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The role of A3 adenosine receptors in protecting the myocardium from ischaemia/reperfusion injury

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The role of A_3 adenosine receptors in protecting the myocardium from ischaemia/reperfusion injury

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2-CL-IB-MECA	A_3 adenosine receptor agonist
8(SPT)	A_1 adenosine receptor antagonist
A_1AR	A_1 adenosine receptor subtype
$A2_aAR$	$A2_a$ adenosine receptor subtype
$A2_bAR$	$A2_b$ adenosine receptor subtype
A_3AR	A_3 adenosine receptor subtype
ADP	Adenosine Di Phosphate
AIF	Apoptosis Inducing Factor
AKT	Cellular AKT
AMP	Adenosine Monophospahte
APAF1	Apoptosis Protease Activating Factor 1
ARC	Apoptosis Repressor caspase Recruitment Domain
ATP	Adenosine Triphosphate
BAD	BCL 2 family pro-apoptotic protein
BAK	BCL 2 family pro-apoptotic protein
BAX	BCL 2 family pro-apoptotic protein
BCL2	B-cell leukemia/lymphoma 2
BCLxl	Bcl-2-like 1 protein
BID	BCL 2 family pro-apoptotic protein
BIM	BCL 2 family pro-apoptotic protein
BSA	Bovine Serum Albumin
CABG	Coronary Artery Bypass Grafting
CASPASE	Cysteine Aspartate Albumin
CCPA	A_1 Adenosine Receptor Agonist
CF	Coronary Flow
DAG	Diacetylglycerol
DED	Death Effector Domain
DNA	Deoxyribosenucleic Acid
DPCPX	A_1 adenosine receptor antagonist
ERK	Extracellular Regulated Kinase
eNOS	Endothelial Nitric Oxide Synthase
FACS	Fluorescent Assisted Cell Sorting
FADD	Fas Associated Death Domain
FASL	FAS Ligand
FLIP	FADD Like Inhibitory Proteins
G-Protein	G-protein regulatory protein
GFR	Growth Factor Receptor
GRB	Growth Factor Receptor Binding Protein
H_2O_2	Hydrogen Peroxide
HRK	BCL 2 family pro-apoptotic protein
IAP	Inhibitor of Apoptosis protein
I-CAM	Cell Adhesion Molecule

IAP 1	Inhibitor of Apoptosis 1
IAP 2	Inhibitor of Apoptosis 2
K_{ATP}	ATP sensitive potassium channel
KO	Knock Out
LVDP	Left Ventricular Developed Pressure
MAPK	Mitogen activated protein kinase
MECA	A3 Agonist
MEK1/2	Mitogen Extracellular Kinase 1/2
mPTP	Mitochondrial permeability transition pore
MRS 1191	A_3 adenosine receptor antagonist
MRS 1523	A_3 adenosine receptor antagonist
mTOR	Mammalian Target of Rapamycin
NECA	Non-Specific Adenosine Receptor Agonist
NF-κb	Nuclear factor κB
NIX	BCL 2 family pro-apoptotic protein
NO	Nitric Oxide
NOXA	Proapoptotic member of Bcl 2 family
P70S6K	70S ribosomal protein
PAF	Platelet Activating Factor
PDK1	phosphoinositide-dependant kinase 1
PKA	Protein Kinase A
PKC	Protein Kinase C
PARP	Poly (ADP ribose) polymerase
PD98059	MEK 1 inhibitor
PI3K	Phosphatidylinositol 3 kinase
PLC	Phospholipase C
PLD	Phospholipase D
PTEN	Phosphatase and tensin homologue
PUMA	p53 upregulated modulator or apoptosis
RAF	MAP kinase kinase kinase
RISK	Reperfusion injury Salvage kinase pathway
ROS	Reactive Oxygen Species
SEM	Standard Error of the MEAN
SER	Serine
SMAC/DIABLO	Second mitochondrial activator of caspases
SOS	Ras Guanine Exchange Factor
TYR	Tyrosine
UO126	MEK1/2 Inhibitor
UV	Ultraviolet
VDAC	Voltage dependant anion channel
WORT	Wortmannin
WT	Wild type
XIAP	Linked inhibitor of apoptosis protein

ABSTRACT

Activation of A_3 adenosine receptors has been shown to protect the myocardium from ischaemia reperfusion injury in a number of animal models. The PI3K - AKT and MEK1/2 - ERK1/2 cell survival pathways have been shown to play a critical role in regulating myocardial ischaemia reperfusion injury. In this study we investigated whether the A_3 adenosine receptor agonist 2-CL-IB-MECA protects the myocardium from ischaemia reperfusion injury, when administered at reperfusion or post reperfusion and whether the protection involved the PI3K – AKT or MEK 1/2 – ERK1/2 cell survival pathways. In the Langendorff model of ischaemia reperfusion injury isolated perfused rat hearts underwent 35 minutes of ischaemia and 120 minutes of reperfusion. Administration of 2-CL-IB-MECA (1nM) at reperfusion significantly decreased infarct size to risk ratio compared to non-treated ischeamic reperfused control hearts. This protection was abolished in the presence of the PI3K inhibitor Wortmannin or MEK1/2 inhibitor UO126. Western blot analysis determined that administration of 2-CL-IB-MECA (1 nM) upregulated ERK1/2 phosphorylation. In the adult rat cardiac myocyte model of hypoxia/reoxygenation cells underwent 6 hours of hypoxia and 18 hours of reoxygenation. Administration of 2-CL-IB-MECA (1 nM) at the onset of reoxygenation significantly decreased cellular apoptosis and necrosis. Administration of 2-CL-IB-MECA (1nM) in the presence of the Wortmannin or UO126 significantly reversed this anti-apoptotic effect and anti-necrotic effect.

Our data further showed that 2-CL-IB-MECA protects myocytes subjected to hypoxia/reoxygenation injury via decreasing cleaved-caspase 3 activity that was abolished in presence of the PI3K inhibitor but not in the presence of the MEK1/2 inhibitor UO126.

Administration of 2-CL-IB-MECA (100nM) at the onset of reperfusion also significantly decreased infarct size to risk ratio in the ischaemic reperfused rat heart compared to controls that was reversed in the presence of Wortmannin or Rapamycin. This protection was associated with an increase in PI3K-AKT / p70S6K / BAD phosphorylation. 2-CL-IB-MECA (100nM) administered at reoxygenation also significantly protected adult rat cardiac myocytes from hypoxia/reoxygenation injury

in an anti-apoptotic and anti-necrotic manner. This anti-apoptotic/necrotic effect of 2-CL-IB-MECA was abolished in the presence Wortmannin. Furthermore, that this protection afforded by 2-CL-IB-MECA (100nM) when administered at reoxygenation was associated with a decrease in cleaved caspase 3 activity that was abolished in the presence of the Wortmannin

Interestingly, postponing the administration of 2-CL-IB-MECA to 15 or 30 minutes after the onset of reperfusion significantly protected the isolated perfused rat heart from ischaemia reperfusion injury in a Wortmannin and UO126 sensitive manner. This protection was associated with an increase in AKT and ERK1/2 phosphorylation.

Administration of the A_3 agonist 2-CL-IB-MECA 15 or 30 minutes after the onset of reoxygenation significantly protected isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation from injury in an anti-apoptotic/necrotic manner. This anti-apoptotic was abolished upon PI3K inhibition with Wortmannin or MEK1/2 inhibition with UO126. The anti-necrotic effect of 2-CL-IB-MECA when administered 15 or 30 minutes post-reperfusion was **not** abolished in the presence of the inhibitors. Delaying the administration of 2-CL-IB-MECA to 15 or 30 minutes after reoxygenation was associated with a decrease in cleaved-caspase 3 activity that was abolished in the presence of Wortmannin but not in the presence of the MEK 1/2inhibitor UO126.

Collectively, we have demonstrated for the first time that administration of 2-CL-IB-MECA at the onset of reperfusion protects the ischaemic reperfused rat myocardium from lethal ischaemia reperfusion injury in a PI3K and MEK1/2 sensitive manner. Delaying the administration of 2-CL-IB-MECA to 15 or 30 minutes after the onset of reperfusion of reoxygenation also significantly protects the isolated perfused rat heart from ischaemia reperfusion injury and the adult rat cardiac myocyte from hypoxia/reoxygenation injury in an anti apoptotic / necrotic manner. Furthermore, that this protection is associated with recruitment of the PI3K-AKT and MEK1/2 – ERK1/2 cell survival pathways.

Chapter 1: Literature Review

1.0. Ischaemic Heart disease

Coronary heart disease is set to be the leading cause for increased morbidity and mortality within the developing world by 2020 (Lopez and Murray. 1997; Goldberg et al., 2004; Kloner and Rezkalla. 2004). Currently, significant primary interventions have evolved against atherosclerosis, hypertension, and hyperlipidaemia all working to limit the onset of coronary artery occlusion and hence injury to the myocardium. Although these interventions are necessary to limit the onset of myocardial infarction there is still an imperative need to develop robust secondary interventions that may be applied after the onset of ischaemia to decrease myocardial injury (Yellon et al., 2000).

From a clinical perspective patients often present to primary care services after the onset of ischaemia (heart attack) and rarely before an ischaemic insult. Research has historically focussed on preconditioning the myocardium with pharmacological agents that are cardioprotective when given before the onset of ischaemia but in a clinical setting these would be no use because it is seldomly possible to predict when someone is going to have a heart attack. Therefore, pharmacological agents administered before an ischaemic episode would rarely be used in a clinical setting. Pharmacological agents being administered during ischaemia and reperfusion would be of more clinical significance whereby agents can be administered to suspected heart attack patients presenting with angina symptoms and / or showing ST elevation on a electrocardiogram although not all heart attack patients show ST elevation. Furthermore, cardioprotective agents could also be used to limit the manifestations of

ischaemia reperfusion injury when administered as an adjunctive therapy during coronary artery bypass grafting or during thrombolytic therapy (Maddock et al., 2002).

1.1.1 Atherosclerosis

Atherosclerosis is a progressive inflammatory disease that affects arterial blood vessels that can manifest into coronary artery occlusion resulting in myocardial infarction. Atherosclerosis occurs as a result of the development of atherosclerotic plaques that protrude into the lumen of the coronary arteries resulting in the obstruction of blood flow (Ross. 1999). Plaque formation is a gradual process that can occur primarily due to a range of insults on the vascular endothelium as well the accumulation white blood cells and lipoproteins. Vascular insults are implicated due to smoking, obesity, hyperlipidaemia, hypertension, diabetes and genetic predisposition (Altman. 2003).

These factors can lead to endothelial damage and dysfunction. Over time the plaque recruits platelets, macrophages, smooth muscle cells, lipids like cholesterol leading to hardening (also known as furring) and narrowing of the arteries leading to the insufficient supply of blood to the myocardium a process referred to as ischaemia. Therefore, understanding the mechanisms involved during a heart attack is imperative and further developing pharmacological therapies to ameliorate myocardial injury remains to be achieved.

1.1.2. Ischaemia

The inability of the myocardium to meet oxygen and nutrient supply with respect to demand is referred to as myocardial ischaemia (Asano et al., 2003). At rest residual flow may be sufficient to the demand but upon exertion the demand for nutrient rich blood may not be met. As the myocardium is unable to meet oxygen demand the ischaemic tissue reverts from aerobic to anaerobic respiration as a means of generating energy. This process involves the conversion of glucose to lactic acid to yield energy leading to lactic acid accumulation in the ischaemic zone (Solani and Harris. 2005). Sustained ischaemia can have deleterious consequences leading to irreversible damage to myocytes.

Prolonged ischaemia has profound effects on the intracellular milieu including: morphological changes - cell swelling (Lichtig and Brooks. 1974), disruption of oxidative phosphorylation, adenosine tri-phosphate (ATP) depletion, collapse of the ATP dependant Na^+/K^+ and Ca^{2+} pumps, decrease in intracellular pH, reversible and irreversible myocyte damage (Graham et al., 2004), development of the calcium and oxygen paradox (Braunwald and Kloner. 1985) and free radical mediated injury (Wall. 2000).

Soon after the onset of myocardial ischaemia, ATP levels normally maintained by mitochondria quickly become depleted in the ischaemic region having profound effects on intracellular moieties (Kang et al., 2000). For instance, ATP is required by ion pumps like the Na^+/K^+-ATPase pump to maintain intracellular ion concentrations. Consequently, ATP depletion leads to a failure of ATP dependant ion pumps leading to an influx of Na^+ and Ca^{2+} ions due to the Na^+/Ca^+ exchanger (NCE) operating in a

reverse mode. This increase in Ca^{2+} ions leads to calcium overload (Inserte et al., 2004).

ATP is rapidly degraded to adenosine monophosphate (AMP) and adenosine during ischaemia (Jeroudi et al., 1994). Adenosine is further degraded leading to the formation of free radicals (Figure.1.1). Free radicals released by neutrophils have also been shown to further hasten myocardial injury (discussed later).

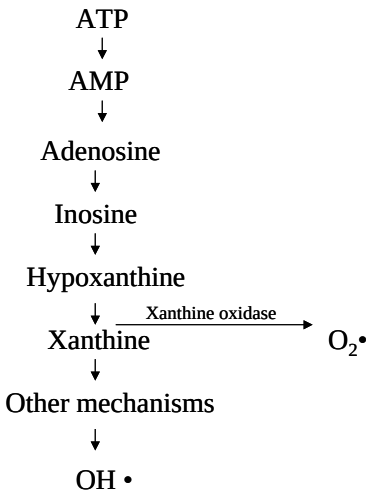

Figure 1.1 Shows the mechanisms involved in ATP degradation and superoxide radical O_2• and OH • radical generation.

Prolonged myocardial ischaemia leads to myocardial injury where the cardiac myocytes are non-reversibly (myocardial necrosis) damaged, referred to as myocardial infarction (Braunwald and Kloner. 1985). Myocardial ischaemia is reversible by thrombolytic therapy, angioplasty or CABG. The restoration of myocardial blood flow is referred to as reperfusion.

1.1.3. Reperfusion

A number of techniques have been developed to reperfuse the previously ischaemic myocardium including thrombolysis, percutaneous transluminol coronary angioplasty and coronary artery bypass graft (CABG). Reperfusion although crucial to salvaging reversibly damaged myocytes paradoxically has been shown to have deleterious consequences and therefore has been referred to as lethal reperfusion injury (Van der Vusse et al., 1985; Yellon et al., 2000). Reperfusion has been referred to as a double-edged sword in the context where without reperfusion, tissue salvage cannot take place but with reperfusion paradoxically hastening the cellular injury process referred to as lethal reperfusion injury (Braunwald and Kloner. 1985; Zhao et al., 2002).

Lethal reperfusion injury can lead to the development of reperfusion-induced arrhythmias, enzyme release, myocardial stunning, reversible myocardial stunning, reversible and irreversible cell damage (Bolli et al., 1999; Yellon et al., 2000). The mechanisms underlying lethal reperfusion injury remain to be fully elucidated but a number of proposed contributors have been put forward.

Reperfusion injury by removal of coronary artery occlusion results in a number of physiological events including development of: oxygen derived free radicals, no reflow phenomenon, cell swelling, neutrophil recruitment, calcium overload, apoptosis, necrosis, myocardial haemorrhage that could possibly contribute to reperfusion injury, these shall be discussed in turn.

1.1.4. Oxygen derived free radicals

The re-introduction of oxygenated blood to the ischaemic myocardium leads to the generation of injurious oxygen derived free radicals (Bolli et al., 1989) (Figure 2). Free radicals exist within cells and are generally generated by mitochondria. These are quickly catalysed by free radical scavengers like superoxide dismutase and glutathionine peroxidase (Van der Vuess et al., 1985, Forde et al., 1997). In the non-ischaemic myocardium endogenous free radical scavengers exist and via their antioxidant abilities limit free radical dependant injury. During ischaemia/reperfusion these antioxidant enzymes become overpowered and unable to limit free radical dependant cellular injury. Initially proposed by Hearse et al., (1973) free radical levels were quantified by Zweier et al., (1987) who showed free radical levels to peak during myocardial reperfusion. Reoxygenation of the ischaemic myocardium results in the development of oxygen derived free radical like superoxide anion (O_2-) and the hydroxyl radical. Other potential sources of free radical generation include neutrophils and the displacement of electrons from mitochondria. These radicals can interact with superoxide dismutase to form hydrogen peroxide H_2O_2, but H_2O_2 can interact with O_2- and generate highly reactive hydroxyl free radicals.

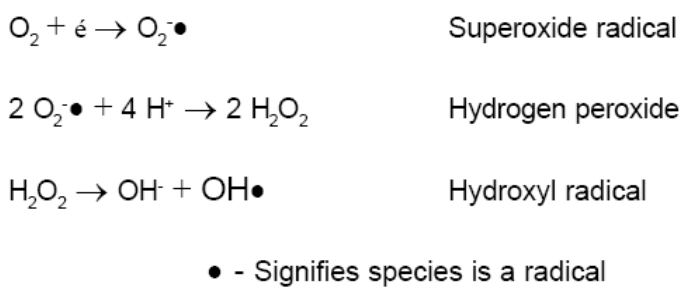

Figure 1.2. Oxygen derived free radical generation (adapted from Wall. 2000).

Although free radical generation is seen in the ischaemic myocardium due to ATP degradation, at reperfusion the source of free radical generation is oxygen and nitric oxide (NO). In a number of studies free radicals have been shown to contribute to reperfusion injury (Van den Hoek et al., 1996; Simpson et al., 1987; Zweier et al., 1988). Studies have shown that reoxygenation leads to a highly significant increase in oxygen derived free radicals and mediate functional injury to the myocardium as well as impaired contractile function in the isolated heart (Zweier. 1988; Bolli et al., 1989). Zweier (1988) measured superoxide derived free radicals in the reperfused heart concluding that free radical concentrations peaked within the first minute of reperfusion, and that administration of recombinant superoxide dismutase improved post ischaemic contractile function as well as decreasing free radical concentrations in the rabbit.

These radicals can attack numerous targets ranging from the endothelium, smooth muscle and damage DNA leading to apoptosis and disruption of mitochondrial ATP synthesis. Free radicals have also been shown to generate lipid peroxides that can lead to disruption of membrane integrity, inhibition of membrane enzymes and structural changes (Forde et al., 1999). Jolly et al., (1984) have shown that the administration of the antioxidants superoxide dismutase and catalase decrease free radical dependant injury in the ischaemic reperfused myocardium in the dog. Studies have shown the beneficial effects of using free radical scavengers in reducing reperfusion injury in the canine model of hypothermic global ischaemia (Stewart et al., 1983).

Interestingly, Bognar et al., (2006) have shown HO-3538, a modified superoxide dismutase and mitochondrial permeability transition inhibitor to significantly decrease

the release of reactive oxygen species in the micro-environment. They also show HO-3538 to limit the opening of the mitochondrial permeability transition pore thereby preventing the release of mitochondrial pro-apoptotic proteins like cytochrome c. They also showed in the ischaemic reperfused rat heart that the administration of HO-3538 significantly decreased the degree of myocardial infarction. To date no study has shown antioxidants to mediate cardioprotection in the human heart.

ROS have also been shown to damage the vascular endothelium by modifying proteins and membrane lipids as well as affecting the function of endothelial cells and their ability to vasodilate in response to endothelial derived relaxation factor and NO (Gin et al., 2007).

ROS generation although viewed as injurious has now been shown to play a role in protecting the ischaemic myocardium. Ischaemic preconditioning (IP) has been shown to be a trigger for ROS generation, whereby brief periods of ischaemia and reperfusion (IP) before index ischaemia reduced ROS generation compared to the control group (Kevin et al., 2005). ROS generation may have profound deleterious consequences within the myocardium; IP may activate cardioprotective mechanisms and limit injury (Kevin et al., 2005).

A range of publications support the role of free radicals in ischaemia reperfusion injury and further the cardioprotective role of free radical scavengers in limiting myocardial injury. Despite the ability of free radical scavengers to limit myocardial injury in animal models they still need to be translated into human clinical models.

1.1.5. Myocardial Stunning

The stunned myocardium has been defined as reversible post-ischaemic myocardial contractile dysfunction that is reversible over-time providing perfusion is not hindered (Bolli, 1990). There are two main theories which have been proposed to explain myocardial stunning. The first theory suggests that injury is mediated by free radicals and the second, that the stunning is mediated by calcium overload, both acting to disrupt cell contractility.

1.1.6. No-Reflow Phenomenon

The no-reflow phenomenon is described as the lack of coronary flow to previously ischaemic tissue upon reperfusion. No-reflow is thought to occur as a result of microvascular damage like endothelial swelling, plugging of capillaries, haemorrhage, microvascular compression due to swelling and contracture of myocytes (Braunwald and Kloner 1985; Rezkalla and Kloner. 2002).

1.1.7. Calcium Overload

The re-introduction of blood into the myocardium at reperfusion has been shown to lead to calcium overload. Calcium in the perfusate has been shown to lead to the development of cellular contracture (Vaselle. 2004). Calcium overload may also contribute to the development of free radicals adding to the injury process. Elevated levels of calcium have also been shown to activate proteases and phospholipases that can mediate damage of the sarcolemma (Barry. 1987). Reperfusion mediated calcium overload had also been shown to lead to the development of hypercontracture where myofibrils are considerably shortened leading to cytoskeletal damage. Hypercontracture has been shown to be the preliminary cause of cardiomyocyte

necrosis and is referred to as contraction band necrosis (Piper et al., 2003). Shine et al., (1983) have shown that reperfusion with reduced calcium in the perfusate to ameliorate the effects of calcium overload which supports further a role of calcium in ischaemia reperfusion injury.

Ankarcrona et al. (1995) showed neurones subjected to extreme calcium overload, results in neuronal necrosis whereas lower levels of calcium overload results in neuronal apoptosis. During myocardial ischaemia ATP levels are depleted and the levels of inorganic phosphate and cytosolic Ca^{2+} rise significantly (Duchen. 2000).

1.2.0. Cell Death

Cell death is part of the body's innate mechanism of removing cells no longer required whether in response to injury or cells that are senescent. Cell death has been shown to be divided into two separate distinct pathways referred to as necrosis and apoptosis. Necrosis is an ATP-independant process occurring in response to cellular injury (Braunwald and Kloner. 1985; Piper and Gracia-Dorado. 1999). Cellular necrosis can occur in response to cellular stressors like myocardial ischaemia, reperfusion, heat and infection. These stressors can lead to either cellular necrosis or apoptosis depending on the level of injury and the environmental conditions.

1.2.1. a. Necrosis

Cellular necrosis results in swelling of organelles like mitochondria leading to the release of cytochrome c and further leading to the rupture of the cell membrane releasing the intracellular content into the extra cellular space (Braunwald and Kloner, 1985). Historically necrosis has been viewed as a passive accidental process but

recent studies have suggested that cellular necrosis is an active form of cell death that is regulated (Henriquez et al., 2008).

1.2.1.b. Apoptosis

Apoptosis also known as programmed cell death has been the focus of much attention in past decade. Apoptosis is a highly ordered sequence of events leading to cell death. Characteristic of apoptotic cell death is chromatin condensation, nucleosomal ladders of DNA fragments (200 base pairs), cellular shrinkage, endonucleolytic digestion of cellular DNA but not mitochondrial DNA and is an energy-dependant process (Gublins et al., 2000; Gottlieb et al., 1994).

Apoptotic cell death is initiated in response to many different stimuli like ROS, hydrogen peroxide, heat shock, specific receptor molecules like tumour necrosis factor receptor and necrosis. Apoptosis is inhibited by endogenous proteins like apoptosis repressor with caspase recruitment domain (ARC), and inhibitor of apoptosis (IAP) and FADD (Fas-Associated Death Domain) - FADD like inhibitory proteins (FLIP) (Gustafsson et al., 2004; Stephanou et al., 2001) . These proteins have been shown to bind and inactivate caspases limiting apoptotic cell death.

Apoptosis has been shown in numerous studies to play an active role in ischaemia reperfusion injury, although there has been significant controversy as to whether apoptosis is activated during ischaemia, reperfusion or both (Gottlieb et al., 1994; Bailik et al., 1999). Studies have shown apoptosis to be active after the onset of index ischaemia and to be accelerated during reperfusion (Chakrabarti et al., 1997; Black et al., 1998; Borutaite et al., 2003). Gottlieb et al., (1994) have shown cardiomyocytes

to die by apoptosis only after ischaemia followed by reperfusion and that apoptosis did not occur during a period of sustained ischaemia. Controversy still remains as to whether apoptosis occurs at ischaemia or reperfusion, but it is feasible that apoptosis occurs at reperfusion because during ischaemia there is a period of depletion of high-energy phosphates like ATP as well as nutrients like glucose and oxygen therefore the reintroduction of coronary blood flow (reperfusion) consisting of glucose, essential amino acids and oxygen may initiate apoptosis.

There are several key players in apoptosis including caspases, mitochondria, and the sarcoplasmic reticulum (Borutaite et al., 2003). Caspases are cytokine-dependant proteases that have been shown to cleave structural proteins in the nucleus and cytoplasm (Nicholson et al., 1997) initiating the apoptotic process. Caspases become active by the binding of specific ligands to their respective receptors triggering apoptosis.

There are two main pathways that regulate apoptosis that are referred to as the intrinsic and extrinsic pathways. The death receptor pathway (extrinsic) involves the activation of death receptor located on the extracellular surface of the cell by apoptotic stimuli. The death receptors are closely related to the tumour necrosis factor gene super family and play divergent regulatory roles apart from regulating apoptosis. Activation of the death receptor can result in the initiation of the apoptosis processes resulting in receptor association with the adaptor protein FADD (Fas associated death domain). FADD has a death effector domain (DED) that associates with pro-caspase 8. The formation of the FADD-pro-caspase 8 complex results in the immediate cleavage of pro-caspase 8 to active caspase 8. Activated caspase 8 can bind to pro-

caspase 3 resulting in activation of caspase-3 and execution of cellular apoptosis. Caspase 3 results in cleavage of various death substrates leading to a range of biochemical and morphological changes characteristic of apoptosis (Figure.1.3).

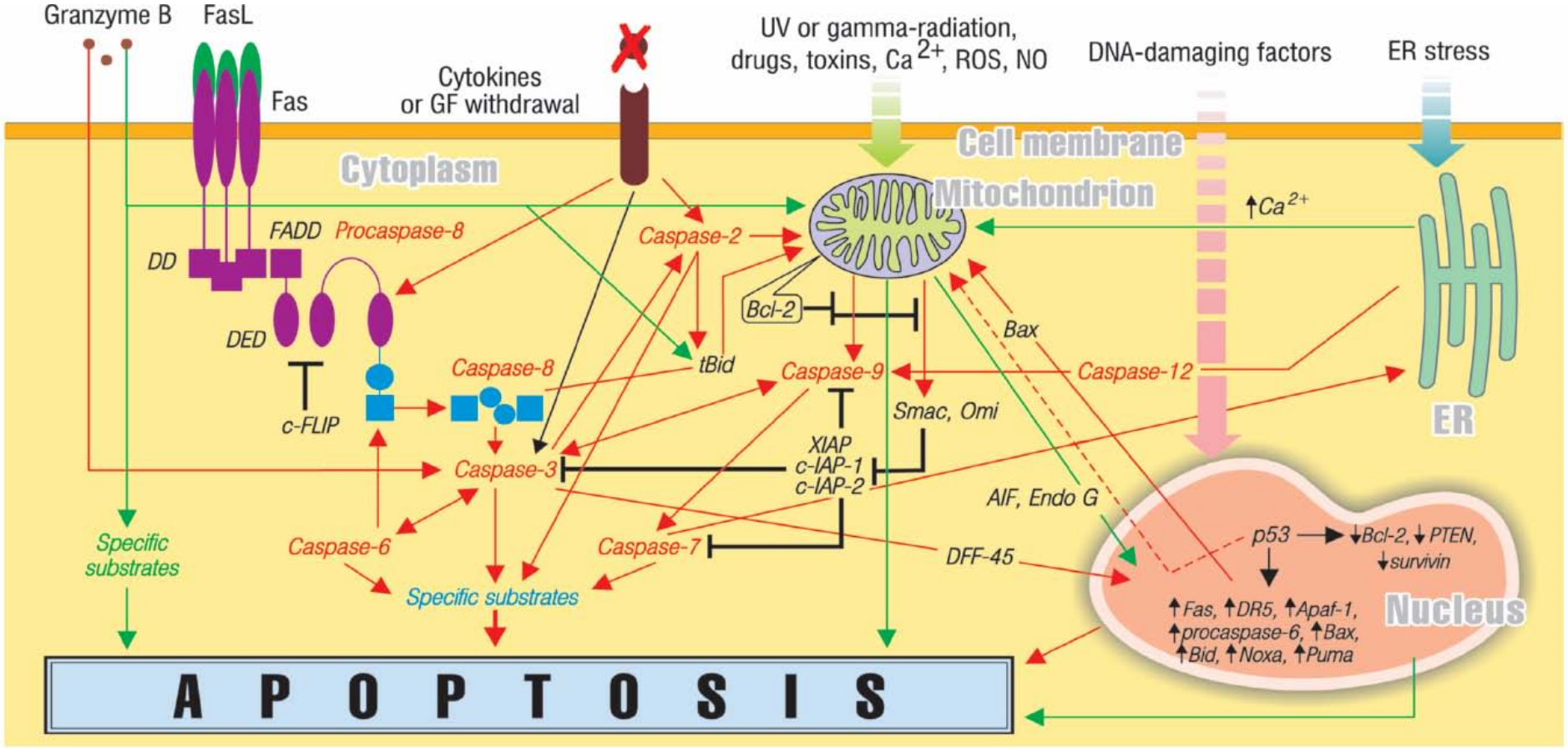

Figure 1.3. Schematic representation of Caspase dependent (in red) and independent (in green) mechanisms of apoptosis

The intrinsic death pathway is regulated by the mitochondrial activity in response to GPCR's stimulated in response to environmental stress like hypoxia, oxidants, nutrient deprivation and DNA damage leading to the release of cytochrome c and intra-mitochondrial proteins like apoptosis inducing factor (AIF), second mitochondria-derived activator of caspases (SMAC/DIABLO) and endonuclease G (Borutaite et al., 2003; Regula et al., 2004). These factors act on effector caspases like caspase 3 and execute the apoptotic cell death machinery.

The intrinsic death pathway also involves the Bcl-2 family of proteins that can promote or suppress apoptosis. Members of the Bcl-2 family that promote apoptosis include Bax, Bak, Bad, Bim, Nix and Hrk and members that inhibit apoptosis are Bcl-2 and $Bclx_L$ (Korsmeyer. 1993; Yin et al., 1994; Regula et al., 2004). Proteins like Bak and Bax are required to make the mitochondrial membrane permeable to release molecules like cytochrome c, SMAC and AIF (Cheng at al., 2003).

Several studies have shown caspase 3 to mediate a pivotal role in the regulation of cellular apoptosis. In the cerebral model of ischaemia reperfusion injury Namura et al., (1998) have shown increased levels of cleaved (activated) caspase 3 following reperfusion with levels peaking between 30 – 60 minutes of reperfusion. Black and colleagues (1998) showed by immunohistochemical staining elevated levels of caspase 3 in the risk region in the left ventricle of the ischaemic reperfused rat heart.

Cytochrome c has been implicated in many studies as an initiator of apoptosis in the ischaemic reperfused heart (Honda et al., 2005). Cytochrome c has been shown to disassociate from mitochondria in response to cellular stresses like ROS and calcium

overload. Cytochrome c released into the cytoplasm in response to the cell death signals has been shown to activate apoptosis by binding to Apaf-1 (apoptotic protease activating factor 1) that binds to ATP and caspase 9 and undergoes apoptosome formation (Lundberg and Szweda. 2004). The apoptosome becomes the caspase activation complex that can activate caspase 3 as an end effector of the intrinsic pathway (Gottlieb et al., 1994; Bernardi et al., 1999).

Cytochrome c is released by mitochondria by two mechanisms 1) pore formation (Gross et al., 1998; Haunstetter and Izumo. 1998; Antonsson et al., 2000) and 2) through the mitochondrial permeability transition pore (MPTP) (Halestrap et al., 2004; Hausenloy et al., 2002; 2004). During cellular stress cellular Bcl-2 homologues Bid, Bak and Bax have been shown to oligomerise and form a pore in the outer mitochondrial membrane. Bid has been shown to translocate to mitochondria and release cytochrome c.

The MPTP is formed between the inner and the outer mitochondrial membrane and has been shown to be composed of three main subunits, cyclophilin D, voltage dependant anion channel (VDAC) and adenine nucleotide (Halestrap et al., 2004). The MPTP has also been implicated in cytochrome c release. Under normal conditions the MPTP remains closed and mitochondrial permeability transition is thought to occur in response to cellular stresses like calcium overload and oxidative stress.

Investigations by Weiss et al., (2003) and Halestrap et al., (2004) have shown mitochondrial permeability transition to occur at reperfusion, where the MPTP is

primed during ischaemia. Opening of the MPTP results in MPT (mitochondrial permeability transition) leading to subsequent matrix swelling, depression of the membrane potential and initiation of cell death via cytochrome c release.

Research over the past decade has shown that apoptosis is a key player in the development of myocardial infarction in the ischaemic reperfused myocardium. Therefore, it is crucial that we understand the potential mechanisms that activate or inactive cellular apoptosis. Agents like caspase inhibitors have been shown to protect the ischaemic reperfused myocardium from reperfusion injury when administered at the onset of reperfusion (Mocanu et al., 2000).

Despite recent advances in our understanding of the mechanisms involved in the cell death our understanding is still unclear. Therefore, it seems feasible to further research the cell death machinery to understand the potential mechanisms involved and to identify potential targets to promote cell survival.

1.3.0 Adenosine Receptors

To date four adenosine receptors have been identified and cloned and characterised as A_1, $A2_a$, $A2_b$ and A_3 (Tucker and Linden. 1993; Fredholm et al., 2001). Adenosine receptors have been shown to be expressed within the cardiovascular system including the coronary artery, pulmonary artery, aorta and smooth muscle cells, mast cells and more importantly cardiac myocytes (Auchampach and Bolli. 1999). Adenosine receptors belong to the superfamily of G protein coupled receptors (GPCR) exerting a dynamic range of responses and have been classified upon their stimulatory or inhibitory actions on adenylate cyclase and on selectivity of agonists

and antagonists (Fredholm et al., 2001; Mubagwa and Fleming. 2001; Klinger et al., 2002).

Adenosine A_1 receptors have been shown to be coupled to pertussis toxin sensitive G_i and G_o proteins, inhibiting adenylate cyclase activity, activating phospholipase C and opening K_{ATP} channels (Ababe and Mustafa. 1993; Auchampach and Gross. 1993; Fredholm et al., 2000; Germack and Dickenson. 2004). Adenosine A_2 receptors have been shown to couple to G_s proteins, stimulating adenylate cyclase (Germack and Dickenson. 2004). Adenosine A_3 receptor (A_3AR) inhibits adenylate cyclase similarly to the A_1 receptors. A_3AR also couple to G_i and G_o proteins and possibly G_q as characterised by Palmer et al., (1995). A_3AR activation has been implicated to activate phospholipase C and D (Abbracchio et al., 1995; Germack and Dickenson. 2004; Headrick and Peart.2005) See Figure 1.4.a.

Figure 1.4.a Cell signalling pathways involved in A_3AR mediated cardioprotection. (Headrick and Peart. 2005)

1.3.1. Adenosine

Adenosine released from cells interacts with sarcolemmal membrane adenosine receptors. Having a short half-life, adenosine exerts its physiological effects in an autocoid manner. The purine nucleoside adenosine has long been implicated to play a regulatory role within the heart. Adenosine has been shown to exist within all tissues of the mammalian body were it has been shown to regulate key physiological processes like regulating heart rate and vasculature tone (Tabrizchi and Bedi. 2001). Although the key role of adenosine is to undergo phosphorylation by adenosine kinase

to form adenosine mono phosphate (AMP) and further phosphorylation to generate adenosine tri-phosphate (ATP) the universal energy molecule (Figure 1.4.a) (Mullane and Bullough. 1995). Adenosine has also been shown to exert other effects independent of energy regulatory processes including upregulation cell growth and differentiation by activating cellular signalling pathways (Mubagwa and Flameng. 2001; Schulte and Fredholm. 2003; Fitz. 2007). Other effects of adenosine include the regulation of heart rate, hypotension, coronary blood flow, bronchoconstriction and mast cell degranulation (Fredholm et al.,2000; 2005)

Adenosine receptors are widely expressed but expression can be species dependant (Liden at el., 1993; Salvatore et al., 1993; Dixon et al., 1996). In the rat adenosine A_1 receptor has been shown to widely expressed in the brain, heart, aorta, liver, kidney, eye and bladder (Dixon et al., 1996). The same group have shown A_3AR to be widely expressed in the heart, central nervous system, lung uterus and testis. A_{2A} were also shown to be expressed in the lung, brain and uterus although the A_{2B} mRNA was also expressed in the jejunum and colon.

During conditions of cellular stress adenosine levels rise significantly above basal levels and have been shown to play a pivotal role in terms of improving tolerance to ischaemia during periods of ischaemic injury by limiting necrosis and apoptosis (Van Wylen. 1994; Vinten-Johanson et al., 1995). The key processes involved in the formation and metabolism of adenosine are summarised in figure 1.4.b. Deussen et al., (1999) have shown elevated levels of adenosine in hearts perfused with lower levels of oxygen, supporting adenosine elevation during periods of hypoxia.

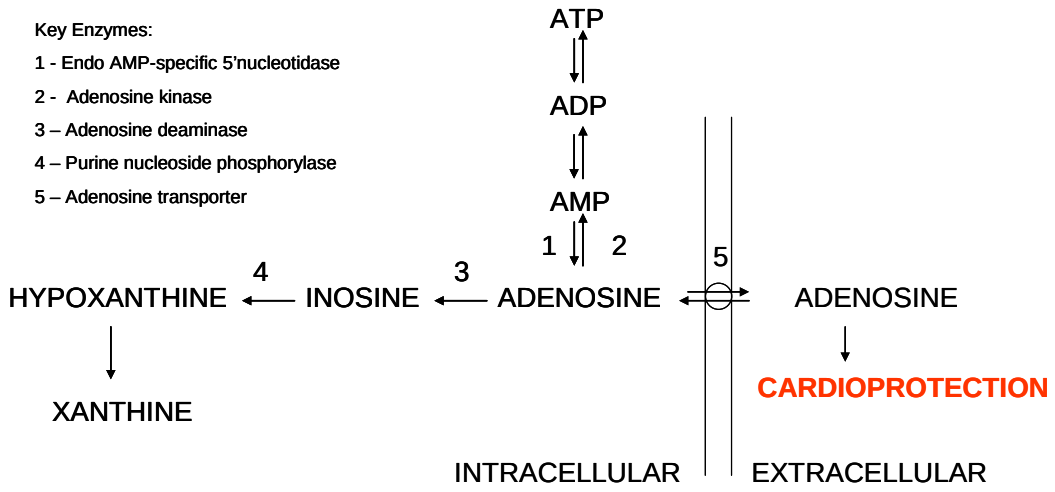

Figure 1.4.b. Shows the pathways involved in the formation and metabolism of adenosine (Adapted from Mullane and Bullough. 1995).

Adenosine plays a direct role in the cardiovascular system regulating blood flow through the vascular system via activating adenosine receptors. The assessment of individual receptor effect on the cardiovascular system was determined by using receptor specific agonists. Activation of A_1 adenosine receptors with the A_1AR agonist cyclopentyladenosine (CPA) has been shown to decrease blood pressure and cardiac output after systemic administration in hypertensive rats (Webb et al., 1990; Monopoli et al., 1998). Activation of A_1 adenosine receptors is generally associated with the regulation of heart rate where the administration of A_1AR agonists decreases heart rate. Hoffman et al., (1997) showed the administration of the A_1AR agonist N6-cyclopentyladenosine to significantly decrease heart rate in mammalian embryos. This group also showed A_{2a} and A_{2b} adenosine receptor agonists to have no effect on heart rates. Furthermore, they showed that the activation of A_3ARs mildly decreased heart rate in mammalian embryos. A_{2a} and A_{2b} adenosine receptor activation has been shown to mediate coronary artery blood flow regulating vasodilation (Trochu et al., 2003; Hinschen et al., 2003).

Activation of A_3 receptors with different A_3 adenosine receptor agonists has been shown to cause different haemodynamic effects in different species. Lasley et al., (1999) reported IB-MECA and CL-IB-MECA to have no effect on cardiac function in the rat and rabbit and both where seen to have no effect on coronary flow in the rabbit. IB-MECA was seen to increase coronary flow in the rat heart. This increase in coronary flow was completely blocked by the A_{2a} antagonist Sch-58261 suggesting IB-MECA-mediated increases in coronary flow were mediated by activation of A_{2a} adenosine receptors. 2-CL-IB-MECA at concentrations below 50nM was seen to have no effect on coronary flow in the rat heart, but when the concentration was increased to 100 nM coronary flow increased by 18%. Maddock et al., (2002) have also reported 2-CL-IB-MECA to cause vasodilation and increase coronary flow at the 100 nM concentration although at lower concentrations were seen to have little effect on coronary flow.

1.4.1. Ischaemic Preconditioning

Ischaemic preconditioning (I/P) is a non-pharmacological mechanistic approach defined as brief cycles of ischaemia reperfusion before the index of index ischaemia that has been shown to reduce myocardial ischaemia/reperfusion injury (Murray et al., 1986). This group showed that four cycles of 5 minutes ischaemia before 40 minutes index ischaemia significantly reduced infarct size in the canine model of ischaemia reperfusion injury. Subsequently, ischaemic preconditioning has been shown in a number of models to confer cardioprotection via reduction of infarct size in the swine (Schott et al., 1990), and rabbit (Liu et al., 1991).

Studies by Thourani et al., 1999 showed that ischaemic preconditioning resulted in preservation of post ischaemic endothelial function as well as a decrease in neutrophil medicated injury. IP has also been associated with reducing the energy demands during an ischaemic insult as well as reduced calcium overload (Jennings et al., 2001). Attenuation of neutrophil dependant injury and apoptosis by ischaemic preconditioning was also seen by Nakamura and colleagues (2000) in the ischaemic reperfused rat heart.

Ischaemic preconditioning is thought to protect against the ischaemic reperfused myocardium via endogenous preformed agents released locally like adenosine, bradykinin, opioids and catecholamines that act on cell surface G protein coupled receptors (Cohen at al., 2000). Activation of these receptors has been shown to activate intracellular signalling pathways promoting cell survival and inhibiting apoptosis. The exact signalling pathways via which ischaemic preconditioning confers protection remain elusive although research has implicated protein kinase C and tyrosine kinase to be key mediatory kinases (Ytrehus et al., 1994; Vahlhaus et al., 1998). Yellon and colleagues (2002; 2003; 2004) have recently shown IP to confer myocardial protection from ischaemia reperfusion injury via recruitment of the PI3K-AKT and the MEK1/2 – ERK1/2 cell survival pathways where inhibition of these pathways abolished IP dependant cardioprotection. Interestingly Solenkova et al (2006) have shown ischaemic preconditioning to protect the heart via the release of endogenous adenosine in rabbit ischaemic reperfused heart.

Further research after the discovery of IP and its ability to protect the ischaemic heart led to the discovery of a second window of protection that exists between 24-96 hours

after the initial preconditioning stimulus (Marber et al., 1993). Although the signalling pathways involved in delayed preconditioning are different to IP and involve protein kinase C, nitric oxide and cyclooxygenase 2 (Bolli. 2000; Guo et al., 2000).

1.4.2. Pharmacological Preconditioning

Administration of pharmacological agents before index ischaemia has also been shown to induce cardioprotection a phenomenon referred to as pharmacological preconditioning. (Yellon and Downey. 2003). Acetylcholine and opioid receptor agonists have been shown to trigger a preconditioned like cardioprotective effect when administered prior to myocardial ischaemia and reperfusion. Downey and colleagues (2006) have shown administration of acetylcholine 30 minutes before ischaemia significantly reduced myocardial infarction in isolated rabbit hearts. In the same study they showed the protection involved recruitment of MEK1/2 – ERK 1/2 and PI3K – AKT cell survival pathway.

Lasley et al., (1995) have shown pharmacological preconditioning with adenosine and adenosine analogues improved post ischaemic ventricular dysfunction where this protection was lost by co-administration of A_1AR antagonist in the rat and rabbit. Pharmacological preconditioning with A_3AR agonists has been shown mediate cardioprotection in the rat (DeJonge et al., 2002; Hochhauser et al., 2007), mice (Zhao and Kukreja. 2002) and rabbit (Kodani et al., 2001; Tracey et al., 1997). Germack and Dickenson. (2005) showed preconditioning with adenosine (AR agonist), CPA (A_1AR agonist) or the A_3 adenosine receptor agonist 2-CL-IB-MECA significantly protected neonatal rat cardiac myocytes from hypoxia reoxygenation injury. Pharmacological preconditioning with A_1AR agonists has also been shown to mediate cardioprotection

in the rat (Hochhauser et al., 2007; DeJonge et al., 2002) and rabbit (Hill et al., 1998). Preconditioning hearts with adenosine analogues has been shown to be cardioprotective. Safran et al., (2001) have shown administration of A_1AR agonist 2-chloro-N6-cyclopentyl-adenosine (CCPA) or A_3AR agonist CL-IB-MECA prior to hypoxia significantly attenuated rat myocyte injury where the protection was abrogated by pre-treatment with A_1 and A_3 antagonist DPCPX and MRS 1523, respectively. Studies by Tracey et al., (1997) have shown that preconditioning with the A_3AR agonist IB-MECA attenuated ischaemia reperfusion injury in the rabbit heart. This group later showed that the protection mediated by the A_3AR agonist CB-MECA involved activation of K_{ATP} channels where protection was blocked by K_{ATP} channel antagonist glibenclamide (Tracey et al., 1998).

In the human atrial model of ischaemia reperfusion injury pharmacological preconditioning with A_1AR and A_3AR agonists has been shown to limit reperfusion injury (Carr et al., 1997). Pharmacological preconditioning with A_{2a}AR agonists has also been shown to protect chick ventricular myocytes from hypoxia/reoxygenation injury (Stickler et al., 1996).

1.4.3. Ischaemic Postconditioning

Recent studies by Zhao et al., (2003) saw the introduction of another cardioprotective mechanism referred to as ischaemic postconditioning. Postconditioning is a mechanism defined as brief periods of coronary artery occlusion followed reperfusion at the onset of reperfusion. Zhao et al., (2000) showed that 3 cycles of 30s reperfusion and 30s occlusion at the onset of reperfusion significantly attenuated the development of infarction compared controls receiving no intervention. Furthermore, they

concluded that postconditioning resulted in a decrease in neutrophil adherence in the at risk area accompanied by a reduction in p-selectin expression.

Other studies have shown that postconditioning can only protect the ischaemic heart where the period of coronary artery occlusion is less than 45 minutes in the conscious rat (Tang et al., 2006). Kin and colleagues (2004) showed that postconditioning can only protect the ischaemic heart when applied within the first minute of reperfusion where the protection was abolished if postconditioning was applied after 1 minute. Their study also showed that postconditioning protected against ischaemia reperfusion injury via decreased creatine kinase and oxidant release in response to injury. Halcos and colleagues (2004) investigated the additive effects of IP with ischaemic postconditioning concluding that there was no additive effect of combining IP with ischaemic postconditioning in the canine model of ischaemia reperfusion injury.

Although ischaemic postconditioning is a powerful mechanism for protecting the ischaemic heart the intracellular signalling pathways that mediate this protection remain to be elucidated (Zhao and Vinten-Jonahson. 2006). Zatta et al., (2006) have shown postconditioning to mediate cardio protection via increasing protein kinase C ε expression and inhibiting the translocation of protein kinase C δ. Interestingly, Kin et al. (2005) reported that postconditioning protects the ischaemic heart via the delayed washout of intravascular adenosine in the mouse. They further showed that administration of the non-specific adenosine receptor antagonist 8-SPT abolished the cardioprotective effect of postconditioning implicating a role for adenosine receptors in mediating cardioprotection. The role of adenosine receptors in ischaemic postconditioning was also implicated by Yang and colleagues., (2005) in the rabbit.

Darling and colleagues (2005) have shown postconditioning exerted its cardioprotective effects via upregulation of the MEK1/2 – ERK1/2 cell survival pathway, where the protection was blocked by the MEK1/2 inhibitor PD98059, these results were also seen by Yang et al., (2004). They further determined that postconditioning protects via enhancing nitric oxide bioavailability and opening of mitochondrial K_{ATP} channels and that protection was independent of the PI3K-AKT cell survival pathway. As shown in Figure 1.4.c Yellon and colleagues (2004) have shown in the isolated perfused rat heart that ischaemic postconditioning protects the ischaemic myocardium via recruitment of the PI3K – AKT-eNOS, p70S6K cell survival pathway. These contradictory results may be explained by the use of different inhibitors and animal models used although the exact role remains elusive.

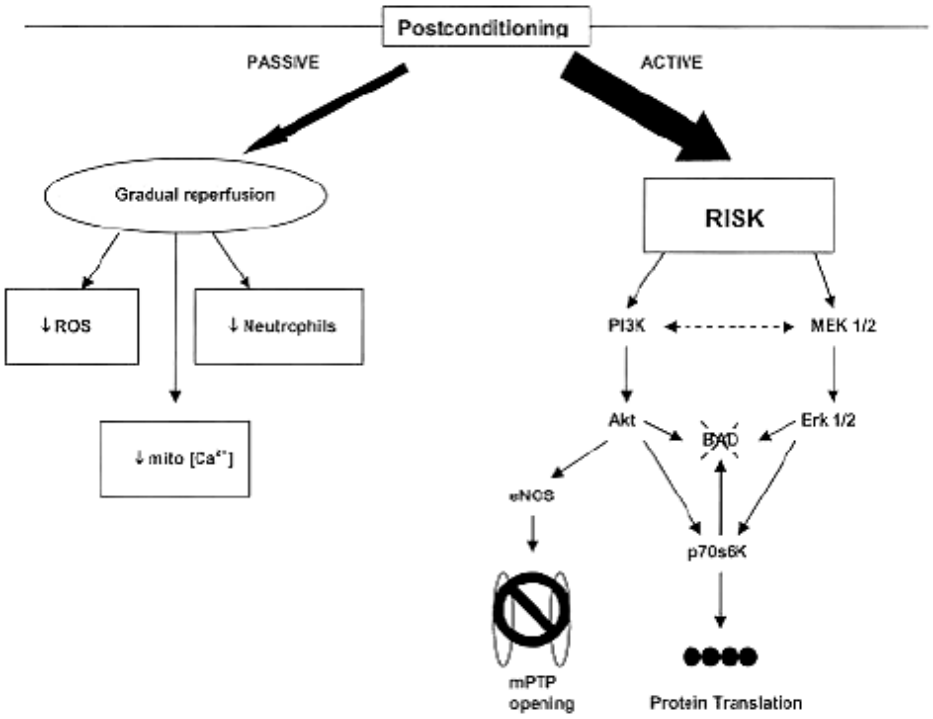

Figure 1.4.c. Schematic representation of the key mediators involved in ischaemic postconditioning mediated cardioprotection. (Adapted from Tsang et al., 2004).

Argaud et al. (2005) recently showed that postconditioning mediates protection via inhibiting opening of the mPTP in the open chest rabbit. Collectively, these data suggest that both ischaemic preconditioning and postconditioning protect the ischaemic myocardium from ischaemia reperfusion injury via recruitment of similar cell survival signalling pathways (Yellon and Hausenloy. 2006). Further intense research is required to establish the poorly characterised mechanisms involved in ischaemic postconditioning.

Research has shown that that ischaemic preconditioning or postconditioning can significantly protect the ischaemic reperfused myocardium from ischaemia reperfusion injury in a number of animal models. Many of the problems faced by researchers include the lack of translation of findings from laboratory experiments to

clinical settings. Ischaemic postconditioning has opened an exciting and promising paradigm for researchers where its application seems feasible in reducing ischaemia reperfusion injury in the human heart. A pilot study carried out by Laskey and colleagues (2005) has shown that that postconditioning can protect the failing human heart from ischaemia reperfusion injury. They showed that postconditioning reduced ischaemic injury and improved blood perfusion in the myocardium.

Another study by Staat et al., (2005) showed ischaemic postconditioning to reduce infarct size by 36% in patients admitted for coronary angioplasty. These preliminary studies show promise for application of ischaemic postconditioning in the clinical setting. Clinicians may consider using ischaemic postconditioning because its practicality as an adjunct therapy during revascularisation procedures like coronary artery bypass grafting or coronary angioplasty (Kloner and Rezkalla. 2006).

1.5.0. Cardioprotection via activation of Adenosine receptors at reperfusion.

Adenosine has been implicated to protect the myocardium from ischaemia reperfusion injury in numerous studies. The classic investigation by Zhao et al., (1993) demonstrated that blockade of adenosine A_1, A_2 and A_3 adenosine receptor antagonist 8-(p-sulfophenyl)-theophylline (8-SPT) at reperfusion limited endogenous adenosine mediated cardioprotection signifying the role of endogenous adenosine mediated cardioprotection. Similar findings have also been proposed by Toombs et al., (1992) who have shown blockade of adenosine receptors with the non-specific adenosine receptor antagonist 8-p-sulfophenyl theophylline (8-SPT) to significantly

increase infarct size by 24% compared to non-treated ischaemic perfused hearts indicating that endogenous adenosine plays a role in limiting infarct development.

Ely et al., (1985) showed treatment with exogenous adenosine before and during ischaemia and reperfusion significantly improved post-ischaemic dysfunction by sustaining ATP levels. Olfasson et el., (1987) showed that intracoronary administration of exogenous adenosine at reperfusion attenuated infarct size in the dog and this effect was mediated by decreased neutrophil infiltration and stagnation of capillaries. Norton et al., (1991) have shown intravenous administration of adenosine 5 minutes before reperfusion significantly attenuated myocardial infarction.

Zhao et al., (2001) showed administration of adenosine at reperfusion significantly limited infarct development in the dog. Furthermore, they concluded attenuation of ischaemia reperfusion injury by adenosine was mediated by upregulation of the anti apoptotic protein Bcl-2 and down regulation of the pro apoptotic protein Bax, accompanied by a significant decrease in necrotic and apoptotic components of cell death in the myocardium. In isolated myocytes upregulation of Bcl-2 had been attributed to inhibit cellular apoptosis (Hockenbrey et al., 1993; Knudson and Korsmeyer. 1997; Kirshenbaum and De Moissac. 1997). Other investigators have shown the protective effects of adenosine in the dog and rabbit model of ischaemia reperfusion injury (Pitarys II et al., 1991; Thornton et al., 1992; Sekili et al., 1995).

Notably, many investigators have shown protection with adenosine either by preconditioning or administration at reperfusion although Heide et al., (1996) failed to observe any protection when adenosine was administered at reperfusion in the canine

myocardium. Goto et al., (1991) also did not observe any protection when adenosine was administered either before coronary artery occlusion of at reperfusion in the rabbit model of ischaemia reperfusion injury. Earlier studies focussed on the role of A_1AR in terms of cardioprotection primarily due to the lack of characterisation of A_3ARs. Studies by numerous investigators have shown A_1AR mediated protection (Yao and Gross. 1993; Liang and Jacobson. 1998).

Studies conducted by Maddock et al., (2002) using the highly specific A_3AR agonist IB-MECA have shown that it protects the myocardium from ischaemia reperfusion injury when administered at reperfusion and furthermore limiting myocardial stunning in the guinea pig. They also showed protection was abolished in the presence of the A_3AR antagonist MRS1191. Maddock et al., (2003) have also shown that the concomitant administration of 2-Cl-IBMECA (A_3AR agonist) at reperfusion in the isolated perfused rat heart and in cardiomyocyte model of ischaemia reperfusion injury to be cardioprotective. In the same study they also showed that the protection was mediated in an anti-apoptotic and anti-necrotic manner where the protection was lost by the blockade of A_3ARs with MRS-1191 (A_3AR antagonist).

Jordan et al. (1999) have shown that administration of the A_3AR agonist CL-IB-MECA at reperfusion significantly attenuated neutrophil mediated reperfusion injury in the rabbit heart and pre-treatment significantly decreased neutrophil adherence to the endothelium, this anti neutrophil adherence was blocked by the A_3AR antagonist MRS-1220.

Stimulation of A_3ARs has been shown to be pro and anti-apoptotic whereby several studies have shown stimulation with micro molar concentrations of A_3AR agonist can further enhance cell death (Shneyvays et al., 1998; Brambilla et al., 2000; Maddock et al., 2002) whereas nanomolar concentrations can mediate cytoprotection (Gao et al., 2001; Maddock et al., 2002). Contrary to this a recent study by Park et al., (2006) has shown IB-MECA at a concentration of 1 μM to protect the ischaemic perfused rat myocardium from ischaemia reperfusion injury when administered at the onset of reperfusion. They also showed that the protection was abolished in the presence of the A_3 antagonist MRS 1334 and the mitochondrial permeability transition pore opener atractyloside. Previously Maddock et al., (2002) and Brambilla et al., (2000) have shown that at micro molar concentrations the A_3 agonists fail to protect the ischaemic myocardium therefore presenting contradictory results.

Over expression of the A_3 adenosine receptors in mice has shown to protect ischaemic-perfused hearts, preserving ischaemic ATP levels and also decreasing heart rate when compared to wild type (Cross et al., 2002). Ge et al., (2006) showed preconditioning with the A_3 agonist 2-CL-IB-MECA to limit infarct development in the ischaemic reperfused mouse but failed to protect in A_3 adenosine receptor gene knock out mouse. In the rat brain model of ischaemia reperfusion injury cerebral infarction was significantly increased in A_3 adenosine receptor knock out rats compared to wild type (Chen et al., 2006). This group further showed that preconditioning with the A_3 agonist 2-CL-IB-MECA confers significant cerebral protection in the ischaemic reperfused rat brain but failed to protect in A_3 adenosine receptor gene knock out rats. Comparatively, Harrison et al., 2002 have shown A_3 adenosine receptor gene knock out mice to generate an ischaemia tolerant phenotype

that does not affect myocardial energy metabolism or pH. The exact mechanisms generating this phenotype currently are unclear, but are thought to be as a role of cellular compensatory changes.

Stimulation of A_2AR with their respective analogues has been shown to mediate protection from ischaemia and reperfusion injury. Maddock et al., (2003) have shown A_{2a} receptor activation at reperfusion with A_{2a} agonist CGS-216680 confers cardioprotection these findings were similar to Lasley et al., (2001); Xu et al., (2001); and Jordan et al., (1997). More recently, Philipp et al., (2006) showed that administration of the non-selective but A_{2b} potent adenosine agonist 5'-(N-ethylcarboxamido)adenosine (NECA) 5 minutes before to 1 hour after reperfusion attenuated infarct development that was abolished in the presence of the A_{2b} adenosine receptor antagonist or the PI3K inhibitor Wortmannin in the rabbit.

Activation of A_3AR has shown to confer cardioprotection in many different models of myocardial ischaemia reperfusion injury. Stimulation of A_3AR has been shown to have: anti cancer properties (Fishman et al., 2001); a role in cerebro-protection (Von Lubitz et al., 2001); anti apoptotic/necrotic properties (Maddock et al., 2003); inhibit apoptotic cell death in ischaemic reperfused brain (Abbracchio et al., 1999), ability to inhibit A_3 antagonist dependant apoptosis in HL-60 Leukemic and U-937 cell lines (Yoa et al., 1997). Recent studies in the feline lung have also shown A_3 agonists to protect the lung against ischaemia reperfusion injury (Matot et al., 2005).

1.6.0 Administration of cardioprotective agents post-reperfusion

Previous studies have shown pharmacological and non-pharmacological approaches can protect the myocardium from ischaemia reperfusion injury, but it remains to be established whether postponing the administration of pharmacological agents after the onset of reperfusion can protect the ischaemic reperfused myocardium. Interestingly, Von Lubitz et al., (2001) have shown postponing the administration of the A_3 agonist IB-MECA 20 minutes after reperfusion significantly protected the mice brain subjected to ischaemia/reperfusion. Although this group did not determine the pathways that may be involved in IB-MECAs mediated protection. Zhang et al., (2002) showed nitrobenzylthioinosine an equilibrative nucleoside transporter subtype 1 inhibitor to protect rat neurones from forebrain ischaemia when administered before ischaemia by increasing post ischaemic levels of adenosine, suggesting post ischaemic increase in adenosine levels mediate protection. Jonassen et al., (2000) showed that administration of hormone insulin 15 minutes after the onset of reperfusion resulted in loss of protection seen when administered at reperfusion in the rat. Recently, Bolli and colleagues (2006) have showed administration of cytokine therapies 4 hours after reperfusion to limit left ventricular modelling and improve left ventricular performance by promoting cardiac regeneration in the mouse.

More interestingly Armstrong et al., (2001) showed caspase inhibitors when administered 1 hour after reperfusion could still reduce infarct development in the rat heart. Dambrova et al., (2002) showed that administration of the novel guanidine ME10092 5 minutes after the initiation of reperfusion significantly attenuated reperfusion induced ST elevation and cardiac arrhythmias in the rat. This group did not further determine the pathways that may be involved in ME10092 mediated cardioprotection.

The A_{2a} agonist administered at reperfusion is shown to reduce infarct size but delaying administration 5 minutes after reperfusion abolishes its protective effect in the rat (Boucher et al., 2004).

Collectively, research to date has shown adenosine to confer cardioprotection when administered at reperfusion or when administered prior to an ischaemic insult. The A_3 adenosine receptor agonist 2-CL-IB-MECA has previously been shown to limit infarct development in the ischaemic reperfused heart in a number of different species. To date no study has shown the intracellular signalling pathways that mediate 2-CL-IB-MECA mediated cardioprotection when administered at reperfusion or post-reperfusion at nanomolar concentrations. The aim of the current study was to determine whether the A_3 agonist 2-CL-IB-MECA mediated cardioprotection in the Langendorff perfused rat heart or adult rat cardiac myocyte from reperfusion/reoxygenation injury by recruiting the PI3K -AKT or MEK1/2 - ERK1/2 pro-survival pathways.

1.7.1. Mitogen Activated Protein Kinases

Mitogen activated protein kinases (MAPK) are a family of kinases that can respond to extracellular stimuli. MAPKs are serine and threonine kinases that have been shown to regulate cell growth cell differentiation and cell death. They can be activated following receptor tyrosine kinase activation or following G_i, G_o, G_q and G_s coupled receptor stimulation (Pearson et al., 2001). Activation of MAPK has been shown to activate a range of cell signalling pathways leading a dynamic range of responses (Lowes et al., 2002). GFRs and G proteins stimulated by growth factors and survival factors have been shown to be coupled to mitogen activated protein kinases (MAPK)

(Koch et al., 1997; Abe et al., 2000). Activation of the MAPK kinase cascade has been demonstrated to occur in response to growth factors, cytokines, and neurotransmitters and transmit survival signals.

Cellular responses occur after receptor ligand interaction followed by a sequence of events involving adaptor proteins growth factor receptor binding protein (GRB) and Ras guanine exchange factor (SOS) that via Ras can activate Raf (MAP kinase kinase kinase) the first of three MAPK module. Phosphorylated Raf can further activate downstream MAPK (extracellular signal regulated kinase) ERK1/2. Phosphorylation of ERK1/2 at Thr $_{202}$ and Tyr $_{204}$ residues transforms ERK1/2 to its active form. ERK1/2 can activate transcription of proteins by phosphorylating c-Myc and other transcription factors like p70S6K upregulating protein expression (Wechsler et al., 1994).

Punn et al. (2000) have show have shown an increase in ERK1/2 during simulated ischaemia that is further upregulated at the onset of reperfusion in cardiac myocytes. Activation of ERK1/2 following has also been shown to protect cardiomyocytes from oxidative stress following cyclooxygenase-2 induction (Adderley et al., 1999). Schulte et al. (2002) have also characterised the effect of adenosine receptor agonists and show upregulation of extra-cellular regulated kinase (ERK1/2) in Chinese hamster ovary cells (CHO) expressing the human A_3AR.

A number of growth factors have been implicated to be cardioprotective via recruitment of the MEK 1/2 – ERK1/2 signalling cascades including cardiotrophin-1

(Sheng et al., 1997), Transforming growth factor β1 (Baxter et al., 2001) and Urocortin (Schulman et al., 2002) (review Yellon & Baxter., 1999).

Graham et al. (2001) have shown a dose and time-dependant phosphorylation of ERK1/2 with the A_3AR agonist IB-MECA in Chinese hamster ovary cells expressing the human A_3AR. Upregulation of ERK1/2 was sensitive to pertussis toxin (Gi/o inhibitor), PD98059 (MEK 1 inhibitor). Germack and Dickenson., (2004) have characterised the activation of ERK 1/2 by adenosine and adenosine analogues in newborn cardiomyocytes. They characterised both time and dose dependant ERK 1/2 phosphorylation with adenosine and A_1, A_{2a} and A_3 receptor analogues. Analogues were seen to increase ERK 1/2 activation but not to the same degree as seen with adenosine. This is thought to be as a direct consequence of adenosine and its ability to stimulate all adenosine receptor subtypes while individual receptor specific agonists exerted their effect respective to its affinity to a specific adenosine receptor subtype.

P38 and JNK (stress activated protein kinase) are members of the MAPK superfamily of kinases that is responsive to stressful stimuli. Including heat shock, UV radiation, osmotic shock and have been shown to play a role in cell death and cell survival. Mackay et al., (1999) have shown inhibition of p38 to protect cardiac myocytes from ischaemia. Inhibition of p38 has also been shown to inhibit apoptosis and improve contractile function after myocardial ischaemia reperfusion (Ma et al., 1999). Kaiser et al., (2005) have interestingly shown inhibition of p38 to reduce myocardial injury following infarction in the mouse although this protection was not seen in the swine. JNKs play a role in regulating apoptosis in cardiomyocytes and many other cell types. Disagreement remains as to whether JNK are anti or pro apoptotic although Kaiser et

al., (2005) have shown genetic inhibition or activation of JNK to protect the myocardium from ischaemia reperfusion injury.

1.7.2. Phosphatidylinositol-3-Kinase

The phosphatidylinositol-3-kinase (PI3K) is a serine/threonine kinase that plays a significant role regulating cell growth, differentiation and survival (Ban et al., 2008). PI3K become activated by the binding of growth factors to GFRs. Stimulation of GFRs is coupled to PI3K via phosphorylation of its terminal residues. PI3K phosphorylation leads to the subsequent phosphorylation of AKT via cell signalling cascade leading to the dual phosphorylation of its ser_{473} and thr_{308} residues by phosphate-dependant kinase-1 (PDK 1) (Ban et al., 2008).

AKT (Protein kinase B) in its active form can modulate cell survival via recruitment of numerous downstream effector proteins phosphorylating forkhead transcription factors, eNOS (endothelial nitric oxide synthase), and the pro-apoptotic protein belonging to the Bcl 2 (B cell lymphoma/leukaemia 2) family BAD (Bcl-2/Bcl-XL-associated death promoter). BAD in its phosphorylated form binds to 14-3-3- proteins therefore BADs ability to associate to Bcl-2 and Bcl-xl is diminished preventing BAD dependant apoptosis initiation (Hausenloy and Yellon. 2004). Furthermore, AKT can activate numerous effector proteins like glycogen synthase kinase 3β (GSK3β) and stimulate glycogen synthesis as well as proteins involved in cell proliferation and growth (Hausenloy and Yellon., 2004; Park et al., 2006).

In the context of myocardial ischaemia and reperfusion Punn et al. (2000) used a cardiac myocyte model of simulated ischaemia and reperfusion to characterise AKT

expression. They have also shown that during ischaemia p-AKT $_{(ser\ 473)}$ is not expressed and expression is enhanced by reperfusion. The PI3K inhibitor Wortmannin abrogated AKT phosphorylation at reperfusion.

Gao et el., (2001) have shown that activation of A_3AR protects mast cells from UV light induced apoptosis by a mechanism involving protein kinase B (AKT). Furthermore, they showed the A_3AR antagonist MRS1523 abrogated A_3AR mediated phosphorylation of AKT. Hence, implicating that A_3AR mediated protection is via the Gi – PI3K – AKT cell survival-signalling pathway. Studies by Kennedy et al., (1999) further support the role of AKT in cell survival. They showed AKT activation to inhibit cell death by preventing cytochrome c release from mitochondria. They further determined that AKT activation could maintain mitochondrial integrity by inhibiting cytochrome c release.

Activation of adenosine receptors by NECA (non selective adenosine agonist) was shown to upregulate phosphorylation of AKT. The PI3K inhibitor Wortmannin or LY294002 and abrogated phosphorylation of AKT by by NECA (Yang et al., 2004). They also showed phosphorylation of PI3K was sensitive to the Gi/o inhibitor pertussis toxin. Implicating a role for Gi/o- PI3K dependant phosphorylation of AKT. This group have also shown that activation of A_3AR by NECA was shown to increase phosphorylation of the stress activated MAPK p38 and ERK 1/2 in a time and dose-dependant manner. Kis et al., (2003) have shown up regulation of PDK-1 – PI3K - AKT cell survival pathway in a rabbit model of ischaemic preconditioning, where the PI3K inhibitor Wortmannin abolisged the protection.

A number of growth factors have been implicated to be cardioprotective via recruitment of the PI3K - AKT or MEK 1/2 – ERK1/2 cascades including insulin growth factor (IGF-1) (Parrizas et al., 1997), Insulin (Johansen et al., 2003) Transforming growth factor β1 (Baxter et al., 2001) and Urocortin (Schulman et al., 2002) (review Yellon & Baxter., 1999).

Hausenloy et al. (2004) have recently proposed cross talk between the PI3K and the ERK1/2 during early reperfusion. In this novel study they propose that blockade of the PI3K activity using the PI3K inhibitor Wortmannin upregulated ERK1/2 phosphorylation and blockade of ERK1/2 pathway using PD98059 upregulated AKT phosphorylation. They further demonstrated that blockade of either PI3K or MEK1/2 abrogated the protective effect of infarct reduction by ischaemic preconditioning.

Collectively these data clearly demonstrate that adenosine released during myocardial ischaemia and reperfusion plays a putative role in the activation of adenosine receptor dependant cell survival signalling pathways (Haq et al., 1998; Sommerchild & Kirkeboen., 2000). Numerous studies in cell lines and rat cardiomyocytes have provided evidence of adenosine and its ability of activate AKT and ERK1/2 via adenosine receptors. Furthermore, studies have characterised adenosine and adenosine analogues to activate these pathways in a dose and time dependant manner.

A range of growth factors have been shown to up regulate downstream effector kinases mediating cardioprotection. Studies have shown preconditioning the myocardium before an ischaemic insult or administrating exogenous agents during ischaemia or at reperfusion via adenosine receptor stimulation can ameliorate

ischaemia reperfusion injury. Collectively from existing studies it seems feasible that activation of A_3AR at reperfusion by the A_3AR agonist 2-Cl-IBMECA can protect the myocardium from ischaemia reperfusion injury and subsequently that this protection be via recruitment of cell survival kinases PI3K-AKT and or MEK 1/2– ERK 1/2.

Extensive research over the years has seen publication of numerous publications entailing pharmacological and non-pharmacological experimental interventions that can protect the ischaemic reperfused myocardium. The development of pharmacological agents to be administered in a clinical setting to limit ischaemia reperfusion injury in humans is a near reality. Continued research in the field of cardiovascular research and development of agents that could potentially lead to an agent that would one day be used in a clinical setting (Bolli et al., 2004).

1.8. Aims and Objectives

1. To determine whether the A_3AR agonist 2-CL-IB-MECA when administered at reperfusion or post-reperfusion protects the myocardium from ischaemia reperfusion injury via the PI3K-AKT or MEK1/2-ERK1/2 survival pathway in the isolated perfused rat heart.
2. To elucidate the status of the cell survival pathway proteins AKT, ERK1/2, P70S6K, BAD in non-treated control ischaemic reperfused and 2-CL-IB-MECA treated hearts when 2-CL-IB-MECA was administered at reperfusion or post-reperfusion in the presence and absence of their respective inhibitors by western blot analysis.
3. To determine whether the administration of 2-CL-IB-MECA at reoxygenation or post-reoxygenation can attenuate caspase 3, apoptosis and necrosis in an adult rat cardiomyocyte model of hypoxia/reoxygenation injury. To determine the role of the PI3K/AKT and MEK1/2 – ERK1/2 pathway in 2-CL-IB-MECA mediated cytoprotection.

Chapter 2. Materials and Methods

2.1.1. Materials

Bovine serum albumin, Tri-phenyltetrazolium (TTC) (reacts with tetrazolium salts to form a formazan pigment indicating dead tissue) and Evans blue (used to delineate the risk area) were purchased from Sigma Chemical Co (Poole, UK). 2-CL-IB-MECA (1-[2-Chloro-6-[[(3-iodophenyl) methyl] amino]-9H-purin-9- yl]-1-deoxy-N-methyl-b-D-ribofuranuronamide) (Highly specific and selective A_3 adenosine receptor Agonist), U0126 (Potent and selective non-competitive inhibitor of MAP kinase kinase.), Wortmannin (cell permeable and selective phosphatidylinositol 3'kinase inhibitor), Rapamycin (selectively inhibits the phosphorylation and activation mTOR) were purchased from Tocris (Bristol, UK).

Phospho-specific ERK1/ERK2 (Thr^{202}/Tyr^{204}), Pospho-specific Akt (Ser^{473}), Cleaved-Caspase 3 (Asp175) (5A1E), Phospho-p70 S6 Kinase (Thr^{389}), BAD Ser^{136}, BAD Ser^{112}) were purchased from New England Biolabs (Hitchin, UK), Antibodies to β-actin were purchased from Abcam (Cambridge, UK). Vybrant Apoptosis assay kits #10 was purchased from Invitrogen (Paisley, UK).

2.1.2. Animals

Male Sprague Dawley rats (body weight 250 - 350 g) were used in these studies. All procedures were in accordance with UK Home Office guidelines on the Animals (Scientific Procedures) Act 1986. Animals were either purchased from Charles River, UK or bred in house at the institutional animal house and had free access to standard pelleted diet and water.

2.2. Langendorff Perfusion- Isolated rat heart preparation

Animals were sacrificed by cerebral dislocation. The rat heart was quickly excised and placed into ice-cold Krebs Heinsleit buffer. The aortic arch was cut away and the aorta was cannulated. The heart was retrogradely perfused on the Langendorff apparatus with Krebs Heinsleit bicarbonate buffer (NaCl 118.5 mM, $NaHCO_3$ 25.0 mM, KCl 4.8 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 1.7 mM, and glucose 12 mM) gassed with 95% O_2 and 5 % CO_2. The temperature was constantly monitored by inserting a thermo-probe in the pulmonary artery and maintained at 37 °C ± 0.5. The left atrium was cut away and a latex balloon was inserted into the left ventricle and inflated to give an end diastolic pressure of 8 to 10 mmHg, to measure left ventricular developed pressure (LVDP). The latex balloon was attached to a cannula connected to a physiological pressure transducer and a bridge amp connected to a Power lab (AD Instruments. Oxfordshire). Heart rate (HR) was measured by inserting an electrocardiogram probe onto the heart and the electrical activity was recorded using a Bioamp (AD Instruments. Oxfordshire). Coronary flow (CF) was measured by collecting the perfusate for one minute at regular time intervals. Haemodynamic variables were recorded at regular intervals using the data acquisition software Scope 4. Haemodynamic data were analysed statistically using the statistical package SPSS 12.

Hearts were allowed to stabilise for 20 minutes after which regional ischaemia was induced by ligating the anterior descending left coronary artery (LAD). Ligation was achieved by inserting a hooked 6-0 silk suture under the left coronary artery to form a snare and passing the ends of the threads through a pipette tip. Ischaemia was achieved by tightening of the threads and by placing the second pipette tip into the

first. Reperfusion was achieved by releasing the threads and by releasing the tips. Myocardial blanching and changes in ECG confirmed ischaemia.

Reperfusion Studies : At the onset of reperfusion hearts were concomitantly infused with either a) normal buffer (control) b) A_3R agonist 2-Cl-IB-MECA (1 nM) c) A_3R agonist 2-Cl-IB-MECA (100 nM), d) A_3R agonist 2-Cl-IB-MECA (1 nM,100 nM) in the presence of cell signalling cascade inhibitors, PI3K inhibitor – Wortmannin (5 nM, 100 nM), MEK 1/2 inhibitor UO126 (10 μM), mTOR inhibitor – Rapamycin (2 nM) Figure 2.1.a. 2-CL-IB-MECA was used at two different concentrations as previously Germack and Dickenson (2004; 2005) have shown 2-CL-IB-MECA to activate ERK1/2 and AKT in a dose dependant manner. Therefore, we used different concentrations to determine whether a higher concentration of 2-CL-IB-MECA was more cardioprotective when administered at reperfusion.

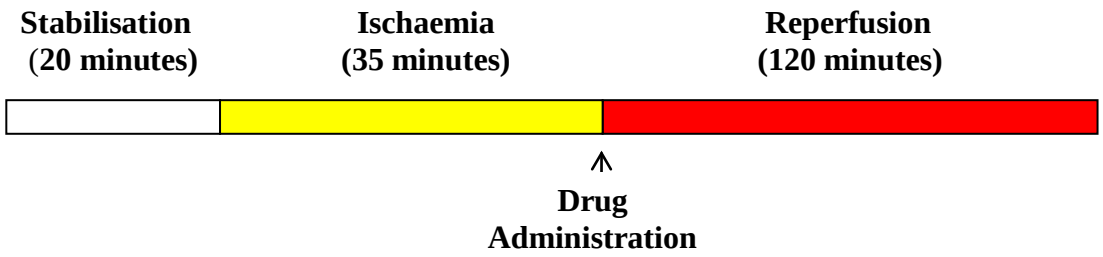

Figure 2.1.a. Diagram shows experimental protocol for isolated perfused rat hearts subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion where drugs were administered at the onset of reperfusion.

Post-Reperfusion Studies: Hearts were concomitantly infused with either a) 2-CL-IB-MECA (1nM) 15 or 30 minutes after the onset of reperfusion b) 2-CL-IB-MECA (1nM) in the presence of the PI3K inhibitor Wortmannin (100nM) or MEK1/2 inhibitor UO126 (10μM) from 15 or 30 minutes post reperfusion (see figure 2.1.b).

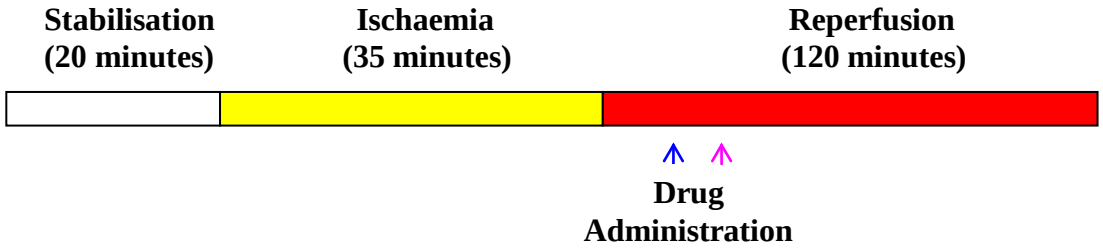

Figure 2.1.b. Diagram shows experimental protocol for isolated perfused rat hearts subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion where drugs were administered 15 or 30 minutes after reperfusion.

At the end of reperfusion the left coronary artery was re-ligated and the heart was infused with 1ml of 0.25% Evans blue, this was to delineate the risk area (figure 2.1.c) .

Figure 2.1.c. Photograph of an isolated rat heart perfused with Evans blue

The hearts were removed from the apparatus weighed and quickly frozen at -20°C. Once frozen the hearts were transversely sliced into 2mm thick slices and incubated at 37°C in 8ml of phosphate buffer 2 (100mM NaH_2PO_4) and 2ml phosphate buffer 1 (100mM $NaH_2PO_4.2H_2O$) containing 0.1g tri-phenyltetrazolium powder. The slices were then placed into 10% formalin for a minimum of four hours in order to enhance the contrast between the viable and non-viable areas (figure 2.1.d)

Figure 2.1.d. Representative photograph of an isolated rat heart slices perfused with Evans blue and TTC stained. The viable tissue is stained blue, risk tissue pink and infarct tissue pale/white.

The slices were then placed between two clear Perspex sheets and squeezed together using bulldog clips. The slices were traced onto an OHP film using different colours for viable, risk and infarct tissue. Risk tissue stained pink/red and the infarct tissue stained pale/white. Infarct to risk ratio was determined by tracing around the acetate sheet using the Summasketch II tablet and areas were determined using the NIH Image software.

2.3. Data Acquisition Equipment

Physiological pressure transducer (AD Instruments™), connected to a bridge Amp (AD Instruments™), PowerLab™ (AD Instruments™), Scope 4™ software (AD Instruments™) for recording haemodynamic data, Quantity One Software™ was used for densitometric analysis of Western blots.

2.4.1. Myocyte Isolation

Animals were sacrificed by cerebral dislocation. Sprague Dawley rat hearts were quickly excised and placed into ice-cold modified Krebs Heinsleit buffer. The aortic arch was cut away and the aorta was cannulated to the cannula. The heart was as mentioned above retrogradely perfused on the Langendorff apparatus with modified Krebs Heinsleit bicarbonate buffer (NaCl 116.0 mM, $NaHCO_3$ 25.0 mM, KCl 5.4 mM, $MgSO_4$ .7.H_2O 0.4 mM, $CaCl_2$ 1.7 mM, and glucose 10 mM, taurine 20 mM, pyruvate 5 mM and Na_2HPO_4.12H_20 0.9 mM) at a speed of 10 ml/min. HPLC grade water was used for all solutions. The buffer was oxygenated for 30 minutes with 95% O_2 and 5% CO_2 (BOC Gases) after which the buffer was heated to 37°C and the pH was corrected to pH 7.4 with NaOH.

Hearts were perfused for 5 minutes with Krebs buffer containing calcium (1.7mM) followed by 5 minutes of perfusion with calcium-free Krebs Heinsleit buffer. Upon perfusion with calcium free buffer the heart ceased contraction and turned pale in colour and shiny on the exterior. 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%, $CaCl_2$ 4.4 μM pH adjusted to pH 7.4 with NaOH. During perfusion with Collagenase the effluent was collected and reused.

After perfusion with the digestion buffer the heart was removed from the cannula and the atriums were cut off. The ventricles were placed into a small beaker and gently the tissue was teased apart and further cut into smaller pieces with a clean pair of scissors. The tissue was incubated with 15 ml of fresh digestion buffer pre-heated to 37 °C for 10 minutes. The digestion buffer was aspirated and was passed through a nylon mesh

into a sterile 50 ml falcon tube. The remaining tissue was removed from the mesh and placed into the beaker with fresh digestion buffer. The tissue was placed again into the orbital shaker at a speed of 150 rpm for 20 minutes. The buffer previously removed was centrifuged (Jouan HS centrifuge) at 400 rpm for 2 minutes. The supernatant was removed using a sterile pipette and the pellet was redistributed in 25 mls of freshly prepared restoration buffer (NaCl 116 mM, $NaHCO_3$ 25 mM, KCl 5.4 mM, $MgSO_4.7.H_2O$ 0.4 mM, glucose 10 mM, taurine 20 mM, pyruvate 5 mM $Na_2HPO_4.12H_20$ 0.9 mM, 1% BSA and 1% Pen-Strep). All processes were carried out in the laminar flow cabinet to reduce the risk of infection.

The cells in suspension were placed into the incubator at 37 °C and received 5 doses of 58 µl of 100 mM $CaCl_2$. The cells were placed into a 90 mm Petri dish and incubated overnight. Cells retrieved from the second isolation also underwent the same protocol. Figure 2.1.e shows a photograph of isolated adult rat cardiac myocyte.

Figure 2.1.e. Photograph shows an isolated adult rat cardiac myocyte

(Hein et al., 2006)

2.4.2. Exclusion Criteria

To ensure reproducibility of experiments the following exclusion criteria was applied:

- Myocytes showing less then 65 % viability were disregarded
- Myocytes not exhibiting clear striations were also disregarded

2.4.3. Hypoxia and Reoxygenation Protocol

The next day cells underwent 6 hours of hypoxia followed by 18 hours of reoxygenation. The cells were retrieved from the incubator and transferred to a falcon tube. The cells were centrifuged at 500 rpm for 5 minutes and the supernatant was carefully pipetted out. The cells were redistributed in restoration buffer and 1 ml aliquots were taken for the normoxic group. The cells were then centrifuged at 500 rpm for 5 minutes and the supernatant was removed. The cells were incubated in 15 mls of hypoxic buffer (12 mM KCL, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$, 4 mM HEPES, 10 mM Deoxyglucose and 20 mM lactate and placed into a hypoxic chamber pre-heated at 37 °C. The air in the chamber was removed by a suction pump and replaced the 5% CO_2 balanced in Argon. After 6 hours of hypoxia the cells were removed from the chamber and centrifuged at 500 rpm for 5 minutes. The supernatant was removed and replaced with 24 ml of restoration buffer. The pellet was redistributed and 1ml aliquots were pipetted into a 24 well sterile plate. While the cells were being centrifuged the A_3 agonist 2-Cl-IB-MECA (1 nM, 10 nM, 100 nM) and cell signalling pathway inhibitors (Wortmannin 5 nM, 100 nM; Rapamycin 2 nM; UO126 10 μM) were defrosted and aliquotted into the appropriate wells in the 24 well plate as described in figure 2.1.f. Cells were also aliquotted for the normoxic group, unstained group and the vehicle control group.

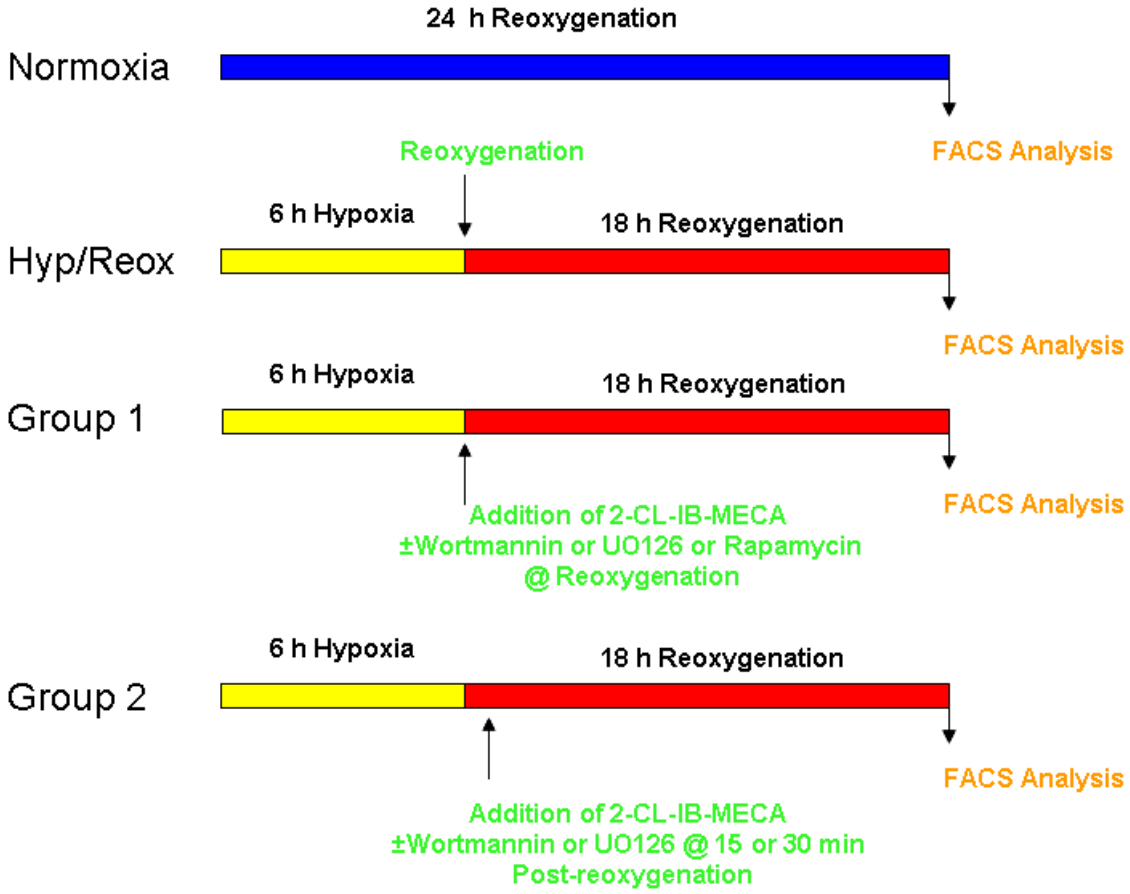

Figure 2.1.f. Protocol for FACS analysis of isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia and 18 hours reoxygenation. The A_3AR agonist 2-CL-IB-MECA was administered at reoxygenation or post-reoxygenation in the presence or absence of the PI3K inhibitor Wortmannin or the MEK1/2 inhibitor UO126.

Where agonists and inhibitors were added together the inhibitor was added 1 minute prior to adding the agonist. Once the cell suspension was added to each well the suspension was pipetted twice to ensure the drugs are fully distributed. Cells were reoxygenated in the incubator for 18 hours before undergoing Fluorescence Activated Cell Sorting Analysis (FACS) for assessment of cellular apoptosis, Cleaved-caspase-3 activity and necrosis.

2.5.0. Preparation for Fluorescence Activated Cell Sorting Analysis - Cell Death

Adult rat cardiomyocytes were exposed to 6 hours of hypoxia followed by 18 hours of reoxygenation after which the myocytes were labelled with fluorochromes purchased from Invitrogen (Paisley, UK). SYTOX green dye binds to cellular nucleic acids in necrotic cells and is also impermeable to live and early apoptotic cells. Apoptotic myocytes were measured using Annexin V Allophycocyanin that has high affinity for phosphatidylserine (PS) exposed on the outer membrane of apoptotic cells. Resazurin C_{12} is reduced by viable cells. Cells were analysed by flow cytometric analysis using the FACS Calibur™ flow Cytometer.

To prepare the cells for FACS analysis the cells need to undergo a set of processes. 1 x Annexin V buffer was prepared from 5x stock by diluting 4 ml 5X Annexin V (50 mM HEPES, 700mM NaCL, 12.5 mM CaCL2, pH 7.4) with 16 ml ddH_20. Fluorchromes were prepared as follows: 50 μM C_{12} resazurin (component B) was prepared by adding l μl of 1 mM C_{12} resazurin stock into 19 μl ddH_20, 1 μM Sytox Green stain (component C) was prepared by adding 5 μl of 10 μM Sytox Green stain into 45μl 1X Annexin V buffer. Eppendorffs containing cells were wrapped in foil to prevent loss of fluorescence. Eppendorffs were labelled and the cell suspensions were transferred from the 24 well plates to their respective Eppendorffs.

Eppendorffs were centrifuged at 500 rpm for 5 minutes, supernatant was removed and the cells were washed by resuspending with 300 μl Annexin V buffer to remove non-attached fluorochromes. Cells were centrifuged again at 500 rpm for 5 minutes. The supernatant was removed followed by the addition of 100 μl Annexin buffer. The

pellet was resuspended followed by the addition of 1 µl Component B, 1 µl Component C and 5µl Component A. Once the components were added the Eppendorffs were wrapped in foil and incubated at 37 °C for 15 min.

Finally, 400 µl Annexin V buffer added the Eppendorffs preparing the cells for analysis on the FACS Calibur flow cytometer. The fluorochromes were chosen because they have different excitation and emission wavelengths their emission peaks did not overlap. Therefore, the cell population were analysed on the FL-2 and FL-4 channels suitable for the different fluorochromes.

The software was setup to count 10,000 events. Quadrants were plotted as the different fluorochromes would bind specifically to live, apoptotic and necrotic cell populations. The protocol was carried out according to the manufacturer's instructions (Invitrogen. Paisley).

2.6.0. Preparation for FACS Analysis – cleaved-caspase 3

To determine the activity of cleaved caspase 3 in adult rat myocytes, cells underwent hypoxia/reoxygenation protocol as above. At the end of reoxygenation cells were transferred from the 24 well plates into labelled Eppendorffs. Cells were centrifuged at 1200 rpm for 2min. The supernatant was aspirated and the pellet was resuspended in 250 µl phosphate buffered saline. Then the cells were fixed by the addition of 250µl of 6 % formaldehyde to give a final formaldehyde concentration of 3%. This was done in order to fix the cell and prevent further cellular activities. The cells were

placed into the incubator for 10 minutes at 37 °C and immediately after the Eppendorffs were placed on ice for 1 minute.

The Eppendorffs were centrifuged at 1200 rpm for 2 minutes after which the supernatant was aspirated and the cells were permeabilised in order to allow the antibody to enter the cell by resuspending the cells in ice-cold methanol (90%). Cells were incubated on ice for 30 minutes and then centrifuged at 1200 rpm for 2 minutes. The supernatant was removed and the cells were washed in 200 µl incubation buffer (0.5% BSA in PBS stored @ 4°C), this step was repeated once. The Eppendorffs were centrifuged at 1200 rpm for 2 minutes 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 (New England Biolabs. Hitchin) was added to the blocking buffer to give a dilution factor of 1:100 and incubated at room temperature for 60 minutes.

The Eppendorffs were centrifuged again at 1200 rpm for 2 minutes followed by the removal of supernatant and again the cells were washed twice in incubation buffer as before. The cells were resuspended in 200 µl of incubation buffer containing the Alexa Fluor® 488 secondary antibody (Invitrogen) to give a dilution factor of 1:1000. The samples were covered in foil and allowed to incubate for 30 minutes a room temperature. The cells were centrifuged at 1200 rpm for 2 minutes and then washed in incubation buffer. The cells were centrifuged at 1200 rpm and the supernatant was removed.

Finally, the cells were resuspended in 500 µl of phosphate buffered saline and analysed on the flow cytometer on the FL-1 channel. Alexa Flour 488 is excited on FL-1 at 495 nM and emits at 519 nM. Histograms were plotted for each of the groups showing the mean fluorescence for 10,000 cell counts, indicating cleaved-caspase 3 activity. The protocol was carried out according to the manufacturer's instructions (New England Biolabs. Hitchin).

2.7. Tissue preparation for Western Blotting

Isolated rat hearts underwent ischaemia and reperfusion for a specified time period where 2-CL-IB-MECA was administered at reperfusion, 15 or 30 minutes post-reperfusion in the presence and absence of the PI3K inhibitor Wortmannin or MEK1/2 inhibitor UO126 or mTOR/p70S6 kinase inhibitor Rapamycin (See figure 2.1.g.)

At the end of the specified reperfusion period hearts were infused with 0.5ml 0.25% Evans Blue. This was to delineate the non-risk area staining blue and the risk area staining pink/red. Hearts were removed from the apparatus and the risk area was quickly excised using a sterile scalpel. The tissue was freeze clamped in liquid nitrogen using a Wollenberger freeze clamp pre cooled in liquid nitrogen. The tissue was wrapped in silver foil, labelled and stored at –80°C.

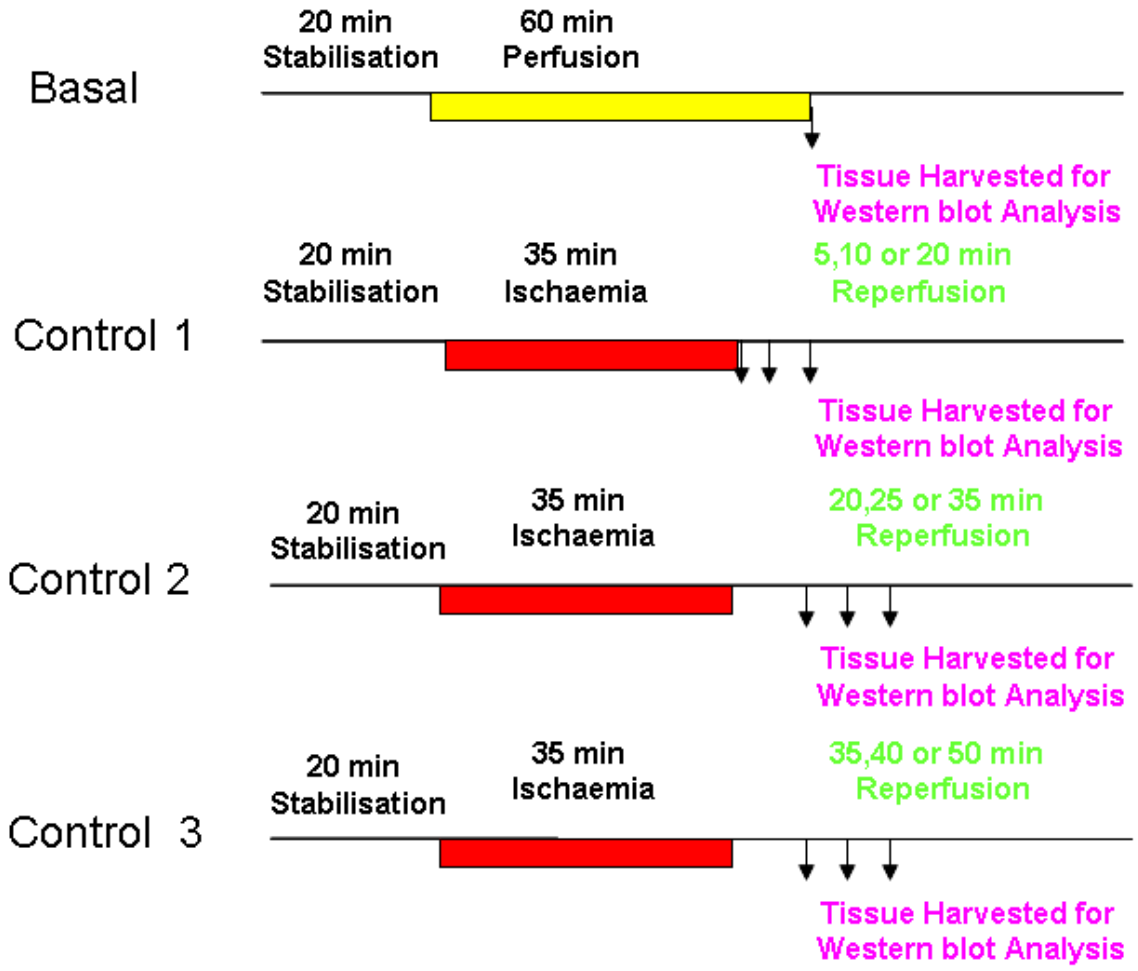

Figure 2.1.g. Protocol used to isolate heart tissue for western blot analysis

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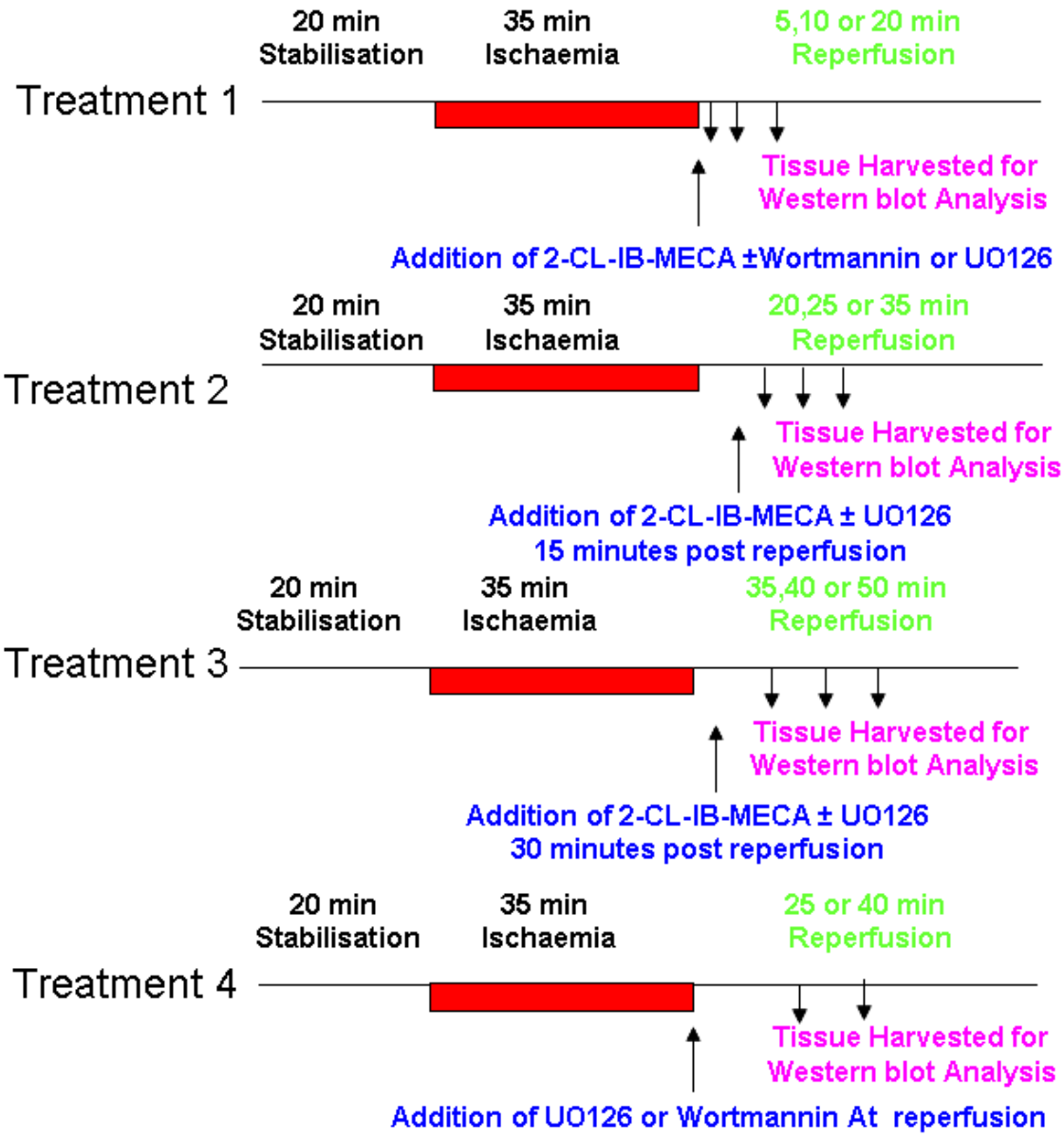


Figure 2.1.g. Protocol used to isolate heart tissue for western blot analysis

2.8. Western blotting

Briefly, 50 mg of frozen ventricular tissue was isolated and homogenised in 250 μl of suspension buffer (NaCl 100 mM, Tris 10 mM (pH 7.6), EDTA 1 mM (pH 8), Sodium pyrophosphate 2 mM, sodium fluoride 2 mM, β-glycerophospahte 2 mM, PMSF 0.1 mg/ml, aprotinin and leupeptin 0.1 μg/ml) using a IKA Labortechnik T25 homogeniser. Samples were centrifuged using a Jouan centrifuge (5 min; 11000 rpm). The supernatant was further diluted in 2 x sample buffer (Tris 100mM (pH 6.8), DTT 200mM, SDS 2 %, Bromophenol blue 0.2 % and glycerol 20 %) and heated to 95 °C. Protein concentrations were estimated using BCA protein assay reagent (Pierce).

A total protein of 40 μg for each sample was separated on a 12.5% SDS-PAGE gel using a BioRad mini protean II system (1 hour at 200V) and transferred to a Hybond Poly vinyl difluude membrane (Amershem Biosciences, UK) using a BioRad trans blot system (1h at 100V in 25 mM Tris, 192 mM Glycine and 20 % methanol. After the completion of transfer the membrane was washed and blocked for 1 hour using blocking buffer (15ml TBST, 1.25g Marvel). Blots were washed and were incubated with primary rabbit polyclonal antibody (phospho-ERK1/2 Thr202/Thr204), Akt (Ser 473), P70S6 kinase (Thr 389), BAD (Ser136) or BAD (Ser 112) and subsequently probed with horseradish peroxidise conjugated anti-rabbit antibody.

Equal loading was confirmed by ponceau S staining of membranes. All antibodies were purchased from New England Biolabs, UK. Proteins were detected by enhanced chemoluminesence ECL Plus (Amersham Biosciences). Blots were exposed to Hyperfilm ECL (Amersham, Buckinghamshire, UK) and developed using Kodak developing/fixing solution (Sigma, Poole. UK). Equal loading and transfer efficiency

was assessed by Ponceau S staining prior to primary antibody application. Figure 2.1.h shows a western blot following autoradiography. The blot shows the molecular marker and ERK1/2. Following Autoradiography blots were stripped and probed with β-actin to confirm equal loading. Films were scanned and densitometry was assessed using the NIH Image J (v1.33) software.

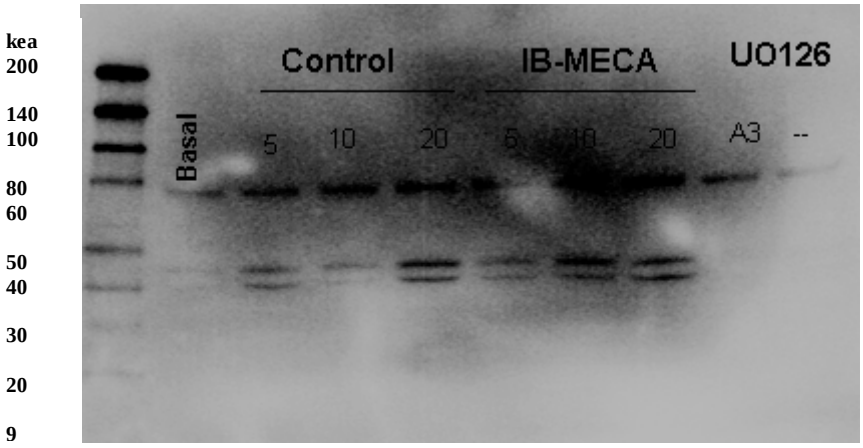


Fig 2.1.h. Illustration of a western blot probed for phosphoERK1/2 with a biotinylated molecular marker.

2.9. Statistical Analysis

All values were expressed as mean ± SEM (Standard Error of the Mean). Infarct size, band densities, cell populations were analysed using SPSS 12 one-way ANOVA with Fishers Protected Least Significant Difference test for multiple comparisons. Differences were considered significant at $P<0.05$.

Haemodynamics where analysed by statistical analysis using the statistical package SPSS version 13. Data were analysed using a two way ANOVA.

Chapter 3: 2-CL-IB-MECA protects the myocardium from ischaemia reperfusion injury via MEK 1/2 – ERK 1/2 cell survival pathway.

3.1.0. Haemodynamics Data Analysis

Haemodynamic data including heart rate, left ventricular developed pressure and coronary flow were collected for all experimental groups. Administration of the A_3 agonist 2-Cl-IB-MECA (1nM) in the presence and absence of cell signalling pathway inhibitors (U0126, Wortmannin and Rapamycin) was seen to have no significant effect on the haemodynamics measured when analysed statistically (Fig 3.1.1, 3.1.2, 3.1.3).

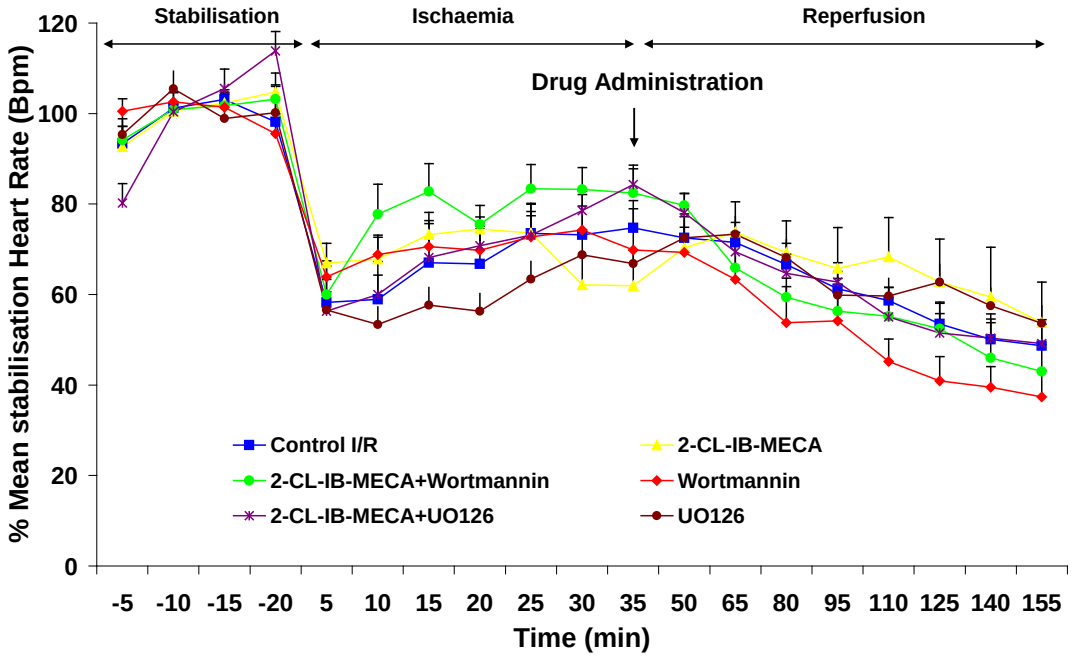


Figure 3.1.1. The chart shows the changes in left ventricular developed pressure in isolated rat hearts subjected to 35 minutes of ischaemia and 120 minutes reperfusion. The A_3AR agonist 2-CL-IB-MECA was administered at reperfusion the presence and absence of the PI3K inhibitor Wortmannin or the MEK1/2 inhibitor UO126. Results are expressed as mean of the stabilisation period ± SEM.

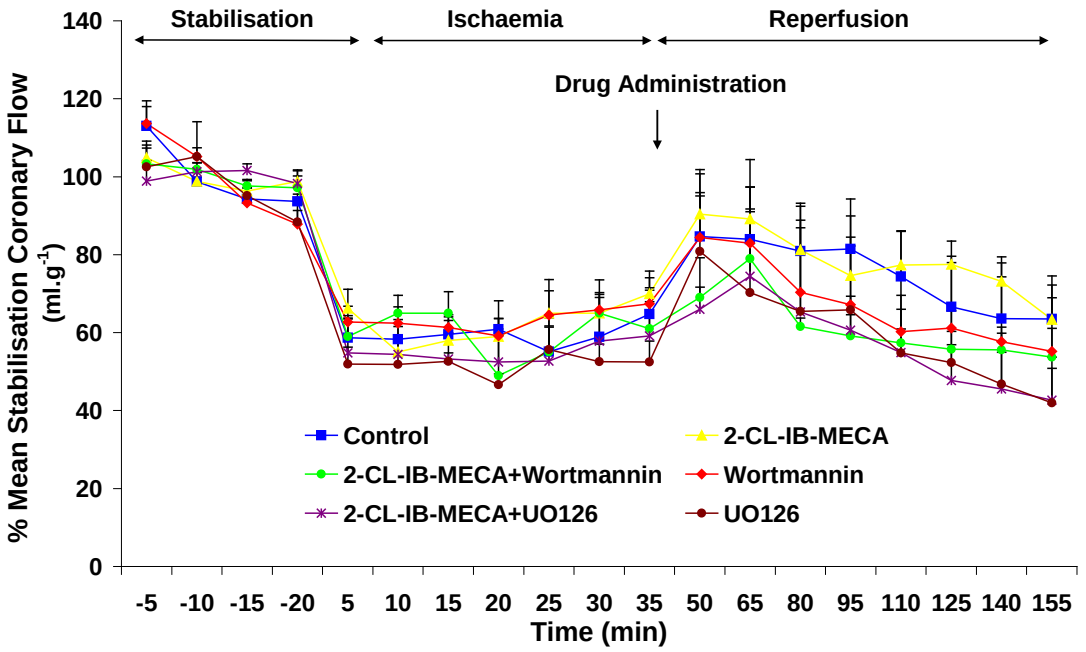


Figure 3.1.2. The chart shows the changes in Coronary flow in isolated rat hearts subjected to 35 minutes of ischaemia and 120 minutes reperfusion. The A_3AR agonist 2-CL-IB-MECA was administered at reperfusion the presence and absence of the PI3K inhibitor Wortmannin or the MEK1/2 inhibitor UO126. Results are expressed as mean of the stabilisation period ± SEM.

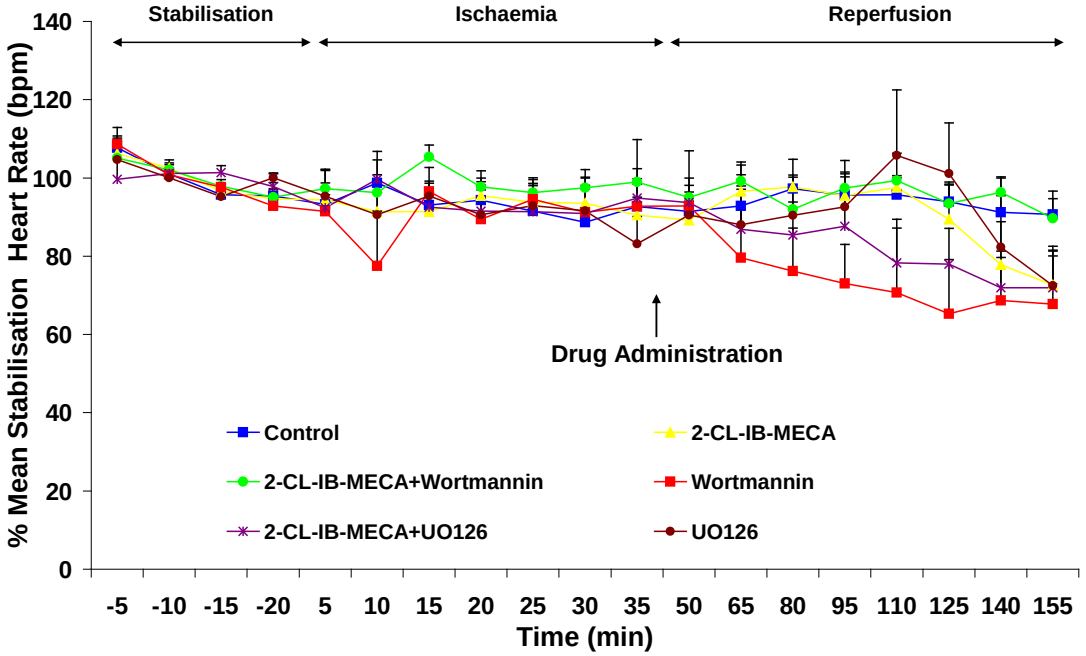


Figure 3.1.3. The chart shows the changes in heart rate in isolated rat hearts subjected to 35 minutes of ischaemia and 120 minutes reperfusion. The A_3AR agonist 2-CL-IB-MECA was administered at reperfusion the presence and absence of the PI3K inhibitor Wortmannin or the MEK1/2 inhibitor UO126. Results are expressed as mean of the stabilisation period ± SEM.

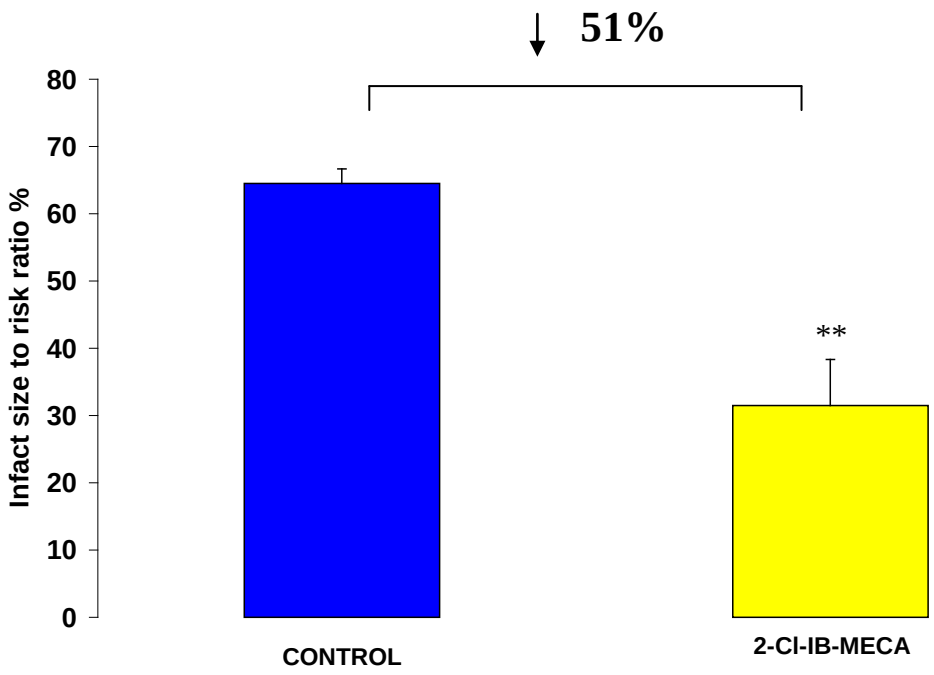
3.2.0. Results - Infarct Size to Risk Ratio Analysis

3.2.1. Effect of the A_3 adenosine receptor agonist 2-CL-IB-MECA when administered at reperfusion in the rat myocardial model of ischaemia reperfusion injury.

In this study we investigated whether the MEK1/2 – ERK1/2 cell survival pathway is involved in protecting the myocardium from ischaemia reperfusion injury when 2-CL-IB-MECA (1nM) was administered throughout reperfusion.

Dimethyl sulphoxide was used as a vehicle to dissolve the A_3AR agonist 2-CL-IB-MECA and the inhibitors UO126, Wortmannin and Rapamycin used in the studies. Studies were carried out to determine the effect of dimethyl sulphoxide (final concentration 0.01%) on infarct size to risk ratio (%) in the isolated perfused rat heart. Our studies are in accordance with previous studies which showed the solvent to have no significant effect on infarct size to risk ratio (%) compared to control hearts (data not shown (Kis et al., 2003).

Isolated perfused rat hearts underwent 35 minutes of ischaemia followed by 120 minutes of reperfusion where the A_3 agonist 2-CL-IB-MECA (1 nM) was administered throughout the reperfusion period. The study showed that the administration of the highly specific A_3AR agonist 2-Cl-IBMECA (1 nM) throughout the reperfusion period significantly decreased infarct size to risk ratio compared to the non-treated control group (32 ± 4% 2-CL-IB-MECA vs. 65 ± 2 % control hearts $P<0.01$) , a reduction by 51 % Figure.3.2.a.



**** P<0.01 2-CL-IB-MECA vs. Control.**
Results are shown as mean ± SEM from 6-9 individual experiments.

Figure 3.2.a. Infarct size to Risk ratio (%) in non-treated control and 2-Cl-IBMECA treated ischaemic reperfused hearts. Isolated perfused rat hearts where subjected to 35 minutes of ischaemia and 120 minutes of reperfusion where the A_3 adenosine receptor 2-Cl-IB-MECA (1 nM) was administered throughout reperfusion.

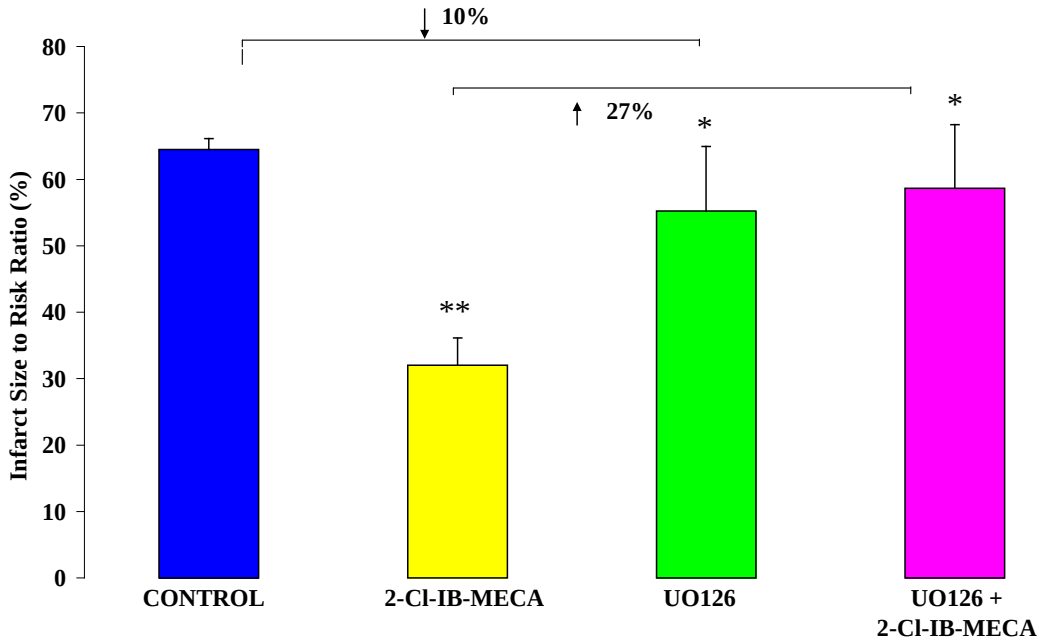
3.3.2. Role of the MEK1/2 – ERK1/2 cell survival pathway in 2-CL-IB-MECA mediated protection when administered at reperfusion in the isolated perfused rat heart.

As mentioned earlier numerous studies have shown that the administration of pharmacological agents at reperfusion to upregulate the MEK 1/2 – ERK 1/2 cell survival signalling pathway conferring cardioprotection Hausenloy and Yellon. 2004).

To determine whether the protection afforded by the A_3AR agonist 2-Cl-IBMECA (1 nM) when administered throughout reperfusion was associated with enhanced MEK1/2 – ERK1/2 activity isolated perfused rat hearts undergoing 35 minutes of ischaemia and 120 minutes of reperfusion were perfused with 2-CL-IBMECA (1 nM)

in the presence and absence of the MEK1/2 inhibitor UO126 (10 μM) throughout reperfusion. Administration of 2-CL-IB-MECA (1 nM) in the presence MEK 1/2 inhibitor UO126 (10 μM) significantly abrogated the protection afforded by 2-CL-IB-MECA (1 nM) when administered alone at reperfusion (32 ± 4% 2-CL-IB-MECA vs. 59 ± 9 % UO126 + 2-CL-IB-MECA P<0.05) (Figure.3.2.b).

Administration of UO126 (10 μM) alone throughout reperfusion had no significant effect on infarct size compared to control (55 ± 8 % UO126 vs. 65 ± 2 % Control P>0.05) (Figure.3.2.b). The data suggest that MEK 1/2 – ERK 1/2 dependant signalling pathways are involved in 2-CL-IB-MECA (1 nM) mediated cardioprotection when administered at reperfusion.



****P<0.01 2-CL-IB-MECA vs. Control *P<0.05 UO126, UO126+2-CL-IB-MECA vs. 2-CL-IB-MECA. Results are shown as mean ± SEM.**

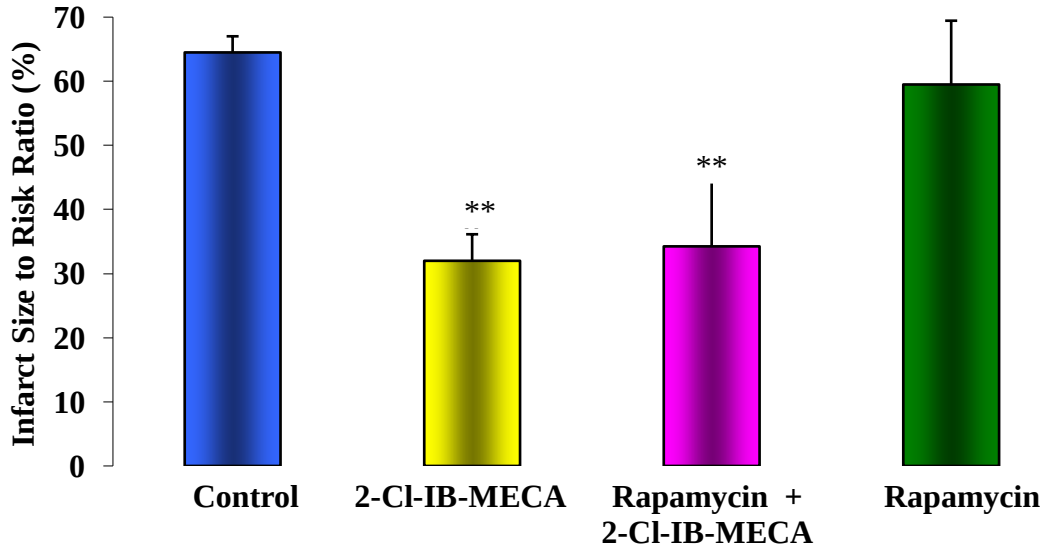
Figure 3.2.b. Infarct size to Risk ratio (%) in non-treated control and 2-Cl-IB-MECA ischaemic reperfused hearts. Isolated perfused rat hearts where subjected to 35 minutes of ischaemia and 120 minutes of reperfusion were the A_3 adenosine receptor 2-Cl-IB-MECA (1 nM) was administered throughout reperfusion in the presence and absence of the MEK 1/2 inhibitor UO126 (10 μM)

3.3.0. Role of p70S6 kinase in 2-CL-IB-MECA mediated protection when administered at reperfusion.

A number of studies have shown the p70S6K to be a downstream effector protein of the MEK 1/2 – ERK 1/2 cell survival pathway. Upregulation of p70S6K activity leads to the increase in protein translation that may be involved in protection.

To determine whether the protection afforded by 2-CL-IB-MECA (1 nM) when administered at reperfusion involved the recruitment of p70S6K, isolated perfused rat hearts undergoing 35 minutes of ischaemia and 120 minutes of reperfusion were perfused with 2-CL-IBMECA (1 nM) in the presence and absence of the mTOR inhibitor Rapamycin (2 nM) throughout reperfusion.

The protection afforded by the A_3 agonist 2-CL-IB-MECA (1 nM) when administered at reperfusion was not abolished in the presence of the mTOR inhibitor Rapamycin (2 nM) (32 ± 4 2-Cl-IB-MECA % vs. 34 ± 10 % 2-CL-IB-MECA + Rapamycin, $P>0.05$) (Figure 3.3). Administration of Rapamycin (2 nM) alone throughout reperfusion had no significant effect on infarct size to risk ratio (%) compared to control (59 ± 2% vs. 65 ± 2 % respectively, $P>0.05$) (Figure 3.3).



****P<0.01 2-CL-IB-MECA, Rapamycin+ 2-CL-IB-MECA vs. Control. Results are shown as Mean ± SEM.**

Figure 3.3. Infarct size to Risk ratio (%) in non-treated control and 2-Cl-IBMECA (1 nM) treated hearts. Isolated perfused rat hearts were subjected to 35 minutes of ischaemia and 120 minutes of reperfusion where the A_3 adenosine receptor 2-Cl-IB-MECA (1 nM) was administered throughout reperfusion in the presence and absence of the mTOR inhibitor Rapamycin (2 nM).

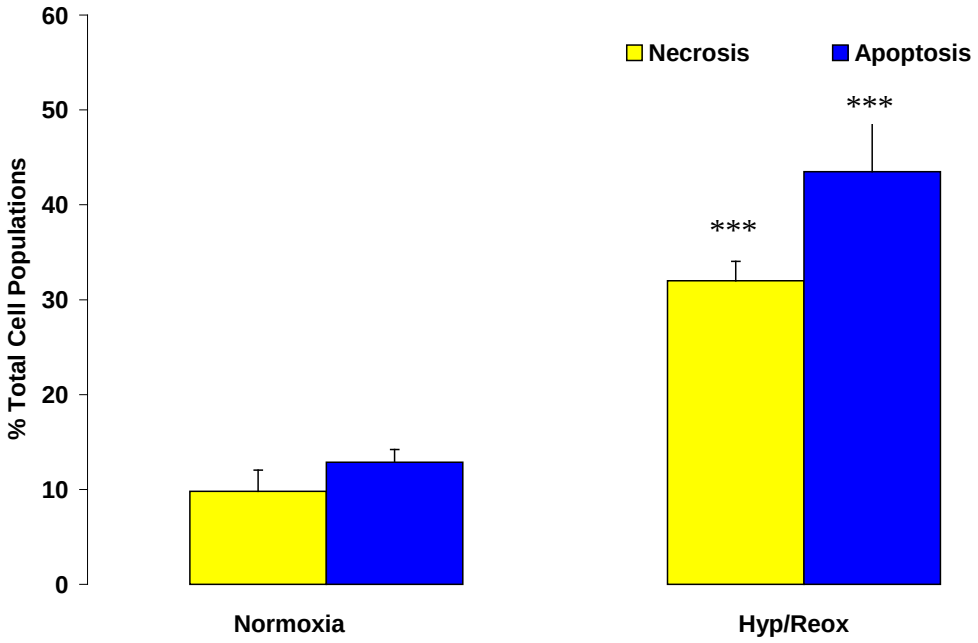
3.4.0. Isolated adult rat cardiomyocyte model of hypoxia reoxygenation injury.

3.4.1. Effect of 2-CL-IB-MECA when administered at reoxygenation in adult rat cardiomyocytes subjected to hypoxia reoxygenation.

Isolated adult rat cardiac myocytes were subjected to different protocols as described in figure 2.1.e. Isolated cells were either allowed to reoxygenate for 24 hours (normoxic group) or exposed to 6 hours of hypoxia followed by 18 hours of reoxygenation (Hyp/Reox group) in the presence and absence of the A_3AR agonist 2-CL-IB-MECA or cell signalling pathway inhibitors that were administered either at reoxygenation or post-reoxygenation. At the end of reoxygenation the myocytes were

assessed on the flow cytometer to determine the percentage of live, apoptotic and necrotic cells.

Isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation resulted in a 320% increase in the number of apoptotic cells compared to the non-hypoxic normoxic group (42 ± 5 % Hyp/Reox vs. 13 ± 1% Normoxia, P<0.001) (Figure 3.4.a). Isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation also resulted in a 310% increase in the number of necrotic cells compared to the non-hypoxic normoxic group (32 ± 2% Hyp/Reox vs. 11 ± 2 % Normoxia, P<0.001) (Figure 3.4.a).



*****P<0.001 Hyp/Reox vs. Normoxia.**

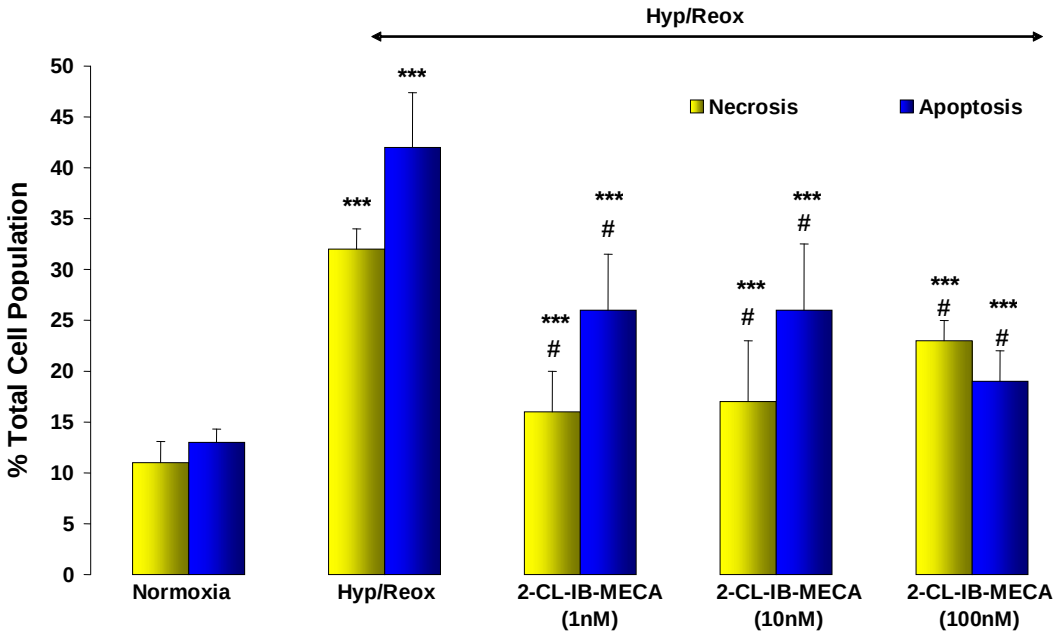
Figure 3.4.a. Assessment of apoptosis and necrosis in isolated adult rat cardiomyocytes subjected to 24 hours of oxygenation (Normoxia) or 6 hours hypoxia and 18 hours of reoxygenation (hyp/reox). Results are shown as Mean ± SEM and are expressed as a percentage of 10,000 cells counted from 9 individual experiments.

To ascertain the role of A_3ARs in limiting the deleterious consequences of reoxygenation injury adult rat cardiac myocytes were subjected to 6 hours of hypoxia and 18 hours of reoxygenation where the A_3AR agonist 2-Cl-IB-MECA (1 nM, 10 nM, 100 nM) was administered throughout the reoxygenation period. Different doses of 2-CL-IB-MECA were administered at reperfusion to determine whether increasing the dose increased 2-CL-IB-MECA's cytoprotective effect.

Administration of the A_3 agonist 2-Cl-IB-MECA (1 nM) throughout reoxygenation significantly decreased the percentage of apoptotic myocytes compared to the Hyp/Reox group (26 ± 6% 2-CL-IB-MECA vs. 42 ± 5 % Hyp/Reox, P<0.001) (Figure 3.4.a). Administration of the A_3 agonist 2-Cl-IB-MECA (1nM) throughout reperfusion also significantly decreased the percentage of necrotic myocytes compared to the Hyp/Reox group (16 ± 4 % 2-CL-IBMECA vs. 32 ± 2 % Hyp/Reox, P<0.001) (Figure 3.4.b). At higher concentrations of the A_3AR agonist 2-Cl-IB-MECA (10 nM and 100 nM) myocytes were significantly protected from reoxygenation injury in an anti-apoptotic and anti-necrotic manner. Administration of 2-CL-IB-MECA (10 nM) throughout reoxygenation significantly decreased the number apoptotic myocytes compared to the Hyp/Reox group (26 ± 8% 2-CL-IBMECA vs. 42 ± 5 % Hyp/Reox, P<0.001) Figure 3.4.b. Administration of 2-CL-IB-MECA (10nM) throughout reoxygenation significantly decreased the number of necrotic myocytes compared to the Hyp/Reox group (17 ± 6% 2-CL-IB-MECA vs. 32 ± 2 Hyp/Reox, P<0.001) (Figure 3.4.b). Administration of 2-CL-IB-MECA (100 nM) throughout reoxygenation significantly decreased the number of apoptotic cells compared to the Hyp/Reox group (19 ± 3 % 2-CL-IB-MECA vs. 42 ± 5% Hyp/Reox P<0.001) (Figure 3.4.b). Furthermore, administration of 2-CL-IB-MECA (100 nM)

throughout reoxygenation significantly decreased the number of necrotic cells compared to the Hyp/Reox group (23 ± 2 % 2-CL-IB-MECA vs. 32 ± 2 % Hyp/Reox, $P<0.001$) (Figure 3.4.b). Characterisation of the effect of the A_3 agonist at different concentrations 2-CL-IB-MECA (1 nM, 10 nM, 100 nM) in attenuating hypoxia reoxygenation injury showed all the concentrations to be cardioprotective. Furthermore, that there was no dose response relationship observed between the different concentrations of 2-CL-IBMECA in protecting myocytes from hypoxia reoxygenation injury when administered at reoxygenation ($P>0.05$) (Figure 3.4.b).

Previous studies have shown concentrations of 2-CL-IB-MECA greater then 10 nM cause vasodilation in the Langendorff isolated perfused rat heart model that were not seen at the 1 or 10 nM concentration (Maddock et al., 2003). Therefore 2-CL-IB-MECA was used at the 1 nM concentration in all experiments unless stated.



***** P<0.001 Hyp/Reox, 2-CL-IB-MECA (1nM, 10nM, 100nM) vs. Normoxia.**
#P<0.001 2-CL-IB-MECA (1nM, 10nM, 100nM) vs. Hyp/Reox.

Figure 3.4.b. Assessment of apoptosis and necrosis in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The A_3AR agonist 2-CL-IB-MECA (1 nM; 10 nM; 100 nM) was added at the onset of reoxygenation. Results are shown as Mean ± SEM and are expressed as a percentage of 10,000 cells counted from 9 individual experiments.

3.5.0. The role of MEK 1/2 – ERK 1/2 cell survival pathway in 2-CL-IB-MECA mediated cardioprotection in adult rat cardiomyocytes subjected to 6h hypoxia and 18h reoxygenation.

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 the non-treated Hyp/Reox group (Figure 3.4.b). To determine which mechanisms were involved in 2-CL-IB-MECA (1 nM) mediated cardioprotection we assessed the role of the MEK 1/2 – ERK 1/2 cell survival pathway using the MEK1/2 inhibitor UO126 (10 μM). Isolated myocytes were subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation and were incubated with the A_3 agonist 2-CL-

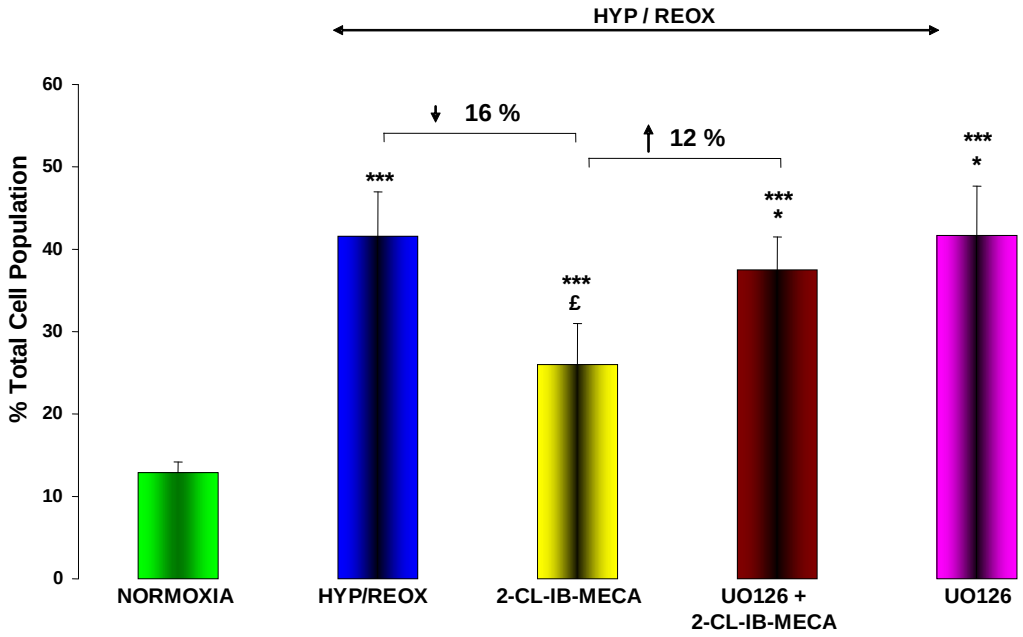
IB-MECA (1 nM) in the presence and absence of the MEK 1/2 inhibitor UO126 (10 μM) throughout the reoxygenation period.

Administration of the A_3 agonist 2-CL-IB-MECA (1 nM) throughout reoxygenation in the presence of the MEK 1/2 inhibitor UO126 (10 μM) significantly abolished the anti-apoptotic effect of 2-CL-IB-MECA (1 nM) compared to when administered alone throughout reoxygenation (38 ± 4 % 2-CL-IB-MECA + UO126 vs. 26 ± 6 % 2-CL-IB-MECA, $P<0.05$) (Figure 3.5.a and 3.6). Administration of the A_3 agonist 2-CL-IB-MECA (1 nM) throughout reoxygenation in the presence of the MEK 1/2 inhibitor UO126 (10μM) also significantly abolished the anti-necrotic effect of 2-CL-IB-MECA (1nM) compared to when administered alone throughout reoxygenation (25 ± 3 % 2-CL-IB-MECA + UO126 vs. 16 ± 4 % 2-CL-IB-MECA, $P<0.05$) (Figure 3.5.b and 3.6.)

Administration of UO126 (10 μM) alone throughout reoxygenation had no significant effect on cellular apoptosis when compared to the Hyp/Reox group (42 ± 6 % UO126 vs. 42 ± 5 % Hyp/Reox, $P>0.05$) (Figure 3.5.a). Administration of UO126 (10 μM) alone throughout reoxygenation was seen to significantly decrease the number of cells dying by cellular necrosis when compared to the Hyp/Reox group (23 ± 3 % UO126 vs. 32 ± 2 % Hyp/Reox $P<0.01$) (Figure 3.5. b).

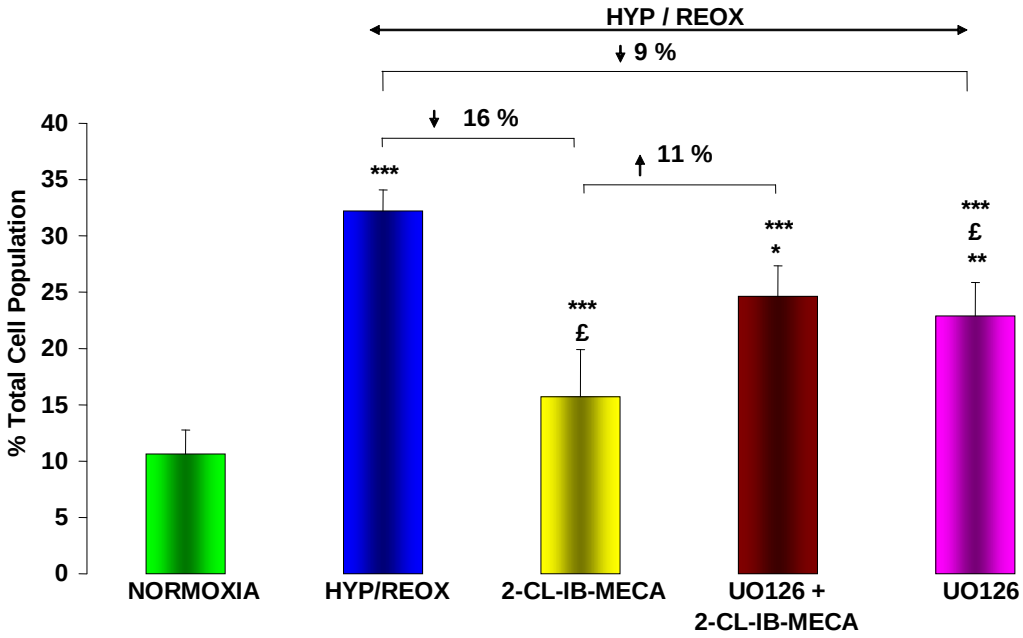
Figure 3.6 shows representative scatter graphs of isolated adult rat cardiac myocytes analysed by flow cytometry. The scatter graphs show myocytes that are normoxic or have undergone 6 hours of hypoxia and 18 hours of reoxygenation. Furthermore, the graphs show hypoxic reoxygenated rat cardiac myocytes where the A_3 agonist 2-CL-

IB-MECA (1 nM) was administered at the onset of reoxygenation in the presence and absence of the MEK 1/2 inhibitor UO126 (10 μM).



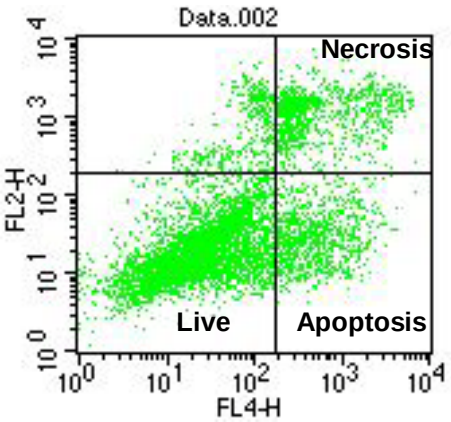
*****P<0.001 Hyp/Reox, 2-CL-IB-MECA, UO126 + 2-CL-IB-MECA, UO126 vs. Normoxia. £ P<0.001 2-CL-IB-MECA vs. Hyp/Reox. * P<0.05 UO126+ 2-CL-IB-MECA vs. 2-CL-IB-MECA.**

Figure 3.5.a. Assessment of Apoptosis in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. Assessing the role of the MEK1/2 – ERK1/2 cell survival pathway in 2-CL-IB-MECA mediated cardioprotection. The A_3AR agonist 2-CL-IB-MECA (1 nM) was administered at reoxygenation in the presence and absence of the MEK 1/2 inhibitor UO126 (10 μM). Results are shown as Mean ± SEM and are expressed as a percentage of 10,000 counted from nine individual experiments.



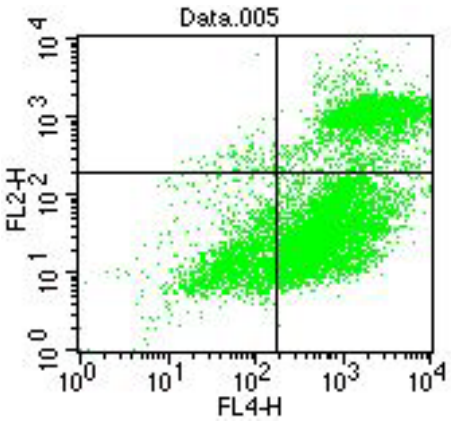
*****P<0.001 Hyp/Reox, 2-CL-IB-MECA, UO126+ 2-CL-IB-MECA, UO126 vs. Normoxia. *P<0.05 UO126 + 2-CL-IB-MECA vs. 2-CL-IB-MECA **P<0.01 UO126 vs. Hyp/Reox £P<0.001 2-CL-IB-MECA vs. Hyp/Reox.**

Figure 3.5.b. Assessment of necrosis in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. Assessing the role of the MEK1/2 – ERK1/2 cell survival pathway in 2-CL-IB-MECA mediated cardioprotection. The A_3AR agonist 2-CL-IB-MECA (1 nM) was administered at reoxygenation in the presence and absence of the MEK 1/2 inhibitor UO126 (10 μM). Results are shown as Mean ± SEM and are expressed as a percentage of 10,000 counted from nine individual experiments.



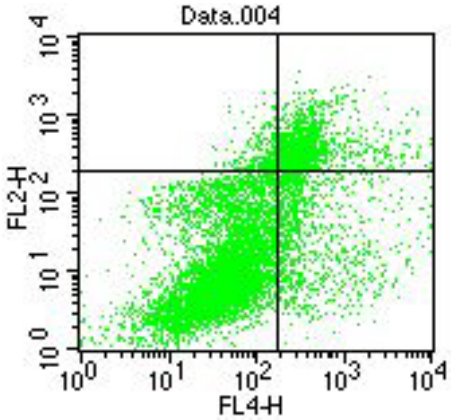
NORMOXIA

Quad	Events	Gated (%)
UL	396	6.4
UR	887	14.4
LL	4057	65.9
LR	815	13.2



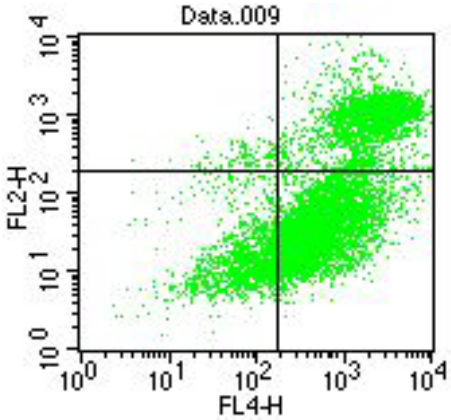
HYPOX/REOX

Quad	Events	Gated (%)
UL	75	1.0
UR	1920	24.7
LL	1473***	19.0
LR	4299	55.4



2-CL-IBMECA (1nM)

Quad	Events	Gated (%)
UL	310	3.8
UR	1934	23.7
LL	4893#	60.1
LR	1010	12.4



2-CL-IBMECA (1nM) + UO126 (10µM)

Quad	Events	Gated (%)
UL	113	1.4
UR	2449	29.4
LL	1363*	16.4
LR	4407	52.9

*** P<0.05 2-CL-IB-MECA+ UO126 vs. 2-CL-IB-MECA (t-test). #P<0.001 2-CL-IB-MECA vs. Hyp / Reox. ***P<0.001 Hyp/Reox vs. Normoxia.**

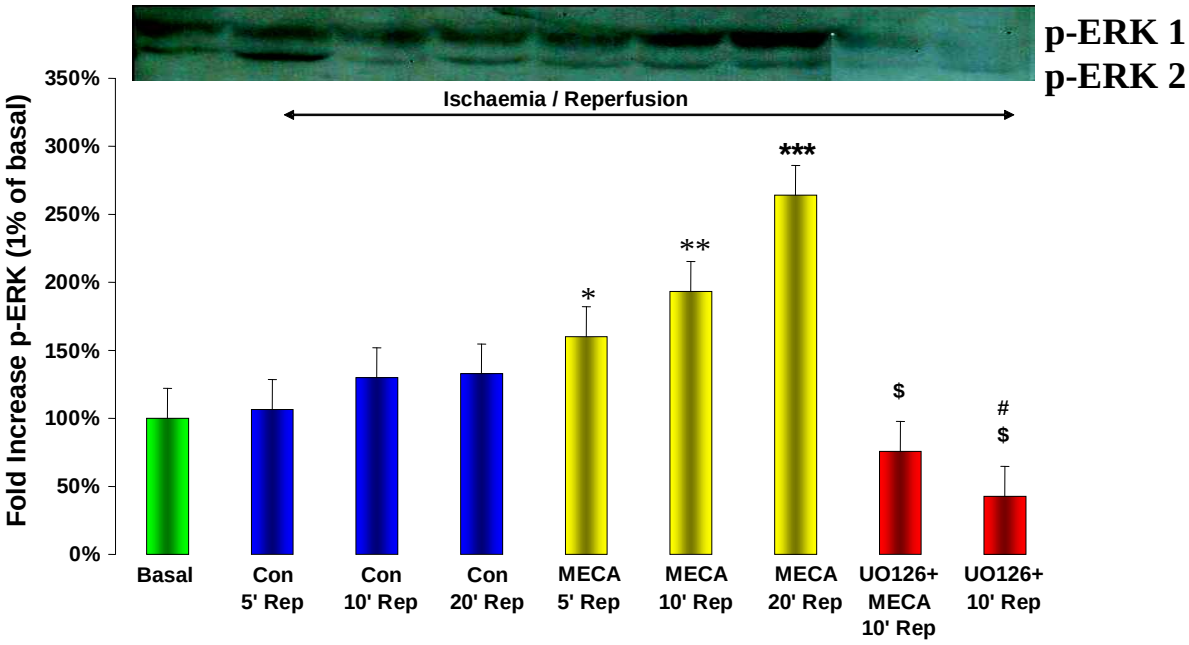
Figure 3.6. Representative scatter graphs from the FACS flow cytometer showing isolated adult rat cardiomyocytes subjected to Normoxia or 6 hours of hypoxia followed by 18 hours of reoxygenation. The A_3 adenosine receptor agonist 2-Cl-IB-MECA (1 nM) was administered at reoxygenation in the presence and absence of the MEK1/2 inhibitor UO126 (10 µM). Myocytes were treated with fluorochromes specific for apoptosis (Lower Right), necrosis (Upper Right) and Live cells (Lower left +Upper Left) (10µM).

3.6.0. Effect of 2-CL-IB-MECA when administered at reperfusion on ERK 1/2 phosphorylation during different time intervals after reperfusion in perfused rat hearts.

In order to investigate whether the ERK 1/2 are activated by 2-CL-IB-MECA (1 nM) isolated perfused rat heart underwent 35 minutes of ischaemia reperfusion for 5, 10 or 20 minutes of reperfusion where the A_3 agonist 2-CL-IB-MECA was administered at the onset of reperfusion. In the control group hearts underwent 35 minutes of ischaemia and 5, 10 or 20 minutes of reperfusion. Basal ERK1/2 activity was assessed by perfusing hearts for 60 minutes.

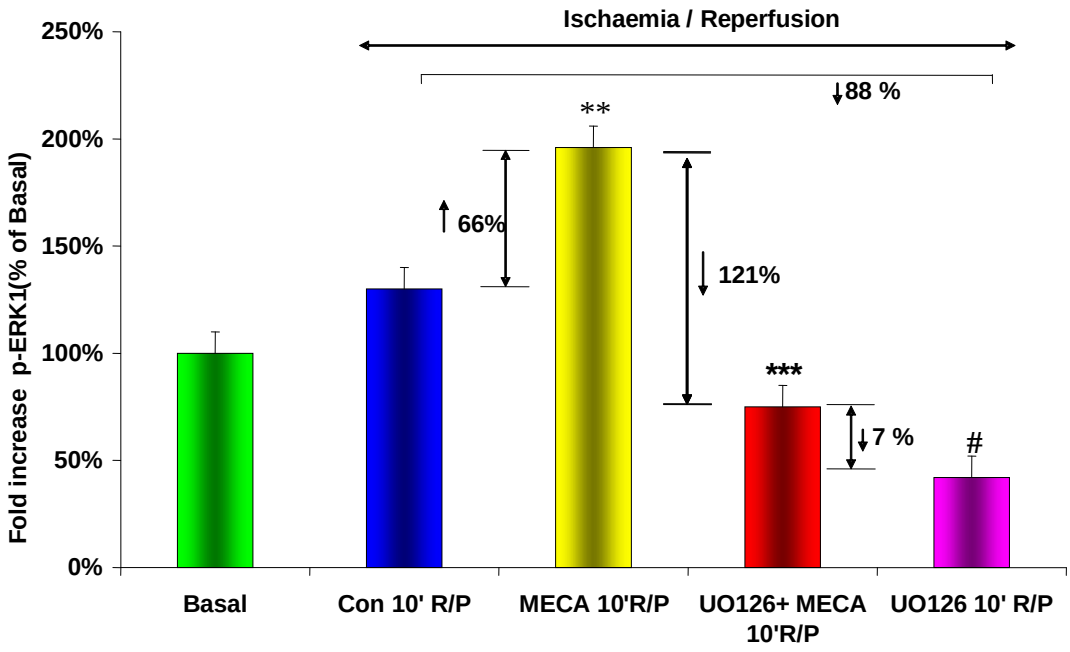
Administration of the A_3 agonist 2-Cl-IB-MECA (1 nM) at reperfusion significantly upregulated the phosphorylation of ERK1 in rat hearts subjected to 35 minutes of ischaemia and 5 minutes ($P<0.05$), 10 minutes ($P<0.01$) and 20 minutes ($P<0.001$) of reperfusion compared to their respective time matched controls (Figure 3.7.a, 3.7.b).

This upregulation of ERK1 phosphorylation by 2-CL-IB-MECA (1 nM) after 10 minutes of reperfusion was significantly abrogated by the co-administration of the MEK1/2 inhibitor UO126 (10 μM) ($P<0.01$) (Figure 3.7.a, 3.7.b). Administration of UO126 (10 μM) alone at reperfusion significantly decreased ERK1 phosphorylation compared to time matched controls $P<0.001$ (Figure 3.7.a, 3.7.b).



*** P<0.05 MECA 5 minutes Reperfusion vs. Control 5 minutes Reperfusion.**
****P<0.01 MECA 10 minutes reperfusion vs. Control 10 minutes Reperfusion.**
***** P<0.001 MECA 10 minutes reperfusion vs. Control 20 minutes Reperfusion.**
$ P<0.001UO126+MECA, UO126 vs. MECA 10 minutes Reperfusion.
 # P<0.001 UO126 vs. Control 10 minutes reperfusion.

Figure 3.7.a. Assessment of ERK 1 phosphorylation in isolated hearts subjected 60 minutes of perfusion, 35 minutes of ischaemia followed by 5, 10 or 20 minutes of reperfusion in the presence (MECA) and absence (control) of the A_3 Agonist 2-CL-IB-MECA (1 nM) (MECA). The A_3AR agonist 2-CL-IB-MECA (1 nM) was administered at reperfusion in the presence and absence of MEK 1/2 inhibitor UO126 (10μM). Results are shown as Mean ± SEM three individual experiments.

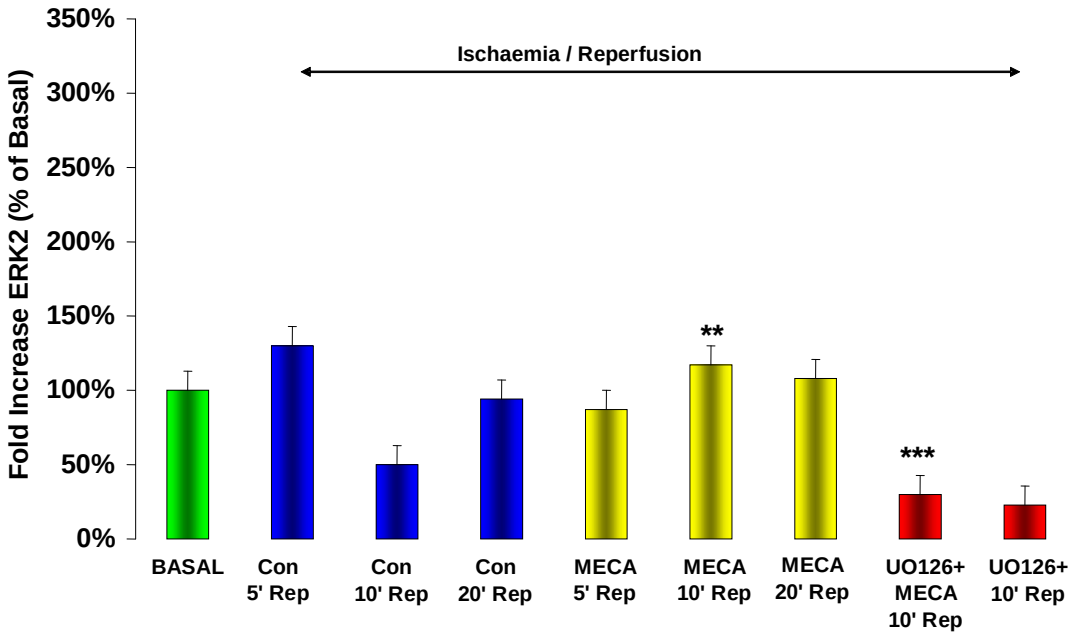


**** P<0.01 MECA 10 minutes Reperfusion vs. Control 10 minutes Reperfusion.**
***** P<0.001 UO126 +MECA vs. MECA 10 minutes Reperfusion.**
 # P<0.001 UO126 vs. Control 10 minutes reperfusion.

Figure 3.7.b. Comparison of ERK 1 phosphorylation in isolated hearts subjected 60 minutes perfusion (Basal), 35 minutes of ischaemia followed by 10 minutes of reperfusion in the presence (Meca) and absence (Con) of the A_3 Agonist 2-CL-IB-MECA (1 nM) (MECA) was administered at reperfusion in the presence and absence of MEK 1/2 inhibitor UO126 (10μM). Graph shows percentage change in ERK1 phosphorylation in ischaemic reperfused hearts following different treatments. Results are shown as Mean ± SEM three individual experiments.

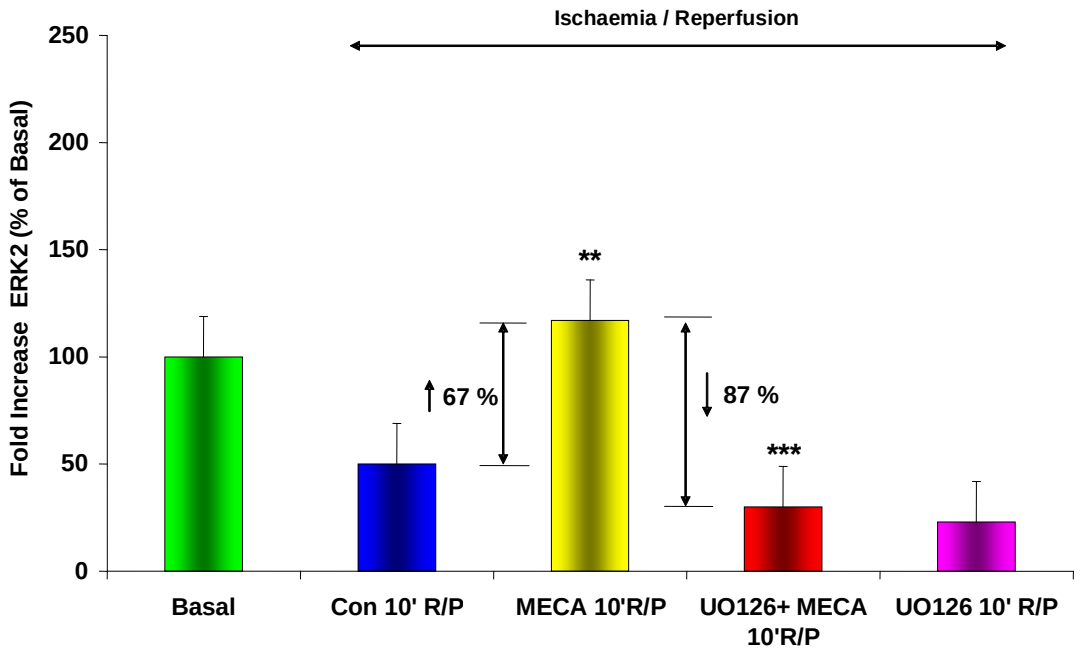
Administration of the A_3 agonist 2-Cl-IB-MECA (1 nM) at reperfusion significantly up regulated the phosphorylation of ERK2 after 10 minutes of reperfusion compared to non-treated time matched control hearts P<0.01 (Figure 3.8.a, 3.8.b). This upregulation of ERK2 phosphorylation by 2-CL-IB-MECA (1nM) after 10 minutes of reperfusion was significantly abrogated by the co-administration of the MEK1/2 inhibitor UO126 (10 μM) P<0.001 (Figure 3.8.a., 3.8.b). Administration of UO126 (10 μM) alone at the onset of reperfusion reduced ERK2 phosphorylation compared

to time matched controls but failed to reach statistical significance (P>0.05) (Figure 3.8.a, 3.8.b).



**** P<0.01 MECA 10 minutes reprfusion vs. Control 10 minutes Reperfusion.**
*****P<0.001 UO126 + MECA vs. MECA 10 minutes Reperfusion.**

Figure 3.8.a. Assessment of ERK2 phosphorylation in isolated hearts subjected 60 minutes of perfusion, 35 minutes of ischaemia followed by 5, 10 or 20 minutes of reperfusion in the presence (Meca) and absence (Con) of the A_3 Agonist 2-CL-IB-MECA (1 nM) (MECA). The A_3AR agonist 2-CL-IB-MECA (1 nM) was administered at reperfusion in the presence and absence of MEK 1/2 inhibitor UO126 (10μM). Results are shown as Mean ± SEM three individual experiments.

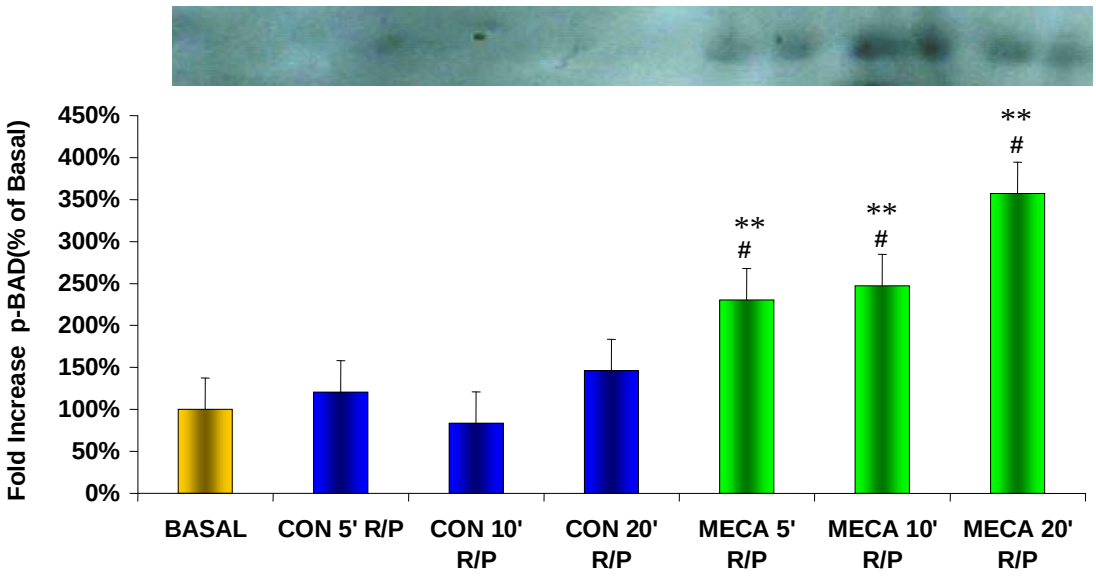


**** P<0.01 MECA 10 minutes reperfusion vs. Control 10 minutes Reperfusion.**
***** P<0.001 UO126 +MECA vs. MECA 10 minutes Reperfusion.**

Figure 3.8.b. Assessment of ERK2 phosphorylation in isolated hearts subjected 60 minutes perfusion (Basal), 35 minutes of ischaemia followed by 10 minutes of reperfusion where the A_3 Agonist 2-CL-IB-MECA (1 nM) was administered at reperfusion in the presence (MECA) and absence (Con) of MEK 1/2 inhibitor UO126 (10μM). Graph shows percentage change in ERK2 phosphorylation in ischaemic reperfused hearts following different treatments. Results are shown as Mean ± SEM three individual experiments.

3.7.1. BAD $_{(Ser112)}$ phosphorylation during different time intervals post reperfusion

Administration of the A_3 agonist 2-Cl-IB-MECA (1 nM) at reperfusion significantly up-regulated the phosphorylation of BAD $_{(Ser112)}$ after 5 minutes, 10 minutes and 20 minutes of reperfusion compared to time matched controls, P<0.01 (Figure 3.9).



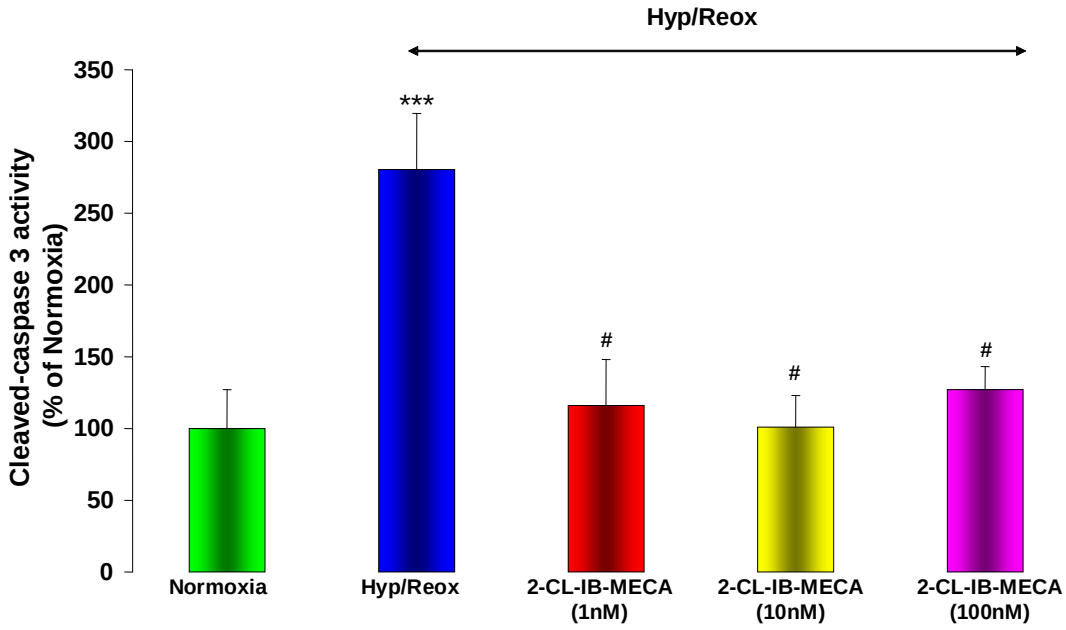
**** P<0.01 MECA 5,10,20 minutes reperfusion vs. Control 5,10,20 minutes reperfusion . #P<0.01 MECA 5,10,20 minutes reperfusion vs. Basal.**

Figure 3.9. Assessment of BAD (Ser 112) phosphorylation in isolated hearts subjected to 60 minutes of perfusion (Basal) or hearts subjected to 35 minutes ischaemia followed by 5, 10 or 20 minutes of reperfusion in the presence and absence (con) of the A_3 Agonist 2-CL-IB-MECA (1 nM) (MECA). Results are shown as Mean ± SEM three individual experiments.

3.8.0 Effect 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 181% increase in cleaved-caspase 3 activity compared to the normoxic group (281 ± 39% Hyp/Reox vs. 100 ± 27 % Normoxia $P<0.001$) (Figure 3.10). Administration of the A_3 agonist 2-Cl-IB-MECA (1 nM) throughout reoxygenation significantly decreased cleaved-caspase 3 activity compared to the Hyp/Reox group (116 ± 32 % 2-CL-IB-MECA (1nM) vs. 281 ± 39 % Hyp/Reox $P<0.001$) (Figure 3.10). 2-CL-IB-MECA (10 nM) administered at reoxygenation was also seen to significantly decrease cleaved-caspase 3 activity compared to the Hyp/Reox group (101 ± 22 % 2-CL-IB-MECA (10 nM) vs. 281 ± 39 % Hyp/Reox $P<0.001$) (Figure 3.10). 2-CL-IB-MECA (100 nM) administered at reoxygenation was also seen to significantly decrease cleaved-caspase 3 activity compared to the Hyp/Reox group (127 ± 16 % 2-CL-IB-MECA (100 nM) vs. 281 ± 39 % Hyp/Reox $P<0.001$) (Figure 3.10).

There was no dose response relationship by administering different doses of 2-CL-IB-MECA (1 nM, 10 nM, 100 nM) on decreasing cleaved-caspase 3 activity when administered at reoxygenation in adult rat cardiomyocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation ($P>0.05$) (Figure 3.10). The data suggest that the cardioprotective effects of 2-CL-IB-MECA are via anti-apoptotic mechanisms.



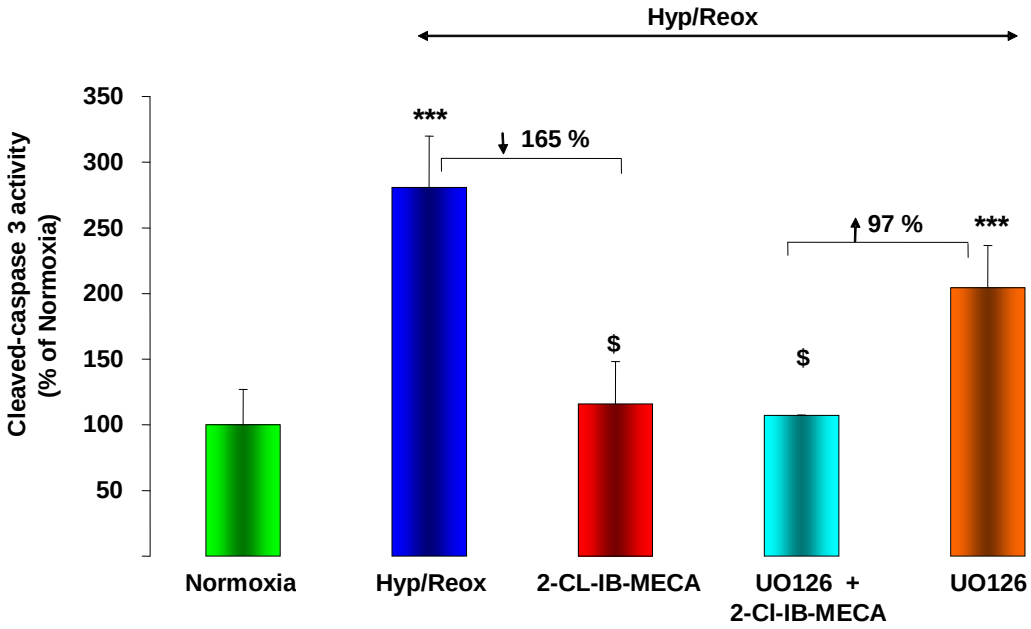
***** P<0.001 Hyp/Reox vs. Normoxia. # P <0.001 2-CL-IB-MECA 1nM,10nM,100nM vs. Hyp/Reox. Mean ± SEM of 5 individual experiments.**

Figure 3.10. Cleaved-caspase 3 activity in isolated adult rat cardiac myocytes subjected to 24 hours of oxygenation (Normoxia) or 6 hours of hypoxia followed by 18 hours of reoxygenation (Hyp/Reox). The A_3 agonist 2-CL-IB-MECA (1 nM, 10 nM, 100 nM) was administered at the onset of reoxygenation.

3.8.1. Role of MEK1/2-ERK1/2 cell survival pathway in cleaved-caspase 3 activity upon administration of 2-CL-IB-MECA at reoxygenation in isolated adult rat cardiomyocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation.

Administration of 2-CL-IB-MECA (1 nM) at reoxygenation significantly reduced cleaved-caspase 3 activity compared to isolated adult rat cardiomyocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation P<0.001, (Figure 3.10). To determine whether the decrease in cleaved-caspase 3 activity by 2-C-IB-MECA (1nM) involved the MEK1/2 – ERK1/2 cell survival pathway the MEK1/2 inhibitor UO126 was used. Isolated adult rat cardiac myocytes were subjected to 6 hours of hypoxia and 18 hours of reoxygenation where the A_3 agonist 2-CL-IB-MECA (1 nM) was administered at reoxygenation in the presence and absence of the MEK 1/2 inhibitor UO126 (10 μM).

Administration of the A_3 agonist 2-CL-IB-MECA (1nM) in the presence of the MEK1/2 inhibitor UO126 (10 μM) throughout reoxygenation failed to block the decrease in cleaved-caspase 3 activity afforded when 2-CL-IB-MECA (1 nM) was administered alone at reoxygenation (107 ± 0.2% 2-CL-IB-MECA + UO126 vs. 116 ± 32 % 2-CL-IB-MECA P>0.05) (Figure 3.11). UO126 (10 μM) when administered alone throughout reoxygenation had no significant effect on cleaved-caspase 3 activity compared to the Hyp/Reox group (204 ± 32% UO126 vs. 281 ± 39% Hyp/Reox P>0.05) (Figure 3.11). Administration of UO126 with 2-CL-IB-MECA at reoxygenation significantly reduced cleaved-caspase 3 activity compared to when UO126 was administered alone at reoxygenation.



***** P<0.001 Hyp/Reox vs. Normoxia.**
$ P<0.001 2-CL-IB-MECA, UO126+ 2-CL-IB-MECA vs. Hyp/Reox.
Mean ± SEM of 5 individual experiments.

Figure 3.11. 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 (Hyp/Reox) of the MEK1/2 inhibitor UO126 (10 μM).

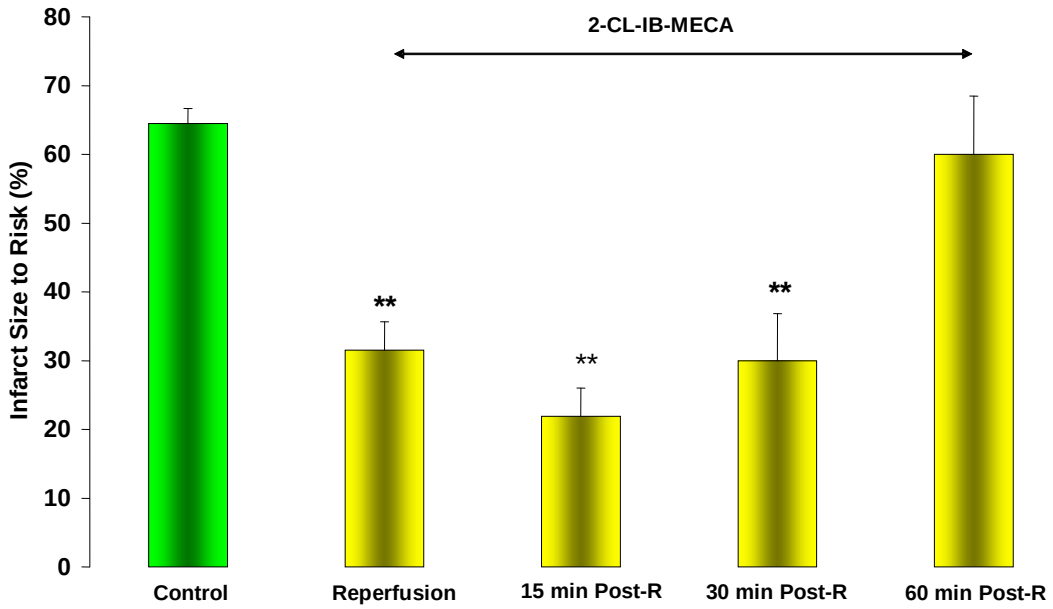
3.9. Conclusion

In conclusion we have presented novel data showing activation of A3ARs at reperfusion protects the isolated perfused rat heart from ischaemia reperfusion injury in a UO126 (MEK 1/2 inhibitor) sensitibe manner. The protection afforded by 2-CL-IB- MECA was associatated with an increase in ERK1/2 at different time point post reperfusion that was abolished in the presence of the MEK1/2 inhibitor UO126. Administration of 2-CL-IB-MECA throughout reoxygenation significantly decreased apoptosis and necrosis in adult rat cardiac myocytes subjected to hypoxia/reoxygenation injury in a UO126 sensitive manner. Furthermore, 2-CL-IB-MECA when administered at significantly decreased cleaved caspase 3 activity although this was not abolished by the MEK1/2 inhibitor UO126.

Chapter 4: Postponing the administration of 2-CL-IB-MECA protects the myocardium from ischaemia reperfusion injury via MEK1/2-ERK1/2 cell survival pathway

4.1. Results-Infarct size to risk ratio analysis

Isolated rat hearts underwent 35 minutes of ischaemia and 120 minutes of reperfusion were the A_3 agonist 2-CL-IB-MECA (1 nM) administration was postponed to 15, 30 and 60 minutes into the reperfusion period. Delayed administration of 2-CL-IB-MECA (1 nM) 15 minutes into reperfusion significantly protected the myocardium from ischaemic reperfusion injury compared to controls (22 ± 4% 2-CL-IB-MECA-Post 15 vs. 65 ± 2% Control $P<0.01$) Figure 4.1. Delaying the administration of 2-CL-IB-MECA (1 nM) to 30 minutes after the onset of reperfusion still significantly protected the myocardium from ischaemia reperfusion injury compared to controls (30 ± 7 % 2-CL-IB-MECA Post 30 vs. 65 ± 2% Control $P<0.01$) Figure 4.1. Postponing the administration of 2-CL-IB-MECA (1 nM) 60 minutes after the initiation of reperfusion abolished 2-CL-IB-MECA dependant cardioprotection seen when administered at reperfusion or at 15 and 30 minutes after reperfusion (60 ± 9 % 2-CL-IB-MECA Post 60 vs. 65 ± 2 Control $P>0.05$) Figure 4.1.



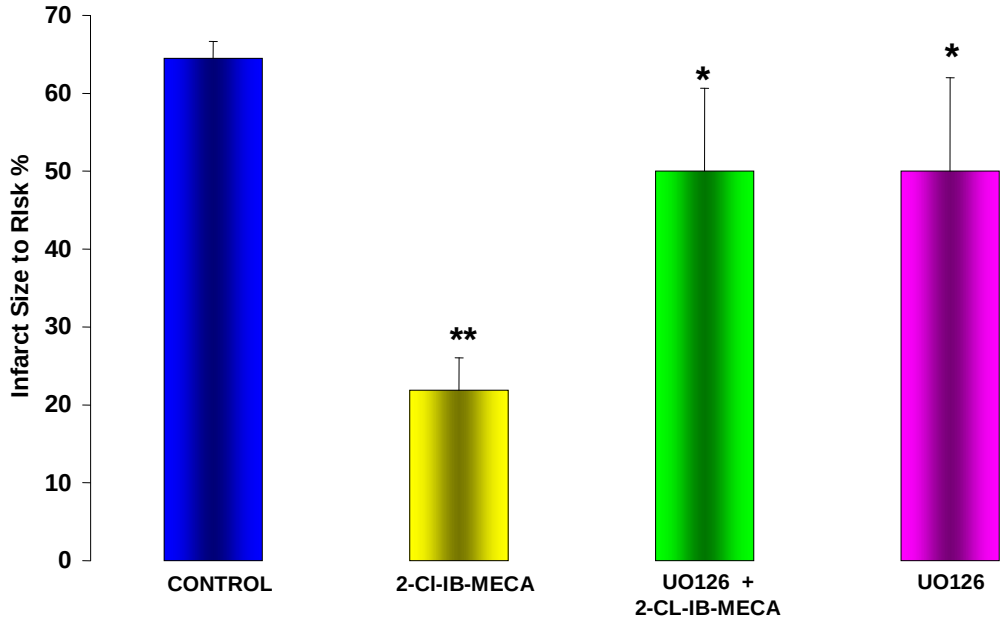
****P<0.01 2-CL-IB-MECA at reperfusion, 15 or 30, min Post-R vs. Control. Results are expressed as Mean ± SEM for 4 – 9 different experiments.**

Figure 4.1. Infarct size to Risk ratio (%) in non-treated control and 2-Cl-IBMECA (1nM) treated ischaemic reperfused hearts. Isolated perfused rat hearts where subjected to 35 minutes of ischaemia and 120 minutes of reperfusion where the A_3 adenosine receptor 2-Cl-IB-MECA (1 nM) was administered at reperfusion or 15 minutes (15 min Post-R) 30 minutes (30 min Post-R) or 60 minutes (60 min Post-R) after reperfusion.

4.2.1. Administration of the A_3 agonist 2-CL-IB-MECA 15 minutes post reperfusion protects the ischaemic myocardium via the MEK1/2–ERK 1/2 cell survival pathway.

We have previously shown that the administration of 2-CL-IB-MECA (1 nM) at reperfusion protects the ischaemic reperfused myocardium from reperfusion injury via recruitment of the MEK1/2 - ERK1/2 cell survival pathway. We assessed the involvement of the MEK1/2 - ERK1/2 pathway when the A_3 agonist 2-CL-IB-MECA was administered 15 minutes after the onset of reperfusion in the presence and absence of the MEK1/2 inhibitor UO126 (10 μM).

Administration of the A_3 agonist 2-CL-IB-MECA (1 nM) 15 minutes post-reperfusion in the presence of MEK1/2 inhibitor UO126 (10 μM) abolished 2-CL-IB-MECA (1 nM) dependant cardioprotection (22 ± 4 % 2-CL-IB-MECA vs. 50 ± 11 % 2-CL-IB-MECA + UO126 P<0.05) Figure.4.2. The MEK1/2 inhibitor UO126 (10 μM) when administered alone had no significant effect on the development of infarction compared to control (50 ± 12 UO126 vs. 65± 2% Control P>0.05) Figure.4.2.



**** P<0.01 2-CL-IB-MECA vs. Control. * P<0.05 UO126 + 2-CL-IB-MECA, UO126 vs. 2-CL-IB-MECA at 15 minutes post-reperfusion. Mean ± SEM.**

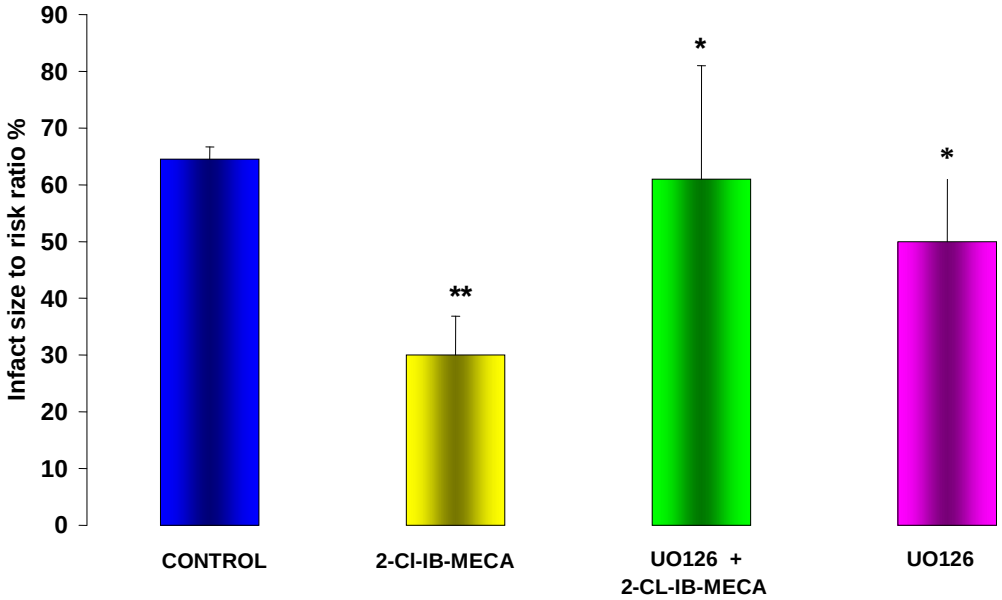
Figure 4.2. Infarct size to Risk ratio (%) in non-treated control and 2-Cl-IBMECA (1 nM) treated ischaemic reperfused hearts. Isolated perfused rat hearts were subjected to 35 minutes of ischaemia and 120 minutes of reperfusion where the A_3 adenosine receptor 2-Cl-IB-MECA (1 nM) was administered at 15 minutes after reperfusion in the presence and absence of the MEK1/2 inhibitor UO126 (10 μM).

Previously, we have shown that the administration of the A_3 agonist 2-CL-IB-MECA (1 nM) protects the ischaemic myocardium via recruitment of the MEK1/2-ERK1/2 cell survival pathway when administered at reperfusion or 15 minutes post reperfusion. To determine if the protection afforded by 2-CL-IB-MECA (1 nM) when

administered 30 minutes post reperfusion is via recruitment of the MEK1/2-ERK1/2 pathway we used the MEK1/2 inhibitor UO126 (10 μM).

4.2.2. Administration of the A_3 agonist 2-CL-IB-MECA 30 minutes post reperfusion protects the ischaemic myocardium via the MEK1/2 – ERK 1/2 cell survival pathway.

Administration of the A_3 agonist 2-CL-IB-MECA (1 nM) 30 minutes post-reperfusion in the presence of MEK1/2 inhibitor UO126 (10 μM) abolished 2-CL-IB-MECA (1 nM) dependant cardioprotection (62 ± 18 % 2-CL-IB-MECA + UO106 vs. 30 ± 7% 2-CL-IB-MECA 30 minutes Post R P<0.05) Figure 4.3. The MEK1/2 inhibitor UO126 (10 μM) when administered alone had no significant effect on the development infarct size to risk ratio (%) compared to control (50 ± 12 UO126 vs. 65 ± 2% Control P>0.05) Figure.4.3.



*** P<0.05 UO126+ 2-CL-IB-MECA vs. 2-CL-IB-MECA at 15 minutes post-reperfusion. ** P<0.01 2-CL-IB-MECA vs. Control. Mean ± SEM.**

Figure 4.3. Infarct size to Risk ratio (%) in non-treated control and 2-Cl-IBMECA (1 nM) treated ischemic reperfused hearts. Isolated perfused rat hearts where subjected to 35 minutes of ischaemia and 120 minutes of reperfusion where the A_3 adenosine receptor 2-Cl-IB-MECA (1 nM) was administered at 30 minutes after reperfusion in the presence and absence of the MEK1/2 inhibitor UO126 (10 μM).

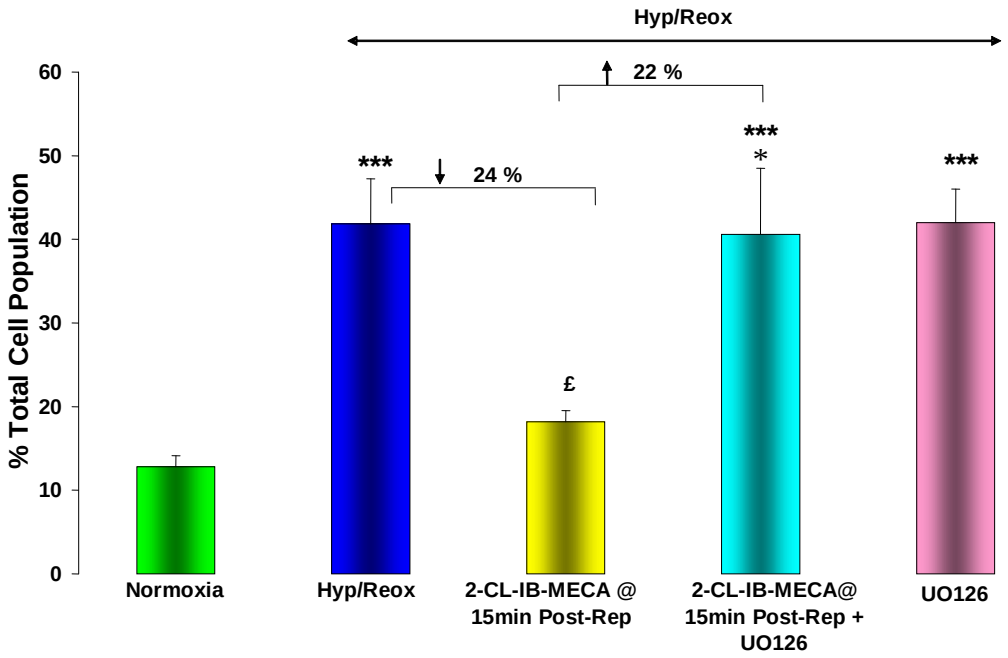
4.3.1. Postponing the administration of 2-CL-IB-MECA 15 minutes after the onset of reoxygenation protects isolated adult rat cardiomyocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation from reoxygenation injury via MEK1/2-ERK1/2 cell survival pathway.

In the previous section we showed that the A_3 adenosine receptor agonist 2-CL-IB-MECA (1 nM) to protect the isolated perfused rat heart from ischaemia reperfusion injury when administered 15 minutes post reperfusion. In this section we determined whether the A_3 agonist 2-CL-IB-MECA when administered 15 minutes after the onset of reoxygenation in isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation. Postponing the administration of the A_3 agonist 2-CL-IB-MECA (1 nM) failed to protect isolated adult rat cardiac myocytes for hypoxia/reoxygenation injury (data not shown). Interestingly, postponing the administration of the A_3 agonist 2-CL-IB-MECA (10 nM) to 15 minutes after reoxygenation significantly decreased the number of apoptotic (18 ± 1 % 2-CL-IB-MECA Post-15 vs. 42 ± 5 % Hyp/Reox $P<0.001$) Figure 4.4.a. Postponing the administration of the A_3 agonist 2-CL-IB-MECA (10 nM) to 15 minutes after reoxygenation significantly decreased the number of necrotic cells (21 ± 1 % 2-CL-IB-MECA Post 15 vs. 32 ± 2 % Hyp/Reox $P<0.001$) Figure 4.4.b.

The anti-apoptotic effect of 2-CL-IB-MECA (10 nM) when administered 15 minutes after the onset of reoxygenation was significantly abolished in the presence of the MEK1/2 inhibitor UO126 (10 μM) (18 ± 1 % 2-CL-IB-MECA Post-15 vs. 41 ± 8 % 2-CL-IB-MECA + UO126 $P<0.05$) Figure 4.4.a. Interestingly, the anti-necrotic effect of 2-CL-IB-MECA (10 nM) when administered 15 minutes after the onset of reoxygenation was not significantly abolished in the presence of the MEK1/2

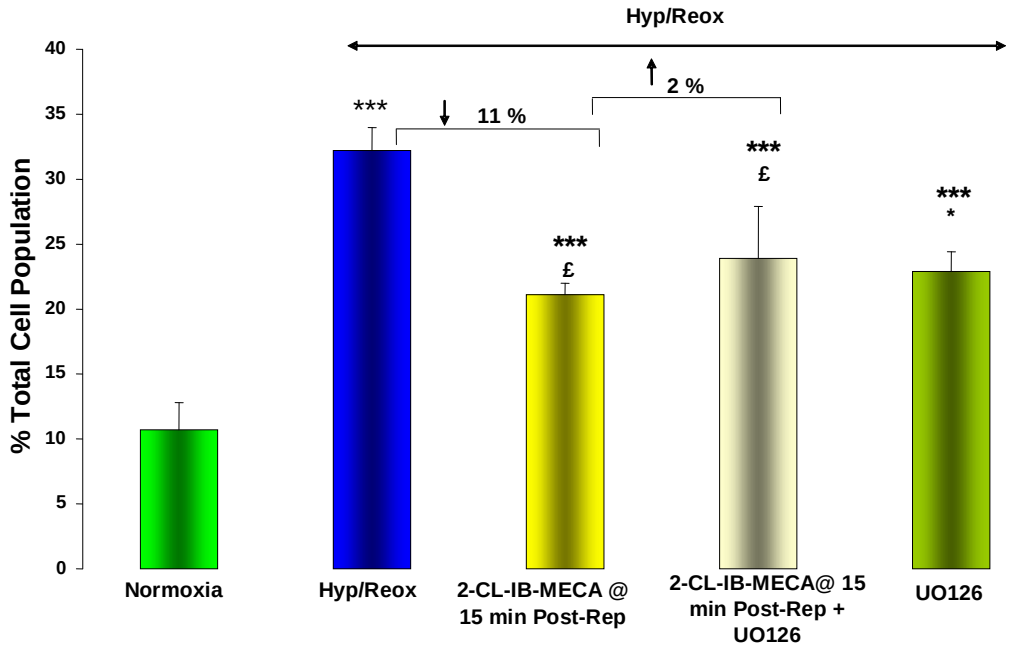
inhibitor UO126 (10 μM) (21 ± 1% 2-CL-IB-MECA vs. 24 ± 4 % 2-CL-IB-MECA + UO126, P>0.05) Figure 4.4.b.

UO126 (10 μM) when administered alone at reoxygenation had no significant effect of cellular apoptosis when compared to the Hyp/Reox group (42 ± 4 % UO126 vs. 42 ± 5 % Hyp/Reox P>0.05) Figure 4.4.a. UO126 (10 μM) when administered alone at reoxygenation was seen to significantly decrease the number of cells dying by cellular necrosis when compared the Hyp/Reox group (23 ± 3 % UO126 vs. 32 ± 2% Hyp/Reox P<0.05) Figure 4.4.b.



*****P<0.001 Hyp/Reox, 2-CL-IB-MECA + UO126, UO126 vs. Normoxia.**
£ P<0.001 2-CL-IB-MECA vs. Hyp/Reox.
*** P<0.05 2-CL-IB-MECA + UO126 vs. 2-CL-IB-MECA.**

Figure 4.4.a. Assessment of apoptosis in isolated adult rat cardiomyocytes subjected to 24 hours oxygenation (Normoxia) or 6 hours hypoxia and 18 hours of reoxygenation (Hyp/Reox). Assessing the role of the MEK1/2 – ERK1/2 cell survival pathway in 2-CL-IB-MECA mediated cardioprotection. The A_3AR agonist 2-Cl-IB-MECA (10 nM) was administered 15 minutes after the onset of reoxygenation in the presence and absence of the MEK 1/2 inhibitor UO126 (10 μM). Results are shown as Mean ± SEM and are expressed as a percentage of 10,000 total cells counted.



*****P<0.001 Hyp/Reox, 2-CL-IB-MECA, 2-CL-IB-MECA+UO126, UO126 vs. Normoxia. £ P<0.001 2-CL-IB-MECA + UO126 vs. Hyp/Reox. * P<0.05 UO126 vs. Hyp/Reox.**

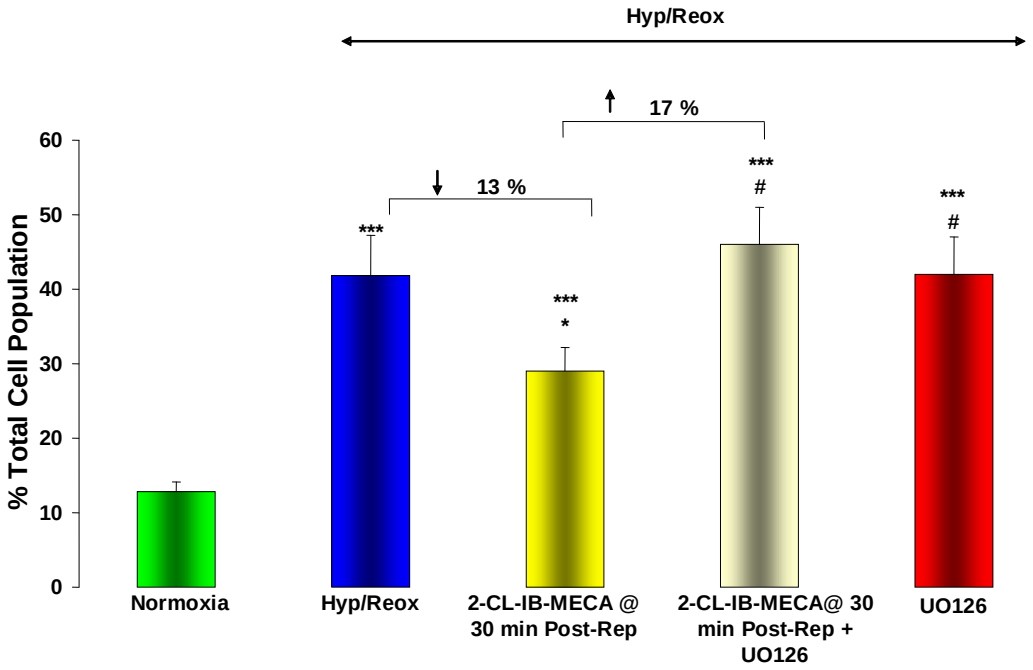
Figure 4.4.b. Assessment of necrosis in isolated adult rat cardiomyocytes subjected to 24 hours oxygenation (Normoxia) or 6 hours hypoxia and 18 hours of reoxygenation (Hyp/Reox). Assessing the role of the MEK1/2 – ERK1/2 cell survival pathway in 2-CL-IB-MECA mediated cardioprotection. The A_3AR agonist 2-Cl-IB-MECA (10 nM) was administered 15 minutes after the onset of reoxygenation in the presence and absence of the MEK 1/2 inhibitor UO126 (10 μM). Results are shown as Mean ± SEM and are expressed as a percentage of 10,000 total cells counted.

4.3.2. Postponing the administration of 2-CL-IB-MECA 30 minutes after the onset of reperfusion protects isolated adult rat cardiomyocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation from reoxygenation injury via MEK1/2-ERK1/2 cell survival pathway.

Postponing the administration of the A_3 agonist 2-CL-IB-MECA (10 nM) to 30 minutes after reoxygenation significantly decreased the number of apoptotic cells compared to the Hyp/Reox group (29 ± 3 % 2-CL-IB-MECA Post-30 vs. 42 ± 5 % Hyp/Reox P<0.01) Figure 4.5.a. Postponing the administration of the A_3 agonist 2-

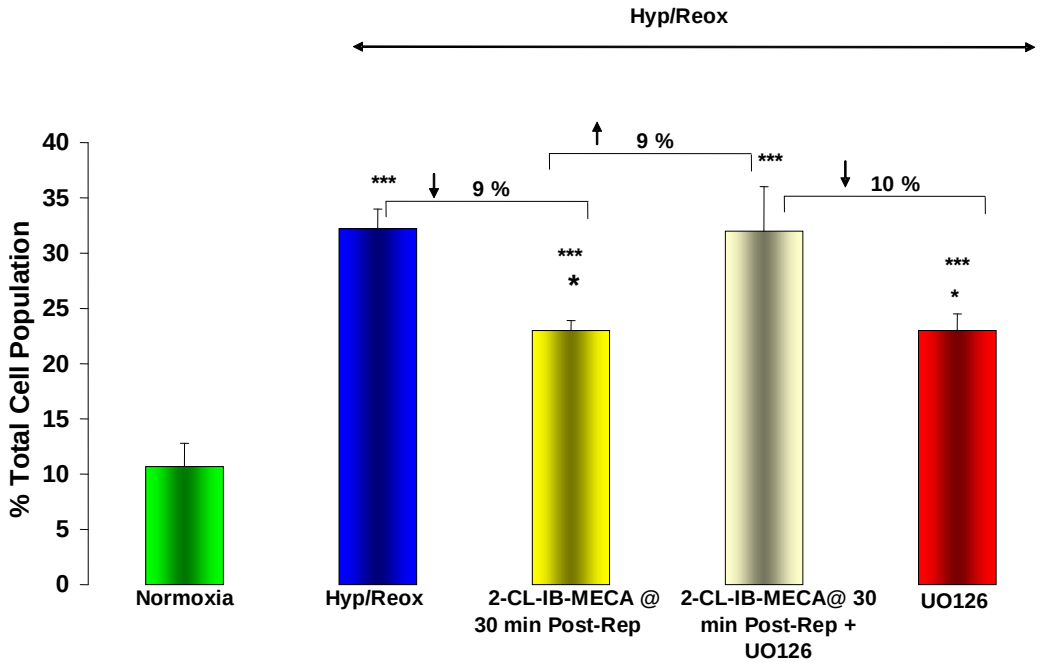
CL-IB-MECA (10 nM) to 30 minutes after reoxygenation significantly decreased the number of necrotic cells compared to the Hyp/Reox group (23 ± 3 % 2-CL-IB-MECA vs. 32 ± 2 % Hyp/Reox $P<0.05$) Figure 4.5.b. This anti-apoptotic effect of 2-CL-IB-MECA (10 nM) when administered 30 minutes after the onset of reoxygenation was significantly abolished in the presence of the MEK1/2 inhibitor UO126 (10 μM) (29 ± 3 % 2-CL-IB-MECA Post-30 vs. 46 ± 5 % 2-CL-IB-MECA Post-30 + UO126 $P<0.05$) Figure 4.5.a. Interestingly as previously shown, the anti-necrotic effect of 2-CL-IB-MECA (10 nM) when administered 30 minutes after the onset of reoxygenation was not significantly abolished in the presence of the MEK1/2 inhibitor UO126 (23 ± 3 % 2-CL-IB-MECA Post-30 vs. 32 ± 3 % 2-CL-IB-MECA Post-30 + UO126 $P>0.05$) Figure 4.5.b.

The MEK1/2 inhibitor UO126 (10 μM) when administered alone at reoxygenation had no significant effect of cellular apoptosis when compared to the Hyp/Reox group (42 ± 6 UO126 vs. 42 ± 5 Hyp/Reox, $P>0.05$) Figure 4.5.a. UO126 (10 μM) when administered alone at reoxygenation was seen to significantly decrease the number of cells dying by cellular necrosis when compared to the hypoxic reoxygenated group (23 ± 3 % UO126 vs. 32 ± 2% Hyp/Reox, $P<0.05$) Figure 4.5.b.



*****P<0.001 Hyp/Reox, 2-CL-IB-MECA, 2-CL-IB-MECA+UO126, UO126 vs. Normoxia. *P<0.05 2-CL-IB-MECA vs. Hyp/Reox. # P<0.05 2-CL-IB-MECA+UO126, UO126 vs. 2-CL-IB-MECA.**

Figure 4.5.a. Assessment of apoptosis in isolated adult rat cardiomyocytes subjected to 24 hours of oxygenation (Normoxia) or 6 hours hypoxia and 18 hours of reoxygenation (Hyp/Reox). Assessing the role of the MEK1/2 – ERK1/2 cell survival pathway in 2-CL-IB-MECA mediated cardioprotection. The A_3AR agonist 2-Cl-IB-MECA (10 nM) was administered 30 minutes after the onset of reoxygenation in the presence and absence of the MEK 1/2 inhibitor UO126 (10 μM). Results are shown as Mean ± SEM and are expressed as a percentage of 10,000 total cells counted.



*****P<0.001 Hyp/Reox, 2-CL-IB-MECA, 2-CL-IB-MECA+UO126, UO126 vs. Normoxia. *P<0.05 2-CL-IB-MECA, UO126 vs. Hyp/Reox.**

Figure 4.5.b. Assessment of necrosis in isolated adult rat cardiomyocytes subjected to 24 hours oxygenation (Normoxia) or 6 hours hypoxia and 18 hours of reoxygenation (Hyp/Reox). Assessing the role of the MEK1/2 – ERK1/2 cell survival pathway in 2-CL-IB-MECA mediated cardioprotection. The A_3AR agonist 2-Cl-IB-MECA (10 nM) was administered 30 minutes after the onset of reoxygenation in the presence and absence of the MEK 1/2 inhibitor UO126 (10 μM). Results are shown as Mean ± SEM and are expressed as a percentage of 10,000 total cells counted.

4.4.1. ERK 1/2 phosphorylation in 2-CL-IB-MECA mediated cardioprotection when administered 15 minutes post-reperfusion.

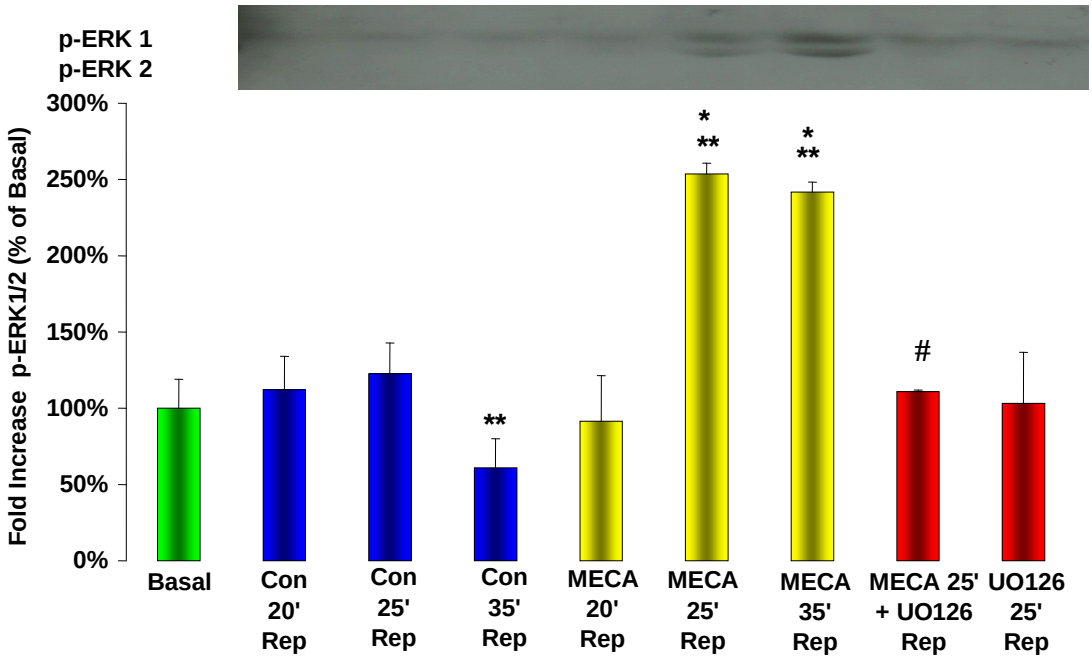
In the isolated perfused rat heart model of ischaemia reperfusion injury we showed that the postponing the administration of the A_3 agonist 2-Cl-IB-MECA (1 nM) to 15 minutes or 30 minutes after the onset of reperfusion protected the heart from ischaemia reperfusion injury. We further showed that this protection was abolished in the presence of the MEK 1/2 inhibitor UO126 (10 μM). In the isolated rat cardiomyocyte model of hypoxia reoxygenation injury we have shown that the delayed administration of 2-CL-IB-MECA (1 nM) 15 and 30 minutes post

reoxygenation confers cardioprotection that was abolished by the MEK 1/2 inhibitor UO126 (10 μM).

To determine the role of the MEK 1/2 – ERK 1/2 cell survival pathway in 2-CL-IB-MECA dependant cardioprotection hearts were treated with 2-CL-IB-MECA (1 nM) in the presence of the MEK 1/2 inhibitor UO126 (10 μM) at various time intervals post-reperfusion.

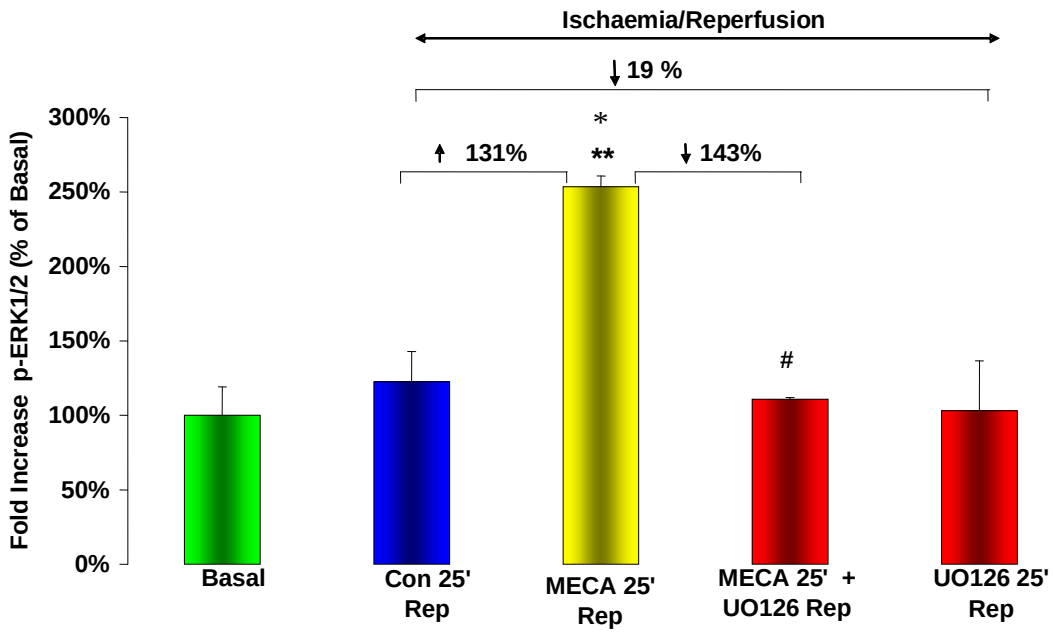
In control hearts ERK 1/2 phosphorylation was observed at 20, 25 and 35 minutes of reperfusion. Administration of 2-CL-IB-MECA (1 nM) after 15 minutes of reperfusion significantly up-regulated the phosphorylation of ERK 1/2 with maximal phosphorylation of ERK 1/2 after 25 minutes of reperfusion compared to time matched control, ($P<0.05$) Figure 4.6.a,b.

The increase in ERK1/2 phosphorylation by the administration of the A_3 agonist 2-CL-IB-MECA (1 nM) 15 minutes post reperfusion was significantly abolished in the presence of MEK1/2 inhibitor U0126 (10 μM) $P<0.05$, Figure.5.6. The administration of the MEK 1/2 inhibitor UO126 (10 μM) alone had no significant effect on ERK 1/2 phosphorylation compared to time matched controls ($P>0.05$), Figure 4.6.a, b.



*** P<0.05 MECA 25, 35 minutes reperfusion vs. Time matched controls. # P<0.05 MECA + UO126 vs. MECA 25 minutes Reperfusion. **P<0.01 Con 35, MECA 25, MECA 35 minutes reperfusion vs. Basal.**

Figure 4.6.a. Assessment of ERK 1/2 phosphorylation in isolated hearts subjected to 60 minutes perfusion (basal), 35 minutes of ischaemia followed by 20, 25 or 35 minutes of reperfusion in non-treated control and 2-CL-IB-MECA treated hearts (1 nM) (MECA). The A_3AR agonist 2-CL-IB-MECA (1 nM) was administered 15 minutes post- reperfusion in the presence and absence of the MEK 1/2 inhibitor UO126 (10 μM). Results are shown as Mean ± SEM of three individual experiments.



*** P<0.05 MECA 25 vs. Time matched controls. # P<0.05 vs. MECA 25 minutes Reperfusion. **P<0.01 MECA 25 vs. Basal.**

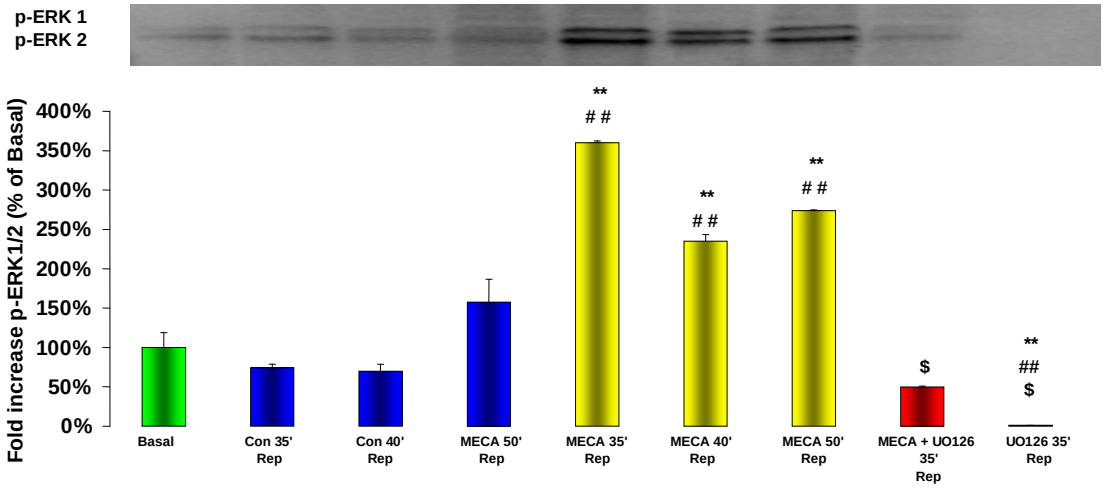
Figure 4.6.b. Assessment of ERK 1/2 phosphorylation in isolated hearts subjected to 35 minutes of ischaemia followed by 25 minutes of reperfusion in non-treated control and 2-CL-IB-MECA treated hearts (1 nM) (MECA). The A_3AR agonist 2-CL-IB-MECA (1 nM) was administered 15 minutes post-reperfusion in the presence and absence of the MEK 1/2 inhibitor UO126 (10 μM). Results are shown as Mean ± SEM of three individual experiments.

4.4.2. Role of MEK 1/2 – ERK 1/2 signalling pathway in 2-CL-IB-MECA mediated cardioprotection when administered 30 minutes post reperfusion.

Previously, we showed that 2-CL-IB-MECA (1nM) protects the ischaemic reperfused rat heart when administered 30 minutes post reperfusion in a MEK 1/2 – ERK 1/2 sensitive manner. To elucidate the cardioprotective cell signalling pathways involved in 2-CL-IB-MECA (1 nM) mediated cardioprotection when administered 30 minutes post reperfusion it was necessary to determine the phosphorylation status of ERK1/2 in heart tissues at various time points post reperfusion. Hearts were treated with 2-CL-IB-MECA (1 nM) in the presence and absence of the MEK 1/2 inhibitor U0126 (10 μM).

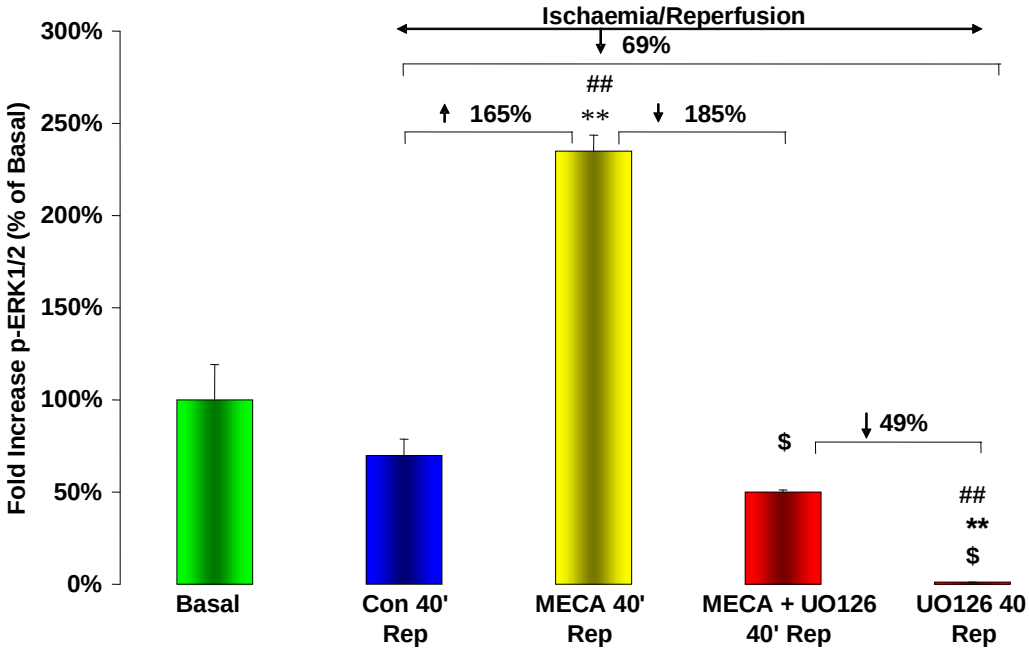
In control hearts ERK 1/2 phosphorylation was observed at 35, 40 and 45 minutes of reperfusion. Administration of the A_3AR agonist 2-CL-IB-MECA (1nM) significantly up regulated the phosphorylation of ERK with maximal phosphorylation of ERK 1/2 at 35 minutes of reperfusion compared to time matched controls, where the A_3 agonist was perfused 30 minutes post reperfusion ($P<0.01$), Figure. 4.7.a,b.

The increase in ERK1/2 phosphorylation after 40 minutes of reperfusion by 2-Cl-IB-MECA (1 nM) when administered 30 minutes after reperfusion was significantly abolished in the presence of the MEK1/2 inhibitor U0126 (10 μM) $P<0.01$, Figure 4.7.a, b. UO126 when administered alone at reperfusion significantly blocked ERK1/2 phosphorylation compared to the time matched control and basal groups $P<0.01$ (Figure 4.7.a, b).



**** P<0.01 MECA 35, 40, 50 minutes reperfusion, UO126 35minutes reperfusion vs. Basal. ## P<0.01 MECA 35, 40 50 minutes reperfusion vs. 35, 40, 50 minute controls. $ P<0.01 UO126+ 2-CL-IB-MECA, UO126 vs. MECA 40 minutes Reperfusion and Basal.**

Figure 4.7a. Assessment of ERK 1/2 phosphorylation in isolated hearts subjected to 60 minutes perfusion (basal), 35 minutes ischaemia followed by 35, 40 or 50 minutes of reperfusion in non-treated control and 2-CL-IB-MECA (1 nM) (MECA) treated hearts. The A_3AR agonist 2-CL-IB-MECA (1 nM) was administered after 30 minutes post reperfusion in the presence and absence of the MEK 1/2 inhibitor UO126 (10 μM). Results are shown as Mean ± SEM of three individual experiments.



**** P<0.01 MECA 40 minutes reperfusion, UO126 vs. Basal.**
P<0.01 MECA 40 minutes reperfusion, UO126 40 minutes reperfusion vs. Time matched controls.
$ P<0.01 MECA 40 minutes reperfusion + UO126, UO126 vs. MECA 40 minutes Reperfusion and Basal.

Figure 4.7.b. Assessment of ERK 1/2 phosphorylation in isolated hearts subjected to 60 minutes perfusion (basal), 35 minutes ischaemia followed by 40 minutes of reperfusion in non-treated control and 2-CL-IB-MECA (1 nM) (MECA) treated hearts. The A_3AR agonist 2-CL-IB-MECA (1 nM) was administered after 30 minutes post reperfusion in the presence and absence of the MEK 1/2 inhibitor UO126 (10 μM). Results are shown as Mean ± SEM of three individual experiments.

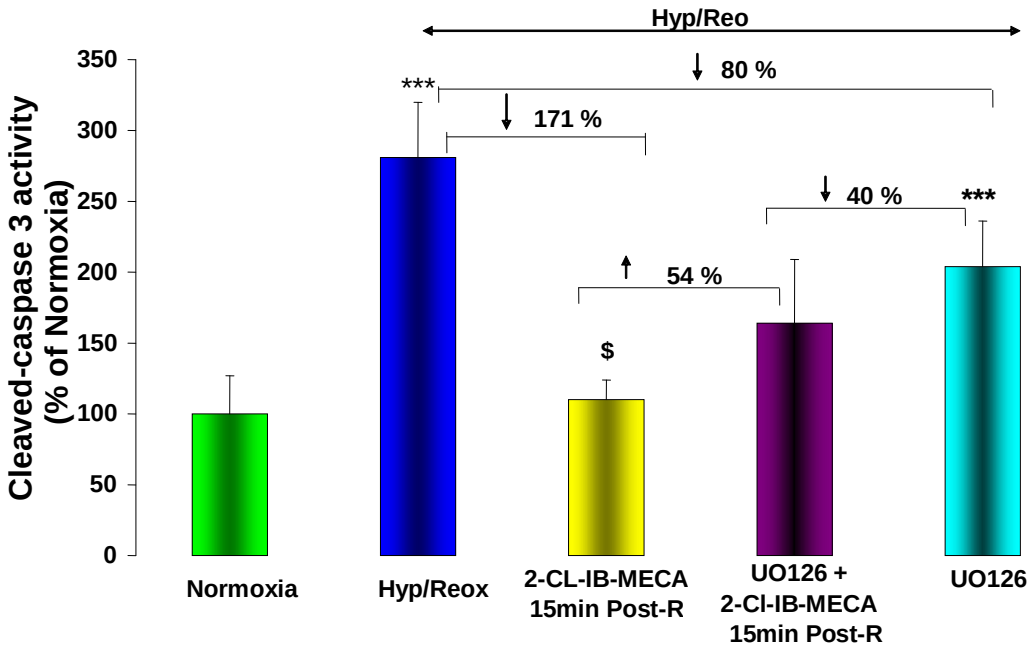
4.5.1. Role of MEK1/2-ERK1/2 cell survival pathway in cleaved-caspase 3 activity upon administration of 2-CL-IB-MECA (10 nM) 15 minutes post-reoxygenation in isolated adult rat cardiomyocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation.

To determine whether delaying the administration of 2-CL-IB-MECA (10 nM) 15 minutes post-reoxygenation could still decrease cleaved-caspase 3 activity adult rat cardiac myocytes were subjected to 6 hours of hypoxia and 18 hours of reoxygenation where 2-CL-IB-MECA (10 nM) was administered 15 minutes post reoxygenation. Administration of 2-CL-IB-MECA (10 nM) 15 minutes post reoxygenation significantly decreased cleaved-caspase 3 activity compared to the Hyp/Reox group (110 ± 14 % 2-CL-IB-MECA Post-15 vs. 281 ± 31 % Hyp/Reox $P<0.001$) Figure 5.8.

To determine whether the decrease in cleaved-caspase 3 activity by 2-CL-IB-MECA (10 nM) when administered 15 minutes after the onset of reoxygenation was via the MEK 1/2 – ERK 1/2 cell survival pathway isolated adult rat cardiomyocytes where subjected to 6 hours of hypoxia and 18 hours of reoxygenation where the A_3AR agonist 2-CL-IB-MECA (10 nM) was administered at 15 minutes post reperfusion in the presence and absence of the MEK 1/2 inhibitor UO126 (10 μM).

Administration of 2-CL-IB-MECA (10 nM) 15 minutes post reoxygenation in the presence of the MEK 1/2 inhibitor UO126 (10 μM) failed to abolish the decrease in cleaved-caspase 3 seen when 2-CL-IB-MECA was administered alone 15 minutes post reperfusion (110 ± 14 % 2-CL-IB-MECA Post-15 vs. 164 ± 61 % 2-CL-IB-MECA Post-15 + UO126 $P>0.05$), Figure 4.8. Administration of the MEK 1/2 inhibitor UO126 (10 μM) at reoxygenation had no significant effect on cleaved-

caspase 3 activity compared to the Hyp/Reox group (204 ± 32 % UO126 vs. 281 ± 39 % Hyp/Reox P>0.05), (Figure 4.8).



*****P<0.001 Hyp/Reox, UO126 vs. Normoxia.**
$ P<0.001 2-CL-IB-MECA vs. Hyp/Reox.
Mean ± SEM of 5 individual experiments.

Figure 4.8. Cleaved-caspase 3 activity in isolated adult rat cardiac myocytes subjected to 24 hours oxygenation (Normoxia) or 6 hours of hypoxia followed by 18 hours of reoxygenation (Hyp/Reox). The A_3 agonist 2-CL-IB-MECA (10 nM) was administered 15 minutes post-reoxygenation in the presence and absence of the MEK 1/2 inhibitor UO126.

4.5.2. Role of MEK1/2-ERK1/2 cell survival pathway in cleaved-caspase 3 activity upon administration of 2-CL-IB-MECA (10nM) 30 minutes post reoxygenation in isolated adult rat cardiomyocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation.

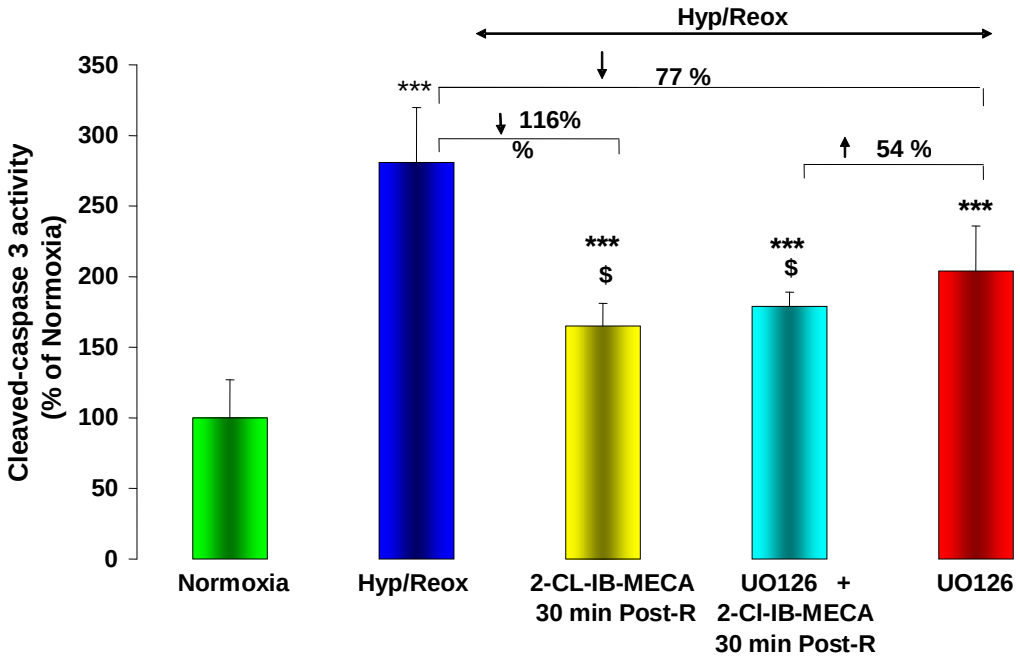
To further assess whether postponing the administration of 2-CL-IB-MECA (10 nM) 30 minutes after the onset of reoxygenation could still decrease cleaved-caspase 3 activity isolated adult rat cardiac myocytes were subjected to 6 hours of hypoxia and

18 hours of reoxygenation where the A_3 adenosine receptor agonist 2-CL-IB-MECA (10 nM) was administered 30 minutes after the onset of reoxygenation.

Administration of 2-Cl-IB-MECA (10 nM) 30 minutes after the onset of reoxygenation significantly reduced cleaved-caspase 3 activity compared to the Hyp/Reox group (165 ± 16 % 2-CL-IB-MECA Post-30 vs. 281 ± 39 % Hyp/Reox $P<0.001$) Figure 4.9.

To determine whether the decrease in cleaved-caspase 3 activity by 2-CL-IB-MECA (10 nM) when administered 30 minutes after the onset of reoxygenation was via the MEK1/2 – ERK1/2 cell survival pathway isolated adult rat cardiomyocytes were subjected to 6 hours of hypoxia and 18 hours of reoxygenation where the A_3AR agonist 2-CL-IB-MECA (10 nM) was administered at 30 minutes post reperfusion in the presence and absence of the MEK1/2 inhibitor UO126 (10 μM).

Administration of 2-CL-IB-MECA (10nM) 30 minutes after the onset of reoxygenation in the presence of the MEK 1/2 inhibitor UO126 (10 μM) failed to abolish the decrease in cleaved-caspase 3 compared to when the A_3 agonist 2-CL-IB-MECA (10 nM) was administered alone 30 minutes after the onset reoxygenation (165 ± 16 % 2-CL-IB-MECA Post-30 vs. 179 ± 10 % 2-CL-IB-MECA Post-30 + UO126 $P>0.05$) Figure 5.9. Administration of the MEK1/2 inhibitor UO126 (10 μM) alone at reoxygenation had no significant effect on cleaved-caspase 3 activity compared to the control Hyp/Reox group (204 ± 32 % UO126 vs. 281 ± 39 % Hyp/Reox $P>0.05$), Figure 4.9.



*****P<0.001 Hyp/Reox, 2-CL-IB-MECA, UO126 + 2-CL-IB-MECA, UO126 vs. Normoxia. $ P<0.001 2-CL-IB-MECA, UO125 + 2-CL-IB-MECA vs. Hyp/Reox. Mean ± SEM of 5 individual experiments.**

Figure 4.9. Cleaved-caspase 3 activity in isolated adult rat cardiac myocytes subjected to 24 hours oxygenation (Normoxia) or 6 hours of hypoxia followed by 18 hours of reoxygenation (Hyp/Reox). The A_3 agonist 2-CL-IB-MECA (10 nM) was administered 30 minutes post-reoxygenation in the presence and absence of the MEK1/2 inhibitor UO126 (10 μM).

4.6. Conclusion

In conclusion activation of A_3ARs with 2-CL-IB-MECA 15 or 30 minutes after the onset of reperfusion significantly reduced infarct size to risk ratio compared to non-treated hearts. This protection was abolished by the co-administreation of the MEK1/2 inhibitor UO126. Activation of A_3ARs 15 or 30 minutes post-reperfusion was associated with an increase in ERK1/2 that was abolished in the presence of hte MEK1/2 inhibitor UO126. Activation of A_3ARs 15 or 30 minutes post-reoxygenation significantly decreased cell death in an anti-apoptotic and anti-necrotic manner. The anti-apoptotic effect was abolished in the presence of the MEK1/2 inhibitor UO126, but did not block teh anti-necrotic effect. Activation of A_3ARs 15 or 30 minutes post

Reoxygenation was associated with a decrease in cleaved caspase 3 activity that was not blocked by the MEK1/2 inhibitor UO126.

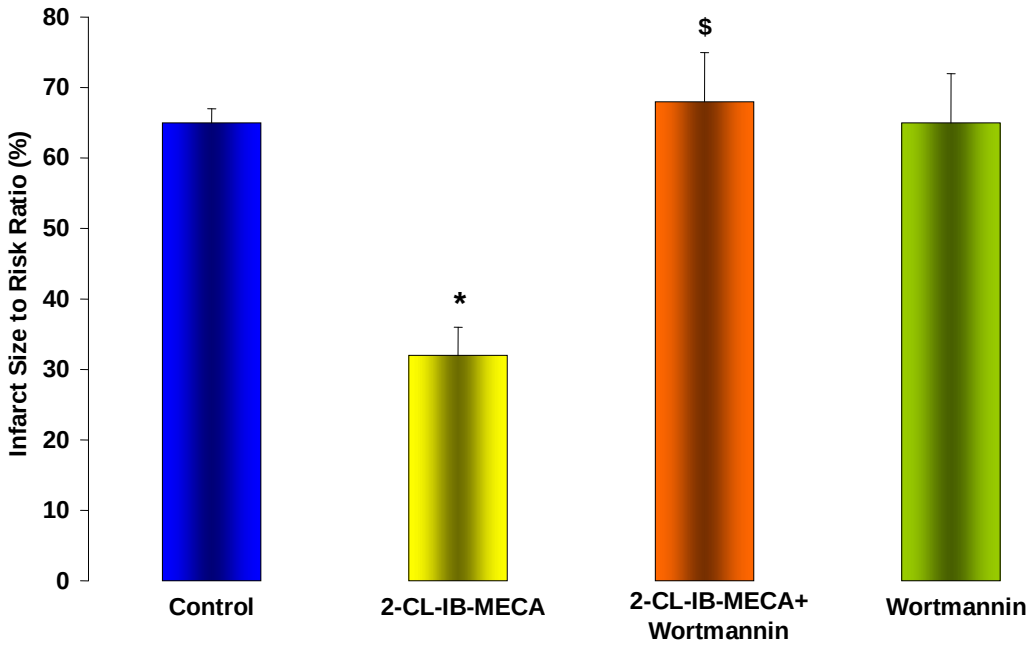
Chapter 5: 2-CL-IB-MECA protects the myocardium from ischaemia reperfusion injury via PI3K-AKT cell survival pathway

5.1.1. Protection afforded by A_3AR agonist 2-CL-IB-MECA at reperfusion is mediated via PI3K - AKT activity in the isolated perfused rat heart

As previously shown in chapter 3 administration of the A_3 agonist 2-CL-IB-MECA (1 nM) throughout reperfusion significantly decreased infarct size to risk ratio (%) compared to non treated controls (65 ± 2 % control hearts vs. 32 ± 4% 2-CL-IB-MECA $P<0.01$) Figure.3.1.

To determine whether the protection afforded by the A_3AR agonist 2-Cl-IB-MECA (1nM) when administered throughout reperfusion was mediated via PI3K - AKT activity, we used the PI3K inhibitor Wortmannin (5 nM). Administration of 2-CL-IB-MECA (1nM) in the presence of the PI3K inhibitor Wortmannin (5 nM) significantly abolished the protection afforded by 2-CL-IB-MECA (1 nM) in comparison to when administered alone throughout reperfusion (68 ± 12% 2-CL-IB-MECA + Wortmannin vs. 32 ± 4% 2-CL-IB-MECA $P<0.05$) Figure.5.1.

Wortmannin when administered alone throughout reperfusion had no significant effect on infarct size to risk ratio (%) compared to non treated control hearts (65 ± 12 % Wortmannin vs. 65 ± 2 % control $P>0.05$. Figure.5.1).



***P<0.05 2-CL-IB-MECA vs. Control. $P<0.05 2-CL-IB-MECA +Wortmannin vs. 2-CL-IB-MECA. Results are shown as Mean ± SEM.**

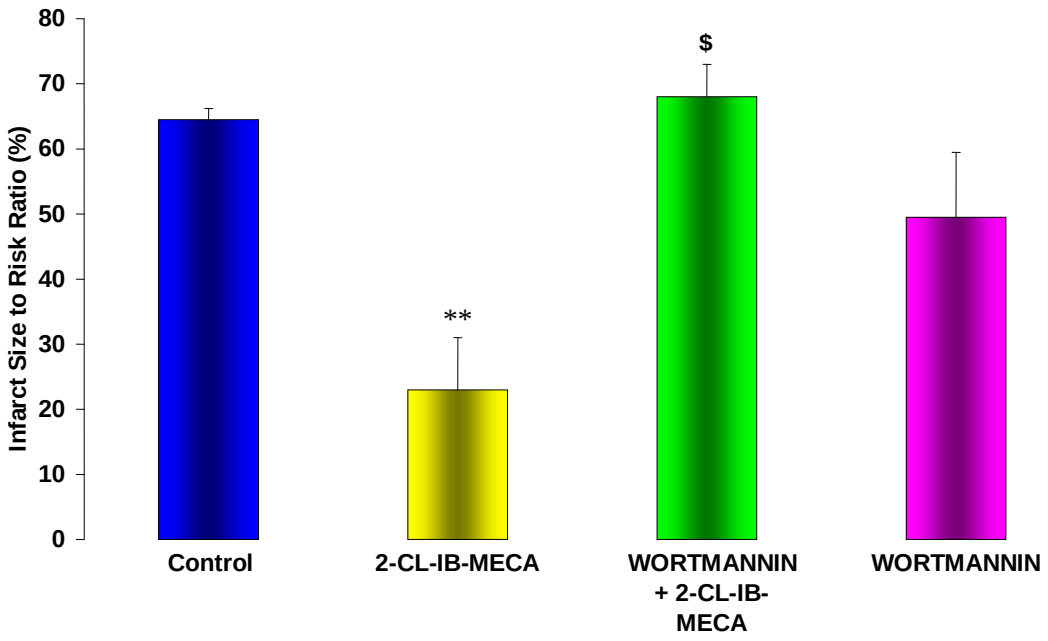
Figure 5.1. Infarct size to Risk ratio (%) in non-treated control and 2-Cl-IBMECA treated ischaemic reperfused hearts. Isolated perfused rat hearts where subjected to 35 minutes of ischaemia and 120 minutes of reperfusion where the A_3 adenosine receptor 2-Cl-IB-MECA (1 nM) was administered throughout reperfusion in the presence and absence of the PI3K inhibitor Wortmannin (5 nM).

Studies by Germack and Dickenson (2004) have shown there to be a dose response relationship between 2-CL-B-MECA concentration and PI3K-AKT activity. Therefore, we next assessed whether a higher concentration of the A_3 agonist 2-CL-IB-MECA could protect the ischaemic reperfused myocardium when administered at reperfusion. The administration of 2-Cl-IB-MECA (100 nM) at reperfusion significantly protects the myocardium from ischaemia reperfusion injury compared to non treated controls (23 ± 8 % 2-CL-IB-MECA vs. 62 ± 2% control P<0.01) Figure 5.2.

To determine whether the protection afforded by the A_3AR agonist 2-Cl-IB-MECA (100nM) when administered throughout reperfusion was mediated PI3K - AKT

activity, we used the PI3K inhibitor Wortmannin (100 nM). Administration of 2-CL-IB-MECA (100 nM) in the presence of the PI3K inhibitor Wortmannin (100 nM) significantly abrogated the protection afforded by 2-CL-IB-MECA (100 nM) when administered alone throughout reperfusion (68 ± 5% 2-CL-IB-MECA + Wortmannin vs. 23 ± 8 % 2-CL-IB-MECA P<0.01) Figure.5.2.

Administration of Wortmannin (100 nM) throughout reperfusion had no significant effect on infarct size to risk ratio (%) compared to controls (49.5 ± 10% Wortmannin vs. 65 ± 2% P>0.05) Figure.5.2.



****P<0.01 2-CL-IB-MECA vs. Control. $ P<0.01 Wortmannin + 2-CL-IB-MECA vs. 2-CL-IB-MECA.**
Results are shown as Mean ± SEM.

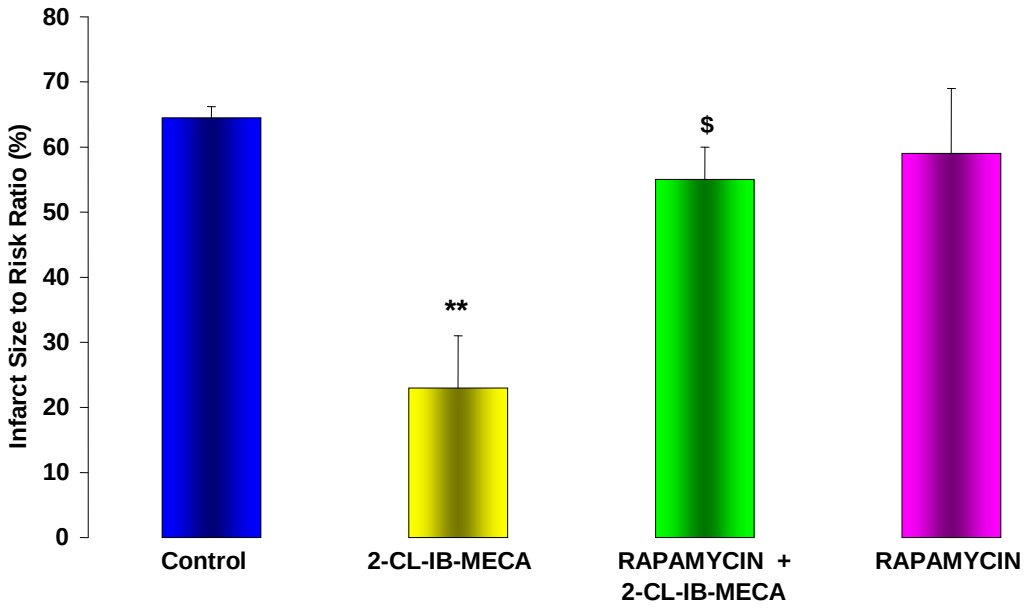
Figure 5.2. Infarct size to Risk ratio (%) in non-treated control and 2-Cl-IBMECA treated ischaemic reperfused hearts. Isolated perfused rat hearts where subjected to 35 minutes of ischaemia and 120 minutes of reperfusion where the A_3 adenosine receptor 2-Cl-IB-MECA (100 nM) was administered throughout reperfusion in the presence and absence of the PI3K inhibitor Wortmannin (100 nM).

5.1.2. Protection afforded by A_3AR agonist 2-Cl-IB-MECA at reperfusion is mediated via PI3K – AKT/ p70S6 kinase activity in the isolated perfused rat heart

We have shown that 2-CL-IB-MECA at 100nM protects the myocardium from ischaemia reperfusion injury via the PI3K – AKT survival pathway. In order to further understand the possible mechanisms via which 2-CL-IB-MECA confers cardioprotection we assessed the role of the downstream translational kinase p70S6.

To determine to role of p70S6 kinase in 2-CL-IB-MECA (100 nM) mediated cardioprotection when administered throughout reperfusion we used the mTOR inhibitor Rapamycin (2 nM). Administration of 2-CL-IB-MECA (100 nM) in the presence of the mTOR inhibitor Rapamycin (2 nM) throughout reperfusion significantly abolished 2-CL-IB-MECA (100 nM) mediated cardioprotection compared when administered alone at reperfusion (55 ± 5% 2-CL-IB-MECA + Rapamycin vs. 23 ± 8 % 2-CL-IB-MECA $P<0.01$) Figure.5.3.

Rapamycin (2 nM) when administered alone throughout reperfusion had no significant effect on infarct size to risk ratio (%) compared to non treated control hearts (59 ± 2% vs. 65 ± 2% $P>0.05$) Figure.5.3.



****P<0.01 2-CL-IB-MECA vs. Control. $ P<0.01 Rapamycin + 2-CL-IB-MECA vs. 2-CL-IB-MECA. Results are shown as Mean ± SEM.**

Figure 5.3. Infarct size to Risk ratio (%) in non-treated control and 2-Cl-IBMECA treated ischaemic reperfused hearts. Isolated perfused rat hearts where subjected to 35 minutes of ischaemia and 120 minutes of reperfusion where the A_3 adenosine receptor 2-Cl-IB-MECA (100 nM) was administered throughout reperfusion in the presence and absence of the mTOR inhibitor Rapamycin (2 nM).

5.2.1. The role of PI3K-AKT cell survival pathway in A_3AR agonist 2-CL-IB-MECA (1nM) cardioprotection in adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours reoxygenation.

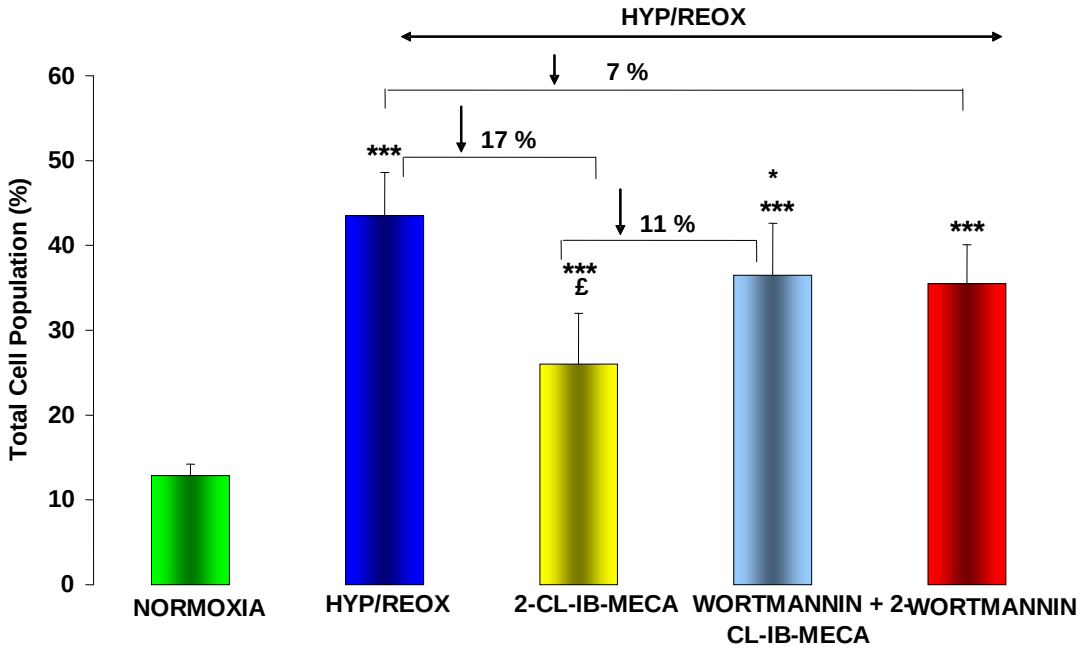
Previously in chapter 3, we showed the administration of 2-CL-IB-MECA (1 nM) throughout reoxygenation significantly protected adult rat cardiac myocytes from hypoxia/reoxygenation injury in an anti apoptotic/necrotic manner.

To elucidate the anti-apoptotic and anti-necrotic mechanisms of 2-CL-IB-MECA (1 nM) when administered at reoxygenation we assessed the role of the PI3K-AKT cell survival pathway using the PI3K inhibitor Wortmannin (5 nM). Isolated myocytes were subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation where the A_3 agonist 2-CL-IB-MECA (1 nM) was administered at the onset of reoxygenation in the presence and absence of 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, $P<0.05$) Figure 5.4.a.

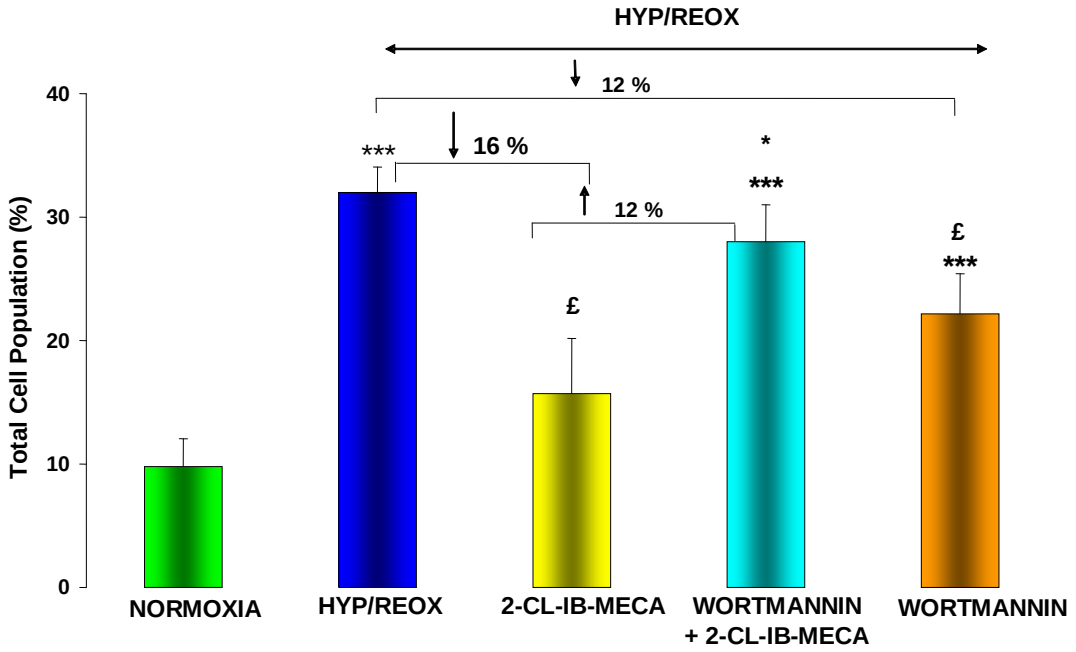
Administration of 2-CL-IB-MECA (1 nM) in the presence of the PI3K inhibitor Wortmannin (5 nM) also significantly abolished the anti-necrotic effect of 2-CL-IB-MECA (1 nM) when administered alone throughout reoxygenation (28 ± 3 % 2-CL-IB-MECA + Wortmannin vs. 16 ± 4 % 2-CL-IB-MECA, $P<0.05$), Figure 5.4.b.

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) Figure 5.4.a. 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<0.05) Figure 5.4. b.



*****P<0.001 Hyp/Reox, 2-CL-IB-MECA, Wortmannin + 2-CL-IB-MECA, Wortmannin vs. Normoxia.**
£ P<0.001 2-CL-IB-MECA vs. Hyp/Reox. *P<0.05 Wortmannin + 2-CL-IB-MECA vs. 2-CL-IB-MECA.

Figure 5.4.a. Assessment of Apoptosis in adult rat cardiomyocytes subjected to 24 hours reoxygenation (Normoxia) or 6 hours hypoxia and 18 hours of reoxygenation(Hyp/Reox). Assessment of PI3K/Akt cell survival pathway in 2-CL-IB-MECa mediated cardioprotection. The A_3AR agonist 2-CL-IB-MECA (1 nM) was administered at reoxygenation in the presence and absence of the PI3K inhibitor Wortmannin (5 nM). Results are shown as Mean ± SEM and are expressed as a percentage of 10,000 cells counted.



*****P<0.001 Hyp/Reox. Wortmannin + 2-CL-IB-MECA , Wortmannin vs. Normoxia. £ P<0.001 Wortmannin vs. Hyp/Reox. * P<0.05 Wortmannin + 2-CL-IB-MECA vs. 2-CL-IB-MECA.**

Figure 5.4.b. Assessment of necrosis in adult rat cardiomyocytes subjected to 24 hours reoxygenation (Normoxia) or 6 hours hypoxia and 18 hours of reoxygenation(Hyp/Reox). Assessment of PI3K/Akt cell survival pathway in 2-CL-IB-MECA mediated cardioprotection. The A_3AR agonist 2-CL-IB-MECA (1 nM) was administered at reoxygenation in the presence and absence of the PI3K inhibitor Wortmannin (5 nM). Results are shown as Mean ± SEM and are expressed as a percentage of 10,000 cells counted.

5.2.2. The role of PI3K-AKT cell survival pathway in A_3AR agonist 2-CL-IB-MECA (100 nM) cardioprotection in adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours reoxygenation.

We further assessed whether 2-Cl-IB-MECA (100 nM) protects isolated adult rat cardiomyocytes from hypoxia/reoxygenation injury via anti apoptotic/necrotic mechanisms. Furthermore, whether cytoprotection was dependant upon recruitment of the PI3K-AKT survival pathway.

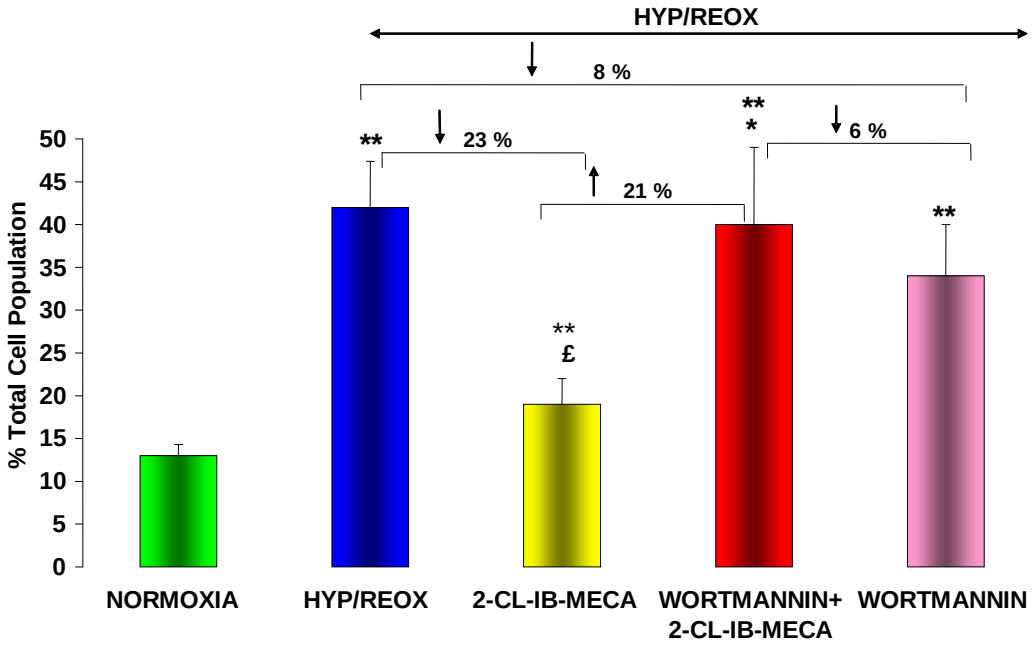
To elucidate the anti-apoptotic and anti-necrotic mechanisms of 2-CL-IB-MECA (100 nM) when administered at reoxygenation, we assessed the role of the PI3K-AKT cell survival pathway using the PI3K inhibitor Wortmannin (100 nM). Isolated myocytes were subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation and were incubated with the A_3 agonist 2-CL-IB-MECA (100 nM) in the presence and absence of PI3K inhibitor Wortmannin (100 nM).

Administration of the A_3 agonist 2-CL-IB-MECA (100 nM) in the presence of the PI3K inhibitor Wortmannin (100 nM) significantly abolished the anti-apoptotic effect of 2-CL-IB-MECA (100 nM) when administered alone throughout reoxygenation (40 ± 9 % 2-CL-IB-MECA + Wortmannin vs. 19 ± 3 % 2-CL-IB-MECA $P<0.05$) Figure 5.5.a.

Administration of 2-CL-IB-MECA (100 nM) in the presence of the PI3K inhibitor Wortmannin (100 nM) throughout reoxygenation also significantly abolished the anti-necrotic effect of 2-CL-IB-MECA (100 nM) when administered alone throughout

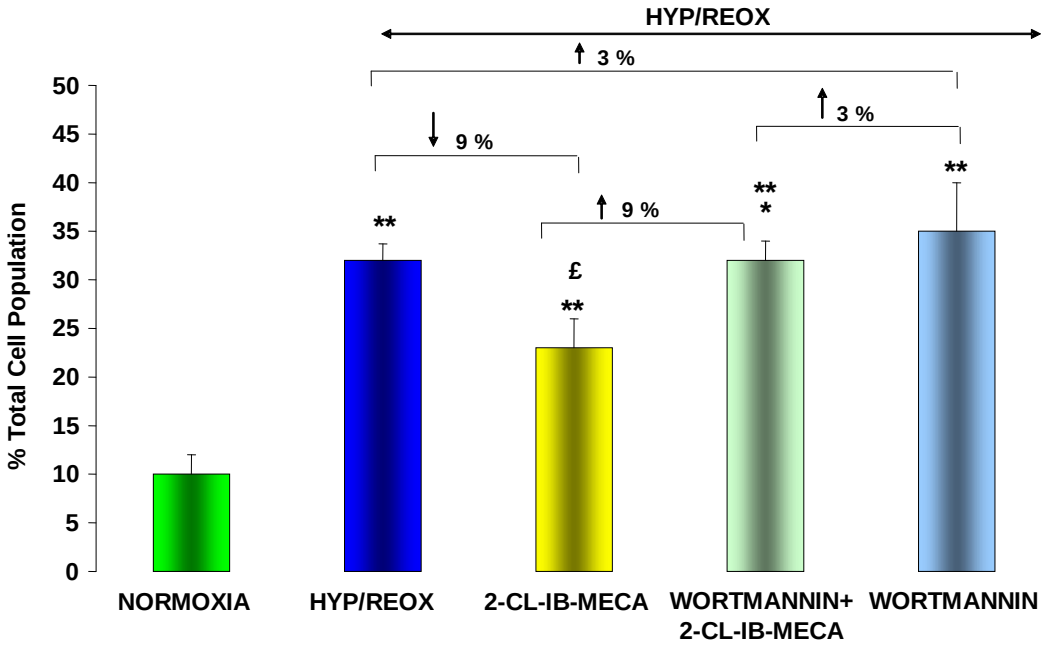
reoxygenation (32 ± 2 % 2-CL-IB-MECA + Wortmannin vs. 23 ± 3 % 2-CL-IB-MECA $P<0.05$) Figure 5.5.b.

Administration of Wortmannin (100 nM) alone throughout reoxygenation had no significant effect on cellular apoptosis in adult rat cardiac myocytes exposed to 6 hours of hypoxia and 18 hours of reoxygenation compared to non treated Hyp/Reox group (34 ± 6 % Wortmannin vs. 42 ± 5 % Hyp/Reox, $P>0.05$) Figure 5.5.a. Administration of Wortmannin (100 nM) throughout reoxygenation had no significant effect on cellular necrosis compared to the non treated Hyp/Reox group (35 ± 5 % Wortmannin vs. 32 ± 2 % Hyp/Reox. $P>0.05$) Figure 5.5.b.



****P<0.01 Hyp/Reox, 2-CL-IB-MECA, Wortmannin+ 2-CL-IB-MECA, Wortmannin vs. Normoxia. £ P<0.01 2-CL-IB-MECA vs. Hyp/Reox. * P<0.05 Wortmannin + 2-CL-IB-MECA vs. 2-CL-IB-MECA.**

Figure 5.5.a. Assessment of apoptosis in isolated adult rat cardiomyocytes subjected to 24 hours oxygenation (Normoxia) or 6 hours hypoxia and 18 hours of reoxygenation (Hyp/Reox). Assessment of PI3k/Akt cell survival pathway in 2-CL-IB-MECA mediated cardioprotection. The A_3AR agonist 2-CL-IB-MECA (100 nM) was administered at reoxygenation in the presence and absence of the PI3K inhibitor Wortmannin (100 nM). Results are shown as Mean ± SEM and are expressed as a percentage 10,000 cells counted.



****P<0.01 Hyp/Reox, 2-CL-IB-MECA, Wortmannin + 2-CL-IB-MECA, Wortmannin vs. Normoxia. £ P<0.01 2-CL-IB-MECA vs. Hyp/Reox. * P<0.05 Wortmannin + 2-CL-IB-MECA vs. 2-CL-IB-MECA.**

Figure 5.5.b. Assessment of necrosis in isolated adult rat cardiomyocytes subjected to 24 hours oxygenation (Normoxia) or 6 hours hypoxia and 18 hours of reoxygenation (Hyp/Reox). Assessment of PI3k-Akt cell survival pathway in 2-CL-IB-MECA mediated cardioprotection. The A_3AR agonist 2-CL-IB-MECA (100 nM) was administered at reoxygenation in the presence and absence of the PI3K inhibitor Wortmannin (100 nM). Results are shown as Mean ± SEM and are expressed as a percentage 10,000 cells counted.

5.3.1 The role of PI3K-AKT-p70S6 kinase cell survival pathway in A_3 adenosine receptor cardioprotection in adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours reoxygenation.

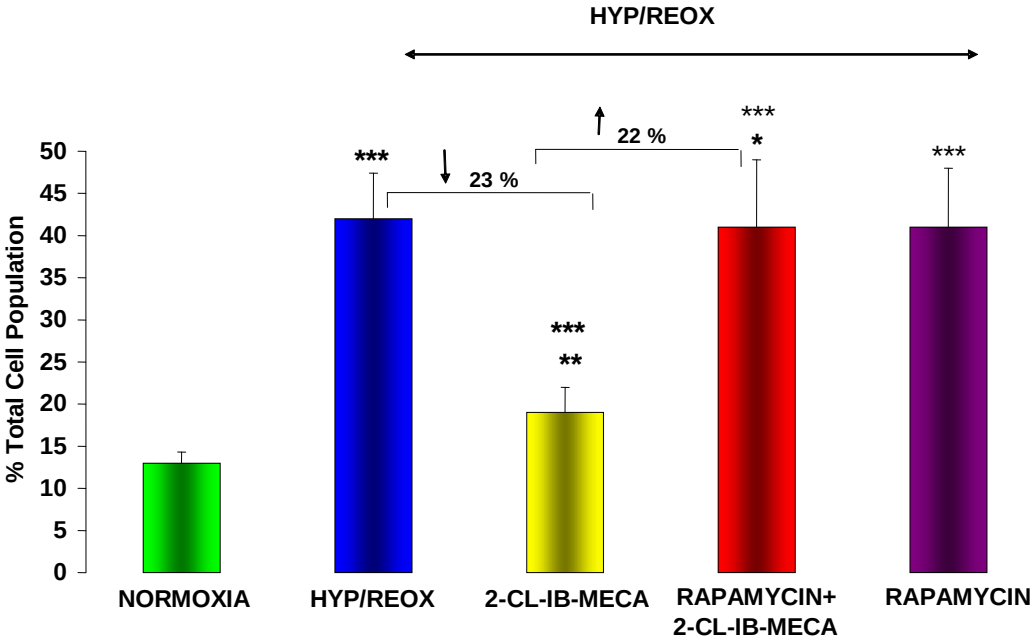
In the isolated perfused rat heart we have shown that the protection afforded by 2-CL-IB-MECA (100 nM) when administered at reperfusion was significantly abolished in the presence of the mTOR inhibitor Rapamycin (2nM) Figure 6.3.

To elucidate the anti-apoptotic and anti-necrotic mechanisms of 2-CL-IB-MECA (100nM) when administered at reoxygenation we assessed the role of the PI3K – AKT - p70S6K cell survival pathway using the mTOR inhibitor Rapamycin (2nM). Isolated myocytes were subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation where the A_3 agonist 2-CL-IB-MECA (100 nM) was administered in the presence and absence of mTOR/p70S6 kinase inhibitor Rapamycin (2 nM).

Administration of the A_3 agonist 2-CL-IB-MECA (100 nM) in the presence of the p70S6 kinase inhibitor Rapamycin (2 nM) significantly abolished the anti-apoptotic effect of 2-CL-IB-MECA (100 nM) compared to when administered alone (41 ± 8 % 2-CL-IB-MECA + Rapamycin vs. 19 ± 3% 2-CL-IB-MECA $P<0.01$) Figure 5.6.a.

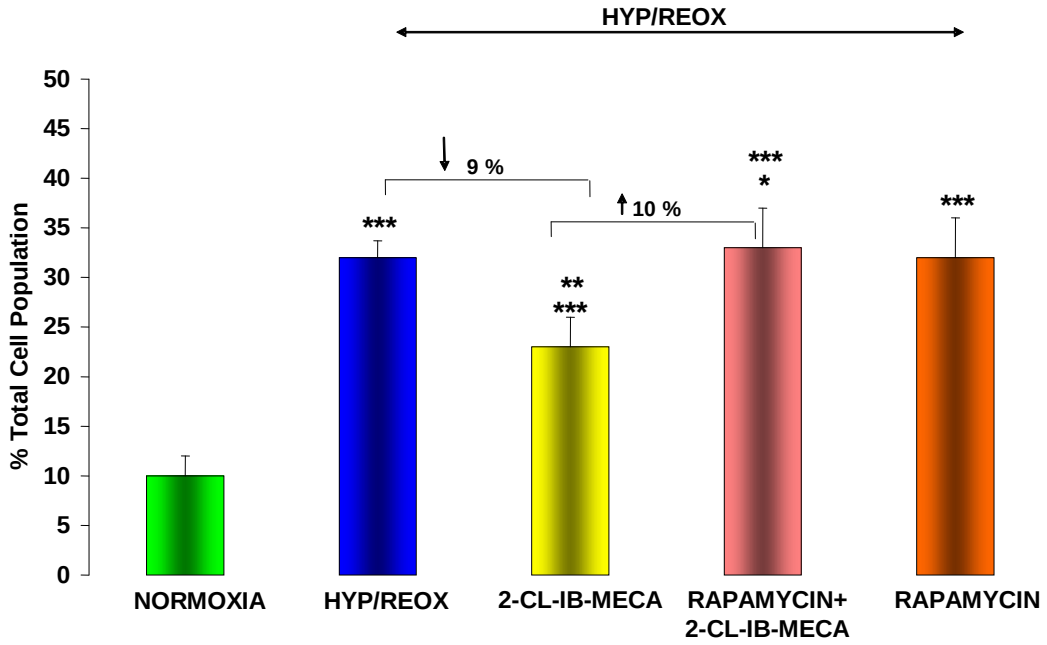
Administration of the A_3 agonist 2-CL-IB-MECA (100 nM) in the presence of the p70S6 kinase inhibitor Rapamycin (2 nM) also significantly abolished the anti-necrotic effect of 2-CL-IB-MECA (100 nM) compared to when administered alone (33 ± 4 % 2-CL-IB-MECA + Rapamycin vs. 23 ± 3 % 2-CL-IB-MECA $P<0.05$) Figure 5.6.b.

Administration of Rapamycin (2 nM) alone throughout reoxygenation had no significant effect on cellular apoptosis compared to the non-treated Hyp/Reox group (41 ± 7 % Rapamycin vs. 42 ± 5 % Hyp/Reox P>0.05) Figure 5.6.a. Administration of Rapamycin (2 nM) alone throughout reoxygenation had no significant effect on cellular necrosis compared to the Hyp/Reox group (32 ± 4 % Rapamycin vs.32 ± 2% Hyp/Reox. P>0.05) Figure 5.6.b.



*****P<0.001 Hyp/Reox, 2-CL-IB-MECA, Rapamycin + 2-CL-IB-MECA, Rapamycin vs. Normoxia. **P<0.01 2-CL-IB-MECA vs. Hyp/Reox. * P<0.05 Rapamycin + 2-CL-IB-MECA vs. 2-CL-IB-MECA.**

Figure 5.6.a. Assessment of apoptosis in isolated adult rat cardiomyocytes subjected to 24 hours oxygenation or 6 hours hypoxia and 18 hours of reoxygenation. Assessment of PI3K – AKT - p70S6 cell survival pathway in 2-CL-IB-MECA mediated cardioprotection. The A_3AR agonist 2-CL-IB-MECA (100 nM) was administered at reoxygenation in the presence and absence of the p70S6 kinase inhibitor Rapamycin (2 nM). Results are shown as Mean ± SEM and are expressed as a percentage 10,000 cells counted.



*****P<0.001 Hyp/Reox, 2-CL-IB-MECA, Rapamycin + 2-CL-IB-MECA, Rapamycin vs. Normoxia. **P<0.01 2-CL-IB-MECA vs. Hyp/Reox. * P<0.05 Rapamycin + 2-CL-IB-MECA vs. 2-CL-IB-MECA.**

Figure 5.6.b. Assessment of necrosis in isolated adult rat cardiomyocytes subjected to 24 hours oxygenation or 6 hours hypoxia and 18 hours of reoxygenation. Assessment of PI3K – AKT - p70S6 cell survival pathway in 2-CL-IB-MECA mediated cardioprotection. The A_3AR agonist 2-CL-IB-MECA (100 nM) was administered at reoxygenation in the presence and absence of the mTOR inhibitor Rapamycin (2 nM). Results are shown as Mean ± SEM and are expressed as a percentage 10,000 cells counted.

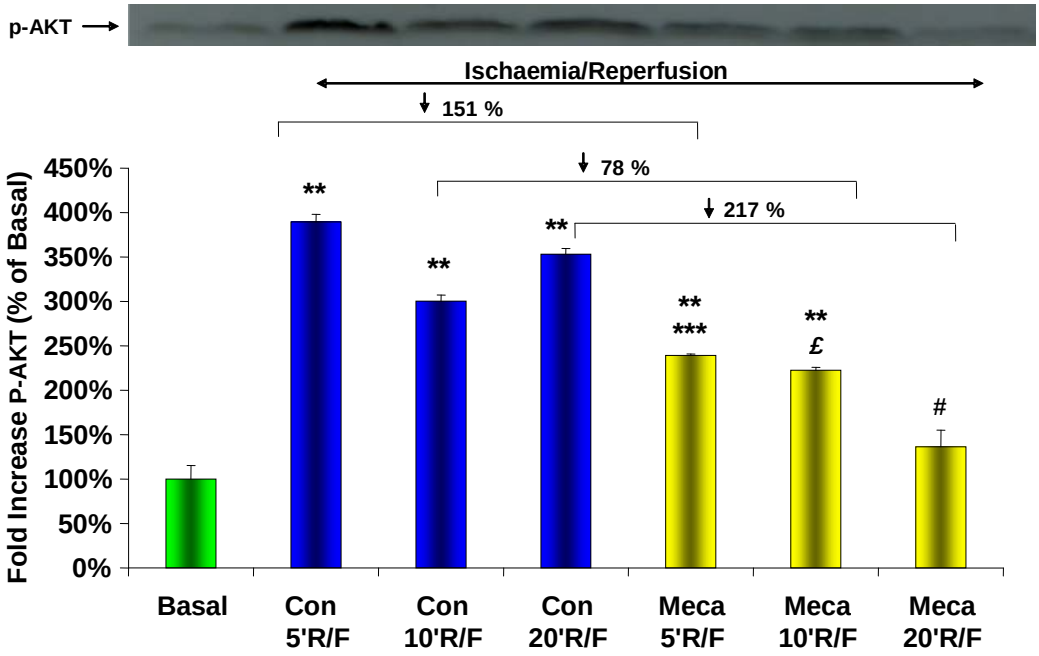
5.4.1. Effect of 2-CL-IB-MECA (1nM) when administered at reperfusion on AKT phosphorylation during different time intervals post reperfusion

Recruitment of the PI3K - AKT intracellular signalling pathway has been shown to promote cell survival by inhibiting cellular apoptosis. A range of cell survival factors like Urocortin, Insulin and cytokines have been shown to recruit the PI3K - AKT intracellular signalling pathway promoting cell survival.

Previously, we have shown that the protection afforded by the A_3 adenosine receptor agonist 2-CL-IB-MECA (1 nM) when administered at reperfusion was abolished by the co-administration of the PI3K inhibitor Wortmannin (5 nM) in the isolated heart model of ischaemia reperfusion injury (Figure 5.1). Therefore we assessed the phosphorylation status of phospho-AKT $_{(Ser\ 473)}$ that is phosphorylated by PI3K at various time intervals post reperfusion in the presence and absence of the A_3 agonist 2-CL-IB-MECA (1 nM).

Phosphorylation of AKT $_{(Ser\ 473)}$ was observed in non treated control and 2-CL-IB-MECA (1 nM) treated ischaemic reperfused hearts. Reperfusion of the ischaemic heart significantly increased the phosphorylation of AKT $_{(ser\ 473)}$ at 5, 10 and 20 minutes of reperfusion compared to the non-ischaemic group (basal) ($P<0.01$ for all time points) Figure 5.7. AKT $_{(ser\ 473)}$ phosphorylation was also observed in 2-CL-IB-MECA (1 nM) treated hearts at 5 and 10 minutes of reperfusion compared to the non-ischaemic group (basal) ($P<0.01$ for both time points) Figure 5.7.

Administration of the A_3 agonist 2-CL-IB-MECA (1 nM) for 5, 10 and 20 minutes of reperfusion significantly decreased the phosphorylation of AKT $_{(Ser\ 473)}$ compared to their respective time matched control hearts (P<0.01 for all time points) (Figure 5.7).



**** P<0.01 Control 5, 10, 20 minutes reperfusion, MECA 5, 10 minutes reperfusion vs. Basal. *** P<0.001 MECA 5 minutes reperfusion vs. Control 5 minutes Reperfusion. £ P<0.01 MECA 10 minutes reperfusion vs. Control 10 minutes Reperfusion. # P<0.001 MECA 20 minutes reperfusion vs. Control 20 minutes reperfusion.**

Figure 5.7. Assessment of Akt phosphorylation in isolated hearts to subjected 60 minutes perfusion (basal), 35 minutes of ischaemia followed by 5, 10 or 20 minutes of reperfusion in the presence and absence (Con) of the A_3 Agonist 2-CL-IB-MECA (1 nM) (MECA). Results are shown as Mean ± SEM of three individual experiments.

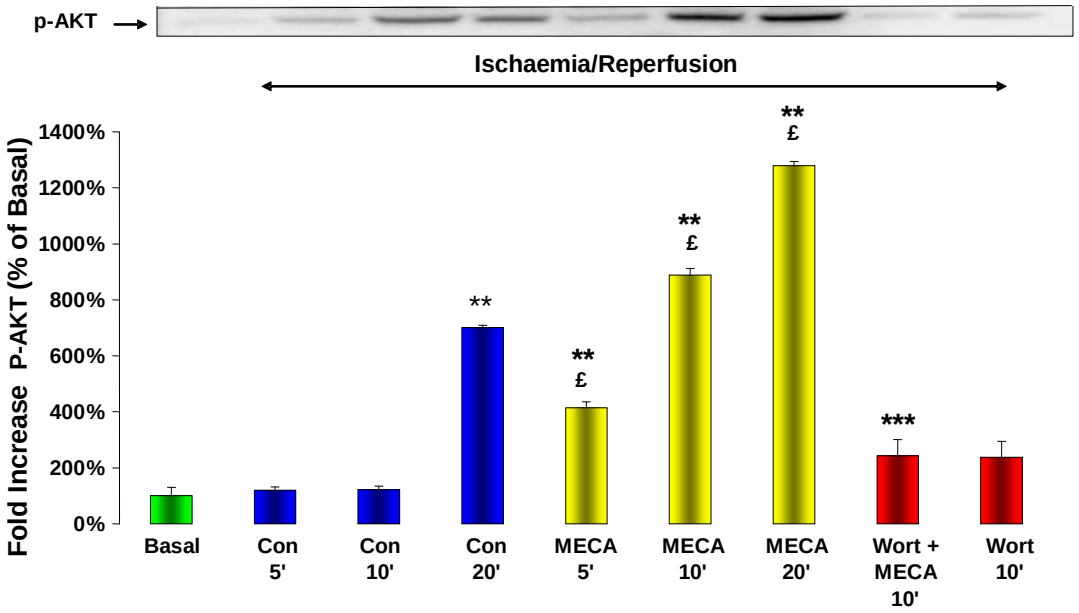
2-CL-IB-MECA (1 nM) when administered at reperfusion was shown to down regulate AKT $_{(ser473)}$ phosphorylation compared to time matched controls, indicating that 2-CL-IB-MECA (1 nM) mediated cardioprotection is independent of the PI3K-AKT pathway at this concentration. Although in the isolated perfused rat heart model the PI3K inhibitor Wortmannin (5 nM) was seen to abolish the protection afforded by 2-CL-IB-MECA (1 nM) when administered at reperfusion.

5.4.2. Effect of 2-CL-IB-MECA (100 nM) when administered at reperfusion on AKT phosphorylation during different time intervals post reperfusion.

We showed that 2-CL-IB-MECA (100 nM) can protect the myocardium from ischaemia reperfusion injury in the isolated perfused rat heart where the protection was abolished in the presence of the PI3K inhibitor Wortmannin (100 nM) Figure 5.2. To understand the signalling pathways involved in 2-CL-IB-MECA (100 nM) mediated cardioprotection when administered at reperfusion it was necessary to look at the phosphorylation status of AKT $_{(ser473)}$ at different time points during reperfusion in the presence and absence of the PI3K inhibitor Wortmannin (100 nM).

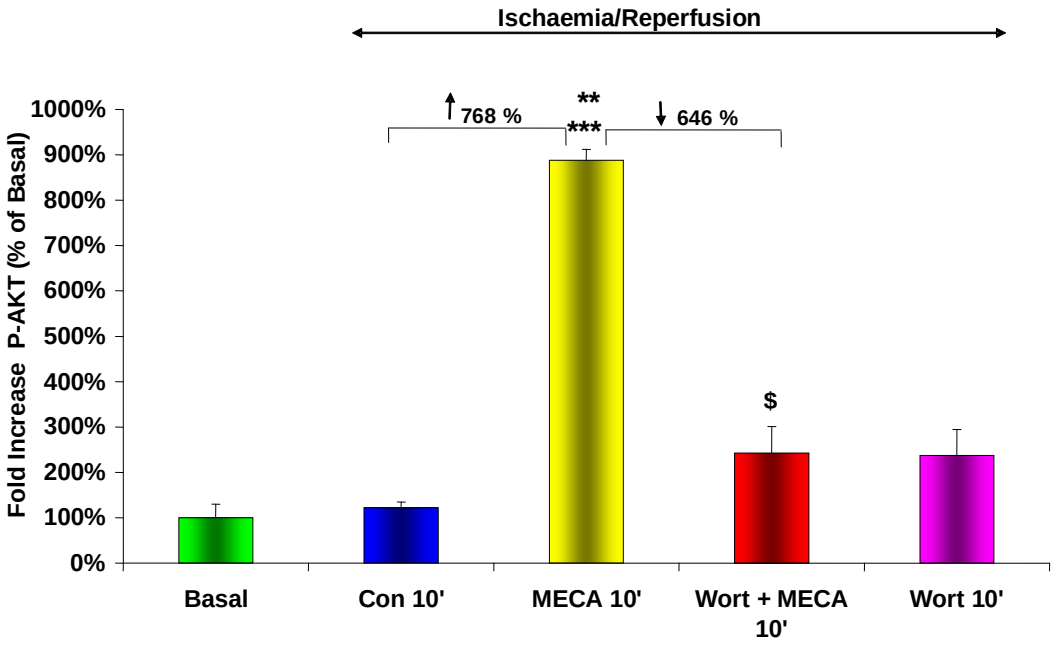
Phosphorylation of AKT $_{(ser473)}$ was observed in non-treated control and 2-CL-IB-MECA (100 nM) treated hearts. Reperfusion of the ischaemic heart in the presence of the A_3 agonist 2-CL-IB-MECA (100 nM) significantly increased phosphorylation of AKT $_{(ser473)}$ at 5 minutes ($P<0.001$), 10 minutes ($P<0.001$) and 20 minutes ($P<0.05$) of reperfusion compared to time matched non-treated control hearts (Figure 5.8.a,b).

Upregulation of AKT $_{(ser473)}$ phosphorylation by the A_3 agonist 2-CL-IB-MECA (100 nM) after 10 minutes of reperfusion was significantly abolished in the presence of the PI3K inhibitor Wortmannin (100 nM) $P<0.001$ (Figure 5.8.a,b). Administration of Wortmannin (100 nM) at the onset of reperfusion alone had no significant effect of AKT $_{(ser473)}$ phosphorylation compared to time matched non-treated control hearts ($P>0.05$) Figure 5.8.a,b.



**** P<0.01 Control control minutes reperfusion, 5, 10, 20 minutes reperfusion MECA vs. Basal. £ P<0.01 MECA 5, 10, 20 minutes reperfusion vs. controls 5, 10, 20 minutes reperfusion respectively *** P<0.001 Wortmannin + 2-CL-IB-MECA vs. 2-Cl-IB-MECA 10 minutes Reperfusion.**

Figure 5.8.a. Assessment of AKT (ser 473) phosphorylation in isolated hearts to subjected to 60 minutes perfusion (Basal) or 35 minutes ischaemia followed by 5, 10 or 20 minutes of reperfusion in non-treated control and 2-CL-IB-MECA treated hearts. The A_3 agonist 2-CL-IB-MECA (100 nM) (MECA) was administered at reperfusion in presence and absence of the PI3K inhibitor Wortmannin (100 nM) (Wort). Results are shown as Mean ± SEM of three individual experiments.



**** P<0.01 MECA 10 minutes reperfusion vs. Basal. *** P<0.001 MECA 10 minutes reperfusion vs. Control 10 minutes reperfusion. $ P<0.001Wort+MECA 10 minutes reperfusion vs. 2-Cl-IB-MECA 10 minutes Reperfusion.**

Figure 5.8.b. Comparison of AKT (ser 473) phosphorylation in isolated hearts to subjected to 60 minutes perfusion (Basal) or 35 minutes ischaemia followed by 10 minutes of reperfusion in non-treated control and 2-CL-IB-MECA treated hearts. The A_3 agonist 2-CL-IB-MECA (100 nM) (MECA) was administered at reperfusion in presence and absence of the PI3K inhibitor Wortmannin (100 nM) (Wort). Results are shown as Mean ± SEM of three individual experiments.

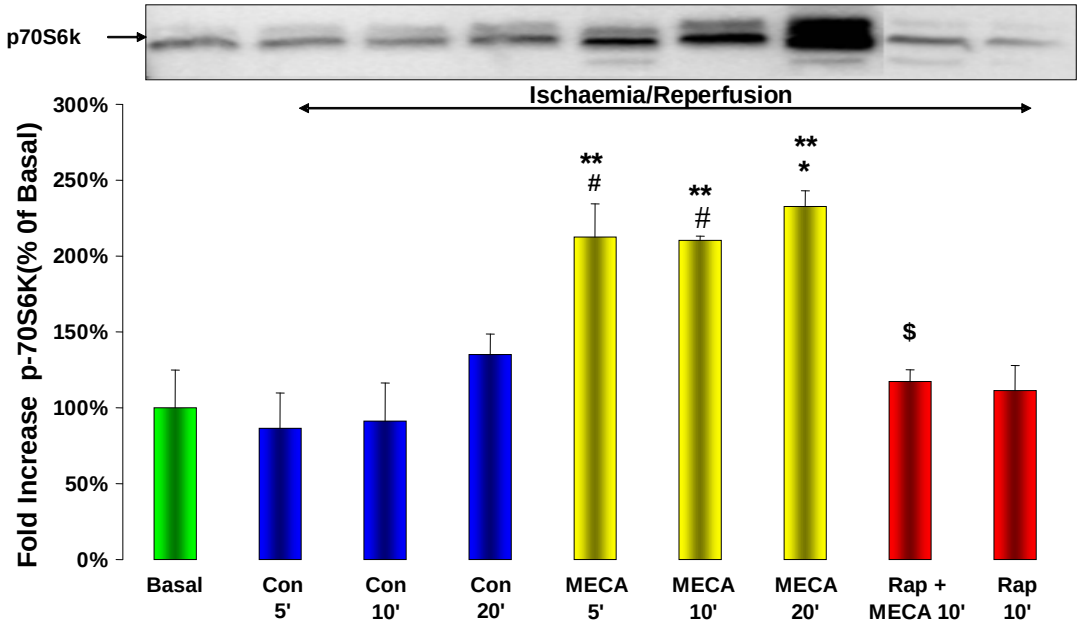
5.4.3. Effect of 2-CL-IB-MECA (100 nM) when administered at reperfusion on p70S6 phosphorylation during different time intervals post reperfusion

Previously we have shown that activation of A_3 adenosine receptors with the A_3 agonist 2-CL-IB-MECA (100 nM) protects the myocardium from ischaemia reperfusion injury in the isolated perfused rat heart. The protection afforded by the A_3 agonist 2-CL-IB-MECA (100 nM) was abolished in the presence of the mTOR inhibitor Rapamycin (2 nM) Figure 5.3.

To understand the role of p70S6 kinase in 2-CL-IB-MECA (100 nM) mediated cardioprotection it was necessary to harvest tissue that had been treated with 2-CL-IB-MECA (100 nM) in the presence and absence of the mTOR inhibitor Rapamycin (2 nM) at various time points post reperfusion.

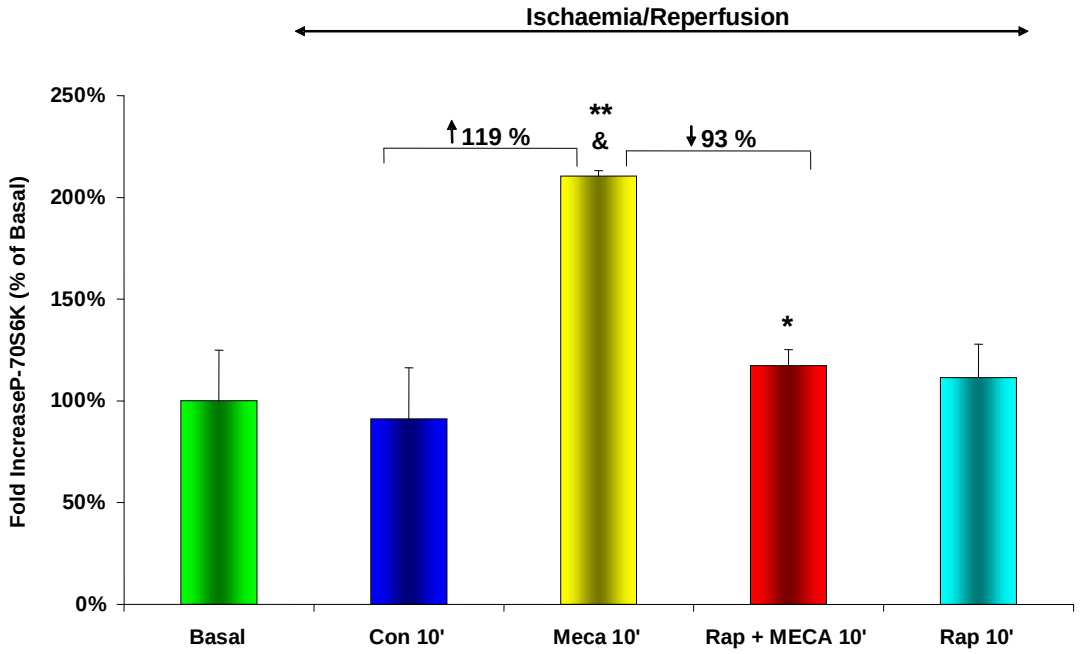
Phosphorylated p70S6 kinase $_{(Thr\ 389)}$ was seen in all tissues and time points assessed. Administration of 2-CL-IB-MECA (100 nM) at the onset of reperfusion was seen to significantly upregulate the phosphorylation of p70S6 $_{(Thr389)}$ kinase after 5 minutes ($P<0.01$), 10 minutes ($P<0.01$) and 20 minutes ($P<0.05$) of reperfusion compared to their time matched non-treated controls hearts (Figure 5.9.a,b).

Administration of the 2-CL-IB-MECA (100 nM) in the presence of the mTOR inhibitor Rapamycin (2 nM) for 10 minutes of reperfusion significantly abolished 2-CL-IB-MECA (100 nM) dependant phosphorylation of p70S6 $_{(Thr\ 389)}$ kinase compared to 2-CL-IB-MECA (100 nM) when administered alone for 10 minutes of reperfusion ($P<0.05$), Figure 5.9.a,b.



**** P<0.01 MECA 5, 10, 20 minutes reperfusion vs. Basal. #P<0.01 MECA 5, 10, 20 minutes reperfusion vs. control 5, 10, 20 minutes reperfusion respectively. * P<0.05 MECA 20 minutes reperfusion vs.control 20 minutes reperfusion. $ P<0.05 Rapamycin + 2-CL-IB-MECA vs. 2-Cl-IB-MECA 10 minutes of Reperfusion.**

Figure 5.9.a. Assessment of p70S6 kinase phosphorylation in isolated hearts to subjected 60 minutes of perfusion (basal) or 35 minutes of ischaemia followed by 5, 10 or 20 minutes of reperfusion in non-treated control (Con) and 2-CL-IB-MECA hearts. The A_3 agonist 2-CL-IB-MECA (100 nM) (MECA) was administered in the presence and absence of the mTOR inhibitor Rapamycin (2 nM) (Rap). Results are shown as Mean ± SEM of three individual experiments.



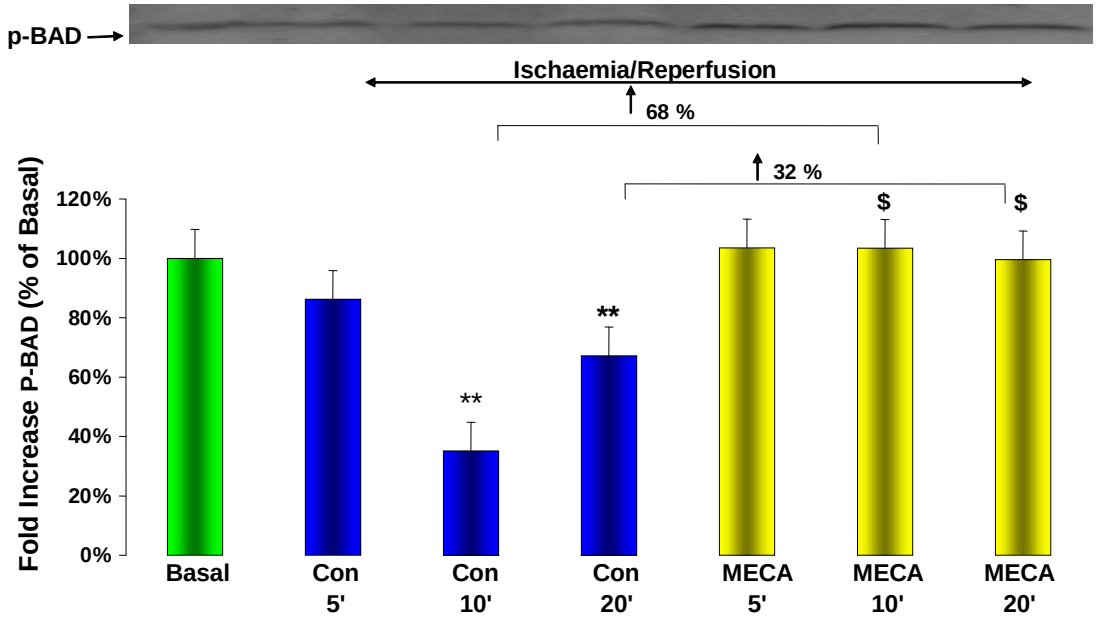
**** P<0.01 MECA 10 minutes reperfusion vs. Basal. & P<0.01 MECA 10 minutes reperfusion vs. Control 10 minutes reperfusion. * P<0.05 Rapamycin + 2-CL-IB-MECA vs. 2-Cl-IB-MECA 10 minutes of Reperfusion.**

Figure 5.9.b. Comparison of p70S6 kinase phosphorylation in isolated hearts to subjected 60 minutes of perfusion (basal) or 35 minutes of ischaemia followed by 5, 10 or 20 minutes of reperfusion in non-treated control (Con) and 2-CL-IB-MECA hearts. The A_3 agonist 2-CL-IB-MECA (100 nM) (MECA) was administered in the presence and absence of the mTOR inhibitor Rapamycin (2 nM) (Rap). Results are shown as Mean ± SEM of three individual experiments.

5.5.0. Effect of 2-CL-IB-MECA (100nM) when administered at reperfusion on BAD $_{(ser136)}$ phosphorylation during different time intervals post reperfusion.

Another downstream target of the PI3K-AKT survival pathway is pro-apoptotic protein BAD. Activation of BAD by PI3K – AKT cell survival pathway can attenuate cellular apoptosis by promoting its association with 14-3-3 proteins abolishing the apoptotic effect.

Phosphorylation of BAD $_{(ser136)}$ was observed in all tissues and time points assessed. The phosphorylation of BAD $_{(ser136)}$ was significantly decreased after 10 minutes of reperfusion compared to the non ischaemic basal group ($P<0.01$) Figure 5.10. Administration of 2-Cl-IB-MECA (100 nM) for the first 10 minutes of reperfusion significantly upregulated the phosphorylation of BAD $_{(ser136)}$ compared to time matched control group ($P<0.01$) Figure 5.10.



**** P<0.01 Control 10, 20 minutes reperfusion vs. Basal. $ P<0.01 MECA 10, 20 minutes reperfusion vs. Control 10, 20 minutes reperfusion repectively.**

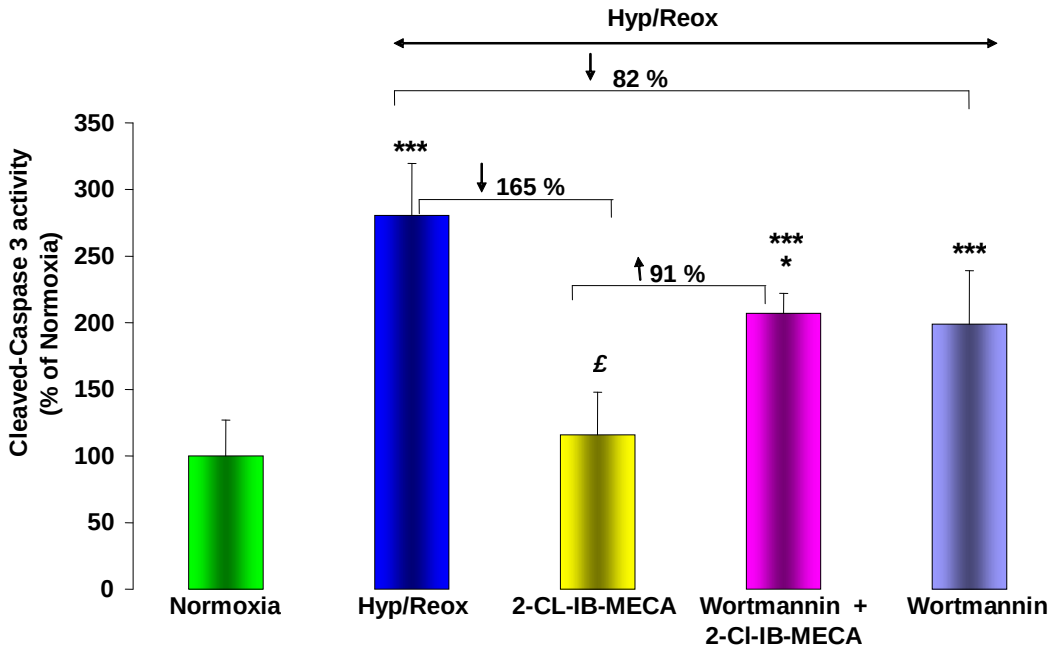
Figure 5.10. Assessment of BAD $_{(ser136)}$ phosphorylation in isolated hearts to subjected 60 minutes of perfusion (basal) or 35 minutes of ischaemia followed by 5, 10 or 20 of reperfusion in non-treated control (Con) and A_3 agonist 2-CL-IB-MECA (100nM) (MECA) treated hearts. 2-CL-IB-MECA was administered at the onset of reperfusion. Results are shown as Mean ± SE of three individual experiments.

5.6.1. Effect of 2-CL-IB-MECA on cleaved-caspase 3 activity and the role of the PI3K – AKT cell survival pathway.

To determine the role of the PI3K-AKT cell survival pathway on cleaved-caspase 3 in 2-CL-IB-MECA (1 nM) medicated cardioprotection, isolated adult rat cardiac myocytes underwent 6 hours of hypoxia and 18 hours of reoxygenation where the A_3 agonist 2-CL-IB-MECA (1 nM) was administered in the presence and absence of PI3K inhibitor Wortmannin (5 nM) throughout reoxygenation.

Administration of 2-CL-IB-MECA (1 nM) throughout reoxygenation significantly decreased cleaved-caspase 3 activity that was significantly abolished in the presence of the PI3K inhibitor Wortmannin (5 nM) (116 ± 32 % 2-CL-IB-MECA vs. 207 ± 15 % 2-CL-IB-MECA + Wortmannin $P<0.05$) Figure 5.11 and Figure 5.14. Administration of Wortmannin (5nM) throughout reoxygenation alone had no significant effect of cleaved-caspase 3 activity compared to the non-treated Hyp/Reox group (199 ± 40 % Wortmannin vs. 281 ± 39 % $P>0.05$) Figure 5.11 and Figure 5.14.

Figure 5.14 shows a representative scatter graph from the FACS flow cytometer showing the difference in cleaved-caspase 3 activity in normoxic myocytes as well as myocytes that have undergone 6 hours of hypoxia and 18 hours of reoxygenation where the A_3 agonist 2-CL-IB-MECA (1 nM) was administered throughout reoxygenation in the presence and absence of the PI3K inhibitor Wortmannin (5 nM).



*****P<0.001 Hyp/Reox, Wortmannin + 2-CL-IB-MECA, Wortmannin vs. Normoxia. £ P<0.001 2-CL-IB-MECA vs. Hyp/Reox. * P<0.05 Wortmannin + 2-CL-IB-MECA vs. 2-CL-IB-MECA. Mean ± SEM of 5 individual experiments.**

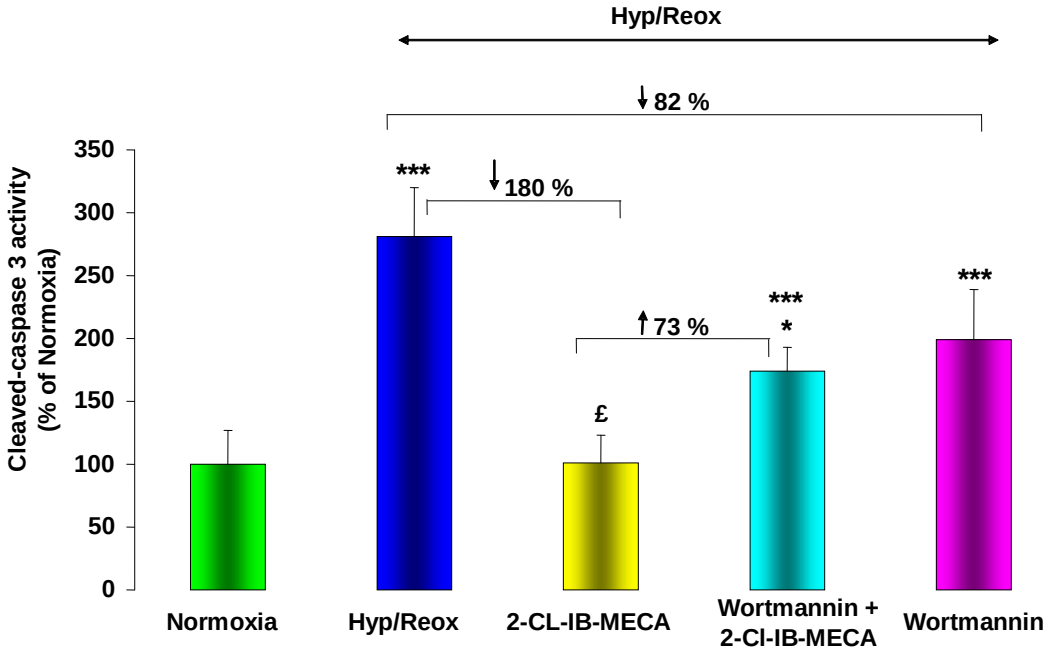
Figure 5.11. Cleaved-caspase 3 activity in isolated adult rat cardiac myocytes subjected to 24 hours oxygenation (Normoxia) or 6 hours of hypoxia followed by 18 hours of reoxygenation. The A_3 agonist 2-CL-IB-MECA (1 nM) was administered throughout reoxygenation in the presence and absence of the PI3 kinase inhibitor Wortmannin (100 nM).

5.6.2. Effect of 2-CL-IB-MECA (10 nM) on cleaved-caspase 3 activity and the role of the PI3K – AKT cell survival pathway.

To determine the role of the PI3K-AKT cell survival pathway on cleaved-caspase 3 in 2-CL-IB-MECA (10 nM) medicated cardioprotection, isolated adult rat cardiac myocytes underwent 6 hours of hypoxia and 18 hours of reoxygenation where the A_3 agonist 2-CL-IB-MECA (10 nM) was administered in the presence and absence PI3K inhibitor Wortmannin (5 nM) throughout reoxygenation.

Administration of 2-CL-IB-MECA (10 nM) throughout reoxygenation significantly decreased caspase 3 activity compared to the non-treated Hyp/Reox group (101 ± 22% 2-CL-IB-MECA vs. 281 ± 32 % Hyp/Reox P<0.001) Figure 5.12.

Administration of 2-CL-IB-MECA (10 nM) throughout reoxygenation in the presence of the PI3K inhibitor Wortmannin (5 nM) was seen to significantly abolish the A_3 agonist dependant decrease in cleaved-caspase 3 activity compared to when administered alone throughout reoxygenation (174 ± 19 % 2-CL-IB-MECA + Wortmannin vs.101 ± 32 % 2-CL-IB-MECA $P<0.05$) Figure 5.12. Administration of Wortmannin (5 nM) throughout reoxygenation alone had no significant effect on cleaved-caspase 3 activity compared to the non-treated Hyp/Reox group (199 ± 40 % Wortmannin vs. 281 ± 39 % $P>0.05$) Figure 5.12.



*****P<0.001 Hyp/Reox, Wortmannin + 2-CL-IB-MECA , Wortmannin vs. Normoxia. £ P<0.001 2-CL-IB-MECA vs. Hyp/Reox. * P<0.05 Wortmannin + 2-CL-IB-MECA vs. 2-CL-IB-MECA. Mean ± SEM of 5 individual experiments.**

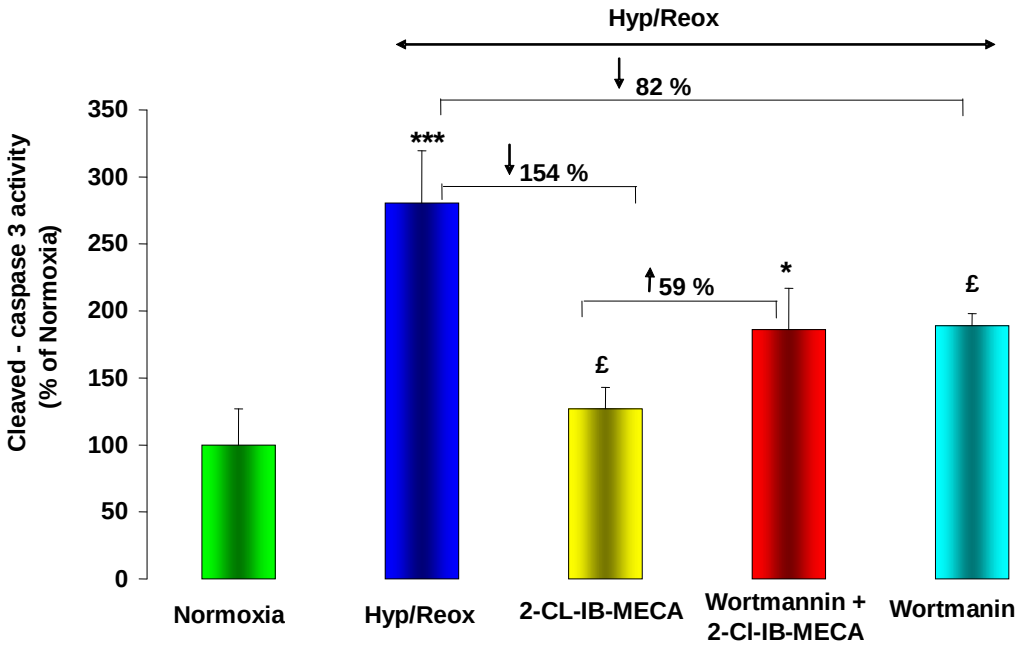
Figure 5.12. Cleaved-caspase 3 activity in isolated adult rat cardiac myocytes subjected to 24 hours oxygenation (Normoxia) or 6 hours of hypoxia followed by 18 hours of reoxygenation. The A_3 agonist 2-CL-IB-MECA (10 nM) was administered at the onset of reoxygenation in the presence and absence of the PI3 kinase inhibitor Wortmannin (5 nM).

5.6.3. Effect of 2-CL-IB-MECA (100 nM) on cleaved-caspase 3 activity and the role of the PI3K – AKT cell survival pathway.

Administration of 2-CL-IB-MECA (100 nM) throughout reoxygenation was also seen to significantly decrease cleaved-caspase 3 activity compared to the non-treated Hyp/Reox group (127 ± 16 % 2-CL-IB-MECA vs. 281 ± 39 % Hyp/Reox $P<0.001$) Figure 5.13.

Administration of 2-CL-IB-MECA (100 nM) throughout reoxygenation in the presence of the PI3K inhibitor Wortmannin (100 nM) was seen to significantly abolish the decrease in cleaved-caspase 3 activity compared to when 2-CL-IB-MECA (100 nM) was administered alone throughout reoxygenation (186 ± 31 % 2-CL-IB-MECA + Wortmannin vs. 127 ± 16 % 2-CL-IB-MECA $P<0.05$) Figure.5.13.

Previous studies have used the PI3K inhibitor Wortmannin at the concentration of 5 nM and 100 nM. The concentration of Wortmannin was increased to 100 nM when the concentration of 2-CL-IB-MECA was increased above 1 nM (Young et al., 2000; Jonassen et al., 2001; Park et al., 2006).



*****P<0.001 Hyp/Reox vs. Normoxia. £P<0.001 2-CL-IB-MECA vs. Hyp/Reox.**
*** P<0.05 Wortmannin + 2-CL-IB-MECA vs. 2-CL-IB-MECA.**
Mean ± SEM of 5 individual experiments.

Figure 5.13. Cleaved-caspase 3 activity in isolated adult rat cardiac myocytes subjected to 24 hours of oxygenation or 6 hours of hypoxia followed by 18 hours of reoxygenation. The A_3 agonist 2-CL-IB-MECA (100 nM) was administered at the onset of reoxygenation in the presence and absence of the PI3 kinase inhibitor Wortmannin (100 nM).

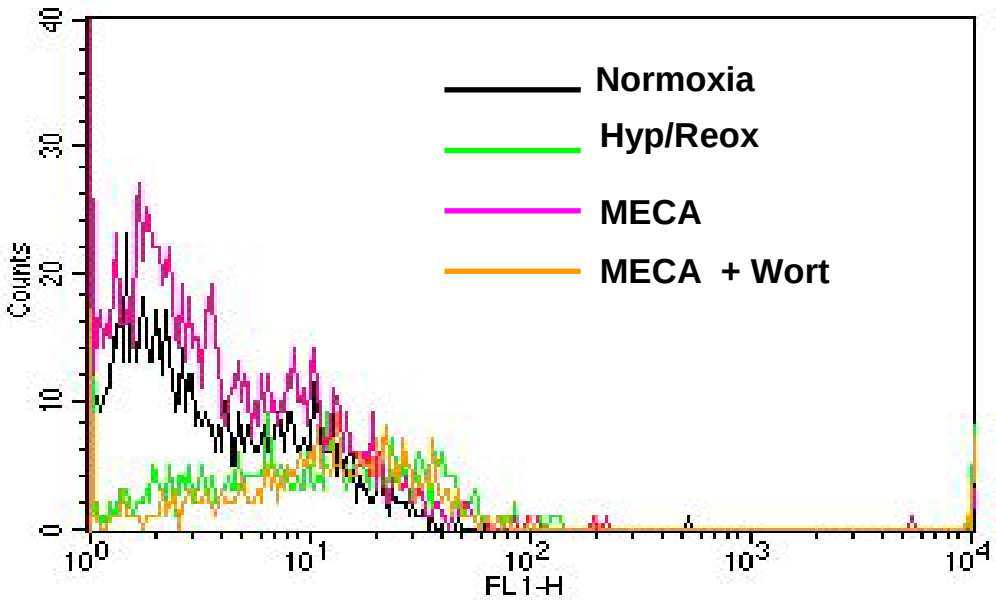


Figure 5.14. Representative graph from the FACS flow cytometer FL-1 channel showing mean fluorescence of cleaved-caspase 3 in isolated adult rat cardiac myocytes subjected 24 hours oxygenation (Normoxia), 6 hours of hypoxia followed by 18 hours of reoxygenation (Hyp/Reox). The A_3 agonist 2-CL-IB-MECA (1 nM) (Meca) was administered at the onset of reoxygenation in the presence of the PI3 kinase inhibitor Wortmannin (5 nM) (Wort). The graph shows the changes in the expression of cleaved-caspase 3. Hypoxia/reoxygenation resulted in a significant increase in the expression of cleaved-caspase 3 that was reversed by the administration of 2-CL-IB-MECA at the onset of reoxygenation.

5.7 Conclusion

In conclusion activation of A_3ARs at the onset of reperfusion with 2-CL-IB-MECA (1 nM or 100 nM) significantly decreased infarct size to risk ratio compared to non-treated controls where the protection was abolished by the PI3K inhibitor Wortmannin (5 nM or 100nM) or the mTOR inhibitor Rapamaycin (2 nM).

Administration of 2-CL-IB-MECA (1 nM or 100 nM) at reoxygeantion significantly decreased apoptosis and necrosis that was abolished in the presence of the PI3K inhibitor Wortmannin or the mTOR inhibitor Rapamycin (2 nM).

Administration of of 2-CL-IB-MECA (1 nM) was associated with a decrease in AKT phosphorylation compared to time matched controls. Administration of 2-CL-IB-

MECA (100 nM) was associated with an increase in AKT and P70S6K phosphorylation compared to their time matched controls that was abolished in the presence of their respective inhibitors. Activation of A_3ARs at reperfusion by 2-CL-IB-MECA (100nM) was associated with increased phosphorylation of BAD.

Isolated cardiac myocytes were subjected to hypoxia/reoxygenation injury resulting in a significant increase in cleaved capsase 3 activity that was abolished by the presence of the A_3AR agonist 2-CL-IB-MECA (1 nM or 100 nM). This cytoprotective anti-cleaved caspase 3 activity was abolished by the co-administration of the PI3K inhibitor Wortmannin (5 nM or 100nM).

Chapter 6. Postponing the administration of 2-CL-IB-MECA protects the myocardium from ischaemia reperfusion injury via PI3K-AKT cell survival pathway

6.1.0. Results-Infarct size to Risk Ratio Analysis

We have previously shown in chapter 4, that the administration of 2-CL-IB-MECA (1nM) 15 minutes and 30 minutes post reperfusion to protect the ischaemic myocardium from reperfusion injury. To determine whether this protection was dependant on recruitment of PI3K/AKT cell survival pathway isolated perfused rat hearts underwent 35 minutes of ischaemia and 120 minutes of reperfusion where the A_3 agonist 2-CL-IB-MECA (1nM) was administered 15 minutes or 30 minutes after the onset of reperfusion in the presence or absence of the PI3K inhibitor Wortmannin

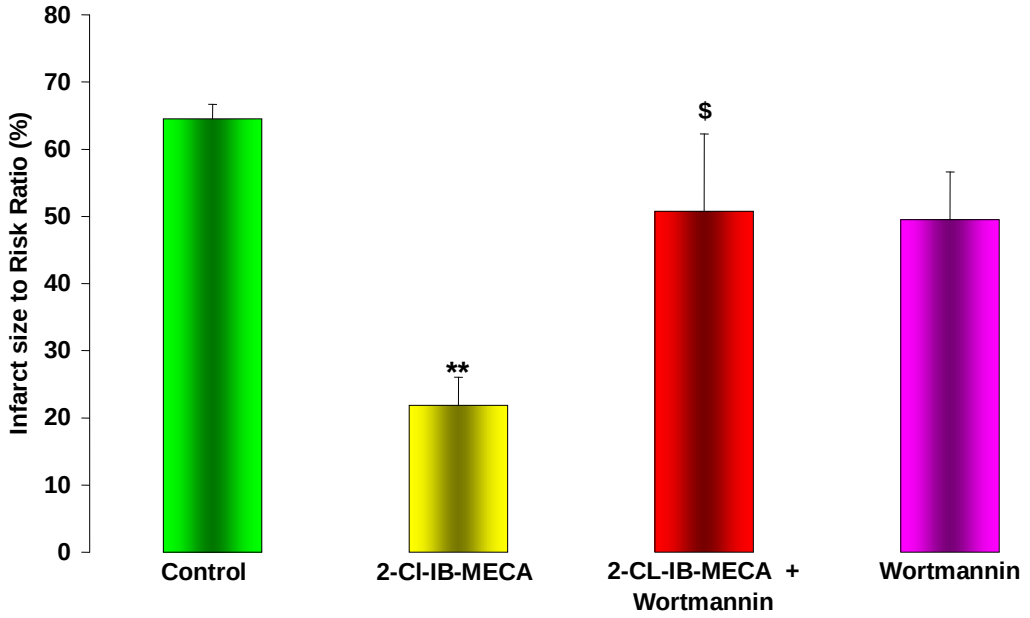
6.1.1. Administration of the A_3 agonist 2-CL-IB-MECA 15 minutes post reperfusion protects the ischaemic myocardium via the PI3K-AKT cell survival pathway.

Postponing the administration of the A_3 agonist 2-CL-IB-MECA (1nM) to 15 minutes after the onset of reperfusion significantly protected the ischaemic reperfused myocardium. To elucidate the intracellular signalling pathways via which this protection was being mediated hearts were perfused with 2-CL-IB-MECA (1 nM) in the presence and absence of the PI3K inhibitor Wortmannin (100 nM) 15 minutes after the start of reperfusion.

Administration of the A_3 agonist 2-CL-IB-MECA (1 nM) in the presence of the PI3K inhibitor Wortmannin (100 nM) 15 minutes after the onset of reperfusion significantly abolished the protection of 2-CL-IB-MECA (1 nM) when administered alone at

reperfusion (51 ± 9 % 2-CL-IB-MECA Post-15 + Wortmannin vs. 22 ± 4 % 2-CL-IB-MECA Post-15, P<0.05) Figure 6.1.

Wortmannin (100 nM) alone had no significant effect of the development of infarct size to risk ratio in the ischaemic reperfused myocardium compared to control (50 ± 10 % Wortmannin vs. 65 ± 2 Control, P>0.05) Figure 6.1.



**** P<0.01 2-CL-IB-MECA vs. Control. $ P<0.01 Wortmannin + 2-CL-IB-MECA vs. 2-CL-IB-MECA at 15 minutes Post-Rep. Mean ± SEM.**

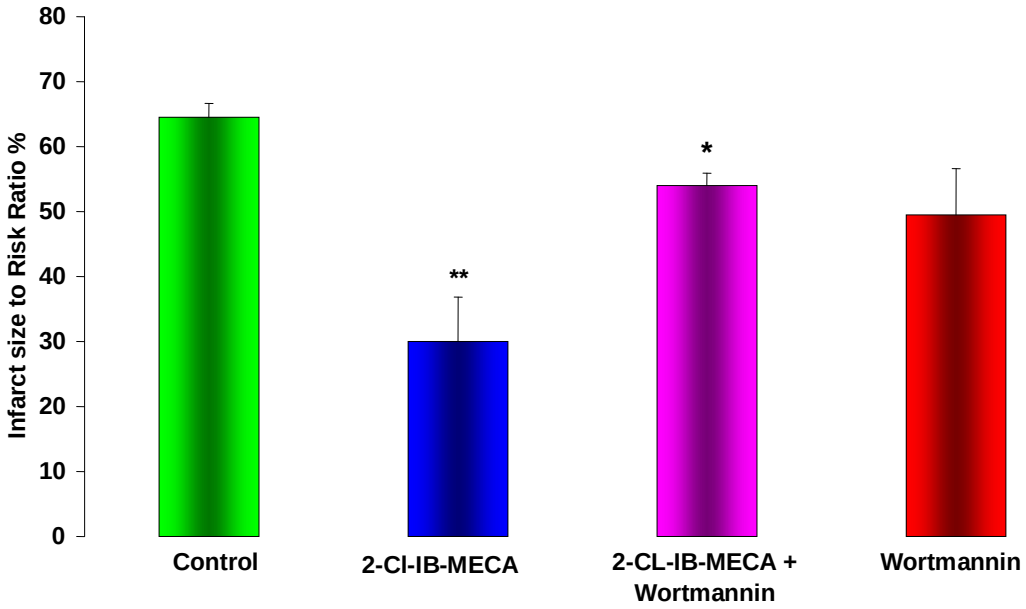
Figure 6.1. Infarct size to Risk ratio (%) in non-treated control and 2-Cl-IBMECA (1 nM) treated ischaemic reperfused hearts. Isolated perfused rat hearts where subjected to 35 minutes of ischaemia and 120 minutes of reperfusion where the A_3 adenosine receptor 2-Cl-IB-MECA (1 nM) was administered at 15 minutes after reperfusion in the presence and absence of the PI3K inhibitor Wortmannin (100 nM).

6.1.2. Administration of the A_3 agonist 2-CL-IB-MECA 30 minutes post reperfusion protects the ischaemic myocardium via the PI3K-AKT cell survival pathway.

Administration of A_3 agonist 2-CL-IB-MECA (1 nM) when administered at reperfusion or 15 minutes post reperfusion was seen to protect the ischaemic

myocardium from ischaemia reperfusion injury via recruitment of the PI3K pathway. To determine whether the protection afforded by 2-CL-IB-MECA (1 nM) when administered 30 minutes after the onset of reperfusion was via recruitment of the PI3K-AKT pathway 2-CL-IB-MECA (1 nM) was administered at reperfusion in the presence of the PI3K inhibitor Wortmannin (100 nM). Administration of 2-CL-IB-MECA (1 nM) 30 minutes after the onset of reperfusion in the presence of the PI3K inhibitor Wortmannin (100 nM) significantly abolished the protection when 2-CL-IB-MECA (1 nM) was administered alone 30 minutes post reperfusion (54.0 ± 2 % 2-CL-IB-MECA Post-30 + Wortmannin vs. 30 ±7 % 2-CL-IB-MECA Post-30 $P<0.05$) Figure 6.2.

Wortmannin (100 nM) alone had no significant effect on the development of infarct size to risk ratio in the ischaemic reperfused myocardium compared to control (50 ± 10 % Wortmannin vs. 65 ± 2 Control, $P>0.05$) Figure 6.2.



**** P<0.01 2-CL-IB-MECA vs. Control. * P<0.05 Wortmannin + 2-CL-IB-MECA vs. 2-CL-IB-MECA at 30 minutes Post-Rep.**
Mean ± SEM.

Figure 6.2. Infarct size to Risk ratio (%) in non-treated control and 2-Cl-IBMECA (1 nM) treated ischaemic reperfused hearts. Isolated perfused rat hearts where subjected to 35 minutes of ischaemia and 120 minutes of reperfusion where the A_3 adenosine receptor 2-Cl-IB-MECA (1 nM) was administered at 30 minutes after reperfusion in the presence and absence of the PI3K inhibitor Wortmannin (100 nM).

6.2.1. Postponing the administration of 2-CL-IB-MECA 15 minutes post reoxygenation protects isolated adult rat cardiomyocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation from reoxygenation injury via the PI3K-AKT cell survival pathway.

To further determine the cardioprotective role of the A_3 agonist 2-CL-IB-MECA when administered 15 minutes post reperfusion isolated adult rat cardiomyocytes when subjected to 6 hours of ischaemia followed by 18 hours of reoxygenation. 2-CL-IB-MECA (10 nM) was administered 15 minutes after the initiation of reoxygenation in the presence of the PI3K inhibitor Wortmannin (100 nM).

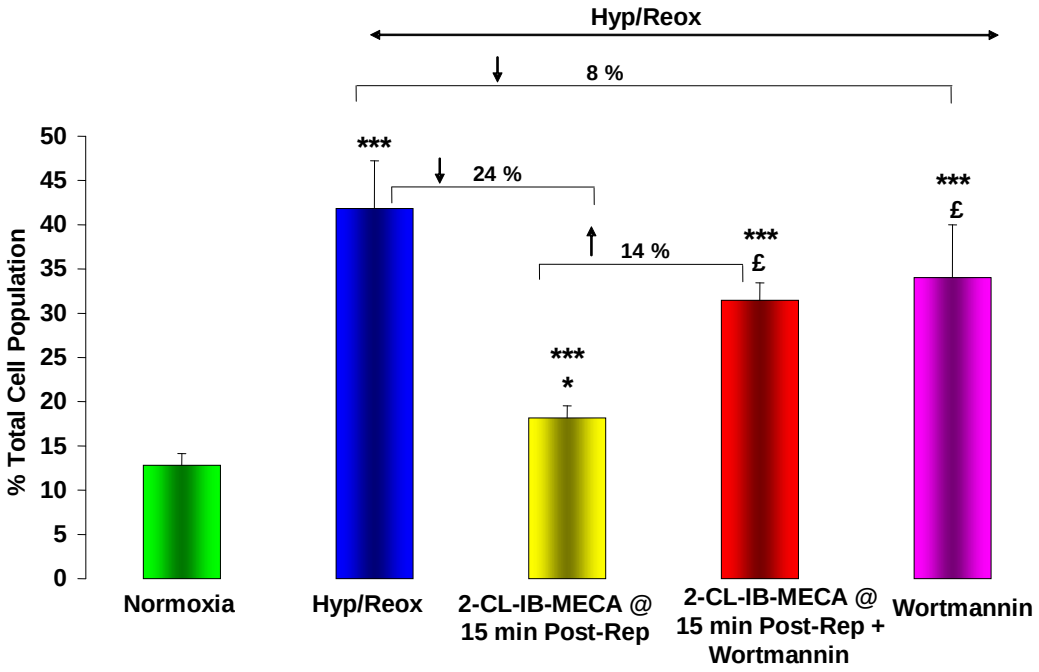
In previous experiments we used 2-CL-IB-MECA at a concentration of 1 nM to initiate protection, but when this concentration was used at 15 and 30 minutes post reperfusion it failed to induce protection, however protection was seen when the concentration was increased 10 nM. Furthermore, the protection observed with 2-Cl-IB-MECA (10 nM) was not blocked by Wortmannin at 5 nM, but was blocked by Wortmannin at 100 nM concentration (data not shown).

Postponing the administration of the A_3 agonist 2-CL-IB-MECA (10 nM) to 15 minutes post reoxygenation significantly decreased the number of apoptotic myocytes compared to the Hyp/Reox group (18 ± 1% 2-CL-IB-MECA Post-15 vs. 42 ± 5 % Hyp/Reox $P<0.05$) Figure 6.3a.

Postponing the administration of the A_3 agonist 2-CL-IB-MECA (10 nM) to 15 minutes post reoxygenation significantly decreased the number of necrotic myocytes compared to the Hyp/Reox group (21± 1% 2-CL-IB-MECA Post-15 vs. 32 ± 2 % Hyp/Reox $P<0.05$) Figure 6.3.b.

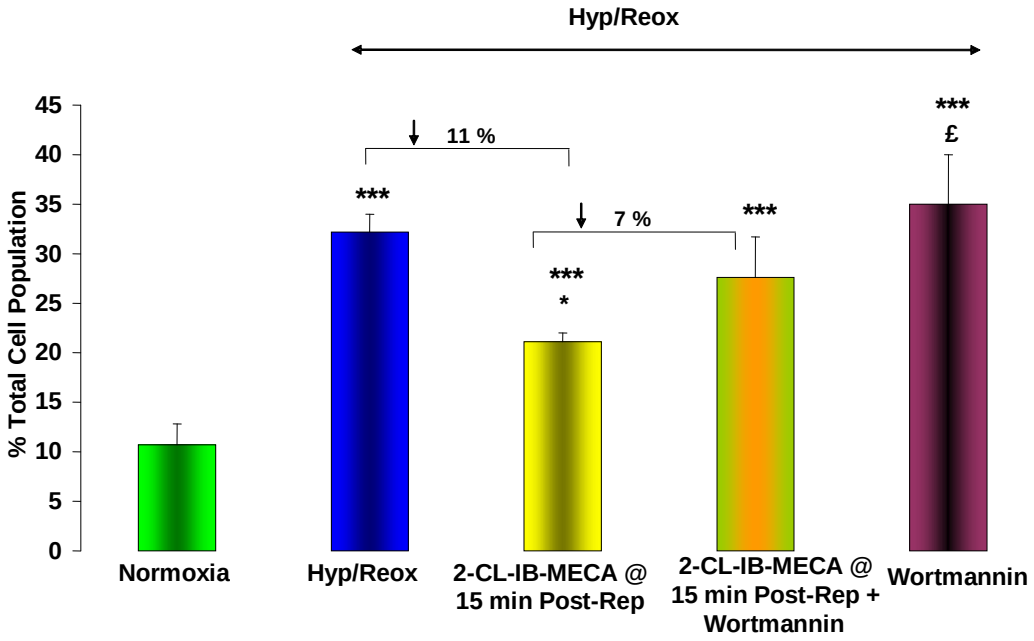
Administration of 2-CL-IB-MECA (10 nM) 15 minutes post reperfusion in the presence of the PI3K inhibitor Wortmannin (100 nM) significantly abolished the anti-apoptotic effect compared to when 2-CL-IB-MECA (10 nM) was administered alone 15 minutes post reperfusion (18 ± 1% 2-CL-IB-MECA Post 15 vs. 32 ± 2 % 2-CL-IB-MECA Post 15 + Wortmannin $P<0.05$) Figure 6.3.a. Furthermore, the anti-necrotic effect of 2-CL-IB-MECA (10 nM) when administered alone 15 minutes post reperfusion was partially abolished in the presence of the PI3K inhibitor Wortmannin (100 nM), but did not reach statistical significance (21 ± 1 % 2-CL-IB-MECA Post 15 vs. 28 ± 4 % 2-CL-IB-MECA Post 15 + Wortmannin $P>0.05$) Figure 6.3.b.

Administration of Wortmannin (100 nM) alone throughout reoxygenation had no significant effect on myocyte apoptosis compared to the Hyp/Reox group (34 ± 6 % Wortmannin vs. 42 ± 5% Hyp/Reox P>0.05) Figure 6.3.a. Administration of Wortmannin (100 nM) alone throughout reoxygenation had no significant effect on myocyte necrosis compared to the Hyp/Reox group (35 ± 5 % Wortmannin vs. 32 ± 2 % Hyp/Reox P>0.05) Figure 6.3.b.



*****P<0.001 Hyp/Reox, 2-CL-IB-MECA, Wortmannin + 2-CL-IB-MECA, Wortmannin vs. Normoxia. *P<0.05 2-CL-IB-MECA vs. Hyp/Reox. £ P<0.05 2-CL-IB-MECA + Wortmannin, Wortmannin vs. 2-CL-IB-MECA.**

Figure 6.3.a. Assessment of apoptosis in isolated adult rat cardiomyocytes subjected to 24 hours oxygenation (Normoxia) or 6 hours hypoxia and 18 hours of reoxygenation. Assessment of the PI3k / Akt cell survival pathway in 2-CLIB-MECA mediated cardioprotection. The A_3AR agonist 2-CL-IB-MECA (10 nM) added 15 minutes post-reperfusion in the presence and absence of the PI3K inhibitor Wortmannin (100 nM). Results are shown as Mean ± SEM and are expressed as a percentage of the total cells counted.



*****P<0.001 Hyp/Reox, 2-CL-IB-MECA at 15 minutes post-rep vs. Normoxia. *P<0.05 2-CL-IB-MECA at 15 minutes post-rep vs. Hyp/Reox. £ P<0.05 Wortmannin vs. 2-CL-IB-MECA.**

Figure 6.3.b. Assessment of necrosis in isolated adult rat cardiomyocytes subjected to 24 hours oxygenation (Normoxia) or 6 hours hypoxia and 18 hours of reoxygenation. Assessment of the PI3k / Akt cell survival pathway in 2-CLIB-MECA mediated cardioprotection. The A_3AR agonist 2-CL-IB-MECA (10 nM) added 15 minutes post-reperfusion in the presence and absence of the PI3K inhibitor Wortmannin (100 nM). Results are shown as Mean ± SEM and are expressed as a percentage of the total cells counted.

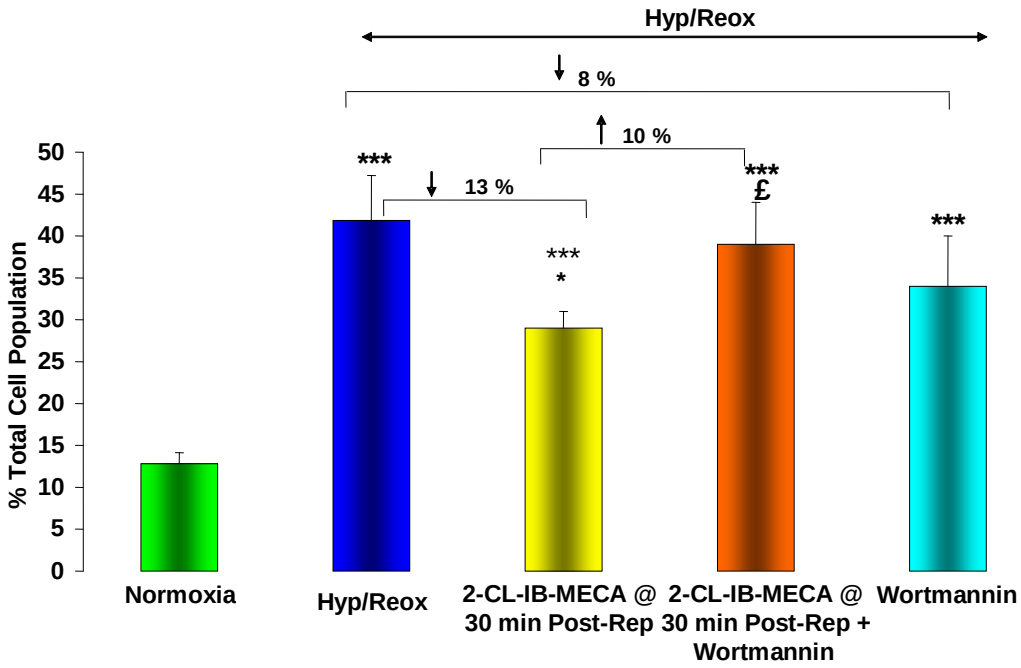
6.2.2. Postponing the administration of 2-CL-IB-MECA 30 minutes post reoxygenation protects isolated adult rat cardiomyocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation from reoxygenation injury via the PI3K - AKT cell survival pathway.

Postponing the administration of the A_3 agonist 2-CL-IB-MECA (10 nM) to 30 minutes post reoxygenation significantly decreased the number of apoptotic cells compared to the non-treated Hyp/Reox group (29 ± 3% 2-CL-IB-MECA Post 30 vs. 42 ± 5 % Hyp/Reox $P<0.01$) Figure 6.4.a. Postponing the administration of the A_3 agonist 2-CL-IB-MECA (10 nM) to 30 minutes post reoxygenation also significantly decreased the number of necrotic cells compared to the non-treated Hyp/Reox group (23 ± 3% 2-CL-IB-MECA Post-30 vs. 32 ± 2 % Hyp/Reox $P<0.05$) Figure 6.4.b.

Administration of 2-CL-IB-MECA (10 nM) 30 minutes post reperfusion significantly decreased cellular apoptosis that was abolished in the presence of the PI3K inhibitor Wortmannin (100 nM) (29 ± 3% 2-CL-IB-MECA Post-30 vs. 39 ± 5 % 2-CL-IB-MECA Post-30 + Wortmannin $P<0.05$) Figure 6.4.a. Furthermore, the anti-necrotic effect of 2-CL-IB-MECA (10 nM) when administered 30 minutes post reperfusion was partially abolished in the presence of the PI3K inhibitor Wortmannin (100 nM), but did not reach statistical significance (23 ± 3 %2-CL-IB-MECA Post-30 vs. 28 ± 2 % 2-CL-IB-MECA Post 30 + Wortmannin $P>0.05$) Figure 6.4.b.

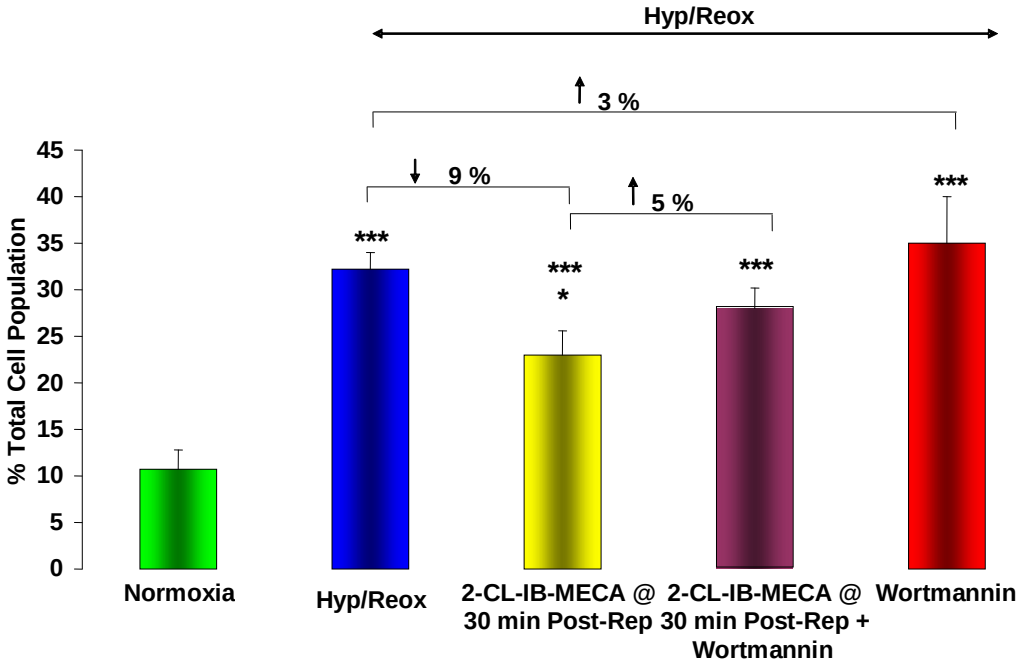
Administration of Wortmannin (100 nM) alone throughout reoxygenation had no significant effect on myocyte apoptosis compared to the Hyp/Reox group (34 ± 6 % Wortmannin vs. 42 ± 5% Hyp/Reox $P>0.05$) Figure 6.4.a. Administration of Wortmannin (100 nM) alone throughout reoxygenation had no significant effect on

myocyte necrosis compared to the Hyp/Reox group (35 ± 5 % Wortmannin vs. 32 ± 2 % Hyp/Reox P>0.05) Figure 6.4.b.



*****P<0.001 Hyp/Reox, 2-CL-IB-MECA at 15 minutes post-rep, 2-CL-IB-MECA+Wortmannin, Wortmannin vs. Normoxia.*P<0.05 2-CL-IB-MECA at 30 minutes post-rep vs. Hyp/Reox. £ P<0.05 2-CL-IB-MECA + Wortmannin vs. 2-CL-IB-MECA.**

Figure 6.4.a. Assessment of apoptosis in isolated adult rat cardiomyocytes subjected to 24 hours oxygenation (Normoxia) or 6 hours hypoxia and 18 hours of reoxygenation. Assessment of the PI3K / Akt cell survival pathway in 2-CL-IB-MECA mediated cardioprotection. The A_3AR agonist 2-CL-IB-MECA (10 nM) was added at 30 minutes post-reperfusion in the presence and absence of the PI3K inhibitor Wortmannin (100 nM). Results are shown as Mean ± SEM and are expressed as a percentage of 10,000cells counted.



*****P<0.001 Hyp/Reox, 2-CL-IB-MECA, 2-CL-IB-MECA + Wortmannin, Wortmannin vs. Normoxia. *P<0.05 2-CL-IB-MECA vs. Hyp/Reox.**

Figure 6.4.b. Assessment of necrosis in isolated adult rat cardiomyocytes subjected to 24 hours oxygenation (Normoxia) or 6 hours hypoxia and 18 hours of reoxygenation. Assessment of the PI3K / Akt cell survival pathway in 2-CL-IB-MECA mediated cardioprotection. The A_3AR agonist 2-CL-IB-MECA (10 nM) was added at 30 minutes post-reperfusion in the presence and absence of the PI3K inhibitor Wortmannin (100 nM). Results are shown as Mean ± SEM and are expressed as a percentage of 10,000cells counted.

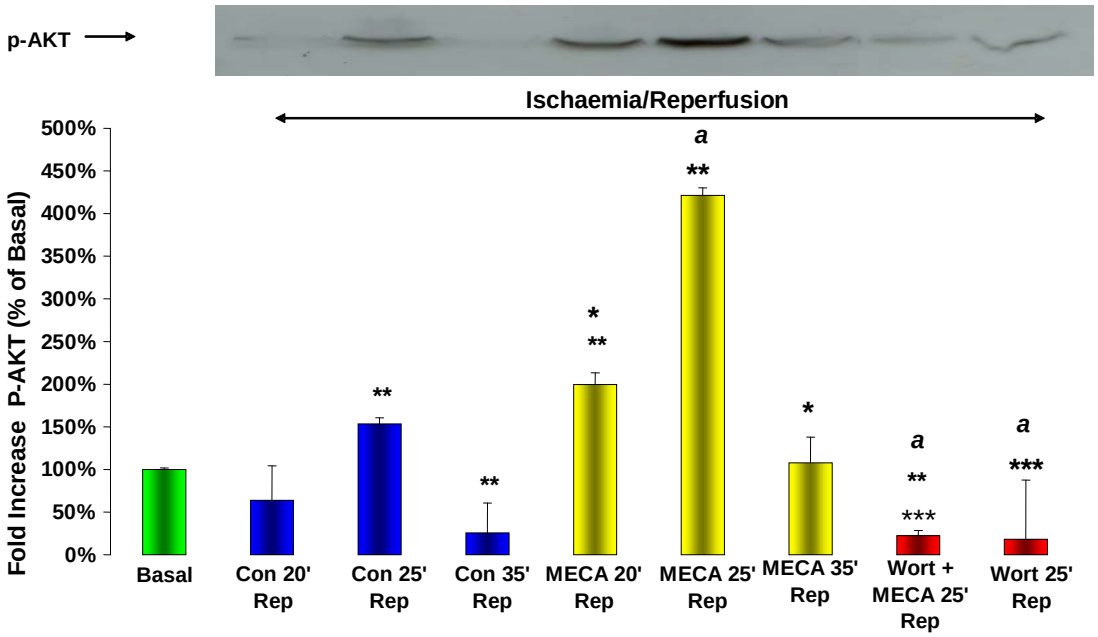
6.3.1. Role of AKT in 2-CL-IB-MECA mediated cardioprotection when administered 15 minutes post reperfusion.

Having determined that 2-CL-IB-MECA (1 nM) protects the isolated perfused rat heart from ischaemia reperfusion injury when administered 15 minutes after the onset of reperfusion via the PI3K-AKT pathway it was important to examine the effect of the A_3 agonist and the PI3K inhibitor Wortmannin (100 nM) on AKT $_{(ser\ 473)}$ phosphorylation. Hearts were harvested 20, 25 and 35 minutes after reperfusion where the A_3 agonist 2-CL-IB-MECA (1 nM) was administered 15 minutes after the onset of reperfusion.

In control hearts AKT $_{(ser\ 473)}$ phosphorylation was observed at 20, 25 and 35 minutes of reperfusion. Administration of 2-CL-IB-MECA (1 nM) significantly up regulated the phosphorylation of AKT$_{(ser\ 473)}$ with maximal phosphorylation of AKT$_{(ser\ 473)}$ at 25 minutes of reperfusion compared to time matched non-treated control, where A_3 agonist 2-CL-IB-MECA (1 nM) was perfused after 15 minutes of reperfusion $P<0.001$ (Figure. 6.4..a,b). Despite the presence of 2-CL-IB-MECA (1nM) tissues harvested at 35 minutes of reperfusion did not show a further increase in AKT phosphorylation although there was a significant increase compared to its time matched non-treated control ($P<0.001$) (Figure 6.5.a,b).

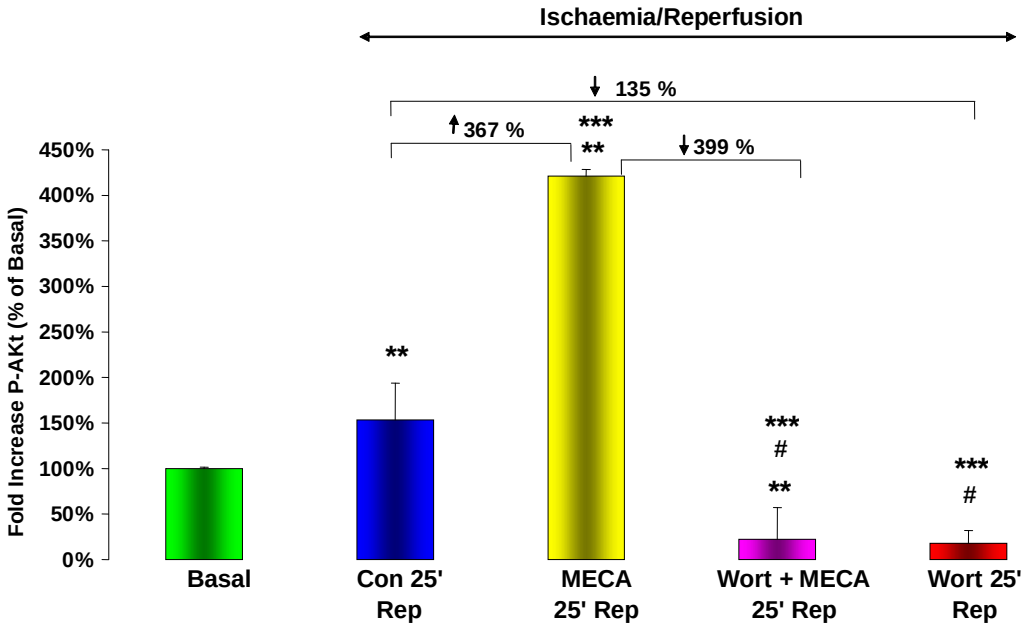
Administration of 2-CL-IB-MECA (1 nM) 15 minutes post-reperfusion upregulated AKT $_{(ser\ 473)}$ phosphorylation after 25 minutes of reperfusion that was significantly abolished in the presence of PI3K inhibitor Wortmannin (100 nM) ($P<0.001$) Figure 6.5.a,b. Administration of the PI3K inhibitor Wortmannin (100 nM) alone for 25

minutes of reperfusion significantly decreased AKT $_{(ser\ 473)}$ phosphorylation compared to the time matched non-treated control group (P<0.01) (Figure 6.5.a,b).



***P<0.05 MECA 20, 35 vs. Control 20, 35. ***P<0.001Wort+MECA 25, WORT 25 vs. MECA 25 minutes post reperfusion. **P<0.01 Control 25,35, MECA 20,25, WORT+MECA 25 vs. basal. *a* P<0.001 WORT+ MECA, WORT vs. control 25 minutes of reperfusion.**

Figure 6.5.a. Assessment of AKT $_{(ser473)}$ phosphorylation in isolated hearts subjected to 60 minutes perfusion (basal) or 35 minutes of ischaemia followed by 20, 25, 35 minutes of reperfusion in the presence and absence of the A_3 Agonist 2-CL-IB-MECA (1 nM) (MECA). The A_3 Agonist 2-CL-IB-MECA (1 nM) was administered 15 minutes post-reperfusion in the presence and absence of the PI3K inhibitor Wortmannin (100 nM) (Wort). Results are shown as Mean ± SEM of three individual experiments.



****P<0.01 Con 25, MECA 25, WORT+MECA 25 vs. basal. *** P<0.001 MECA 25, WORT+MECA 25, WORT 25 vs. control 25 minutes of reperfusion. #P<0.001 WORT+MECA 25, WORT vs. MECA 25 minutes post reperfusion**

Figure 6.5.b. Comparison of AKT (ser473) phosphorylation in isolated hearts subjected to 35 ischaemia followed by 25 minutes of reperfusion in the presence and absence of the A_3 Agonist 2-CL-IB-MECA (1 nM) (MECA). The A_3 Agonist 2-CL-IB-MECA (1 nM) was administered 15 minutes post-reperfusion in the presence and absence of the PI3K inhibitor Wortmannin (100 nM) (Wort). Results are shown as Mean ± SEM of three individual experiments.

6.3.2 Role of PI3K-AKT signalling pathway in 2-CL-IB-MECA mediated cardioprotection when administered 30 minutes post reperfusion.

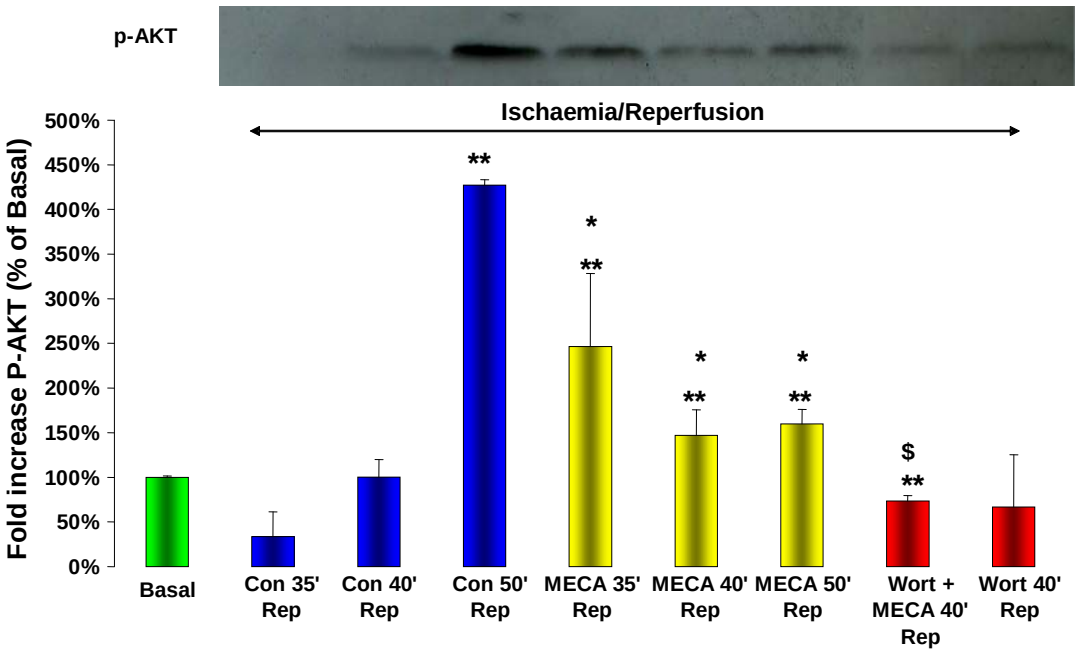
Previously, we have shown that 2-CL-IB-MECA (1nM) when administered 30 minutes post reperfusion can limit the development of infarction in the ischaemic reperfused heart where the protection was blocked by the presence of the PI3K inhibitor Wortmannin (100 nM).

To determine whether this protection afforded by 2-CL-IB-MECA (1 nM) when administered 30 minutes post reperfusion is via the PI3K –AKT cell survival pathway

heart tissues treated with 2-CL-IB-MECA (1 nM) in the presence and absence of the PI3K inhibitor Wortmannin (100 nM) at various time points post reperfusion.

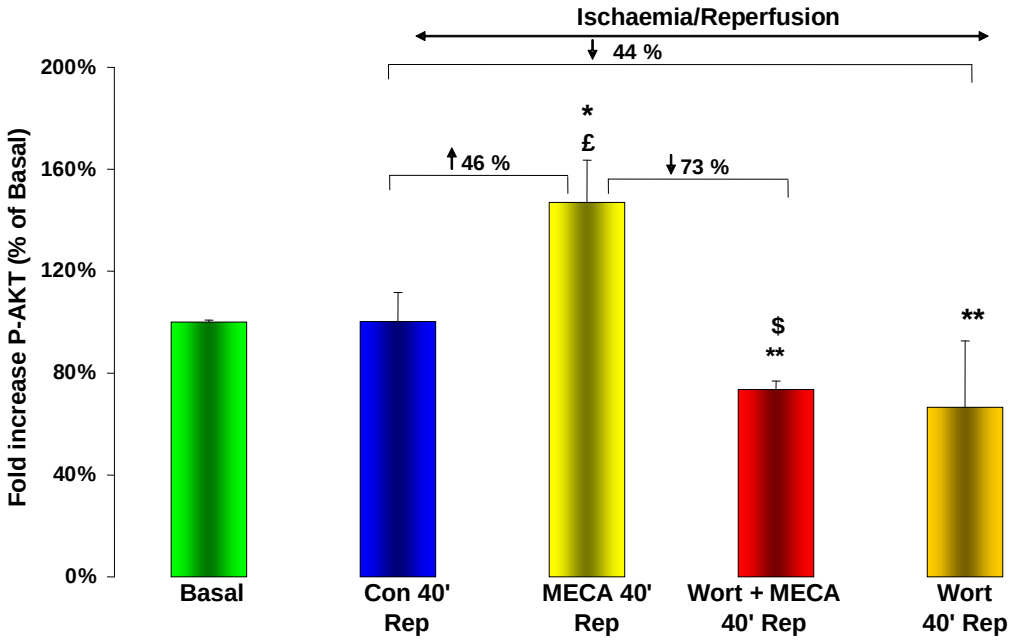
In control hearts AKT $_{(ser473)}$ phosphorylation was observed at 35, 40 and 50 minutes of reperfusion. Administration of 2-CL-IB-MECA (1 nM) 30 minutes after the onset of reperfusion significantly up regulated the phosphorylation of AKT $_{(ser473)}$ at 40 minutes of reperfusion compared to time matched controls. ($P<0.05$) (Figure 6.6.a). Administration of 2-CL-IB-MECA (1 nM) 30 minutes after the onset of reperfusion in the presence of the PI3K inhibitor Wortmannin (100 nM) abolished the increase in AKT $_{(ser473)}$ phosphorylation by 2-Cl-IB-MECA after 40 minutes of reperfusion ($P<0.05$) (Figure 6.6.a,b).

Administration of 2-CL-IB-MECA (1 nM) 30 minutes after reperfusion showed significant down regulation of AKT$_{(ser473)}$ phosphorylation after 50 minutes of reperfusion compared to time matched controls ($P<0.05$) Figure 6.6.a,b.



***P<0.05 MECA 35, 40, 50 vs. Control 35, 40, 50 respectively. $ P<0.05 WORT+MECA 40 vs. MECA 40 minutes post reperfusion. ** P<0.01 Con 50, MECA 35, 40, 50, WORT+MECA 40 vs. Basal.**

Figure 6.6.a. Assessment of Akt phosphorylation in isolated hearts subjected 60 minutes perfusion (basal) or 35 minutes of ischaemia followed by 35, 40 or 50 minutes of reperfusion in the presence and absence (cont) of the A_3 Agonist 2-CL-IB-MECA (1 nM) (MECA). The PI3K inhibitor Wortmannin (100 nM) (Wort) was administered at reperfusion in the presence and absence of the A_3AR agonist 2-CL-IB-MECA (1 nM). Results are shown as Mean ± SEM of three individual experiments.



***P<0.05 MECA 40 vs. Control 40. $ P<0.05 WORT+ MECA vs. MECA 40 minutes post reperfusion. £ P<0.01 MECA 40, WORT+MECA vs. Basal.**

Figure 6.6.b. Comparison of Akt phosphorylation in isolated hearts subjected 60 minutes perfusion (basal) or 35 minutes of ischaemia followed by 40 minutes of reperfusion in the presence and absence (con) of the A_3 Agonist 2-CL-IB-MECA (1 nM) (MECA). The PI3K inhibitor Wortmannin (100 nM) (Wort) was administered at reperfusion in the presence and absence of the A_3AR agonist 2-CL-IB-MECA (1 nM). Results are shown as Mean ± SEM of three individual experiments.

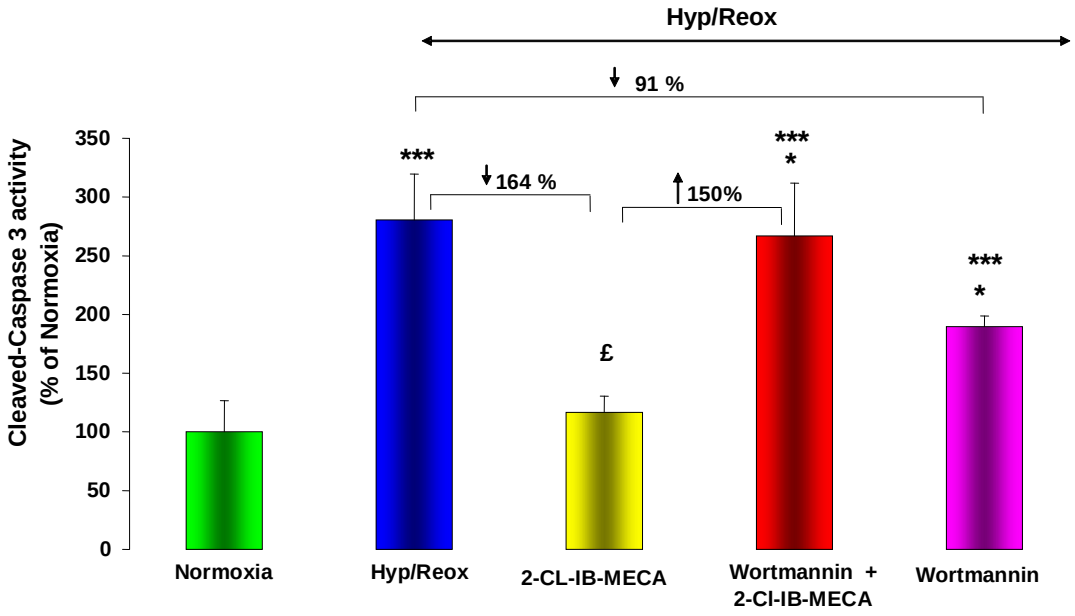
6.4.1. Effect of 2-CL-IB-MECA (10 nM) when administered 15 minutes post reoxygenation on cleaved-caspase 3 activity in isolated adult rat cardiomyocytes.

We have shown that the A_3 agonist 2-CL-IB-MECA (1 nM; 10 nM; 100 nM) significantly decreased cleaved-caspase 3 activity when administered at reperfusion in the presence of the PI3K inhibitor Wortmannin (100 nM). To determine whether 2-CL-IB-MECA (10nM) when administered 15 minutes after the onset of reoxygenation could still confer protection via decreasing cleaved-caspase 3 activity isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation where the A_3 agonist was administered 15 minutes after the onset of reoxygenation. Furthermore, to determine whether this protection was via recruitment of the PI3K – AKT cell survival pathway 2-CL-IB-MECA (10 nM) was administered 15 minutes after the onset of reoxygenation in the presence and absence of the PI3K inhibitor Wortmannin (100 nM).

Administration of 2-CL-IB-MECA (10 nM) 15 minutes after the onset of reoxygenation significantly decreased cleaved-caspase 3 activity compared to the non-treated Hyp/Reox group (117 ± 14% 2-CL-IB-MECA Post-15 vs. 281 ± 39 % Hyp/Reox $P<0.001$) (Figure 6.7).

Administration of 2-CL-IB-MECA (10 nM) decreased cleaved-caspase 3 activity when administered 15 minutes after the onset of reoxygenation that was significantly abolished in the presence of the PI3K inhibitor Wortmannin (100nM) (117 ± 14% 2-CL-IB-MECA Post-15 vs. 267 ± 66% 2-Cl-IB-MECA Post-15 + Wortmannin $P<0.05$) (Figure 6.7).

Administration of Wortmannin (100 nM) alone throughout reoxygenation resulted in a significant decrease in cleaved-caspase 3 activity compared to the Hyp/Reox group (190 ± 9 % Wortmannin vs. 281 ± 39 % Hyp/Reox P<0.05) (Figure 6.7).



***** P<0.001 Hyp/Reox, Wortmannin + 2-CL-IB-MECA, Wortmannin vs. Normoxia. £ P<0.001 2-CL-IB-MECA vs. Hyp/Reox. *P<0.05 Wortmannin+2-CL-IB-MECA, Wortmannin vs. 2-CL-IB-MECA 15 minutes Post-Reoxygenation.**

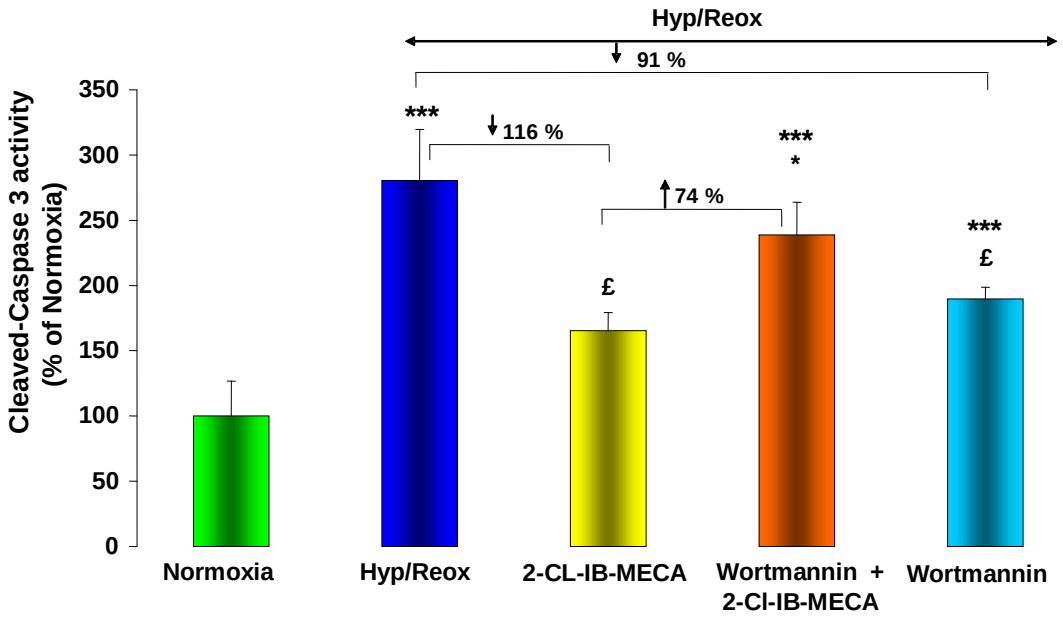
Figure 6.7. Cleaved-caspase 3 activity in isolated adult rat cardiac myocytes subjected to 24 hours oxygenation (Normoxia) or 6 hours of hypoxia followed by 18 hours of reoxygenation (Hyp/Reox). The A_3 agonist 2-CL-IB-MECA (10 nM) was administered 15 minutes after the onset of reoxygenation in the presence and absence of the PI3K inhibitor Wortmannin. Mean ± SEM of 5 individual experiments.

6.4.2. Effect of 2-CL-IB-MECA when administered 30 minutes post reoxygenation on cleaved-caspase 3 activity in isolated adult rat cardiomyocytes.

Administration of 2-CL-IB-MECA (10 nM) 30 minutes after the onset of reoxygenation significantly decreased caspase 3 activity in isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation compared to the non-treated Hyp/Reox group (165 ± 16% 2-CL-IB-MECA Post-30 vs. 281 ± 39 % Hyp/Reox $P<0.001$) (Figure 6.8).

To determine whether the decrease in cleaved-caspase 3 by 2-CL-IB-MECA (10 nM) when administered 30 minutes after reoxygenation was via the PI3K – AKT cell survival pathway we used the PI3K inhibitor Wortmannin (100 nM). Administration of 2-CL-IB-MECA (10 nM) 30 minutes after the onset of reoxygenation in the presence of the PI3K inhibitor Wortmannin (100 nM) significantly abolished the decrease in cleaved-caspase 3 compared with when 2-CL-IB-MECA (10 nM) was administered alone 30 minutes after reoxygenation (165 ± 16 % 2-CL-IB-MECA Post-30 vs. 239 ± 35 % 2-CL-IB-MECA Post-30 + Wortmannin $P<0.05$) (Figure 6.8).

Administration of Wortmannin (100 nM) alone throughout reoxygenation resulted in a significant decrease in cleaved-caspase 3 activity compared to the Hyp/Reox group (190 ± 9 % Wortmannin vs. 281 ± 39 % Hyp/Reox $P<0.05$) (Figure 6.8).



*****P<0.001 Hyp/Reox, Wortmannin + 2-CL-IB-MECA, Wortmannin vs. Normoxia. £ P<0.001 2-CL-IB-MECA vs. Hyp/Reox. * P<0.05 Wortmannin + 2-CL-IB-MECA vs. 2-CL-IB-MECA 30 minutes Post-Reoxygenation.**

Figure 6.8. 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 (10 nM) was administered 30 minutes after the onset of reoxygenation in the presence and absence of the PI3K inhibitor Wortmannin administered at the onset of reperfusion. Mean ± SEM of 5 individual experiments.

6.5. Conclusion

To conclude administration of of 2-CL-IB-MECA (1 nM) 15 or 30 minutes post-reperfusion significantly decreased infarct size to risk ratio compared to non-treated controls. This protection was abolished by the co-administration of the PI3K inhibitor Wortmannin. Administration of 2-CL-IB-MECA (1 nM) 15 or 30 minutes was associated with an increase in AKT phosphorylation compared to time matched controls. This increase in AKT was abolished in the presence of the PI3K inhibitor Wortmannin.

Activation of A_3ARs 15 or 30 minutes post-reoxygenation significantly decreased apoptosis and necrosis in hte isolated cardiac model of hypoxia/Reoxygenation injury.

This anti-apoptotic effect was abolished by the presence of Wortmannin. The anti-apoptotic effect of 2-CL-IB-MECA was not abolished by Wortmannin.

Delaying the administration of 2-CL-IB-MECA to 15 or 30 minutes after the onset of reoxygenation significantly decreased cleaved caspase 3 activity that was abolished in the presence of Wortmannin in the cardiac myocyte model of hypoxia/reoxygenation injury.

7. General Discussion

Ischaemic heart disease is a leading factor in the development of myocardial infarction in the world today. To reduce the burden of ischaemic heart disease related events on the National Health Service the health service has adopted a policy to prevent the development of ischaemic heart disease by identifying high risk patients and treating them early on (Skinner et al., 2007). Despite these changes in clinical practice a large number of patients still suffer from the deleterious consequences of ischaemic heart disease. In the clinical setting patients are often admitted on the development of angina or after suffering a myocardial infarction where blood troponin and creatine kinase levels are assessed to confirm a diagnosis of myocardial infarction as well an ECG reading showing ST segment depression (Pasternak *et al*., 1988; Collinson and Gaze. 2007). Although some patients who are suffering from a heart attack may not exhibit ST segment depression on ECG analysis. Depending on the nature and extent of injury patients undergo thrombolytic therapy, primary angioplasty or coronary artery bypass grafting to restore blood flow to the ischaemic region.

Restoration of coronary flow to the ischaemic myocardium remains the only mechanism of salvaging reversibly damaged cardiac myocytes, but itself can further hasten the injury process (Braunwald and Kloner. 1985). It is of utmost importance to elucidate the mechanisms that may be attributed to hastening the injury process to identify potential targets when developing cardioprotective agents. Therefore there is an imperative need for the development of pharmacological agents that can be used as adjunct therapies to limit ischaemia reperfusion injury to be administered in a clinical setting. Recent advances in molecular pharmacology have led to increased

understanding of cellular signalling processes that mediate myocardial ischaemia reperfusion injury. A multitude of studies have shown endogenous and exogenous agents to mediate ischaemia reperfusion injury, some having detrimental effects and others having innate cardioprotective abilities (Hausenloy and Yellon. 2004).

Adenosine levels increase in the ischaemic myocardium due to insufficient availability of oxygen and nutrients to maintain oxidative phosphorylation (Van Wylen et al., 1994; Vinten-Johansen et al., 1995; Deussen et al., 1999). Studies have shown that administration of exogenous adenosine at reperfusion can protect the ischaemic reperfused myocardium from reperfusion injury and was dependant upon adenosine receptor activation (Olfasson et al., 1987; Norton et al., 1991; Toombs et al., 1992; Zhao et al., 1993; 2001; Jordan et al.,1999). Numerous studies have also shown preconditioning with the A_3AR agonist 2-CL-IB-MECA to mediate cardioprotection in a number of different models (Olfasson et al., 1987; Norton et al., 1991; Germack and Dickenson. 2005). 2-CL-IB-MECA has also been shown to mediate cardioprotection by delayed preconditioning (Zhao et al., 2002; 2003; Tokano et al., 2001)

More recently, studies showed that ischaemic postconditioning protects the myocardium from ischaemia reperfusion injury where the protective abilities were attributed to the delayed washout of endogenous adenosine that activated adenosine receptors, inhibition of these receptors abolished protection (Kerendi et al., 2005). Adenosine analogues have been developed to show increased affinity to adenosine receptor subtypes to determine individual adenosine receptor subtype contribution to physiological processes (Tucker and Linden. 1993; Fredholm et al., 2001).

Our data showed that activation of A_3 adenosine receptors at reperfusion with 2-Cl-IB-MECA significantly attenuated myocardial infarction development in the risk zone. These findings are consistent with previous studies by Maddock et al., (2003), who showed that activation of A_3 adenosine receptors protected the ischaemic reperfused rat heart against ischaemia/reperfusion injury when administered at reperfusion. Studies by Park et al., (2006) have also shown protection with 2-CL-IB-MECA when administered 5 minutes before reperfusion till the end of reperfusion in the isolated perfused rat heart.

Cellular injury results in activation of the immune response and results in the migration of neutrophils and mast cells to the site of injury were they release intracellular components like bradykinin and histamine initiating the inflammatory response. A_3AR activation results in inhibiting neutrophil accumulation and limiting immune cell dependant cardiac injury as shown by Jordan et al., (1999).

The protection associated with A_3ARs activation by 2-CL-IB-MECA can be directly attributed to A_3AR activation and not other receptor subtypes. Ge et al., (2006) interestingly showed that cardioprotection associated with 2-CL-IB-MECA is directly attributed to A_3AR activation in wild type mice, as 2-CL-IB-MECA failed to induce protection in the ischaemic reperfused heart in A_3AR knock out mice. This study further supports our theory that 2-CL-IB-MECA protects by directly acting on A_3ARs.

Previous studies have implicated activation of A_3ARs to be both pro and anti-apoptotic. In this study we used 2-CL-IB-MECA at a concentration of 1 nM – 100 nM

which have previously been shown to be anti-apoptotic. Previously, Maddock et al., (2002) showed protection in isolated rat cardiomyocytes exposed to hypoxia/reoxygenation with nanomolar concentrations of 2-CL-IB-MECA (1-30nM) but concentrations above >30nM failed to attenuate injury in myocytes or the isolated rat heart. Our study showed 2-CL-IB-MECA to protect cardiac myocytes at the concentration of 100 nM previously shown by Maddock et al., (2002) to be not protective. These contradictory results may be explained as a result of the use of highly specific fluorochromes not available before. Abbaracchio et al., (2000) previously showed 2-CL-IB-MECA when administered at micro-molar concentrations reduced cell number in naïve Chinese hamster ovary cells transfected with the human A_3AR compared to parent cells not expressing the A_3AR. Although, the reduction in cell number was reversed by the A_3AR antagonist MRS1191 (Brambilla et al., 2000), attributing high A_3 agonist concentrations to cellular apoptosis. Shneyvays et al., (1998) have also shown IB-MECA used at >10μM induced apoptosis in neonatal cardiomyocytes, induced DNA breaks and morphological changes attributed to apoptosis. Although, the same group later showed activation of A_3ARs with nanomolar concentration of 2-CL-IB-MECA attenuated myocyte injury in myocytes exposed to hypoxic injury reversed by the AR antagonist MRS1523. They also showed that the A_3 agonist delayed the onset of irreversible injury as well as delaying the collapse of mitochondrial membrane potential (Safran et al., 2001).

Our study further demonstrated that postponing the administration of 2-CL-IB-MECA 15 minutes or 30 minutes after reperfusion still conferred cardioprotection via infarct size reduction in the isolated perfused rat heart. Postponing the administration of cardioprotective agents after the onset of reperfusion is a novel hypothesis and

therefore has not been extensively studied. Von Lubitz et al., (2001) interestingly showed IB-MECA when administered 20 minutes **after** reperfusion to attenuate infarct development in the mice brain subjected to ischaemia/reperfusion. Further, they conclude that the post ischaemic treatment resulted in improved neuronal preservation, decreased intensity of reactive gliosis and a significant reduction in microglial infiltration.

These findings cardioprotective effects of post ischaemic administration of caspase inhibitors were supported by the findings of Armstrong et al., (2001). Al-Rajaibi et al., (2006) have also shown delaying the administration of caspase inhibitors to 15, 30 or 60 minutes after the onset of reperfusion to protect the isolated perfused rat from ischaemia reperfusion injury and protect adult rat cardiac myocytes from hypoxia/reoxygenation injury. Jin et al., (2003) have shown chymase inhibitor administration 24 hours after myocardial infarction significantly improved survival rates in Syrian hamsters.

This study furthered showed activation of A_3ARs with 2-CL-IB-MECA when administered at reoxygenation attenuated apoptosis and necrosis in isolated adult rat cardiomyocytes compared to the non-treated group. These results are in accordance with Maddock et al., (2002) who showed 2-Cl-IB-MECA administration at reoxygenation attenuated apoptosis and necrosis and also that the cytoprotection was abolished by the A_3AR antagonist MRS1191.

Rivo et al., (2004) support these findings as in the feline lung model of ischaemia reperfusion injury they showed administration of IB-MECA at reperfusion

significantly reduced injury as seen by a reduction in apoptosis. The administration of the A_3AR antagonist MRS 1191 abolished this protective effect. Liang and Jacobson. (1998) have also shown 2-CL-IB-MECA to protect chick cardiac myocytes from ischaemic injury.

Despite the knowledge that activation of A_3ARs can reduce injury in response to myocardial ischaemia/reperfusion very little is known about the intracellular signalling pathways that may be involved in mediating this protection.

The literature shows programmed cell death (apoptosis) to be a key component in ischaemia reperfusion injury. Therefore, ligands or growth factors that can target apoptosis may afford an opportunity in limiting the consequences of ischaemia reperfusion injury. Innate anti-apoptotic intracellular signalling pathways have been shown to play a role in regulating apoptosis (Kunapuli et al., 2001; Buja. 2005).

Previous studies have shown that adenosine receptors differentially activate cell signalling pathways. Stimulation of A_1ARs has been shown to mediate cardioprotection via Gi dependant activation of phospholipase C (Parsons et al., 2000; Lee et al., 2001; Germack and Dickenson. 2005). A_3AR stimulation has been shown to increase DAG formation via phospholipase D activation dependant on Rho A kinase phosphorylation (Parsons et al., 2000; Lee et al., 2001). Downstream cell signalling pathways activated in response to 2-CL-IB-MECA have been identified in numerous models of myocardial injury (Headrick and Peart. 2005). Activation of A_3ARs has also been shown to modulate the actions of mitogen activated protein kinases like ERK1/2 and PI3K in neonatal rat cardiomyocytes (Germack and

Dickenson. 2004; 2005). Previously A_3AR dependant protection has also been shown to act on downstream effectors like K_{ATP} channels as well as promoting NF-κB binding to nuclear proteins (Tracey et al.,1998; Zhao and Kukreja. 2002).

The PI3K - AKT signalling cascade has been well studied with reference to its role in ischaemia reperfusion injury. AKT the downstream target of PI3K can activate a number of downstream targets including eNOS, p70S6K, BAD and PKC (Cross et al., 2000; Park et al., 2006). The MEK1/2 – ERK1/2 survival pathway has been shown to play a role in cell survival, growth and differentiation. In the context of cell survival phosphorylation of ERK 1/2 can activate downstream targets like p70S6 kinase, BAD mediating cardioprotection Mocanu et al., 2002; Germack and Dickenson. 2004, 2005; Bose et al., 2005). The exact cellular mechanisms via which A_3ARs mediate protection in the ischaemic reperfused myocardium remain unclear as studies have shown different pathway are activated in response to acute and delayed A_3AR mediated cardioprotection.

Administration of 2-CL-IB-MECA at reperfusion/reoxygenation decreases infarction and cell death via upregulation of MEK 1/2 – ERK 1/2, where inhibition of MEK 1/2 with UO126 abolished these protective effects. These results are comparable to those of Matot et al., (2006) who showed 2-CL-IB-MECA in the in-vivo feline lung model of ischaemia reperfusion injury to limit infarction via upregulation of ERK 1/2. In dose response studies in neonatal myocytes Germack and Dickenson (2004) have shown 2-CL-IB-MECA to upregulate phosphorylation of ERK1/2 at 1 nM concentration. Our data further show that 2-CL-IB-MECA (1nM) mediated myocardial protection is independent of p70S6K activity as the mTOR inhibitor

Rapamycin did not attenuate protection. Park et al., (2006) recently showed that Rapamycin when administered at reperfusion failed to abolish 2-CL-IB-MECA (1 μM) mediated protection indicating A_3 agonist protection is independent of p70S6 kinase at these concentrations. Interestingly, administration of 2-CL-IBMECA (100 nM) at reperfusion was seen to significantly increase the phosphorylation of P70S6 kinase. In light of our findings and the current literature it seems that 2-CL-IB-MECA does not activate p70S kinase at 1 nM concentrations and at 1μM as Park et al. (2006) did not observe p70S6K phosphorylation with 2-CL-IB-MECA at 1 μM concentration.

Cleaved-caspase 3 activity was also seen to be reduced in response to A_3AR activation in adult rat cardiomyocytes subjected to hypoxia/reoxygenation injury. This protective effect was not abolished by the MEK 1/2 inhibitor UO126 indicating 2-CL-IB-MECA mediated protection is independent of the MEK1/2 – ERK 1/2 survival pathway. In contrast Germack and Dickenson (2005) showed preconditioning with 2-CL-IB-MECA reverses caspase 3 activity in neonatal myocytes subjected to hypoxia/reoxygenation injury in a MEK 1/2 - ERK 1/2 dependant pathway. These contradictory results may be explained as the latter experiments were conducted in neonatal cardiac myocytes whereas our study used fully differentiated adult rat cardiac myocytes. Furthermore, the protection seen by Germack and Dickenson (2005) was attributed to pharmacological preconditioning with the A_3 agonist whereas in our study the A_3 agonist was added at the onset of reoxygenation. Our findings regarding capsase 3 need to be interpretated cautiously as we measured the level of cleaved caspase 3 and not caspase 3 itself. The survival pathways that mediate protection may be dependant upon the time of administration and the model used.

Post-reperfusion activation of A_3ARs was also seen to decrease cleaved-caspase 3 activity but was not blocked by the MEK 1/2 inhibitor UO126 implying that cleaved-caspase 3 activity is independent of the MEK 1/2-ERK1/2 cell survival pathway.

This is the first study to show activation of A_3ARs post reperfusion protects the isolated perfused rat heart and adult rat cardiac myocyte from ischaemic injury via the MEK 1/2 – ERK 1/2 cell survival pathway. These results indicate the alternative pathways are activated in decreasing cleaved-caspase 3 like the PI3K/AKT survival pathway.

The A_3 adenosine receptor agonist 2-CL-IB-MECA (1 nM) was shown to upregulate the phosphorylation of BAD $_{(ser112)}$ via the MEK 1/2 – ERK 1/2 survival pathway whereas 2-CL-IB-MECA (100 nM) was shown to phosphorylate BAD $_{(ser136)}$ via the PI3K-AKT survival pathway. Phosphorylation of the pro-apoptotic protein BAD is deemed cardioprotective as phosphorylation upregulates BAD's association with 14-3-3- proteins attenuating apoptosis by decreasing caspase 3 activity. Insulin when administered at reperfusion has been shown to activate the PI3K-AKT/BAD cell survival pathway (Jonassen et al., 2000).

Furthermore, our data showed 2-CL-IB-MECA (100 nM) when administered at reperfusion significantly decreased cleaved-caspase 3 activity in a Wortmannin sensitive manner in accordance with the findings of Germack and Dickenson., (2005) who showed preconditioning with 2-CL-IB-MECA (100 nM) to decrease caspase 3 activity in a Wortmannin sensitive manner. These data demonstrate that 2-CL-IB-MECA can activate pro-survival kinases when administered at reperfusion protecting

the myocardium/cardiomyocyte from ischaemia reperfusion / hypoxia reoxygenation injury. Protection was also associated with a decrease in cleaved-caspase 3 activity in cardiomyocytes reversed in the presence of Wortmannin. Further studies are required to determine the exact role of AKT phosphorylation and its effect on cleaved-caspase 3 activity when the A_3 agonist 2-CL-IB-MECA is administered at reperfusion or post reperfusion.

Postponing the administration of 2-CL-IB-MECA 15 to 30 minutes after reperfusion significantly attenuated myocardial infarction. Protection was abolished in the presence of the MEK1/2 inhibitor UO126. Stimulation of the A_3ARs 15 and 30 minutes post reperfusion limited hypoxia/reoxygenation injury in cardiac myocytes in an anti-apoptotic/necrotic manner. The anti-apoptotic potential was reversed upon inhibition of MEK 1/2 with UO126. Delayed administration of 2-CL-IB-MECA was accompanied by an increase in ERK 1/2 phosphorylation that was reversed in the presence of the MEK 1/2 inhibitor UO126. Therefore the protection was dependant upon MEK 1/2 activation as inhibition of MEK1/2 abolished protection. Our findings are comparable with those of Von Lubitz et al., (1994; 2001) who have shown administration of 2-CL-IB-MECA when administered at reperfusion or 25 minutes post reperfusion protects against cerebral ischaemia reperfusion injury.

2-Cl-IB-MECA when administered at reoxygenation to isolated cardiac myocytes significantly decrease cleaved-caspase 3 activity compared to the non-treated group. The PI3K inhibitor Wortmannin reversed this process of cleaved-caspase 3 decrease by the A_3AR agonist 2-CL-IB-MECA. These data imply that 2-CL-IB-MECA (1 nM) when administered at low concentrations at reperfusion/reoxygenation activates PI3K

and down regulates cleaved-caspase 3 activity without activating AKT. Administration of the PI3K inhibitor Wortmannin (100 nM) alone at reoxygenation significantly decreased cleaved-caspase 3 activity compared to the Hyp/Reox group. These findings are similar to those of Debiais et al., (2004) who showed that apoptosis induced by low serum concentrations in osteoblasts was reversed by administration of fibroblast growth factor 2 (FGF-2) and that the protection was abolished by the PI3K inhibitor Wortmannin although FGF-2 failed to induce AKT phosphorylation. They further show that FGF-2 induced protection as a result of inhibition of caspase 2 and caspase 3 activity.

Wildmann et al., (1998) have shown caspases to be linked to the degradation of cellular proteins like AKT and ERK1 during cellular injury and caspase inhibitors to block the apoptotic signal by blocking the activity of caspases leading to an increase in these pro-survival proteins may provide an explanation for our findings.

Our findings are comparative with those of Germack and Dickenson (2004) who showed a dose-dependent increase in AKT phosphorylation in naïve unstressed rat cardiac myocytes. They showed 2-CL-IB-MECA at the 1nM concentration to down regulate AKT phosphorylation whereas higher concentrations significantly increased AKT phosphorylation compared to non treated naive cardiac myocytes. Therefore, this study supports our results showing 2-CL-IB-MECA (1 nM) protects cardiac myocytes from hypoxia reoxygenation injury when administered at reoxygenation in a PI3K dependant pathway independent of AKT induction.

Higher concentrations of 2-CL-IB-MECA (100nM) when administered at reperfusion were shown to still limit myocardial infarction development. In the cardiomyocyte model the A_3 agonist was shown to significantly decrease apoptosis and necrosis in myocytes subjected to hypoxia and reoxygenation. The anti-infarct and anti-apoptotic/necrotic ability of 2-CL-IB-MECA were abolished by the PI3K inhibitor Wortmannin or the p70S6K inhibitor Rapamycin. Western blot analysis revealed 2-CL-IB-MECA (100nM) when administered at reperfusion upregulated AKT, p70S6K and BAD phosphorylation and were inhibited, with their respective inhibitors abolishing protection. Although, it should be noted that when 2-CL-IB-MECA (1 nM) was administered at lower concentration at reperfusion in the isolated perfused rat heart the mTOR Rapamycin failed to block the protection (Figure 6.3).

AKT when activated phosphorylates a number of downstream targets including p70S6K that regulates translation. Insulin has been shown to activate the PI3K-AKT / p70S6K / BAD pathway when administered at reperfusion, limiting myocardial infarction (Jonassen et al., 2001). This group further show inhibition of PI3K by Wortmannin or p70S6K by Rapamycin abolished insulin dependant protection. The findings of the study are in accordance with Park et al., (2006) who have shown infarct sparing effects with IB-MECA (1 μM) in the isolated rat heart associated with increased phosphorylation of AKT and showed that the protection was lost upon PI3K inhibition with Wortmannin. Our studies showed that 2-CL-IB-MECA (1 nM) when administered at low concentrations did not activate p70S6K but when 2-CL-IB-MECA was administered at the 100nM concentration p70S6K was activated. Therefore, it seems possible that 2-CL-IB-MECA activated p70S6K in a dose-dependant manner.

The A_3 agonist 2-CL-IB-MECA administered 15 minutes post reperfusion/reoxygenation significantly limited infarct development in the ischaemic reperfused myocardium and cell death (apoptosis and necrosis) in adult rat cardiac myocytes subjected to hypoxia/reoxygenation. The protection was associated with recruitment of the PI3K – AKT survival pathway where the administration of 2-Cl-IB-MECA (1 nM) upregulated the phosphorylation of AKT compared to control hearts. The critical role of PI3K for 2-CL-IB-MECA cardioprotection when administered 15 minutes after reperfusion was determined by using the PI3K inhibitor Wortmannin. Wortmannin abolished protection implicating PI3K activity being necessary to salvage the myocardium when administered 15 minutes post reperfusion. 2-CL-IB-MECA when administered 15 minutes post reperfusion significantly attenuated myocyte apoptosis and necrosis where the PI3K inhibitor Wortmannin abolished this cytoprotection. The A_3 agonist was also seen to decrease the activities of cleaved-caspase 3 in isolated cardiomyocytes that were reversed by Wortmannin indicating PI3K activity to be critical for A_3 agonist dependant cardioprotection. Postponing the administration of 2-CL-IB-MECA to 30 minutes post reperfusion was still capable of attenuating myocardial infarction in the isolated rat heart in a Wortmannin sensitive manner where 2-CL-IB-MECA upregulated the phosphorylation of AKT

Previous studies have shown preconditioning with 2-CL-IB-MECA to protect neonatal rat cardiac myocytes from hypoxia/reoxygenation injury via the PI3K-AKT/caspase 3 survival pathway. Park et al (2006) have shown the administration of 2-CL-IB-MECA at reperfusion to protect the isolated perfused rat heart from ischaemia reperfusion injury where the protection was abolished in the presence of the

PI3K inhibitor Wortmannin. The data collectively show that 2-CL-IB-MECA can attenuate infarct development when administered at reperfusion, 15 or 30 minutes post reperfusion.

Further experiments are required to determine the role of cellular crosstalk for example, if the PI3K pathway is inhibited then would there be activation of the MEK1/2 cell survival pathway and vice versa.

Mitochondria play a fundamental role in regulating cellular energetics as well as being an end effector of the cell death machinery. Griffiths et al., (1993;1995) showed that during myocardial ischaemia the mPTP remains closed and that it opens within the first few minutes of reperfusion and furthermore administration of Cyclosporin A (a mPTP inhibitor) deemed cardioprotective. This group showed that Cyclosporin A administration significantly improved left ventricular function in comparison to non treated ischemic reperfused hearts and was seen to reduce mitochondrial damage induced by calcium overload. Another mPTP inhibitor Sangliferin A has also been shown to protect the isolated ischaemic reperfused rat heart from reperfusion injury via improving left ventricular developed pressure after reperfusion and significantly reducing LDH release, an indicator of necrotic damage (Clarke et al., 2002).

Yellon and colleagues recently showed mPTP to be functional target in the human myocardium. Interestingly this group showed the mPTP inhibitors Cyclosporine A and Sangliferin A administered to human atrial tissue subjected to hypoxia and reoxygenation resulted in significant improvement in cardiac function and improved

cell survival. In another set of experiments they show the mPTP inhibitors to delay the opening of mPTP in response to stress conditions (Shanmuganathan et al., 2005).

More recently, Park et al., (2006) showed the infarct sparing ability of 2-CL-IB-MECA to be abolished in the presence of the mPTP opener atractyloside implying cardioprotection associated with 2-CL-IB-MECA is dependant on closing the mPTP. They further showed in isolated mitochondria that Ca^{2+} induced swelling was reversed by the administration of 2-CL-IB-MECA. Their data implicates 2-CL-IB-MECA dependant cardioprotection to closure of the mPTP. 2-CL-IB-MECA related mPTP closure may be associated the activation of the pro-cell survival signalling pathways. Activation of the PI3K and or MEK1/2 can phosphorylate downstream targets like BAD and BIM preventing their association with Bcl-xl proteins that are initiators of cellular apoptosis. Ischaemic postconditioning has been shown to confer cardioprotection in a number of studies. Recently postconditioning has been shown to inhibit the opening of the mPTP via upregulation of the reperfusion injury salvage kinases (Kin et al., 2005).

In light of the current literature and the data from the current study we hypothesise cardioprotection associated with delayed activation of A_3ARs in the ischaemic reperfused myocardium could involve upregulation of survival kinases.

A large number of studies have ligands and growth factors to protect the ischaemic reperfused myocardium. Despite an urgent need for the development of adjunctive therapies to limit the deleterious consequences of ischaemia reperfusion injury studies have failed to translate laboratory findings into a clinical setting. Therefore, further

studies are required to identify novel targets for ischaemia reperfusion injury as well their translation into a clinical environment.

Chapter 8. Further Investigations and Limitations.

Our Langendorff studies were carried out in the in vivo model. Further studies are required in an ex vivo model to determine whether the protection is still observed in the live animal model. This would allow us to determine whether the effect of 2-CL-IB-MECA is dependant on blood factors as our model was a blood free model.

Delaying the activation of A_3ARs with 2-CL-IB-MECA (1 nM) to 60 minutes after the onset of reperfusion failed to protect the Langendorff perfused rat heart from ischaemia reperfusion injury. Therefore, further studies are required to determine whether higher concentration of 2-CL-IB-MECA when administered 60 minutes after the onset of reperfusion can protect the ischaemic reperfused heart.

A number of other cell survival pathways have been shown to be involved in mediating cell survival. Therefore, further studies are required to determine the role of other cell signalling cascades like p38 and PKC that may be activated by the A_3 Agonist 2-CL-IB-MECA mediating cardioprotection

New A_3AR agonists that are more potent and selective for the A_3AR have been reported in the literature. Therefore, further studies are required to determine if these agonists are more cardioprotective then 2-CL-IB-MECA.

Previous studies have reported 2-CL-IB-MECA to activate AKT and ERK1/2 in a dose and time dependant manner and also to be pro and anti-apoptotic. Therefore, further studies are required to determine the threshold required for AKT and ERK1/2 activation by 2-CL-IB-MECA to observe cytoprotection.

Our studies determined 2-CL-IB-MECA to phosphorylate p70S6 kinase at the 100 nM concentration but not at the 1 nM concentration. Therefore, further studies are required to determine if 2-CL-IB-MECA activated P70S6k in a dose dependant manner.

2-CL-IB-MECA when administered after 15 or 30 minutes after the onset of reperfusion activated ERK1/2 and AKT. Further studies are required to identify whether this protection involves recruitment of p70S6k and BAD.

The current study determined 2-CL-IB-MECA to upregulate BAD phosphorylation in 2-CL-IB-MECA mediated cardioprotection. To determine whether the increase in BAD phosphorylation was via the PI3k/AKT or MEK1/2-ERK1/2 survival pathway western blot analysis is required assessing BAD phosphorylation when 2-CL-IB-MECA is administered at reperfusion of post-reperfusion in the presence and absence of the PI3K inhibitor Wortmannin or MEK1/2 inhibitor UO126.

Previous studies have determined 2-CL-IB-MECA to delay the opening of the MPTP when administered at reperfusion. Further studies are required are to determine if 2-CL-IB-MECA when administered 15 or 30 minutes after the onset of reperfusion delays the opening of the mitochondrial permeability transition pore.

To determine the activity of caspase 3 in the studies we assessed the activity of active/cleaved caspase 3. Activation of caspase-3 requires protelytic processing of its inactive zymogen into cleaved / activated p16 and p12 fragments. We used the cleaved caspase 3 antibody that detects endogenous levels of cleaved caspase-3. This

antibody does not recognise full length caspase 3 nor other caspases. The data regarding caspase 3 activity needs to be interpretated cautiously as the antibody only detects the cleaved form of caspase-3 and is not a direct measure of caspase 3 itself. Further studies are required to validate our findings. We propose using a flurogenic substrate N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin to assess the activity of capsase 3. Caspase 3 activity can be measured by measuring the generation of the flurogenic cleavage product methylcoumaride on the spectrophotometer. Furthermore, positive and negative controls should be used to validate our findings.

Although, the study identified the cell signalling pathways involved in 2-CL-IB-MECA when administered at reperfusion or post-reperfusion there are limitations of out findings. The experimental models used in our studies where simulated models of ischaemia/reperfusion injury. To accurately determine the role of 2-CL-IB-MECA in mediating cardioprotection in vivo models of ischaemia reperfusion injury should be used. This will allow us to take into account the effect of the vasculature and blood factors in the ischaemic reperfused heart. Furthermore, to determine whether 2-CL-IB-MECA protects the heart via recruitment of pro-survival kinases MEK1/2 and PI3K.

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