of A_3ARs at reperfusion upregulates BAD (Ser_{112}) and BAD (ser136) phosphorylation thereby limiting cellular apoptosis.

Caspase 3, a marker of cellular apoptosis, undergoes a conformational change from its inactive form to its active cleaved form upon receiving apoptotic signals(47). Administration of 2-CL-IB-MECA at the onset of reoxygenation reversed the increase in cleaved-caspase 3 observed in the non-treated Hyp/Reox group. These findings further support our findings that activation of A_3ARs protects the myocardium from reperfusion/reoxygenation injury in the isolated rat heart/myocyte in an anti-apoptotic manner.

To determine whether the decrease in cleaved-caspase 3 was dependent on the recruitment of cell survival kinases MEK1/2-ERK1/2 myocytes were incubated with 2-CL-IB-MECA in the presence and absence of the selective MEK1/2 inhibitor UO126. Our data showed that 2-Cl-IB-MECA dependent decreases in caspase 3 were ameliorated by the PI3K inhibitor Wortmannin and not by the MEK1/2 inhibitor UO126. Previous studies have shown that preconditioning with adenosine (non-specific adenosine receptor agonist) or 2-CL-IB-MECA (highly specific A_3AR agonist) decreased caspase 3 activity in neonatal cardiac myocytes subjected to 4 hours of hypoxia and 18 hours of reoxygenation(32).

We have shown that 2-CL-IB-MECA when administered at reoxygenation protects cardiac myocytes from hypoxia/reoxygenation injury via anti-apoptotic pathways. This protective anti-apoptotic effect of 2-CL-IB-MECA is supported in a number of diverse models of ischaemia/reperfusion including the lung (19-21), heart (8) and brain (23). Furthermore, 2-CL-IB-MECA has also been implicated in reducing cleaved-caspase 3 activity in the ischaemic/reperfused lung (19, 20).

Numerous studies have implicated growth factors, hormones, cytokines and neurotrophic factors to protect against ischaemia reperfusion injury via recruitment of the MEK1/2-ERK1/2/BAD and PI3K/AKT/BADcell survival pathway(28). Urocortin a hormone related to the hypothalamic corticotrophin releasing factor hormone has been shown to protect the ischaemic myocardium from ischaemia reperfusion injury via upregulation of

ERK1/2 in a PD98059 (MEK1/2 inhibitor) sensitive manner as has visfatinanadipocytokine(41, 48, 49). Other studies have shown insulin and Neuregulin-1 mediated cytoprotection involved recruitment of the PI3K/AK/BAD cell survival pathway (50,51).Previous studies have shown that the cAMP Response Element-Binding Protein is downstream target of the PI3K and MAPK cell survival pathway and may play in A_3AR mediated cardioprotection via activation of the transcription factor CREB, which induces the expression of the antiapoptotic factor Bcl-2 (52, 53). Furthermore, studies have shown that A_3AR induced cardioprotection involves ATP-sensitive potassium channels (K_{ATP}) indicating their role A_3AR mediated protection (54). It is feasible that a number of pathways converge including the PI3K/AKT, MEK1-2/ERK1/2/ K_{ATP} channels mediating 2-CL-IB-MECA induced cardioprotection(17, 18).

Interestingly, Ge and colleagues have shown A_3AR mediated cardioprotection to be dependent on activation of A_3AR on bone marrow cells via supressing inflammatory reactions in an *in vivo* model of ischaemia/reperfusion injury (55).The current in-vitro study shows 2-CL-IB-MECA to mediate protection for ischaemia/ reperfusion in a cell free model. Our findings may be explained as A_3ARs have been shown to functionally present on cardiomyocytes where upon activation elicit protection via cell survival signalling pathways.

The A_1/A_2 AR agonist 5'-(N-ethylcarboxamido) adenosine (NECA) has been shown to protect the ischaemic reperfused rabbit heart from reperfusion injury when administered at reperfusion via upregulation of ERK1/2 whereby inhibition of MEK1 with PD98059 blocked the protection(56).

In conclusion we show for the first time that 2-CL-IB-MECA protects the ischaemic reperfused rat heart from ischaemia reperfusion injury when administered at reperfusion via recruitment of the MEK1-2/PI3K cell survival pathway. Furthermore, activation of A_3ARs on the onset of reoxygenation significantly protected the hypoxic / reoxygenated adult rat cardiac myocyte from reoxygenation injury in an anti-apoptotic/necrotic manner via the MEK1/2 - PI3K cell survival pathway. Finally, 2-CL-IB-MECA reduced caspase 3 activityin a PI3K dependent and MEK1/2 independent manner. The potential pathways involved in A_3AR mediated cardioprotection are summarised in Fig 11.

Acknowledgements

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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Figure
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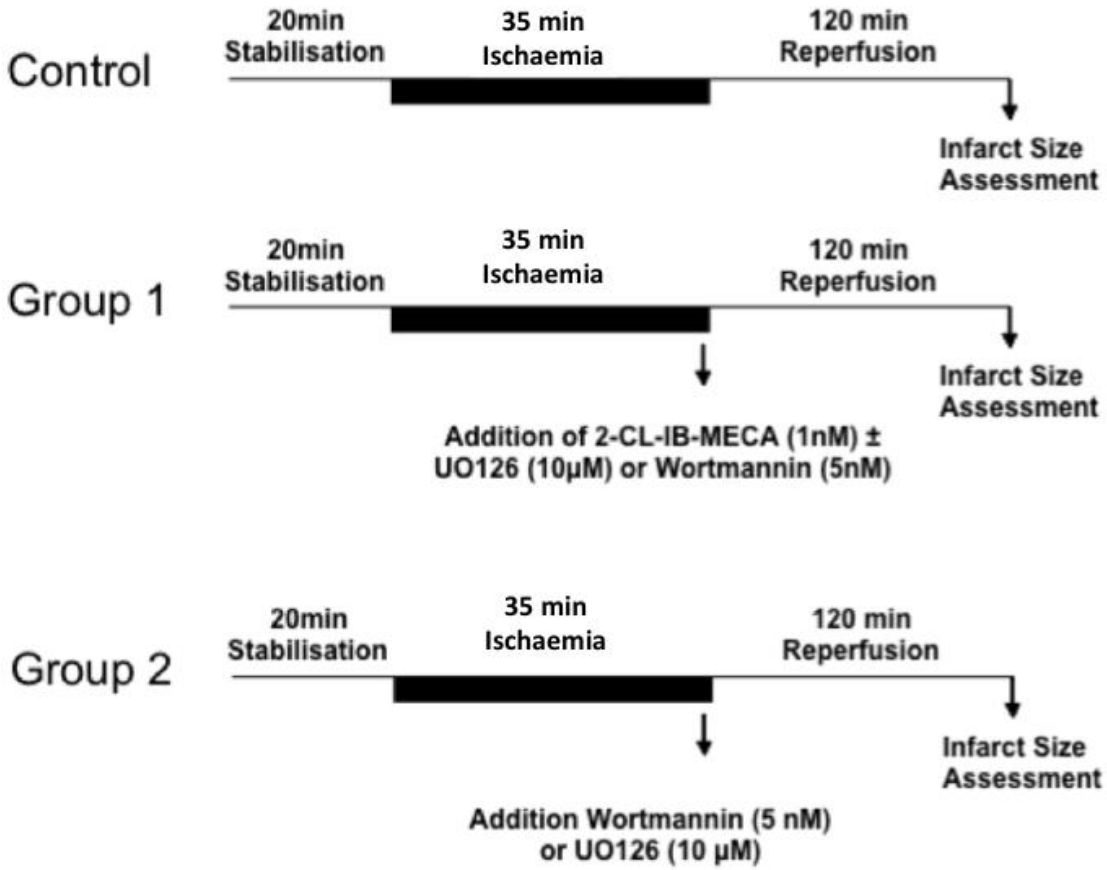

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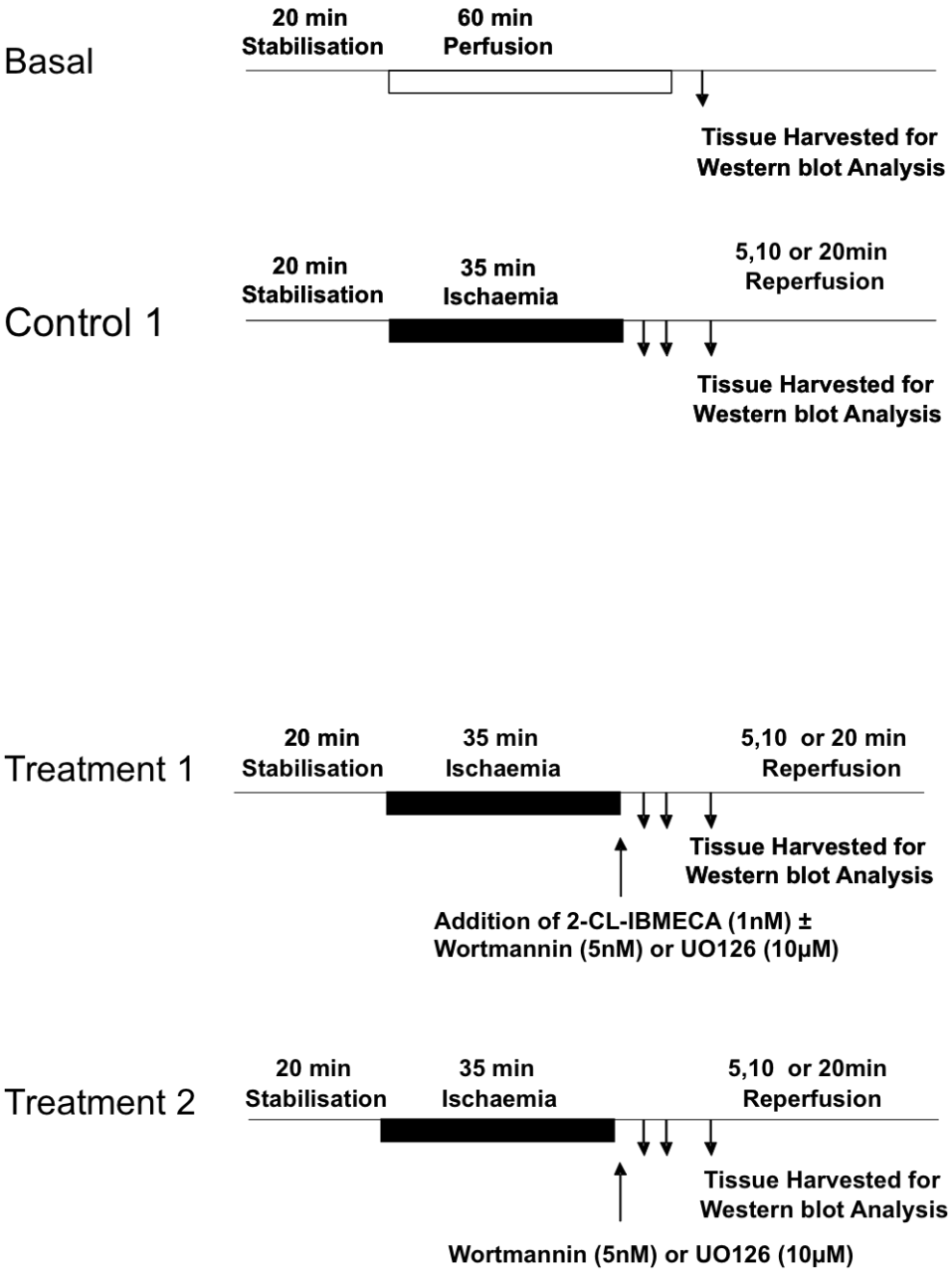
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Western blot Analysis
Treatment 1
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Stabilisation
35 min
Ischaemia
5,10 or 20 min
Reperfusion
Tissue Harvested for
Western blot Analysis
Addition of 2-CL-IBMECA (1nM) ±
Wortmannin (5nM) or UO126 (10μM)
Treatment 2
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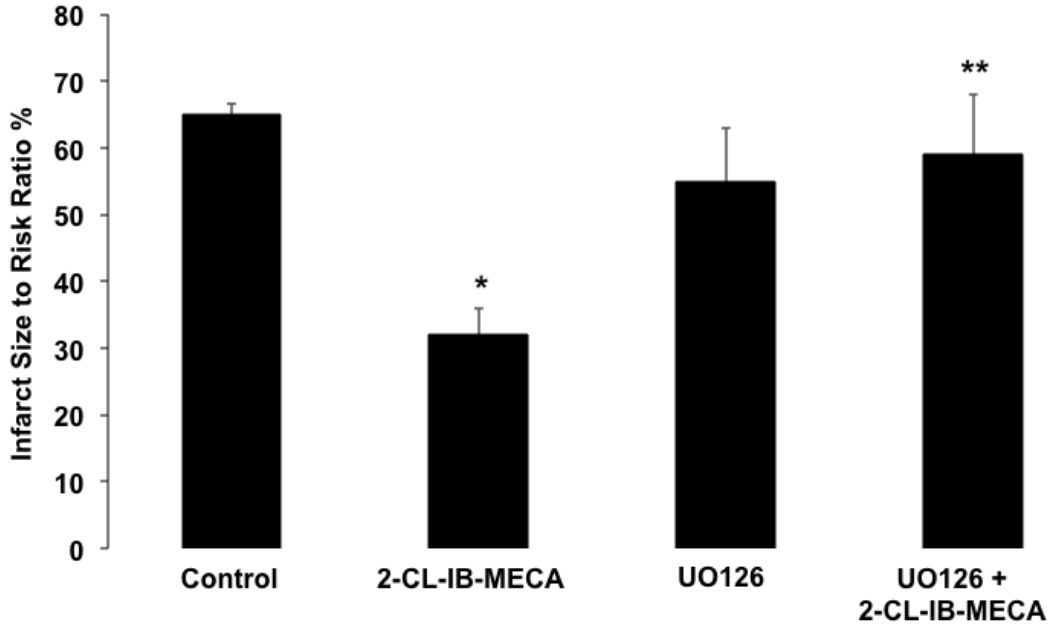
Figure 1b



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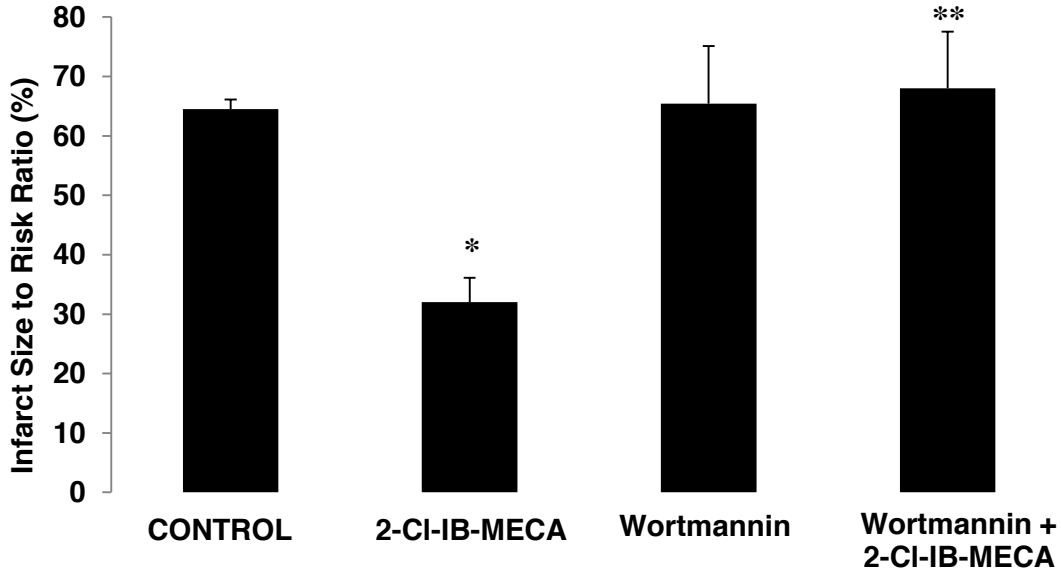

Figure 3

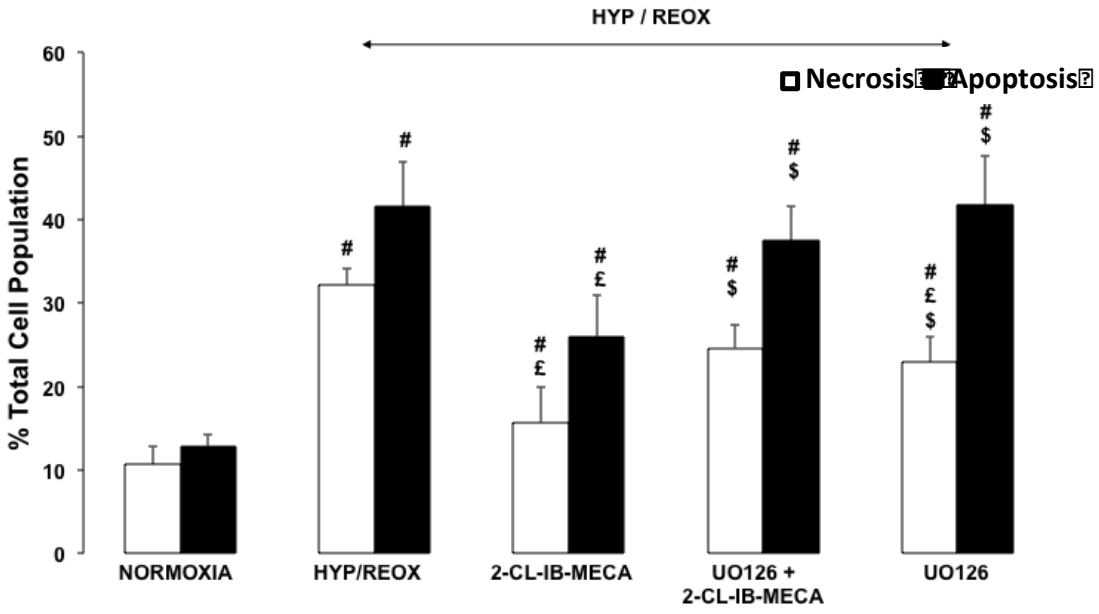

Figure 4

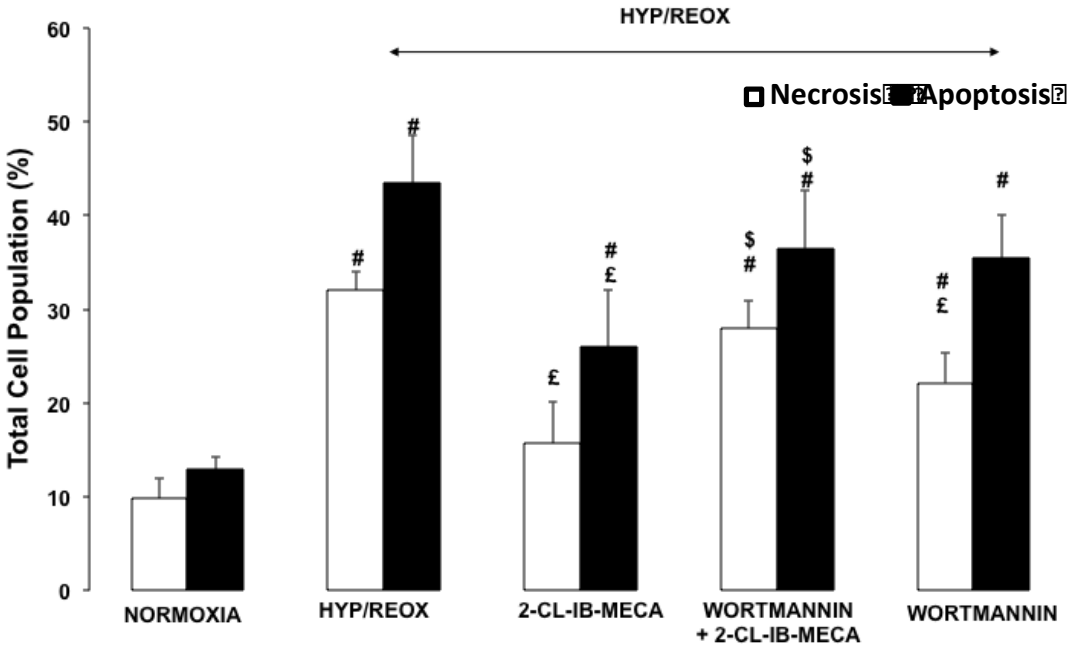

Figure 5

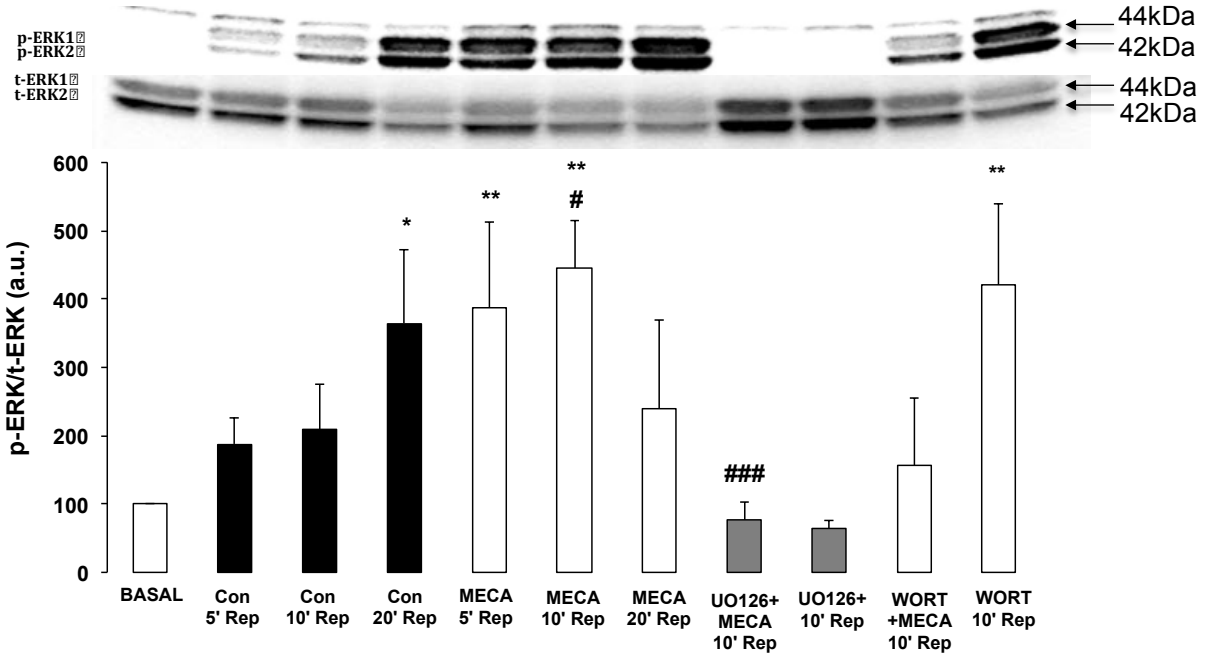

Figure 6

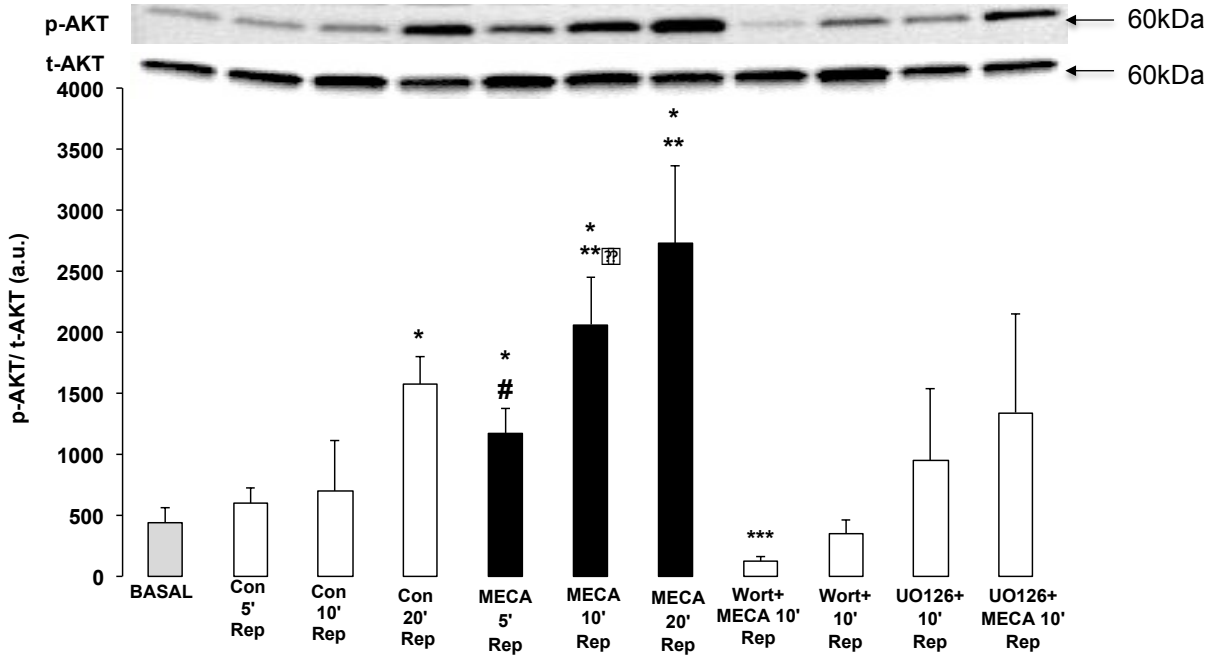

Figure 7

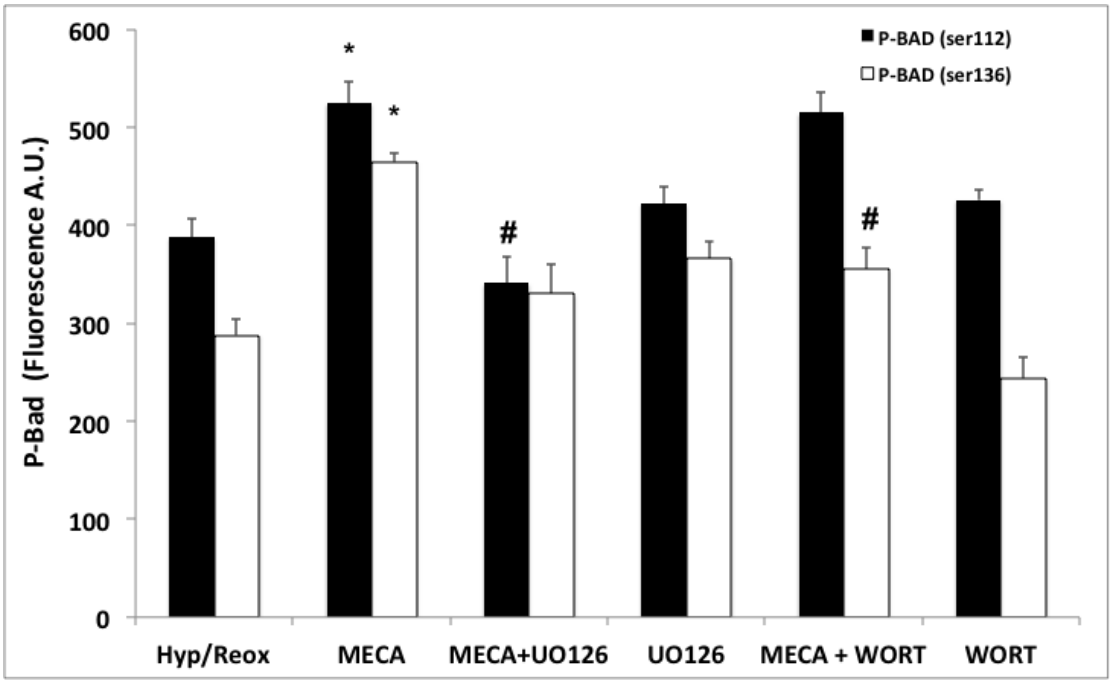

Figure 8

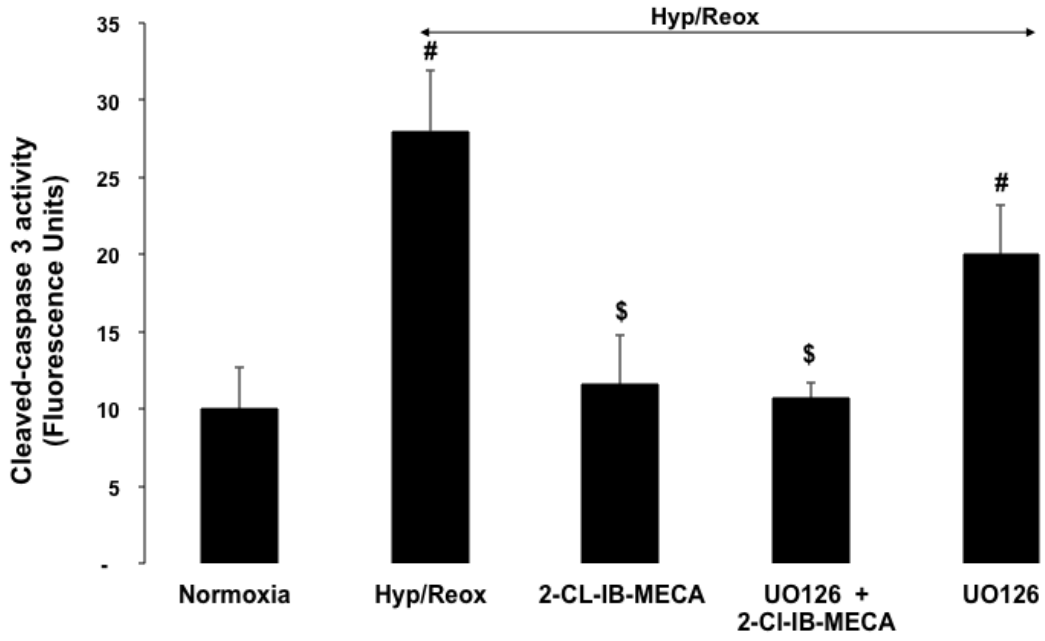

Figure 9

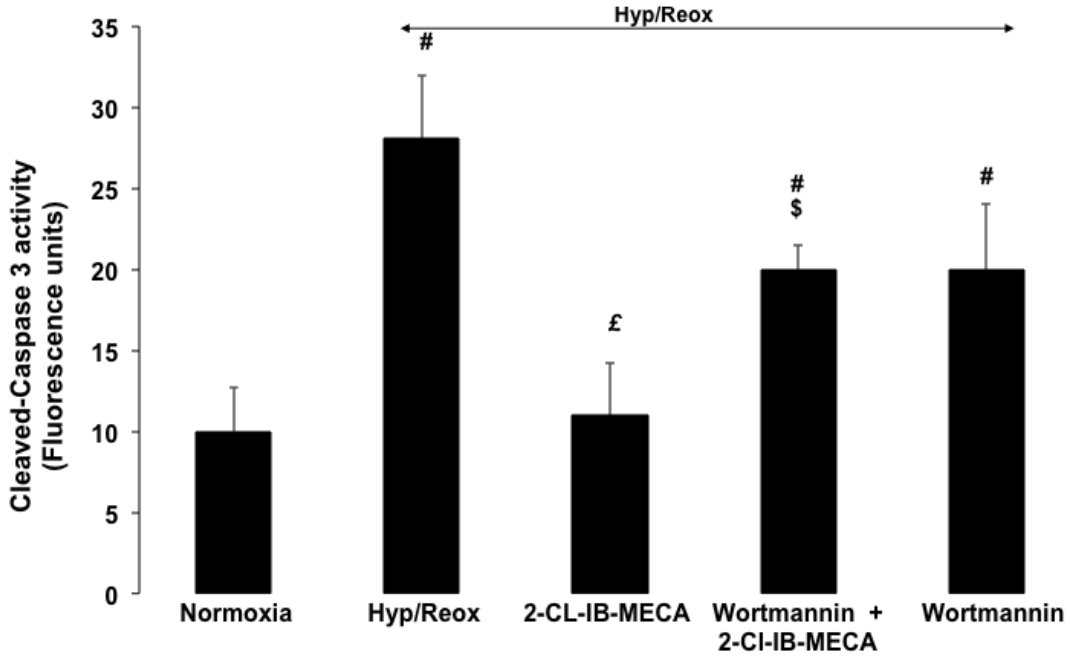

Figure 10

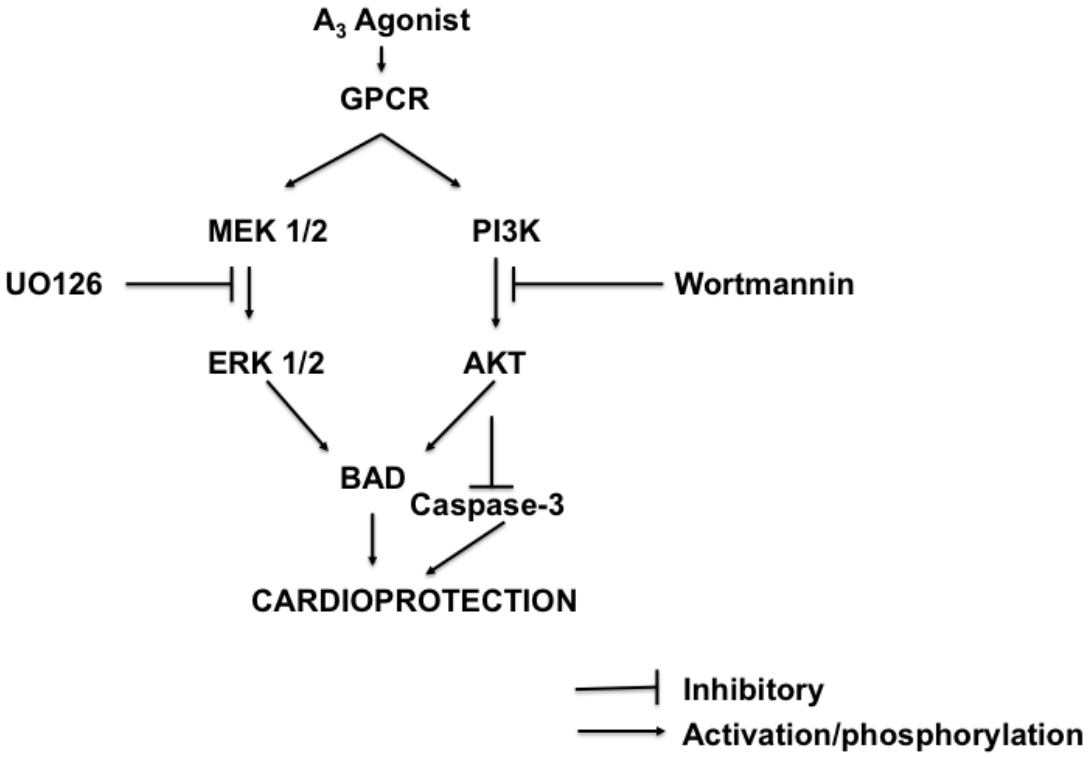

Figure 11

List of Figures

Control 10 10'R/F. ***P<0.001 vs. MECA 10'R/F. ###P<0.01 vs. MECA 10min Reperfusion.

Figure 8. Assessment of p-BAD $_{(Ser\ 112)}$ and p-BAD $_{(ser136)}$ activity in isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation. The A_3 agonist 2-CL-IB-MECA (1 nM) was administered at the onset of reoxygenation in the presence and absence of the MEK1/2 inhibitor UO126 (10 μM) or the PIK inhibitor Wortmannin (5nM). *P<0.05 vs. Hyp/Reox. # P<0.05 vs. M2-CL-IB-MECA. Mean ± SEM of 4 individual experiments.

Figure 9. Cleaved-caspase 3 activity in isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation. The A_3 agonist 2-CL-IB-MECA (1 nM) was administered at the onset of reoxygenation in the presence and absence of the MEK1/2 inhibitor UO126(10 μM). #P<0.001 vs. Normoxia. $P<0.001 vs. Hyp/Reox. Mean ± SEM of 5 individual experiments.

Figure 10. Cleaved-caspase 3 activity in isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation. The A_3 agonist 2-CL-IB-MECA (1 nM) was administered throughout reoxygenation in the presence and absence of the PI3 kinase inhibitor Wortmannin (5 nM). # P<0.001 vs. Normoxia. £ P<0.001 vs. Hyp/Reox. $ P<0.05 vs. 2-CL-IB-MECA. Mean ± SEM of 5 individual experiments.

Figure 11. Shows a hypothetical schematic representation of the cell survival signalling cascades that may be involved in 2-CL-IB-MECA mediated cardioprotection when administered at reperfusion.