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Investigation into the cardiotoxic effects of β- adrenergic receptor agonists in myocardial ischaemia/reperfusion injury

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Investigation into the cardiotoxic effects of β- adrenergic receptor agonists in myocardial ischaemia/reperfusion injury

By

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Abbreviations

%	**Percentage**
7TM	**7 transmembrane region**
αARs	**α Adrenergic Receptor**
βAR	**β Adrenergic Receptor**
β_1AR	**β_1 Adrenergic Receptor**
β_2AR	**β_2 Adrenergic Receptor**
µM	**Micromole**
AC	**Adenylate Cyclase**
ACh	**Acetylcholine**
ACOS	**Asthma-Chronic Obstructive Pulmonary Disorder**
AIF	**Apoptosis inducing factor**
Akt	**Protein Kinase B**
ANT	**Adenine nucleotide translocator**
APAF-1	**Apoptotic Protease Activating Factor-1**
ASK1	**Apoptosis signal regulated kinase 1**
ATP	**Adenosine triphosphate**
CABG	**Coronary artery bypass graft**
CAD	**Coronary artery disease**
CaMKII	**Calmodulin dependent Protein Kinase II**
cAMP	**Cyclic Adenosine Monophosphate**
CF	**Coronary flow**
CHF	**Chronic Heart failure**
CICR	**Calcium induced calcium release**
CL	**Cytoplasmic loop**

COPD	**Chronic Obstructive Pulmonary Disease**
CsA	**Cyclosporin A**
CVD	**Cardiovascular diseases**
Cyp D	**Cyclophilin D**
DISC	**Death-inducing-signalling-complex**
DMSO	**di-methyl sulfoxide**
ER	**Endoplasmic Reticulum**
Erk 1/2	**Extracellular signal-regulated kinase 1 and 2**
FADD	**Fas-associated via death domain**
Form	**Formoterol**
GAPDH	**Glyceraldehyde 3-phosphate dehydrogenase**
GDP	**Guanosine diphosphate**
GPCR	**G protein coupled receptors**
GSK3β	**Glycogen synthase kinase 3β**
GTP	**Guanosine triphosphate**
H^+	**Hydrogen ions**
H_2O_2	**Hydrogen peroxide**
HF	**Heart Failure**
HR	**Heart Rate**
IHD	**Ischaemic Heart Disease**
IL-3, 4, 5	**Inflammatory cytokines**
IMM	**Inner mitochondrial membrane**
IR	**Ischaemia reperfusion**
IsoP	**Isoproterenol**
JNK	**c-Jun N-terminal kinase**

KHB	**Krebs Henseleit Buffer**
LABA	**Long acting β agonist**
LVDP	**Left ventricular diastolic pressure**
M_{1-5}	**Muscarinic Receptor**
MAPK	**Mitogen activated protein kinases**
MI	**Myocardial infarction**
mPTP	**Mitochondrial permeability transition pore**
MTT	**(3−[4,5−dimethylthia−zol−2−yl]−2,5−diphenyl tetrazolinum bromide)**
NADH	**Nicotinamide adenine dinucleotide phosphate**
NCX	**Na^+/Ca^{2+} exchanger**
OMM	**Outer mitochondrial membrane**
PDK	**Phosphoinositide dependent kinase-1**
PKA	**Protein Kinase A**
PI3K	**Phosphoinositide 3-kinase**
PIP_2	**Phosphatydilylinositol 3,4-trisphate**
PIP_3	**Phosphatidylinositol 3,4,5-trisphosphate**
PVDF	**Hybond-P Polyvinyl Difluoride**
RISK	**Reperfusion injury salvage kinase**
RyR	**Ryanodine Receptors**
ROS	**Reactive oxygen species**
SABA	**Short acting β agonist**
SalB	**Salbutamol**
SalM	**Salmeterol**
SMAC	**Second mitochondria-derived activator of caspases**

SERCA	**Sarcoendoplasmic reticulum calcium ATPase**
SOD	**Superoxide dismutase**
SR	**Sarcoplasmic reticulum**
TBS	**Tris-buffered saline**
TMRM	**Tetramethylrhodamine methyl ester**
TNFα	**Tumor necrosis factor**
VDAC	**Voltage dependent anion channel**
WHO	**World Health Organization**

Abstract

The treatment of asthma still relies on primary therapy with bronchodilators; in particular β adrenergic receptor (βAR) agonists with a diverse range of short acting and long acting βARs available. An increase in the number of cardiovascular events with the use of bronchodilators have recently been reported including hypertrophy, heart failure, myocardial ischaemia and infarction. Several subtypes of βAR receptors exist including the β_1 Adrenergic Receptor (β_1AR) and β_2 Adrenergic Receptor (β_2AR), both located in the heart. The effects of selective β_2AR agonists were investigated in the Langendorff model of myocardial ischaemia reperfusion injury, isolated perfused rat hearts underwent 35 minutes of ischaemia and 120 minutes of reperfusion.

The selective β_2AR long acting β agonists Formoterol and Salmeterol had no significant effect on infarct to risk ratio or time taken to depolarisation and hypercontracture in isolated cardiomyocytes. The non-selective β_1AR agonist Isoproterenol has been show to induce myocardial ischaemia and infarction in rat hearts previously, here we demonstrated Isoproterenol (0.5μM) significantly decreased time taken to depolarisation and hypercontracture in isolated cardiomyocytes. The short acting β_2AR agonist Salbutamol (0.01μ-1μM) significantly increased infarct to risk ratio in the Langendorff in addition to significantly decreasing time to hypercontracture in cardiomyocytes in the oxidative stress model highlighting a potential role of the mitochondrial permeability transition pore (mPTP). Activation of phosphorylated Akt and phosphorylated Erk1/2 via the PI3K/Akt signalling pathway and p44/p42 MAPK pathway were investigated by western blot analysis. Salbutamol significantly elevated expression of p-Akt in rat hearts exposed to reperfusion for 20 and 120 minutes whilst reducing expression of p-Erk. Recorded elevated cleaved caspase

3 expression in Salbutamol treated hearts can be associated as a marker of increased in cardiomyocyte cell death.

The β_1AR antagonist CGP 20712 was administered in the presence of Salbutamol with minimal reduction in infarct size in rat hearts recorded and no significant change in time taken to hypercontracture in isolated cardiomyocytes suggesting that Salbutamol mediated toxicity is via β_2AR activation. Confirmation of this was verified with the β_2AR antagonist ICI 118, 551. Significant decrease in infarct size was recorded in addition to a significant increase in time to hypercontracture in the oxidative stress model. Further to this, caspase 3 expression was significantly reduced in addition with p-Akt expression.

With a potential role of the mitochondria and the mPTP contributing to Salbutamol induced myocardial injury, the Cyclophilin D inhibitor Cyclosporin A was administered in hearts and cardiomyocytes in the presence of Salbutamol. Infarct size was significantly reduced whilst time taken to hypercontracture significantly increased, suggesting that CsA treatment inhibits Salbutamol mediated injury via Cyclophilin D inhibition of the mPTP.

To conclude, our results demonstrated that Salbutamol caused cardiotoxicity at tissue, cellular and protein level in conditions of ischaemia reperfusion injury. Further to this, inhibition of Cyclophilin D by CsA, or the use of the β_2AR antagonist ICI 118, 551 inhibits Salbutamol induced toxicity.

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1 Introduction

1.1 Respiratory Disorders

The human airways and lungs play a vital role in internal and external respiration, however their ability to function correctly and efficiently can be impeded by various pathophysiological disorders. Respiratory disorders can be grouped into several categories that include obstructive diseases (asthma, bronchitis, asthma), restrictive conditions (cystic fibrosis, pleural effusion, sarcoidosis) and vascular diseases (pulmonary oedema, pulmonary embolism). The more common respiratory disorders observed in patients worldwide according to the World Health Organisation (WHO), include chronic bronchitis, emphysema, pneumonia, chronic obstructive pulmonary disease and asthma. Within the UK 1 in 7 individuals are affected by a form of chronic lung disease that may include chronic obstructive pulmonary diseases (WHO, 2004).

These wide ranges of respiratory disorders can be treated broadly however in respect of disorders affecting the airways by bronchoconstriction, the use of a bronchodilator is required. In particular, Chronic Obstructive Pulmonary Disease (COPD) and Asthma are the two of the main respiratory disorders that contribute to an on going mortality effect

1.2 Chronic Obstructive Pulmonary Disease

Various respiratory issues cause constriction of the bronchioles due to inflammation of tracheal epithelial cells, which can be caused by a variety of extrinsic factors that include smoke, alcohol, dust and anaphylactic reactions (Elliott et al., 2007, Zhang et al., 2012). In more severe cases, diseases of the respiratory system can present as being acute or chronic in the form of asthma and COPD. A 2004 report from the WHO estimated 64 million people worldwide to be affected by COPD (WHO, 2004). An estimated 3 million people in the UK suffer from COPD, often unknowingly as stated by the Department of Health (DH, 2011).

The NHS reported to admitting 115,000 emergency patients with COPD in 2014 with 25,000 of those patients dying (NHS, 2014). Typical symptoms include wheezing, increased mucus production (also seen is asthmatic patients), coughing and increased residual lung capacity in COPD patients (Aduen et al., 2007, Tantisuwat and Thaveeratitham, 2014).

COPD has been defined as a "preventable and treatable disease state characterised by airflow limitation that is not fully reversible" by the American Thoracic Society and European Respiratory Society (Celli and MacNee, 2004). The structure and function of airway smooth muscle does not play a critical role in the development of COPD, however they do contribute to symptoms. The key mechanisms associated with COPD involve the thickening of the airway wall that is non-reversible (Brusasco et al., 2006). In comparison to asthma, COPD airway inflammation is driven by neutrophilic actions and an increase in numbers of macrophages (Brusasco, 2006).

1.3 Asthma

Asthma as an allergic disease, in contrast to COPD, is capable of airway inflammation reversal (Gibson and Simpson, 2009). The Global Initiative Report for Asthma (GINA) estimated worldwide asthma cases to be in the region of 300 million in 2004 with an increase to 400 million by the year 2025 (Masoli et al., 2004). More recent estimates with the use of World Health Organisation (WHO) surveys, have re-calculated the current number of reported cases of asthma to be 315 million as of 2014, with an estimated population of 623 million worldwide to present with symptoms of the respiratory disorder (To et al., 2012).

The mechanism of asthma involves mast cells, which are key in acute and chronic inflammation of the airways. The initiation and release of mediators such as inflammatory cytokines including IL-4, IL-5 and IL-3 are responsible for airway constriction (Bousquet et al., 2000, Lemanske and Busse, 2010). The IL-5 cytokine recruits and activates eosinophils,

which are responsible for the release of highly inflammatory granule-associated substances, resulting in continuous inflammation (Figure 1.1)(Jacobsen et al., 2007).

More recently, a new category of respiratory disorder has emerged involving both asthma and COPD. This new category known as Asthma-Chronic Obstructive Pulmonary Disorder Overlap Syndrome (ACOS), presents in patients who demonstrate features of both asthma and COPD symptoms (Barrecheguren et al., 2015, Gibson and Simpson, 2009). The specific features of ACOS are still unclear, however it is accepted that suffers of asthma with eosinophillic inflammation of the airways can develop neutrophillic inflammation, which is usually only seen in COPD patients. Alleviation and primary treatment of COPD, asthma and ACOS is the use of bronchodilators to reduce bronchoconstriction (Dirkje, 2015).

Figure 1.1 Representation of Chronic or Acute inflammation activation of the airways in response to an allergen in relation to asthma (Heinecke, 2000).

1.4 Bronchodilators

Respiratory diseases such as COPD, ACOS and asthma are treated with various categories of bronchodilators. Bronchodilators are the most potent drugs available and the first line of therapy prescribed to patients due to their effectiveness in alleviating symptoms i.e. bronchoconstriction (Dompeling et al., 1992, Donohue, 2004, Cazzola and Matera, 2008).

Several categories of bronchodilators exist and are available for the treatment of asthma, with each category targeting different receptor types found in airway smooth muscles aiming to reduce/inhibit airway inflammation. These categories include anticholinergic muscarinic receptor antagonists, β Adrenergic Receptor Agonists (βARs), leukotriene receptor antagonists and corticosteroids (Wolthers, 2015, Hering, 2015). More specialised and novel categories of bronchodilators exist such as combination therapies of βARs and corticosteroids, phosphodiesterase inhibitors and specific potassium channel openers (Malerba et al., 2010).

1.4.1 Muscarinic Antagonists

The first category of bronchodilators is one of the oldest that exists in medicine, dating back as early as the 17th century, with patients recommended to smoke plant alkaloids (Jackson, 2010). Parasympathetic activity of the vagal nerve in the airways induces constriction and increased mucus secretion from submucosal glands with the secretion of the neurotransmitter Acetylcholine (ACh) (Gross and Skorodin, 1984, Rodrigo and Rodrigo, 2002). However, ACh secretion is not restricted solely to the increased activity of the parasympathetic system, but can also be secreted from inflammatory cells and bronchial epithelial cells (Gosens et al., 2006). The use of anticholinergic drugs as a therapeutic target has proved to be successful to alleviate symptoms of asthma by antagonising muscarinic receptors.

Five different subtypes of muscarinic receptors have been identified that are located in the airway smooth muscle known as M_1, M_2, M_3, M_4 and M_5 (Alvarado-Gonzalez and Arce, 2015). Specifically, M_1, M_2 and M_3 receptors are found on the lining of the trachea and have been targeted to reduce the secretion of ACh. Examples of such drugs include a non-selective muscarinic antagonist of M_1, M_2 and M_3 receptors, Ipratropium Bromide (Ailani et al., 1995). Once occupying the receptor site, secreted ACh is unable to activate the muscarinic receptors to initiate bronchoconstriction thus reducing bronchoconstriction. The onset of bronchodilation with the use of Ipratropium Bromide is approximately 30 minutes and can last up to 6 hours (Scullion, 2007).

1.4.2 β Adrenergic Receptor Agonists

Adrenergic receptors are abundant throughout the body in particular the lungs, heart, airway smooth muscle, lining of the trachea and are the primary targets for adrenergic receptor agonists. Adrenergic receptors are classed into 2 different sub-types, α adrenergic receptors (αARs) and β adrenergic receptors (βARs) (Strosberg, 1993, Strosberg, 1995). In therapeutic approaches to respiratory diseases such as COPD and asthma, βARs in particular have been targeted due to their mechanism of action to inhibit bronchoconstriction. Like muscarinic receptors, βARs are G protein coupled receptors (GPCRs) which will be discussed in detail later (Katritch et al., 2013).

βARs have several different subtypes that are present in the trachea, these are predominantly the β_1 Adrenergic Receptors (β_1AR) and the β_2 Adrenergic Receptors (β_2AR) (Granneman, 2001). βAR agonists have been designed to target the β_2ARs selectively, however there are non-selective capabilities of bronchodilators that act on β_1ARs that also achieve bronchodilation. As described earlier, asthma induces bronchoconstriction in response to an allergen or a non-allergen response such as exercise or cold-induced asthma (McFadden and

Gilbert, 1994, Carlsen et al., 1998). Airway smooth muscles containing mast cells are stimulated by the release of pro-inflammatory cytokines, Immunoglobulin E (IgE), resulting in an increase in histamine production (Bradding et al., 2006). Histamine release in addition to leukotriene release, bind to corresponding receptors on the airway smooth muscle cells resulting in bronchoconstriction (Jarjour and Kelly, 2002). βAR agonists act directly on their respective receptors on airway smooth muscles and mast cells, which inhibit the release of pro-inflammatory mediators thus reducing bronchoconstriction (Grisanti et al., 2010).

βARs are not only classified as selective and non-selective for their respective βAR subtypes, but can also be classed as long acting β agonists (LABA), short acting β agonists (SABA) and more recently, ultra long acting agonists (Anderson et al., 1994). Each of these classifications of drugs refers to the time taken to the onset of their effect and duration of time the effect lasts for. The specificity of βAR agonists is dependent on two properties of the drug, firstly the ability of the drug to bind to the receptor and secondly to produce a response i.e. affinity and efficacy (Baker, 2010).

1.4.3 Isoproterenol

One of the first βAR agonist to go to market was the non-selective bronchodilator Isoproterenol also known as Isoprenaline (Pearce and Hensley, 1998). Its non-selective ability to bind to either β_1ARs or β_2ARs, made it a very potent bronchodilator, however its affinity for β_1ARs is considerably higher than that of β_2ARs. Isoproterenol has been shown to induce myocardial ischaemia that is associated with increased oxidative stress, calcium overload, heart failure, ventricular hypertrophy and myocardial infarction and is used experimentally to induce myocardial ischaemia and heart failure in cardiomyocytes of varying species (Chen et al., 2014, Leenen et al., 2001, Zhang et al., 2005). The use of Isoproterenol is now limited for the treatment of asthma but is still used in rare circumstances

to treat torsades de pointes and bradycardia (Kim et al., 2014, Viskin, 1999b). Isoproterenol induced calcium overload will be discussed later on (see section 1.12).

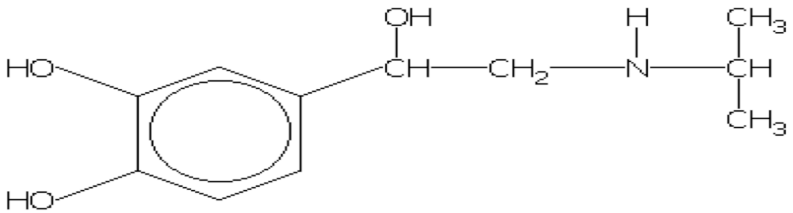
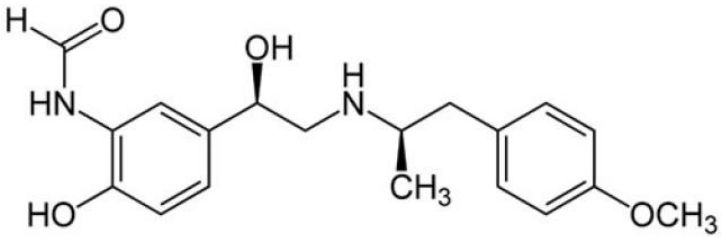
1.4.4 Salbutamol

The SABA Salbutamol (also known as Albuterol) was the first designed selective β_2AR agonist and has a high affinity for β_2ARs in contrast to other SABAs (Bandaru et al., 2015, Dougall et al., 1991). The onset of the action of Salbutamol can be as quick as 5-30 minutes in asthmatic and COPD patients, and last for approximately 2-4 hours. Administration of Salbutamol causes bronchodilation in addition to the other β_2AR agonists mentioned, can also cause positive inotropic and chronotropic effects on the heart and affect haemodynamics (Fowler et al., 2013). An interesting aspect of the chemical structure of Salbutamol has been investigated in regards to enantiomers. The asymmetric structural shape of βARs agonists in general can exist as optical isomers (Johnson, 2006). The S-Albuterol enantiomer is the most commonly form of the bronchodilator on the market, however affinity studies have shown that the R-Albuterol enantiomer is up to 100 times more potent than S-Albuterol.

1.4.5 Formoterol & Salmeterol

Both Formoterol and Salmeterol are LABAs that are selective for β_2ARs in the trachea and bronchioles, however they do also affect haemodynamics of the heart. Initial studies of the novel drug Salmeterol demonstrated it as a partial agonist and to have a high selectivity for β_2ARs when compared to Isoproterenol and Salbutamol (Ball et al., 1991). In contrast to Salmeterol, Formoterol is a full agonist that is more readily available to β_2ARs and is less lipophilic allowing faster onset of action (Anderson, 1993). An advantage of Formoterol being a full agonist when compared to Salmeterol, is its ability to bring the same efficacy as Salmeterol but with occupying less receptors (Johnson, 2006).

Both of these LABAs have been shown to effect both potassium and glucose serum levels in rat and humans (Guhan et al., 2000). Of the 2, Salmeterol's effects have been shown to last longer contributing to its novelty as a longer acting β agonist than Formoterol. Both of these LABAs can be used to alleviate symptoms of asthma and COPD for up to 8 hours at a time. Like Isoproterenol, both Salmeterol and Formoterol have been shown to cause inotropic and chronotropic effects in the heart, demonstrating its capability to act on β_2ARs in the heart as well (Watson et al., 2013).

β Adrenergic Receptor Agonist Chemical Structure	βAR Selectivity	Short or Long Acting β Agonist
Isoproterenol 	β_1, β_2	Short acting β Agonist
Formoterol 	β_2	Long Acting β Agonist
Salmeterol	β_2	Long Acting β Agonist

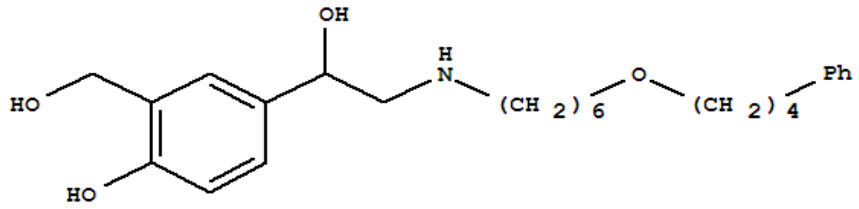
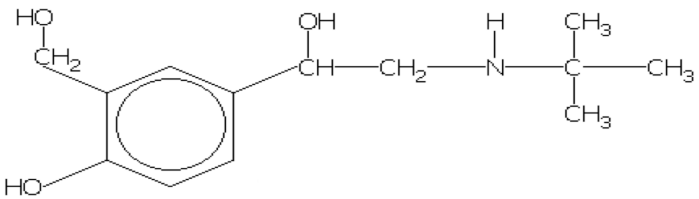
		
Salbutamol 	β_2	Short acting β Agonist

Table 1.1 Chemical structures of Isoproterenol, Formoterol, Salmeterol and Salbutamol with their respective β Adrenergic Receptor selectivity (Ball et al., 1991)

1.5 Cardiovascular effects of β Adrenergic Receptor Agonists

The use of βAR agonists for the treatment of asthma or COPD is not without its adverse effects. Bronchodilators, in particular βAR agonists, have been shown to have positive chronotropic and inotropic effects of the heart (Ball et al., 1991, Carlsson et al., 1977, Watson et al., 2013). As mentioned earlier, several subtypes of βARs exist, however, of the 4 subtypes (β_1-β_4), there is an abundance of β_1ARs and β_2ARs present in the myocardium (Nikolaev et al., 2010). Expression of both β_1ARs and β_2ARs have been identified in the heart with mRNA studies in rodent hearts and also in human ventricular myocytes (Brodde and Michel, 1999).

The ratio of β_1ARs:β_2ARs in rat hearts has been approximated at a 60%:40% ratio in favour of β_1ARs in the rat heart (Xiao and Lakatta, 1993). In the human heart, this ration is even more favourable to β_1ARs with an approximate density of 75-80%, 15-18% β_2AR density with the remainder as β_3ARs (Lymperopoulos and Bathgate, 2013). The location of the βARs in the heart have been specifically located to the myocardium of the heart that is made up of individual cardiomyocytes, where at the cell crest surface, the β_1ARs reside (Steinberg, 2004). β_2ARs have been identified to localise deep within the t-tubules of cardiomyocytes within invaginations known as caveolae (Cros and Brette, 2013, Calaghan and White, 2006). The activation of either of these receptors will be discussed in detail in section 1.4.

Investigations into the distribution of βARs in the heart have shown varying concentrations of βARs dependent on the conditions in which the heart has been exposed to (Nikolaev et al., 2010). In 'healthy' human and rat hearts, distribution of βARs exists as mentioned above in a favourable ratio of β_1ARs:β_2ARs, however, should an insult to the heart occur such as myocardial infarction (MI), ischaemic heart disease (IHD), heart failure (HF) or hypertrophy,

redistribution of the βARs has been recorded with patch clamp techniques identifying calcium release, a characteristic of βAR activation (Chen-Izu et al., 2000, Calaghan and White, 2006, Sutton and Sharpe, 2000). Overstimulation of βARs can occur due to poor management of patients administering metered dose inhalers of βAR agonists (Lavorini et al., 2008, Melani et al., 2004). βARs, in particular the β_2ARs have the ability to protect cardiomyocytes from overstimulation through receptor desensitisation (Johnson, 2006). This unique feature can occur in several ways, firstly, internalisation of the receptor and secondly direct uncoupling of the receptor to inhibit signalling (Johnson, 1998). β_2 and β_3 adrenergic receptors have also been associated with the heart however their exact functions are still not eleudicated. Recent studies have purported potential actions of either of these βARs. Moens and colleagues (2010) have described that the β_3AR is similar to that of the β_2AR that upon activation it can couple to either G_s or G_i subunits of G protein coupled receptors. However, unlike β_2AR activation having to activate the G_s subunit before being able to initiate coupling of the G_i subunit, β_3ARs can initiate either subunit directly, with suggestion that initiation of β_3ARs occurs during over stimulation (Moens et al., 2010). Activation of β_3ARs has been shown to cause vasodilation in rats and dogs and in β_1/β_2 knockout mice in addition to initiating negative inotropic effects when activated (Moens et al., 2010).

An increased number of incidences of mortality have been reported with the use of bronchodilators. A meta-analysis of clinical trials involving treatment of COPD patients associated a link to the use of bronchodilators to increased numbers of mortality and morbidity, with underlying heart abnormalities (Singh et al., 2008). Further studies investigating the use of βARs have identified a similar finding with the use of the non-selective βAR agonist Isoproterenol, with a link to inducing MI and IHD in patients and

increase mortality (Udelson et al., 1989, Senthil et al., 2007). Other βAR agonists have also been identified to increase mortality such as Salbutamol, with a multicentre randomised controlled trial abandoned due to the increase in mortality with the administration of Salbutamol (Gao Smith et al., 2012).

Hospital cases have also been presented with patients presenting with MI upon salbutamol administration. However, further investigation into patient histories identified patients as having pre-existing cardiac diseases including IHD, cardiomyopathies and hypertrophy contributing deaths linked with the use of Salbutamol (Fisher et al., 2004). The Salmeterol Multicentre Asthma Research Trial (SMART) showed an increased number of deaths (805 vs. 604) of the 25,180 participants with the use of the LABA Salmeterol when compared to Salbutamol although not statistically significant (Nelson et al., 2006, Hasford and Virchow, 2006).

1.6 G Protein Coupled Receptors

GPCRs are the largest family of membrane proteins and for this reason are targeted for therapeutics in the pharmaceutical industry. GPCRs are capable of binding to guanosine nucleotides of which there are 3 types of subunits, alpha (α), beta (β) and gamma (γ). Briefly, GPCRs when inactive are bound to guanosine diphosphate (GDP) and upon activation GDP is released from the α subunit and binds to guanosine triphosphate (GTP) to become activated. Once activated, the α subunit separates from the other subunits and moves to its target protein (Neumann et al., 2014, Hamm, 1998).

Several different families of GPCRs exist, all carrying common features between each group, one of which is the sharing of homology of a 7 transmembrane region (7TM) (Howard et al., 2001). Further to this, the 7TM regions also contains 2 ends, one intracellular carboxyl

terminus spanning the TM5 and TM6 regions and extracellular amino terminus (Figure 1.2) (Katritch et al., 2013).

2 key basic classes of GPCRs that we will be focussed on for the purpose of this thesis include the stimulatory (G_s) and inhibitory (G_i) proteins. These particular proteins when activated, release their α subunit, which binds to amplifier enzymes to either inhibit or activate the enzyme. The amplified enzyme is responsible for the production or inhibition of secondary messengers, which target specific protein kinases (Neumann et al., 2014). The amplifier enzyme to be addressed is the Adenylate Cyclase (AC) enzyme which produces cyclic Adenosine Monophosphate (cAMP) from Adenosine Triphosphate (ATP) (Kobilka, 2007).

Figure 1.2 Illustration of a G Protein Coupled Receptor with its 7 transmembrane region, amino terminus and carboxy terminus. Taken from (Neumann et al., 2014)

1.6.1 β_1 Adrenergic Receptor

As mentioned earlier, βARs exist in a number of forms β_1 β_2 and β_3, all of which signal through G protein coupled receptors. Unique features of these receptors upon activation are associated with their signalling cascades to bring about their desired effect, which tend to be influential on contractility of the heart and heart rate (Lymperopoulos and Bathgate, 2013). In reference to cardaic βARs activating via agonist stimulation, βARs are prone to desnesitisation meaning the response of the receptor becomes diminised. This is controlled by a regulatory protein kinase known as the GPCR kinase (GRKs) and β arrestins (Capote et al., 2015)

The β_1ARs located on the cell crest of cardiomyocytes are capable of binding to only the G_s subunit of GPCRs (Madamanchi, 2007). Human βARs share 51% sequence homology between each subtype of βARs in their amino acid sequencing, meaning structural features are very similar (Warne et al., 2008). An integral part of GPCRs is the interaction they have with agonists, in this case βAR agonists such as Isoproterenol. The cytoplasmic loops 2 and 3 (CL2, CL3) have been targeted, as they are responsible for the selectivity and activation of the G proteins. More specifically, the CL2 is responsible for the strength of the interaction with an agonist whilst CL3 is targeted for specificity of an agonist (Warne et al., 2008). Specific to β_1ARs, formation of a α helix of the CL2 next to the surface membrane is believed to be structurally responsible, to activate the receptor (Fredriksson and Schioth, 2005, Baker, 2005b).

βARs agonists, selective or non-selective, have previously been demonstrated to influence the haemodynamics via activation of βARs present in the heart. Activation of the β_1AR-G_s pathway increases cardiac contractility (inotropic effect) (Steinberg, 2004). Studies with

Isoproterenol acting on β_1ARs demonstrated an increase in cAMP accumulation, after AC converts ATP into cAMP, which is responsible for influencing transportation of calcium (discussed in section 1.7) (Ponicke et al., 2006, Sadana and Dessauer, 2009). Increased levels of cAMP initiate the start of a signalling cascade initiated by Protein Kinase A (PKA). PKA has 2 subunits, regulatory and catalytic. cAMP bound to the regulatory subunit of PKA dissociates the catalytic subunit which can go on to phosphorylate intracellular proteins such as phospholamban, sarcoplasmic reticulum, calmodulin, ryanodine receptors (RyR), sarcoplasmic reticulum ATPase and L-type calcium channels (Hudecova et al., 2013, Yoo et al., 2009, Zhu et al., 2005).

1.6.2 β_2 Adrenergic Receptor

Detailed research of the β_2AR is plentiful as it was one of the first and most stable GPCRs to be characterised by radio ligand binding in addition to being the first βAR to be determined structurally by crystallography (Rasmussen et al., 2011, Warne et al., 2008). Having discussed the ability of β_2ARs able to re-distribute throughout the heart under strenuous conditions, another unique ability of the β_2ARs is its signalling pathways, as it is able to activate not only to the G_s subunit, but also the G_i subunit (Ponicke et al., 2006, Xiao et al., 2003). β_2AR-G_S pathway is identical to that described in the previous section of the β_1AR-G_s pathway. Activation of the β_2AR-G_i pathway is not dissimilar from that of the G_S subunit pathway, however once the α subunit of the G_i pathway binds to GTP, direct inhibition of AC occurs thus stopping the signalling cascade to activate PKA, causing a reduction in activation of the previously mentioned proteins (Figure 1.3). This has been shown to occur in both human and rodent ventricular cardiomyocytes (Zhu et al., 2005).

Figure 1.3 Illustration of activation of either Stimulatory G Protein Coupled receptor (β_1ARs/β_2AR-Gs pathway). Activation of the β adrenergic receptore (βAR) initiates depolarisation spreading throughout the cardiomyocyte and deep within T-tubules initiating L-type Ca^{2+} channels (I_{Ca}). Increased Ca^{2+} can occur via revered Na^{+}/Ca^{2+} exchanger (NCX rev). Influx of Ca^{2+} initates further release of Ca^{2+} from the Sarcoplasmic Reticulum (SR) via Ryanodine Receptors (RyR). Intracellular Ca^{2+} is removed from cytosol of cardiomyocyte through the SR Ca ATPase (SERCA) regulated by Phospholamban (PLB). Further removal of Ca^{2+} is removed via the sarcolemmal NCX (Brum et al., 2006)

1.7 Cardiovascular Disease

With increased mortalities reported in asthmatic patients as a result of treatment with bronchodilators, it is important to recognise underlying cardiovascular diseases (CVD) that may exist in patients in the wider population as was suggested by several trials (Singh et al., 2007, Nelson et al., 2006). CVDs cover a wide range of heart irregularities that effect the myocardium, vasculature or rhythm of the heart. CVDs are the number 1 cause of death worldwide with an approximate 17.5 million people dying from a form of a CVD in 2012 (WHO, 2015). Interestingly, analysis of these deaths identified 6.7 million as a result of ischaemic heart disease (IHD). New statistics from the British Heart Foundation (BHF) have identified IHD to be responsible for approximately 70,000 deaths in the UK each year, and currently 2.3 million people in the UK are living with Coronary Heart Disease (BHF, 2015). Risk factors that can contribute to CVDs are considered to be modifiable and non-modifiable, the latter includes age and sex whilst the former includes hypertension, smoking, obesity and diabetes mellitus (Sekhri et al., 2014).

1.8 Ischaemic Heart Disease & Interventions

IHD affects the myocardium due to a build-up of plaques in the coronary arteries. The most commonly affected arteries of the heart are the left descending coronary arteries, which provide the left ventricle with oxygenated blood and nutrients (Libby and Theroux, 2005) . Left ventricular function is paramount to blood flow to the whole body. The composition of plaque within arteries consists of fibrin, cholesterol, calcium and fats (Somers and Dawson, 1997). Build-up of plaques line the walls of arteries causing them to become atherosclerotic resulting in stenosis of the lumen. Stenosis leads to a reduction in blood flow to targeted areas of the heart leading to the myocardium being at risk of becoming ischaemic known as myocardial ischaemia (Figure 1.4) (Fisher et al., 2014).

Interventions of IHD and CAD can come in a range of therapies. Firstly, diagnosis of IHD can be achieved by electrocardiograph stress test analysis or coronary angiography (Qaseem et al., 2012). Current pharmaceutical therapies can involve a range of thrombolytic drugs (Warfarin), ACE inhibitors, Nitrates and β blockers (Qaseem et al., 2012). Surgical intervention may also be necessary in more severe cases of IHD, which include angioplasty or coronary artery bypass graft (CABG).

During an ischaemic period preceding reperfusion, the pathophysiology of the heart and blood vessels are altered significantly. During ischaemia, deprivation of nutrients and oxygen to areas of the myocardium cause biochemical and metabolic changes (Hausenloy and Yellon, 2013). A key mediator is the lack of oxygen which nullifies oxidative phosphorylation, which has a 'downward spiral' of events including depolarisation of the mitochondrial permeability pore (mPTP), ATP depletion and eventual inhibition of myocardial contractions (Hausenloy and Yellon, 2013). Compensatory mechanisms are initiated during ischaemic periods, however they are short lived. To maintain mitochondrial membrane potential, all available ATP is broken down as a result of ATP hydrolysis thus increasing inorganic phosphate (Hausenloy and Yellon, 2013, Pham et al., 2014).

Aerobic respiration in ischaemic hearts becomes laboured and eventually switches to inefficient anaerobic respiration, accumulating lactate and increasing H^+ ions thus decreasing pH leading to acidosis (Avkiran and Marber, 2002). Increased activity of the Na^+/H^+ exchanger compensates for elevated H^+ ions within the myocardium. Ischaemic mediated ATP depletion maladaptive effects of Na^+/K^+ ATPase result in Na^+ increase intracellularly. To compensate for this increase, activation of the Na^+/Ca^{2+} exchanger is reversed, causing a detrimental increase in intracellular calcium concentration (Avkiran and Marber, 2002, Blaustein and Lederer, 1999, Herchuelz et al., 2002)

Figure 1.4 Illustration demonstrating plaque build up in left descending coronary arteries leading to Ischaemic Heart Disease and Myocardial Infarction (NLHBI, 2015)

1.9 Myocardial Infarction

Following untreated ischaemic injury, such as IHD, cardiomyocytes are unable to function appropriately due to the lack of oxygen and nutrients they require for respiration (Libby and Theroux, 2005). Continuing IHD with lack of any interventions results in cardiomyocyte cell death and increased damage to the myocardium, known as myocardial infarction (MI), commonly known as a heart attack. MI has been described as two classes, type 1 and type 2 respectively (Alpert et al., 2014). With reference to type 1 MI, this is a result of atherosclerotic coronary arteries resulting in plaque rupture, leading to MI and necrotic cell death. Type 2 MI, on the other hand, can refer solely to the increase in demand of oxygen and decrease in myocardial blood flow (Alpert et al., 2014). The purpose of

pharmaceutical/surgical interventions of MI and IHD is the restoration of blood supply to the area of the myocardium that has become ischaemic. Successful intervention and restoration of blood flow to that area is known as reperfusion. Reperfusion, although imperative for cardiomyocyte and myocardium survival, has detrimental complications.

1.10 Reperfusion Injury

Highlighted in the previous sections, reperfusion is imperative to restore blood flow to areas of the myocardium that are ischaemic to re-establish oxygen and nutrient supply for correct cardiomyocyte function. However, as important as reperfusion is after ischaemia, further damage to cardiomyocytes ultimately leading to cell death, can be caused and is known as reperfusion injury (Moens et al., 2005). Reperfusion injury can have several effects on the myocardium ranging from arrhythmias, myocardial stunning, microvascular obstruction and lethal reperfusion injury (Hausenloy and Yellon, 2013, Garcia Gonzalez and Dominguez Rodriguez, 2006). Ventricular arrhythmias caused by reperfusion have been recorded in patients suffering from IHD and can be treated with adenosine or β blockers, however they can be self-terminated as well (Kin et al., 2004).

Upon reperfusion, increased oxidative stress and calcium overload can cause irregular contractile function of cardiomyocytes, known as myocardial stunning, which is reversible (Kloner et al., 1998). Lethal reperfusion injury, being non-reversible, has the most detrimental effect on the myocardium. Contributing to this type of reperfusion injury is the combination of oxidative stress, mitochondrial permeability pore, calcium overload and hypercontracture of cardiomyocytes (Halestrap, 2010, Ong et al., 2015b). Measurement of MI size has been shown to increase in conditions of reperfusion injury, contributing up to 50% of the size of the total damage (Yellon and Hausenloy, 2007).

1.11 Oxidative Stress

One of the main stress mediators of ischaemia reperfusion injury is the production of oxygen free radicals known as Reactive Oxygen Species (ROS). Re-introduction of ROS has been shown to cause further detrimental effects to the myocardium such as lethal reperfusion injury (Ansley and Wang, 2013). Free radicals released upon reperfusion include the superoxide anion O_2^-, hydroxyl radical OH^- and peroxynitrite, all of which have been demonstrated to occur in reperfusion of myocardium in humans (Beard et al., 1994). Additional sources of ROS can include enzymes such as xanthine oxidase, cytochrome oxidase and cyclo-oyxgenase in addition to the oxidation of catecholamines (Moens et al., 2005).

During normal physiological conditions of the heart, mitochondria within cardiomyocytes respire producing ROS that are tolerable to the myocardium due to an effective antioxidant defence mechanism. Included in this defence mechanism are ROS scavengers such as superoxide dismutase (SOD), glutathione peroxidase and catalase (Tsutsui et al., 2011). The 2 most effective scavengers are SOD and glutathione peroxidase, with SOD dismutating O_2^- to H_2O_2, whilst glutathione peroxidase catalyses the reduction of H_2O_2 (Tsutsui et al., 2011, Venditti et al., 2014). In circumstances when ROS levels exceed that of the tolerability of the antioxidant defence mechanism, as seen in reperfusion injury, damage by ROS can occur in the myocardium.

ROS can alter the phospholipid bilayer and proteins of cell membranes, causing lipid peroxidation, resulting in loss of membrane integrity and ultimately leading to cell death via necrosis or apoptosis (Braunersreuther and Jaquet, 2012). Interestingly, the importance of lipid peroxidation in lethal reperfusion injury was highlighted in studies of rats exposed only to ischaemia and not reperfusion, reducing MI with other studies demonstrating the reduction

of MI with the use of ROS scavengers (Moens et al., 2005). Further to direct detrimental effects of ROS, the O_2^- anion is also capable of inhibiting the protective role of nitric oxide (NO), which is active to attempt to protect an ischaemic myocardium from further damage (Tsutsui et al., 2011). In addition to the production of ROS by reperfusion injury, calcium ions (Ca^{2+}) also contribute to the mechanism by which myocardial damage is caused. Normal mitochondrial calcium concentration is critical to the production of ATP via the respiratory chain with activation of mitochondrial dehydrogenases to increase supply of Nicotinamide adenine dinucleotide phosphate (NADH) (Denton, 2009, Halestrap and Pasdois, 2009).

1.12 Calcium Regulation

Calcium regulation is important for cardiomyocyte contraction playing a crucial role in excitation contraction coupling (Jafri, 2012). Depolarisation of individual cardiomyocytes occurs with activation of the Sodium/Calcium Exchanger (NCX), causing opening of L-type Calcium channels of the sarcolemma and release calcium into the cytosol (Fearnley et al., 2011).

Maladapted calcium regulation leading to uncontrolled calcium release can result in overload that is detrimental to cardiomyocytes (Baumgartner et al., 2009). Regulation of calcium stores, responsible for cardiac contraction, is stored within the sarcoplasmic reticulum (SR), with elevated calcium concentration Calcium Induced Calcium Release (CICR) causing the opening of Ryanodine Receptors (RyR) located on the SR membrane allowing an efflux of calcium ions from deep within t-tubules of the myocyte initiating cardiac contraction (Inesi and de Meis, 1989, Periasamy et al., 2008). An illustration of the organisation of these channels can be seen in Figure 1.5. Regulation of calcium concentration is maintained by the Sarcoplasmic Reticulum Calcium-ATPase pump (SERCA) and is influenced by concentrations of intracellular calcium and the inhibitory protein phospholamban (Louch et

al., 2012). As mentioned earlier, PKA phosphorylation of phospholamban prevents inhibition of SERCA thus allowing uptake of calcium ions into the SR. Another signalling protein Calmodulin dependent Protein Kinase II (CaMKII) is also capable of activating SERCA by inhibition of phospholamban (Diaz et al., 2005, Louch et al., 2012).

An increase in cytosolic calcium resulting in calcium overload has a detrimental effect on cardiomyocytes in particular on the mitochondria (Orrenius et al., 2015). Swelling of the mitochondria as a result of calcium overload is one method of pro-apoptotic signalling upon rupture and release of the caspases into the cytosol (see Intrinsic Death Pathway) (Giorgi et al., 2012). The effects of calcium overload specifically on the mitochondria are described in the next section.

Activation of βARs has been linked to increased intracellular calcium concentrations. This has also been demonstrated with the β_1AR agonist Isoproterenol. Upon βAR activation, activation of the cAMP/PKA signalling cascade results in activation of L-type Calcium channels. Prolonged activation of βARs as a result of calcium overload causes an increase in ATP depletion within cardiomyocytes due to the inhibition of the N^+/K^+ATPase resulting in increased Na^+ concentration, inhibiting the NCX leading to maladaptive calcium homeostasis (Garcia-Dorado et al., 2012).

Figure 1.4 Illustration of the location of L-type Calcium channels (Ca^{2+} channel), Sodium Calcium Exchanger (NCX), Potassium channel (K^{+}) located on the surface membrane and within t-tubules of cardiomyocytes. Location of Ryanodine Receptors (RyR) and the Sarcoplasmic Reticulum Calcium ATPase pump (SERCA) located on the surface of the Sarcoplasmic Reticulum (SR). SERCA is regulated by the inhibitor phospholamban (PLB).

Taken from (Louch et al., 2012).

1.13 Mitochondrial Permeability Transition Pore

With increased ROS and calcium production as a direct result of ischaemia reperfusion injury, studies have identified these stressors to directly affect the integrity of mitochondria by increasing the permeability of the mitochondrial membrane, in particular affecting the mitochondrial permeability transition pore (mPTP) (Halestrap and Pasdois, 2009, Halestrap and Richardson, 2015). This phenomenon of sudden permeabilisation of the inner mitochondrial membrane is known as mitochondrial permeability transition (Zoratti and Szabo, 1995). The mPTP is a non-selective pore and is permeable to molecules less than

1.5kDa in size, controlling the opening of the inner mitochondrial pore, particularly during physiological stressed conditions such as ischaemia reperfusion (Hung and Lee, 1998, Riojas-Hernandez et al., 2015). The structure of the mPTP has not fully been elucidated, however several components have been suggested to make up the mPTP. Studies inhibiting the opening of the mPTP with the use of ATP, ADP, and bongkrekic acid, all of which inhibit the Adenine Nucleotide Translocase (ANT) (Halestrap and Brenner, 2003). The most abundant protein identified in the mPTP are Voltage Dependent Anion Channels (VDAC) (McCommis and Baines, 2012). Unlike ANTs, VDACs have been identified to be located on the outer mitochondrial permeability pore linking the cytoplasm of the cardiomyocyte to the matrix of the mitochondria (McCommis and Baines, 2012, Halestrap, 2010) (Figure 1.5). Support for VDACs involvement of opening of the mPTP and structure has been supported with successful blockage of VDACs with monoclonal antibodies that inhibited mitochondrial permeability transition (McCommis and Baines, 2012).

Another proposed component of the mPTP is the matrix protein cyclophilin D (Cyp D) (Halestrap and Pasdois, 2009, Giorgio et al., 2010). Successful inhibition of the mPTP opening with cyclosporin A (CsA) was further investigated and was discovered to inhibit the matrix protein later to be named cyclophilin D (Crompton et al., 1999, Giorgio et al., 2010). The effects of CsA on the mPTP as a whole, and in particular on the Cyp D protein is to desensitise it to calcium thus reducing it from initiating mitochondrial permeability transition from mPTP opening. Studies using Cyp D knockout mice supported this point as isolated mitochondria were shown to have a high resistance to calcium induced mPTP opening (Halestrap, 2010, Nakagawa et al., 2005). The use of CsA in the rat ventricular and atrial cardiomyocytes has been demonstrated to protect against drug induced toxicity by Doxorubicin and prevent premature opening of the mPTP (Gharanei et al., 2013,

Shanmuganathan et al., 2005)

Consequences of premature opening of the mPTP in conditions of ischaemia reperfusion cause the contents of the mitochondria to be released into the cytosol of cardiomyocytes. Contents within mitochondria contain pro-death signalling proteins that can initiate cell death in several ways which are discussed later.

Figure 1.5 Proposed structure of the Mitochondrial Permeability Transition Pore with VDAC penetrating the Outer Mitochondrial Membrane (OMM) with the ANT connecting to the Inner Mitochondrial Membrane (IMM). Cyclophilin D attached to the ANT (D) within the matrix of the mitochondria. Illustration taken from Crow et al., 2004.

1.14 Myocardial Cell Death

Several mechanisms of cell death have been identified that include necrosis and apoptosis, which is also divided into a sub category of autophagy. Each of these cell death mechanisms have been shown to occur in different stages of ischaemia and reperfusion injury, all collaborating to increase the amount on MI tissue. The exact favoured mechanism of cell death in cardiomyocytes remains elusive however strong evidence does support that these cell death types do, in fact, occur.

1.14.1 Necrosis

Necrotic cell death has been considered to be a passive form of cell death as it does not require ATP (Edinger and Thompson, 2004). Characteristics of necrotic cell death include breakdown of the cell membrane, ATP depletion, inflammation resulting from the release of pro-inflammatory cytokines and cellular swelling that can lead to cell rupture (Proskuryakov et al., 2003, Taimor et al., 1999). Necrosis has been described as accidental/unregulated form of cell death when first described, however more recent studies have challenged this view with a concept that necrotic cell death can also be 'programmed' i.e. recognisable signalling proteins to initiate necrosis (Kung et al., 2011, Danial and Korsmeyer, 2004). Interestingly, in cases of acute myocardial infarction, necrotic cell death has been shown to take place in addition to apoptosis as a result of pro-apoptotic mediators, which will be discussed in detail in the apoptosis section (Pasotti et al., 2006).

Necrotic cell death has been strongly linked to ischaemia in the heart and has been demonstrated in rodents and humans, with an increased probability or reversing necrotic cell death in the first 30-40 minutes of ischaemia as shown by Pasotti and colleagues (2006). The use of a β_2AR agonist Clenbuterol in Wistar rat cardiomyocytes were shown to induce cell

death and switch the mechanism of cardiomyocyte death from apoptosis to necrosis, a phenomenon known as secondary necrosis (Silva, 2010, Burniston et al., 2005). Burniston and colleagues (2005) observations in rat cardiomyocytes with this particular βAR agonist, found that cardiomyocytes initiated inflammatory responses as a result of secondary apoptosis increasing myocardial damage and injuring adjacent cardiomyocytes.

1.14.2 Apoptosis

Apoptosis, in contrast to necrosis, is dependent on ATP and is also known as programmed cell death. When cells in the body become un-repairable, are no longer needed or are injured due to a severe insult, an internal signalling cascade can destroy them. Apoptosis is specifically targeted and will not affect neighbouring cells and can be initiated in a variety of ways including extrinsic factors and intrinsic factors. Characteristics of apoptosis include shrinkage of the cell, potential swelling of mitochondria towards the end point of apoptosis and caspase activation (Kung et al., 2011). The main mediators of executing apoptosis are the family of caspases located in the cytosol of cells in particular cardiomyocytes (McIlwain et al., 2013). βAR induced apoptosis has been recorded in rat cardiomyocytes, however apoptosis can be activated via one of two pathways named the extrinsic or intrinsic pathway (Crow et al., 2004). β_1AR activation has been linked to promoting cell apoptosis via the G_S subunit, whilst β_2AR activation has been shown to be anti-apoptotic via G_i subunit activation (Woo et al., 2015). Specifically, pro-apoptotic signalling via β_1AR activation can occur via 2 pathways, firstly as described earlier, activation of β_1AR-G_S initiates the cAMP/PKA pathway increasing calcium concentration which can have a detrimental effect on the opening of the mPTP (see 1.13). Prolonged activation of this particular pathway initiates cardiomyocyte apoptosis via the CaMKII when concentration levels are significantly elevated (Zhu et al., 2003). Activation of CaMKII via calcium signalling initiates apoptosis signalling

kinase 1 which can activate the stress activated MAPK c-Jun N-terminal (JNK) pathway (Olofsson et al., 2008)

In addition to being ATP dependent in contrast to necrosis, myocardial induced apoptosis is not linked to the ischaemic phase, but is a result of the reperfusion phase (McCully et al., 2004). How exactly apoptosis contributes to myocardial injury during reperfusion is linked to one of two pathways that can initiate apoptosis, which will be discussed next.

1.14.3 Autophagy

A subclass of apoptotic cell death is known as autophagy. Autophagy is initiated when there is a requirement for the bulk degradation of proteins and organelles. 3 different types of autophagy have been identified, 1. Macroautophagy, 2. Microautophagy, 3. Chaperon-mediated autophagy (Nishida et al., 2009). Autophagy initiated cell death involves the formation of an autophagosome that can engulf the whole organelle that can then attach to a lysosome to be digested. Autophagy can be seen as cytoprotective when engulfing damaged mitochondria that can release pro-apoptotic factors such as cytochrome c thus inhibiting apoptosis. Enhanced autophagy activity in cultured cardiomyocytes has been demonstrated to protect against ischaemia reperfusion injury (Hamacher-Brady et al., 2006). In severe circumstances of injury and insult, such as ischaemia reperfusion injury, autophagy cell death can still be initiated, however due to the increased number of stimuli (ROS, calcium, endoplasmic reticulum), a large area of the cytosol can damaged releasing pro-apoptotic proteins initiating apoptosis (Hamacher-Brady et al., 2007, Nishida et al., 2009).

1.14.4 Extrinsic Death Pathway

As mentioned previously, apoptotic cell death can be mediated via 2 signalling pathways. The first of these pathways, known as the Extrinsic Death Pathway, involves stimuli external to the cell that interact with receptors on the cell surface such as Death receptors (Muzio et al., 1996). Some examples of the ligands that can interact with such cell surface proteins include, Fas and Tumor Necrosis Factor (TNFα). Upon ligand receptor interaction the formation of the Death-Inducing-Signalling-Complex (DISC) occurs (Petros et al., 2004). Upon Fas ligand interaction with its corresponding receptor, recruitment of Fas-associated via death domain (FADD) initiates recruitment of procaspase-8. Activated cleaved caspase-8 initiates a signalling cascade activating caspase-3 and Bid a pro-apoptotic protein (Crow et al., 2004). Further examples of external stimuli include catecholamine drugs such as Isoproterenol and other βARs.

1.14.5 Intrinsic Death Pathway

Internal stimuli that can initiate the Intrinsic Death Pathway include ROS, calcium overload and DNA damage, all possible outcomes of reperfusion injury. Fundamental to this particular pathway activation is the family of Bcl-2 proteins, which are categorised as anti-apoptotic (Bcl-2, Bcl-Xl) and pro-apoptotic (Bid, Bad, Bim, Bmf, Noxa, Puma, BNip-3, Nix) (Petros et al., 2004). The pro-apoptotic proteins are separated further as multi-domain pro-apoptotics and BH3-only pro-apoptotics (Bid, Bad, Bim) (Crow et al., 2004), (Krautwald et al., 2010).

The pro-apoptotic proteins Bax or Bak are recruited in myocardial infarction induced apoptosis. Bax in its inactive state in response to a stimulus such as ROS, translocates to the mitochondria forming pores in the outer mitochondrial membrane (Basanez et al., 2002). This allows the contents of the mitochondria, crucially cytochrome c, into the cytosol of the

cardiomyocyte initiating apoptosis (Lassus et al., 2002). Mitochondria play a key role in the activation of the Intrinsic Death Pathway as the contents within the mitochondria contain several pro-apoptotic proteins (cytochrome c, DIABLO a second mitochondria-derived activator of caspases (SMAC) and high temperature requirement protein 2 (htrA2)) (Crow et al., 2004, Yang et al., 2003, van Empel et al., 2005) . These proteins, when released into the cytosol, can continue the signalling cascade that will ultimately lead to cardiomyocyte cell death.

Cytochrome c release, as a pro-apoptotic protein, has been associated with mPTP opening and mitochondrial rupture. Once released into the cytoplasm, the Apoptotic Protease Activating Factor-1 (APAF-1) is recruited and further recruits procaspase-9 (Crow et al., 2004). Activation of this particular caspase signals further down the cascade and activates procaspase-3. Activated caspase-3 is then involved in further activation and recruitment of procaspase-9 increasing the caspase concentration thus causing a rapid onset of apoptosis. It has been shown in hearts of transgenic mice deficient of procaspase-9, a remarkable decrease in infarction size when hearts were exposed to conditions of ischaemia reperfusion (Crow et al., 2004).

Having discussed the importance of mitochondria initiating the Intrinsic Death Pathway, another stress mediate of reperfusion injury is oxidative stress acting directly on the mitochondria. Oxidative stress promotes the Poly ADP Ribosome Polymerase (PARP), which recruits the Apoptotic Inducing Factor (AIF) (Hong et al., 2004). AIF is responsible for DNA dismemberment and mitochondrial release of cytochrome c, both of which promote apoptosis (Figure 1.6).

Figure 1.6 Mitochondrial regulation of apoptosis displaying both Intrinsic Death Pathway (Left) and Extrinsic Death Pathway (Right). Taken from (Tait and Green, 2012)

1.15 Signalling Pathways

The activation of the death pathways that are associated with cardiomyocyte apoptosis is one aspect of a signalling cascade. Further signalling pathways can be both pro-apoptotic and anti-apoptotic depending on the length of time and volume of signalling proteins that are recruited. 2 pathways that have been associated promoting anti-apoptotic effects against myocardial injury include the phosphoinositide 3-kinase (PI3K)/Protein Kinase B (Akt) pathway and the extracellular regulatory kinase (Erk) 1/2 pathway.

1.15.1 PI3K/Akt signalling pathway

Activation of the PI3K/Akt pathway has been linked to several cellular functions such as cell proliferation, growth and attenuation of apoptosis induced by myocardial ischaemia reperfusion injury (Hausenloy and Yellon, 2004). Akt (Protein Kinase B), a serine-threonine kinase, mediates several pro-apoptotic proteins via inhibition or phosphorylation to prevent apoptosis, in particular Bad and caspase-9 (Jeong et al., 2008).

Activation of PI3K phosphorylates the membrane of phosphatidylinositol 4,5 bisphosphate (PIP) that then goes on to generate $PIP_{3,4 \text{ and } 5.}$ The recruitment of Akt and phosphoinositide dependent kinase-1 (PDK1) by PIP_2 and PIP_3, moves them to the cell membrane activating Akt (Nagoshi et al., 2005). PI3Ks can be divided into several classes determined by their structure and substrate specificity (Vanhaesebroeck et al., 1997). Of particular interest of the classes is the association of the I_B class with activation by β subunits of GPCRs (activated by βAR agonists) (Naga Prasad et al., 2001). A strong association of activation of Akt has been made with rat hearts in the model of ischaemia reperfusion (Hausenloy et al., 2005).

Akt in its active form can regulate several downstream targets such as the forkhead transcription factor, nitric oxide synthase, glycogen synthase kinase-3, BAD and nuclear

factor kB (Mockridge et al., 2000). The Bcl-2 family member BAD, when phosphorylated at Serine 136 by Akt, binds to 14-3-3 proteins. These particular 14-3-3 proteins interact with BAD at serine 136 but have also been shown to interact at serine 112, both capable of inhibiting pro-apoptotic signalling (Masters et al., 2001). Upon BAD activation, the proteins translocate to the mitochondria bound to Bcl-2 and Bcl-Xl where it can form pores inducing cell apoptosis via leakage of mitochondrial contents. BAD in its active form is found within the cytosol continuously bound to 14-3-3 proteins thus inhibiting apoptotic signalling (Masters et al., 2001). The PI3K inhibitor Wortmannin has been shown to inhibit the anti-apoptotic effects of Akt activation in both rabbit and rat hearts (Armstrong, 2004). Akt is a key signalling protein during ischaemia reperfusion as it allows cells to avoid apoptosis and maintain their cellular function by phosphorylating downstream targets such as Bad at serine^{136} and caspase-9, both pro-apoptotic proteins when activated (Mullonkal and Toledo-Pereyra, 2007).

Activation of Akt has been linked with βARs, in particular with Isoproterenol. Rat cardiac endothelial cells, have been demonstrated to initiate the PI3K/Akt pathway as a result of production or pro-inflammatory cytokines leading to elevated levels of phosphorylated Akt in the presence of the βAR agonist Isoproterenol (Chandrasekar et al., 2004).

Investigations into the acute activation of Akt can be beneficial for anti-apoptotic effects, however more recent studies are exploring the effects of chronic activation (Mullonkal and Toledo-Pereyra, 2007, Nagoshi et al., 2005). Although acute activation of Akt has been demonstrated to promote cell survival, chronic activation of Akt has been linked to induce cell apoptosis and promote cardiac abnormalities such as hypertrophy, heart failure and increased myocardial infarction(Mullonkal and Toledo-Pereyra, 2007), (O'Neill and Abel, 2005). Shiojima and colleagues (2005) in their model with transgenic overexpression of

activated Akt an increase in severe cardiac dysfunction, also supported by a similar model by Nagoshi (Nagoshi et al., 2005, Shiojima et al., 2005).

1.15.2 Mitogen-Activated Protein Kinases

Complex signal transductions within the heart are co-ordinated with the use of signalling proteins in response to stimuli (Yang et al., 2013). The mitogen-activated protein kinases (MAPK) are one group of signalling proteins that are responsible for signalling cascades to execute instructions such as cardiac development, metabolism, cell proliferation and apoptosis (Rose et al., 2010). Associated to the MAPK family are four types of MAPKs that initiate signalling cascades, these include Extracellular Signal Regulated Kinases (ERK 1/2), c-Jun NH_2 Terminal Kinase (JNK), p38 kinase and big MAPK (Vandamme et al., 2014, Yang et al., 2013). Activation of any of these MAPK initiate a signalling cascade involving several different MAPKs including, a MAPK kinase kinase (MAPKKK), MAPK kinase and a MAPK (Figure 1.7)(Rose et al., 2010).

Figure 1.7 Illustration of the Mitogen-Activated Protein Kinase cascade. Activation of the MAPK pathway results in a downward cascade from MAPK kinase kinase (MAPKKK) activating MAPK kinase furhter activating MAPK via phosphorylation (Bak et al., 2012).

1.15.3 ERK 1/2 Signalling Pathway

Two variants of ERK exist in the forms ERK1 and ERK2, both approximately 80% identical to each other with similar signalling capabilities. ERK 1/2 has been demonstrated in a variety of species to regulate cytokinesis, cell death and proliferation. Due to importance and abundance of ERK 1/2 in various processes mentioned, ERK 1/2 has been shown to be involved in the formation of cancers and cardiovascular diseases such as cardiac hypertrophy (Kehat and Molkentin, 2010). Activation of ERK 1/2 can be via GPCRs, insulin growth factors, fibroblast growth factors and cytokines. Several different scaffold proteins are

associated with ERK 1/2 such as Ras and β arrestin (Rose et al., 2010). Activation of the signalling mediator Ras by tyrosine kinase activates Raf. Activated Raf is responsible for the phosphorylation of the MEK1/2 thus activating ERK 1/2 via phosphorylation (Rose et al., 2010).

ERK 1/2 activation has been shown to be a key component in the RISK pathway leading to cardioprotection of cardiomyocytes exposed to ischaemia reperfusion injuries (Hausenloy et al., 2005). ERK 1/2 activation has been shown to protect against drug induced myocardial injury such Doxorubicin (Takemura and Fujiwara, 2007). Elevated ERK 1/2 expression in response to a cardiac injury such as ischaemia reperfusion has shown to elevate expression of nitric oxide synthase, endothelial nitric oxide and Bcl-2 (Rose et al., 2010). Further work with adult rat cardiomyocytes has shown cardioprotective effects of ERK 1/2 with interaction of alternative pathways such as the TNF α induced apoptotic signalling pathway. ERK 1/2 signalling has also been demonstrated to compensate for the loss of Akt signalling in post reperfusion in rat hearts to initiate cardioprotective mechanisms (Miki et al., 2007).

Overexpression of active Ras in transgenic mouse hearts demonstrated to have cardiomyocyte hypertrophy (Hunter et al., 1995, Zhang et al., 2003). Strong links to the influence of calcium concentration by expression of ERK 1/2 have also been investigated, in particular the activation of the Ras/Raf/MEK/ERK pathway. In vitro and vivo experiments with ventricular myocytes have demonstrated the direct effect of Ras activation of the SR, reduces L-type Ca^{2+} channels and decreased SERCA activation and increased activity of phospholamban (Zheng et al., 2004).

Aims

The aims of this thesis were:

1. Investigate the effects of Short and Long acting β adrenergic receptor agonists in a model of ischaemia reperfusion injury.
2. Identify the effects of Short and Long acting β agonists on the mitochondrial permeability transition pore in a model of oxidative stress
3. Identify any signalling pathway protein expressions associated with any observed detrimental/protective effects on the rat myocardium.
4. Confirm activation of a specific subtype of the β adrenergic receptors that results in the observed effect on the rat myocardium.
5. Investigate the role of the mitochondrial permeability transition pore in response to increased reactive oxygen species in a model of oxidative stress.
6. Inhibit the detrimental effects of reactive oxygen species with the use of the cyclophilin D inhibitor, Cyclosporin A.
7. In the presence of an identified toxic β adrenergic receptor agonist, co-administer the cyclophilin D inhibitor, Cyclosporin A to observe if reversal of injury can be obtained.

2 Chapter 2 – Material & Methods

2.1 Animals

Male Sprague Dawley rats, 12weeks (350g ± 50g), 18 month (450g ± 50g) and 24 month (500g ± 25g) old, were initially purchased from Charles River (Margate, UK) and were then raised to the appropriate age with free access to pellet chow and water. The rats were kept and raised in accordance with the Home Office Guidance on the Operation (Scientific Procdures) Act 1986 (The Stationary Office, UK)

2.2 Chemicals & Drugs

Salbutamol hemisulfate, Salmeterol, Formoterol hemifumarate, Isoproterenol, Cyclosporin A (CsA), ICI 118, 551 hydrochloride, CGP 20712 dihydrochloride, Wortmannin and U0126 were all purchased from Tocris (Bristol, UK). Salbutamol, ICI 118, 551, CGP 20712 and Isoproterenol were all dissolved in ultrapure water. Formoterol, Wortmannin and U0126 were dissolved in di-methyl sulfoxide (DMSO) ensuring the final concentration of DMSO was <0.02%. Salmeterol and CsA were dissolved in ethanol ensuring the final concentration of ethanol was, 0.01%. All salts used were acquired from Fisher Scientific (Loughborough, UK).

2.3 Langendorff Perfused Heart Model

2.3.1 Isolation of the Rat Heart

Rats were sacrificed by cervical dislocation in accordance with a schedule 1 Home Office procedure (1986) and their hearts were excised and placed into ice cold Krebs Heinsleit Buffer (KHB). The aorta was exposed and cannulated onto the Langendorff apparatus (Figure 2.1), followed by perfusion with KHB (118mM NaCl, 12mM Glucose, 25mM $NaHCO_3$, 4.8mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 1.7mM $CaCl_2$, gassed with 95% O_2, 5% CO_2 (BOC gases, UK)), pH maintained at 7.4 and temperature kept at 37^{o}C ± 0.5^{o}C throughout

the experiment. Time taken from excision of the heart to perfusion with KHB was kept to an absolute minimum i.e less than 3 minutes. Retrograde perfusion of the heart, forces KHB to flow through the coronary vessels allowing perfusion of the myocardium.

The left atrium was cut away and an iso-volumic latex balloon connected to a physiological pressure transducer (AD Instruments, Oxford, UK), was inserted into the left ventricle via the removed left atrium and inflated between 5-10mmHg. Haemodynamic data such as left ventricular developed pressure (LVDP), heart rate (HR) and coronary flow (CF) were collected at regular intervals throughout the experiment. The LVDP was recorded as measurement of the end diastolic pressure in millimeteres per mercury (mmHg) for a one-minute period. The heart rate was calculated via analysis of a one-minute period of the represented electrocardiogram trace. Coronary flow was measured by collecting the effluent and measuring the volume in millilitres (ml). Haemodynamic data were collected using Chart 5 (v5.1.2) and analysed using Graphpad Prism (v6.0.1). All haemodynamic data was calculated as a percentage of the average stabilisation period.

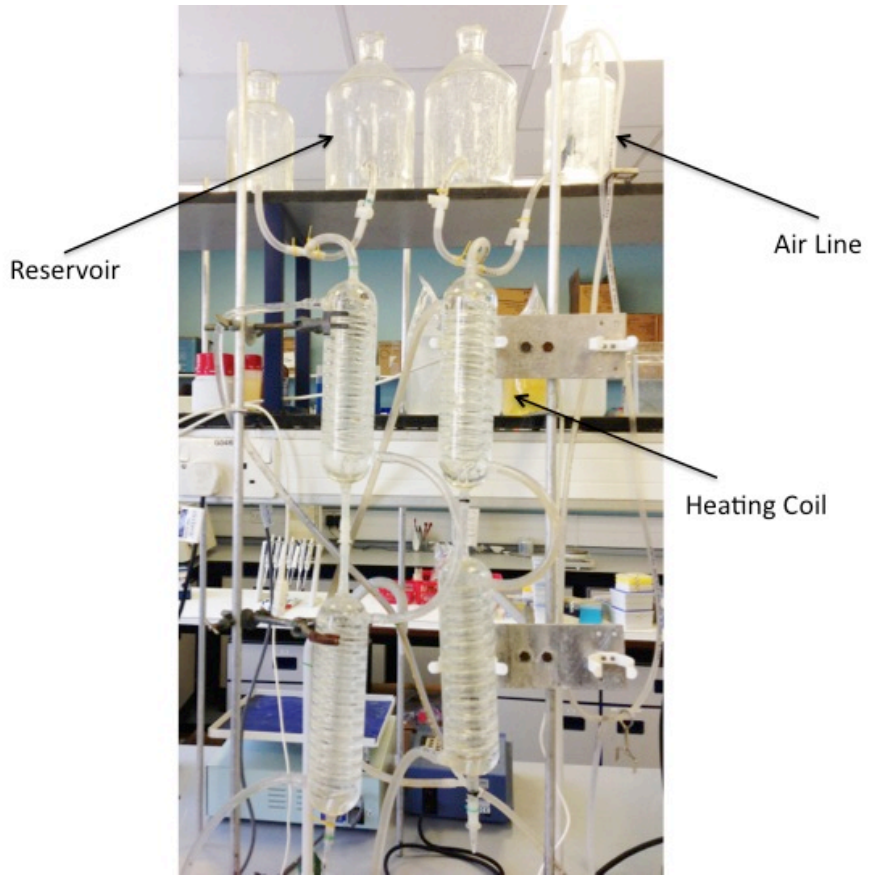


Figure 2.1 Photograph of Langendorff apparatus

2.3.2 Langendorff protocol & Reperfusion studies

The protocol followed is illustrated in Figure 2.2. Hearts were allowed to stabilise for 20 minutes followed by a period of regional ischaemia for 35minutes. Previous studies have indicated periods of ischaemia between 5-20 minutes followed by reperfusion caused myocardial stunning with no myocardial injury but only cellular dsyfunction (Kalogeris et al., 2012). Reperfusion periods greater than 20 minutes have been shown to cause significant irreversible myocardial injury, mimicking injury observed in myocardial infarction (Yellon and Hausenloy, 2007). To induce ischaemia the left anterior descending coronary arteries were occluded. This was achieved by inserting a curved surgical suture under the coronary artery and its thread passed through 2 pipette tips to form a snare, tightening of the snare, initiated ischaemia. Ischaemia was confirmed by a decrease in LVDP. The heart was then reperfused for 120 minutes by removing the snare.

2.3.3 Infarct Size & Data Analysis

After reperfusion the coronary arteries were re-occluded by tightening the snare and infused with 0.25% Evans Blue in saline (Sigma, Dorset, UK) into the heart via the aorta to delineate the non-risk area of the heart (Figure 2.3). Immediately after infusion, the hearts were weighed and frozen overnight at -20^{o}C. The frozen heart was cut into 2mm transverse sections and incubated in 1% triphenyltetrazolium solution (TTC) for 15minutes at 37^{o}C. Following incubation with TTC, the heart slices were fixed in 10% Formalin solution for a

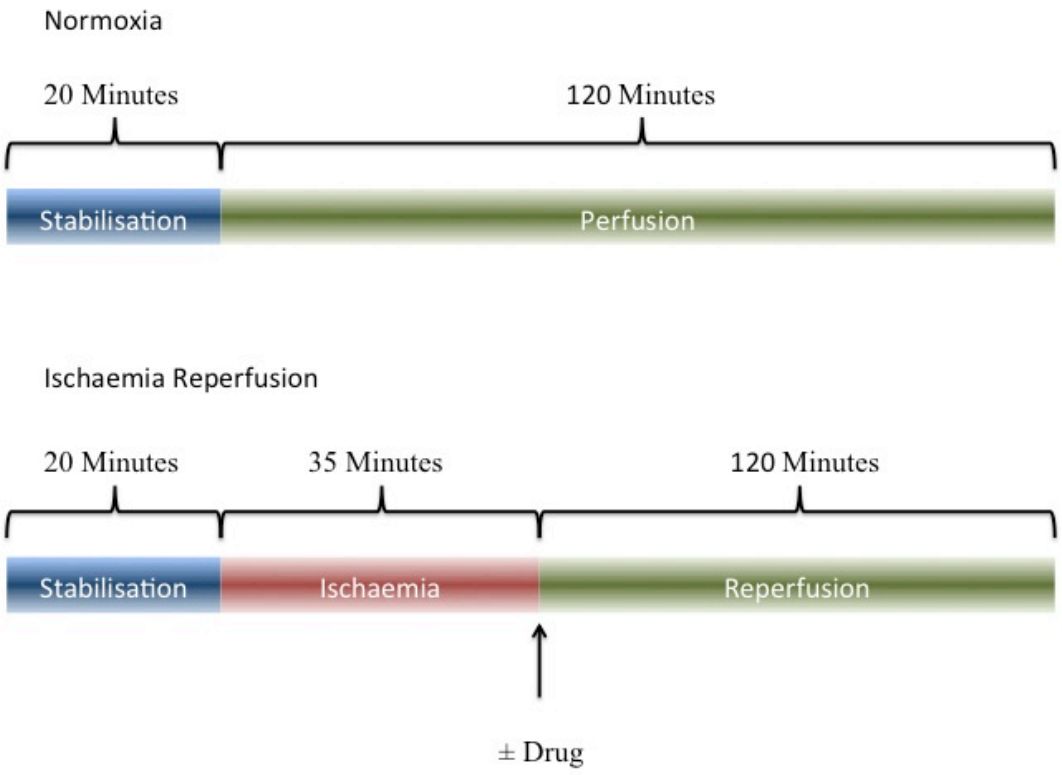


Figure 2.2 Illustration of Langendorff protocol
Hearts underwent stabilisation for 20 minutes followed by 120minutes perfusion for normoxic studies. Ischaemia reperfusion studies were stabilised for 20minutes followed by 35minutes of ischaemia and reperfused for 120minutes. At the onset of reperfusion the drug were administered (± Isoproterenol (0.1µM-0.5µM), salmtereol (0.1µM), formotereol (0.1µM-0.5µM), salbutamol (0.001µM-1µM), ICI 118, 551 (0.0014µM), CGP 20712 (0.0012µM), cyclosporin A (0.2µM), wortmanin (0.1µM), U0126 (10µM)

minimum of 4 hours to fixate the infarct tissue. Heart slices were transferred between 2 perspex blocks held together with bulldog clips and traced onto an acetate sheet. A computerized planimetry package (Image Tool v3.0, NICH) was used to calculate the percentage of infarct tissue compared to the area at risk and expressed as percentage of the Infarct-Risk ratio (I/R) (Figure 2.3.4). This was calculated by measuring each individual 2mm transverse heart slice measuring the percentage of infarct tissue, risk tissue and viable tissue with an overall average for I/R obtained from all individual slices (Figure 2.4). Infarct size was calculated as percentage of the area at risk, which represents the "entire perfusion bed distal to the occluded coronary artery" (Redfors et al., 2012).

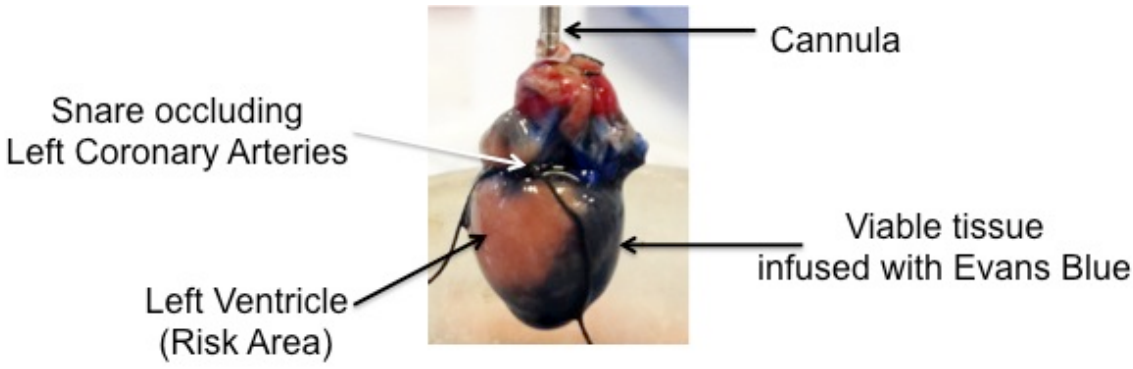


Figure 2.3 Photograph of rat heart after infusion with Evans Blue delineating risk area from non risk area.

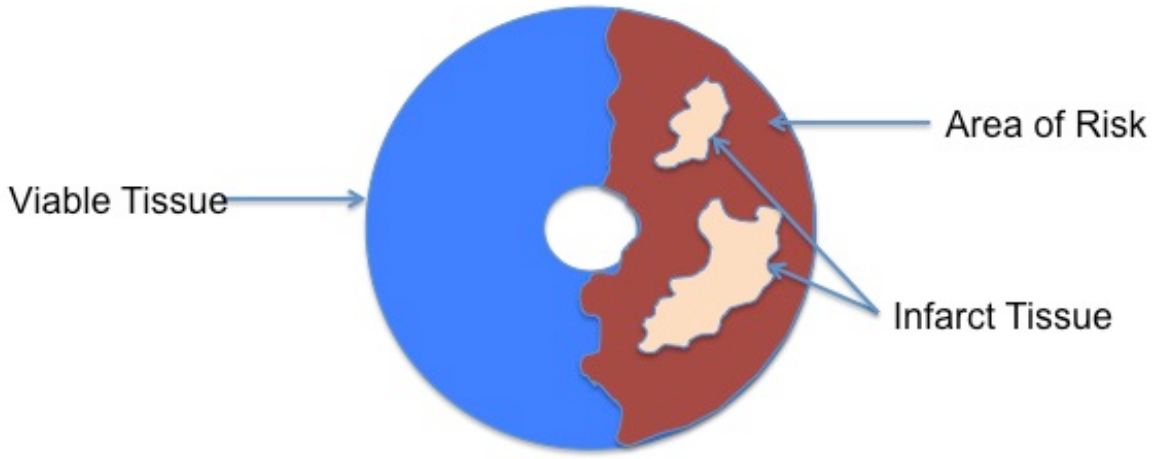


Figure 2.4 Illustration of Heart Slice drawn on acetate sheet.
The blue shaded area shown indicates the area of the heart that was continuously perfused throughout the experiment and is not at risk. The Area of Risk (red) was the portion of the heart subjected to ischaemia containing risk tissue (red) and infarcted tissue (cream).

2.3.4 Exclusion Criteria

Hearts were excluded if coronary flow was less than 8ml/min during stabilisation and also any hearts that had a LVDP less than100mmHg during the same period. Hearts were also excluded if during the reperfusion phase went under fibrillation longer than 5minutes or could not be restored to a normal rhythm in the same amount of time. Hearts that underwent the Langendorff ischaemia reperfusion protocol were excluded from the data if the area at risk was less than 35%,

2.4 Adult Rat Ventricular Myocyte Isolation

Rats were sacrificed by cervical dislocation and the hearts excised and hung on a modified constant flow (14ml/min) Langendorff apparatus (Figure 2.5). Hearts were perfused for 3-4 minutes with calcium free modified Krebs Heinsleit Buffer (116mM NaCl, 25mM $NaHCO_3$, 5.4mM KCl, 0.4mM $MgSO_4$, 10mM glucose, 20mM taurine, 5mM Sodium Pyruvate and 0.9mM Na_2PO_4 as described by (Maddock et al., 2002)). The buffer was then switched and the hearts perfused with digestion buffer (0.075% Worthingtons Type II Collagenase, 4.4µl

$CaCl_2$ and pH adjusted to 7.4 with 1M NaOH). Hearts were perfused for 7 minutes with the digestion buffer and the effluent was collected and reused.

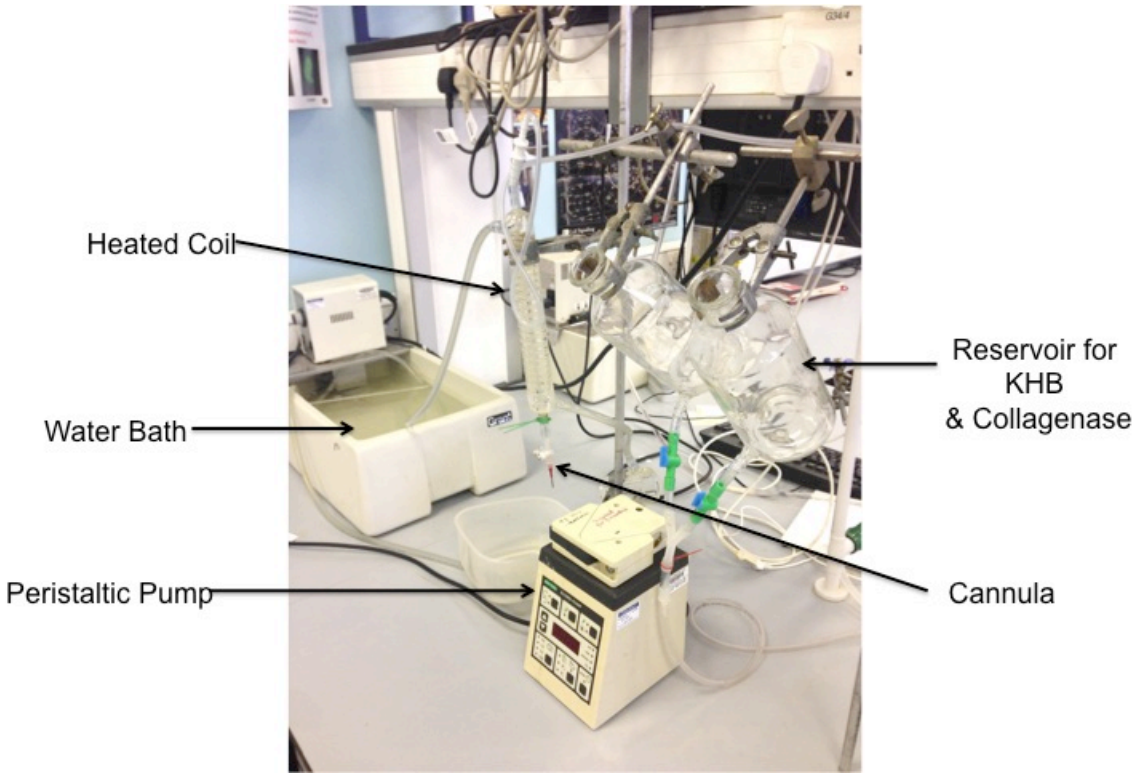


Figure 2.5 Photograph of modified Langendorff rig for Myocyte Isolation

After perfusion with digestion buffer, the atria of the heart was cut away, the ventricles were cut into smaller sections and re-suspended in fresh digestion buffer for 10 minutes on an orbital shaker. The digestion buffer was aspirated and passed through a sterile nylon mesh into a sterile 50ml falcon tube with the undigested tissue re-suspended in fresh digestion buffer and allowed to be further digested on the orbital shaker for 10 minutes. The filtrate was centrifuged for 2 minutes at 600rpm. Using a sterile pipette to remove and discard the supernatant, the pellet was re-suspended in freshly prepared restoration buffer containing: 116mM NaCl, 25.0mM $NaHCO_3$, 5.4mM KCl, 0.4mM $MgSO_4$ .7.H_2O, 10mM glucose, 20mM taurine, 5mM pyruvate 0.9mM Na_2HPO_4.12H_2O, 1% BSA and 1% Pen-Strep, pH 7.4.

Post isolation, the myocytes received 5 doses of $CaCl_2$ over 30 minutes to bring the final concentration to 1.25mM to prevent calcium overload. The myocytes were incubated at 37^{o}C until used in restoration buffer.

2.4.1 Exclusion Criteria

Isolations were discarded if the viability was below 65%. For the oxidative stress model, dishes were discarded if myocytes had not stuck down successfully or less than 3 cells were visible in the field of view during confocal microscopy.

2.5 Hypoxia/Reoxygenation Studies

2.5.1 Hypoxia/Reoxygenation

After myocytes had been isolated, the cells were counted using a haemocytometer. The myocytes were centrifuged at 600rpm for 2 minutes and the restoration buffer discarded. The pellet was re-suspended in 15ml Hypoxic Buffer (12mM KCl, 0.49mM $MgCl_2$, 0.9mM $CaCl_2$, 4mM HEPES, 10mM 2-Deoxy-D-glucose and 20mM Lactate) placed in a petri dish and incubated at 37^{o}C with conditions of 5% CO_2, <1% O_2 using a Galaxy 48R CO_2 incubator, (New Brunswick, Eppendorf, Stevenage, UK). Myocytes were incubated in hypoxic conditions for 2 hours. The cells were re-suspended in the Hypoxic Buffer and centrifuged at 600rpm for 2 minutes. The supernatant was discarded and the cells were re-suspended in restoration buffer. Cells were re-oxygenated for 4 hours in the presence or absence of the drugs and incubated at 37^{o}C.

2.5.2 MTT Assay

The use of the tetrazolium salt MTT (3−[4,5−dimethylthia−zol−2−yl]−2,5−diphenyl tetrazolinum bromide) is widely used to determine cell viability, cytotoxicity and proliferation (Wang et al., 2011). This is determined by the ability of the dehydrogenase enzymes of the mitochondria within the myocytes being able to reduce the MTT to a purple

formazon product that can then be analysed using spectrophotometry (Abe and Matsuki, 2000).

2.5.2.1 Preparation of 96 Well Plate

After the myocytes were successfully isolated and underwent hypoxia, they were counted again using a haemocytometer and re-suspended in the desired volume of restoration buffer to achieve 10,000 cells per 50µl.

Drugs were diluted in restoration buffer and pre-aliquoted into a 96 well plate. The outer wells were left as blanks and 2 columns dedicated to a normoxic control and Hypoxia/Reoxygenation (H/R) control. 50µl of cells were added to the drugs and incubated at 37^{o}C for 4 hours. After incubation with the drugs, 20µl of MTT (5mg/ml) (Sigma, UK) was added to each well and the plate wrapped in foil and incubated for a 2 hours. Following MTT incubation, 100µl lysis buffer (15% SDS in 50% dimethyl formamide) was added to each well and incubated overnight.

2.5.2.2 Data Analysis

Plates were read at an absorbance of 480nm using a plate reader (StingRay v1.1.3). Values were converted as a percentage compared to Normoxic control values. Data was plotted using GraphPad Prism (v6.0.1).

2.5.3 Flow Cytometry – FACS

The Fluorescence Assimilated Cell Sorter (FACS, Becton Dickinson) was used to detect the fluorescence of myocytes when treated in the presence or absence of drugs and detected for cleaved Caspase-3.

2.5.3.1 Cleaved Caspase-3 Activity

Following the Hypoxia/Reoxygenation protocol as described in section 3.7.1, myocytes were treated in the presence or absence of the drugs of interest and re-suspended in restoration buffer. Myocytes were transferred to 1.5ml eppendorf tubes and centrifuged at 1200rpm for 2 minutes. The supernatant was discarded and the cells re-suspended with 500µl of 3% formaldehyde in Phosphate Buffered Saline (PBS) and allowed to fix for 10 minutes at room temperature to prevent any further cellular activities. The eppendorfs were then centrifuged at 1200rpm for 2 minutes followed by discarding the supernatant and replacing it with ice-cold 90% Methanol. Cells were stored overnight at -20^{o}C, or incubated on ice for 30 minutes. Myocytes suspended in methanol were centrifuged at 1200rpm for 2 minutes; supernatant discarded and washed 3 times in 200µl incubation buffer (0.5% BSA in PBS), after each wash, the myocytes were re-suspended and then spun down. The myocytes were blocked for 10 minutes with 200µl incubation buffer followed by incubation for 1 hour at room temperature with the Cleaved Capsase-3 conjugated with Alex Fluor 488 (1:100) (Cell Signalling Technologies, UK) and covered in foil. The myocytes were spun down at 1200rrpm for 2 minutes and the antibody buffer removed and washed 3 times in incubation buffer. After the 3rd wash, the myocytes were re-suspended in 500µl PBS and transferred to FACS tubes for analysis.

2.5.3.2 Data Analysis

Alexa Fluor 488 is excited on FL-1 at 495nM and emits at 519nM. Histograms were plotted for each of the groups showing the mean fluorescence for 10,000 cell counts. The mean fluorescence were normalised against control values and graphs plotted using GraphPad Prism (v6.0.1).

2.6 Oxidative Stress Model

As mentioned earlier the opening of the mPTP results in oxidative stress. To mimic the conditions of oxidative stress the fluorochrome tertramethylrhoadmine methyl ester (TMRM) can be used to investigate the effects of the drugs specifically on the mPTP. TMRM is positively charged, which specifically penetrates and quenches within the mitochondria. Laser stimulation of TMRM creates ROS, which causes the mPTP to open and release its contents into the cytoplasm. This phenomenon can be detected via real time confocal microscopy.

2.6.1 Confocal Microscopy

6 x 60mm sterile petri dishes (Fisher Scientific) were coated with Laminin (1mg/ml diluted in 6.5ml ddH_2O) (Sigma, UK) and isolated myocytes were allowed to adhere to the laminin coated dishes for 2 hours at 37^oC. Dishes were then incubated for 15 minutes with microscopy buffer (Calcium free modified KHB, 10 mM HEPES and 1.2µM $CaCl_2$, pH 7.4) containing 3µM TMRM. The TMRM buffer was aspirated and further incubated for 10 minutes with microscopy buffer in the presence or absence of the drugs of interest.

Using a Zeiss 510 LSM confocal microscope, in turn, each petri dish was placed on the heated stage (37^oC) and the myocytes were observed with a x20 objective lens. The 543-nm channel of the HeNe laser was used to stimulate TMRM. The use of a 585-nm long pass filter was used to collect TMRM fluorescence. Images were analysed using Zeiss software AIM 2.8 (Carl Zeisss Ltd, UK).

2.6.2 Data Analysis

Upon laser stimulation of the TMRM, the time to depolarisation was recorded and represented by the time taken for an increase in light intensity (Figure 2.6). The second reading taken was time to the onset of hypercontracture as a result of opening of the mPTP

and the release of the contents of the mitochondria into the cytoplasm resulting in ATP depletion and eventual cell death.

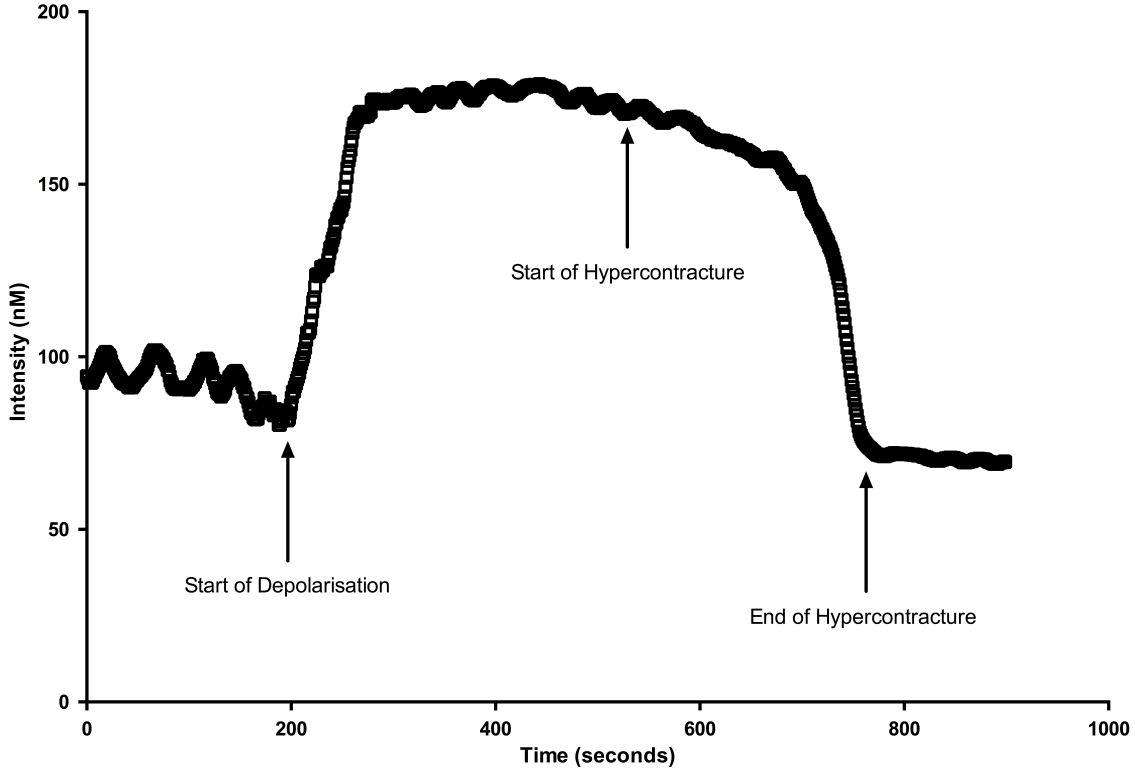


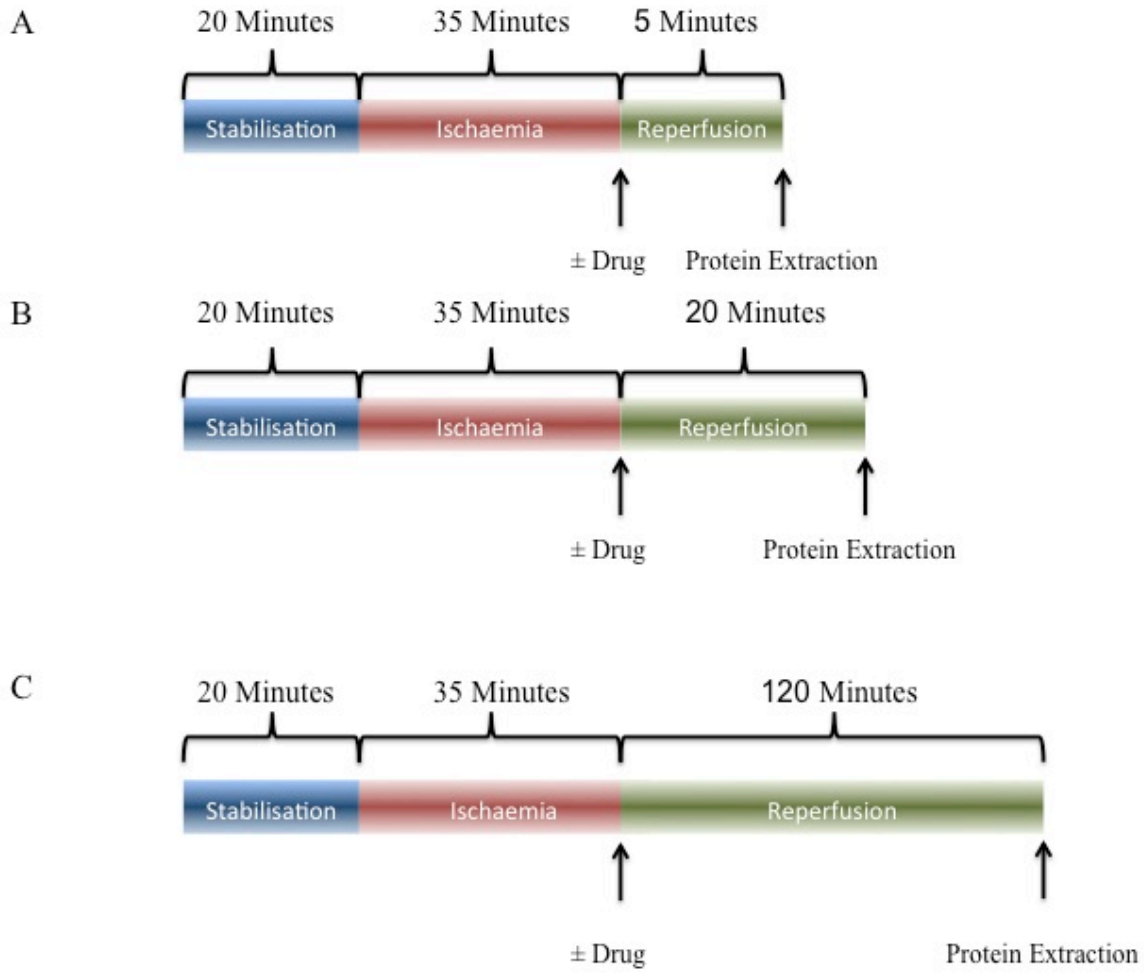
Figure 2.6 Graph indicating the intensity measured of a single cardiomyocyte over time Increase in intensity indicates the release of TMRM into the cardiomyocyte which in turn represents the start of depolarisation. After the plateau phase a decline in intensity represents the start of hypercontracture.

2.7 Western Blotting

2.7.1 Tissue Collection

Hearts were excised and hung as described in section. Hearts were stabilised for 20 minutes followed by 35 minutes of regional ischaemia. The hearts were exposed to different lengths of reperfusion i.e. 5 minutes, 20 minutes or 120 minutes in the absence or presence of the drugs. A 5-minute reperfusion period was used to observe if an onset of exacerbation of

myocardial injury occurred in the presence of the drug. A 20-minute reperfusion period was selected in line with previous studies recording significant myocardial injury during in this time period (Kalogeris et al., 2012). Reperfusion for 120 minutes was used to observe if exacerbation of myocardial injury continued throughout exposure to the heart in the presence of the drug. Once the reperfusion time had elapsed, the heart was cut using a sterile scalpel and the left ventricle isolated. The ventricle was wrapped in silver foil and snap frozen in liquid nitrogen. The heart tissue was labelled and stored at -80^{o}C for future analysis.



2.7.2 Protein Extraction

Approximately 60mg of the frozen samples were cut into smaller pieces and placed in labelled eppendorf tubes without allowing the samples to thaw. Each eppendorf tube contained 400µl of cold lysis buffer (100 mM NaCl, 10 mM (pH 7.6) Tris, 1 mM (pH 8) EDTA, 2 mM Sodium pyrophosphate, 2 mM sodium fluoride, 2 mM β-glycerophosphate, 0.1 mg/ml PMSF, 0.1 µg/ml aprotinin, cocktail tablet, phosSTOP and leupeptin. The samples were homogenised using an IKA Labortechnik T25 homogeniser. Once homogenised the samples were spun at 11,000rpm at 4^{o}C for 10 minutes. The supernatant was removed and aliquoted into newly labelled eppendorf tubes and the pellet discarded. The supernatant protein concentrations were measured using spectrophotometry at 280nm using the NanoDrop system (NanoDrop Technology, Delaware, USA).

100µl of the supernatants were aliquotted to a set of newly labelled eppendorffs and were diluted with 100µl of Sample Buffer (Tris 100mM (pH 6.8), DTT 200mM, SDS 2 %, Bromophenol blue 0.2 % and glycerol 20 %) followed by heating for 10 minutes at 90^{o}C and finally centrifuged at 11,000rpm for 30 seconds. Samples were stored at -20^{o}C for further use.

2.7.3 Gel Electrophoresis

Any kDa Tris-Glycine (4-15%) pre-cast gradient gels were purchased from Bio-Rad, UK. The use of gradient gels allows the migration of proteins until the decreasing pore size (determined by the increasing acrylamide concentration) obstructs any further migration. This allows a broad range of molecular weights to be separated from the sample. The use of gradient gels allows proteins with similar molecular weights to separate more advantageously when compared to fixed concentration gels (Walker, 2014). The gels were placed in the Mini-Protean III system and locked in place. The inner chamber of the 2 gels, were filled

with approximately 125ml running buffer (glycine 14.42 g/l, SDS 1.0 g/l, Tris 3.0 g/l) and the combs removed. 60µg of protein containing sample buffer was loaded into each well. The outside chamber of the system was filled with approximately 400ml running buffer. A dual coloured molecular marker and biotinylated ladder (Bio Rad, UK, Cell Signalling Technologies, UK) were assigned to 2 of the 12 available wells. The gel was run at 130V for 1 hour 30 minutes using the PowerPac 300 (Bio-Rad, UK).

2.7.4 Protein Transfer

Following the gel electrophoresis stage, the gel casket was opened and the gel placed onto a Hybond-P Polyvinyl Difluoride (PVDF) membrane. The PVDF membrane was part of a Trans-Blot Turbo transfer pack (Bio-Rad, UK). Each pack contained Whatman filter paper and a PVDF membrane all pre soaked in transfer buffer (glycine 14.4g/L, tris 3g/L, 30% methanol). The Trans-Blot Turbo modules were assembled as per the manufactures' instructions and transferred for 7 minutes at 1.3A, 25V. The Trans-Blot Turbo modules were disassembled and the polyacrylamide gels were discarded after being stained with Coomassie Blue to ensure successful transfer. The PVDF membrane was cut to size and placed in 15ml Blocking buffer containing 5% Milk in Tris Buffered Saline with 1% Tween-20 (TBS/T) on an orbital shaker for 1 hour.

2.7.5 Immunoblotting

After blocking, membranes were washed for 5 minutes x3 in 15ml TBS/T. Membranes were incubated overnight on an orbital shaker, with antibody buffer (5% milk in TBS/T), at 4^{o}C with the primary antibody of the protein of interest. The proteins of interest for this thesis were phosphorylated-Akt (Ser473), total Akt, phosphorylated p44/42 (Thr/202/Try204), total p44/42 (Cell Signalling Technologies, UK).

Following incubation, membranes were washed for 5 minutes x3 with TBST and incubated with secondary antibody (1:10000) with Anti-rabbit antibody HRP linked IgG and HRP linked anti-biotin, for 1 hour at room temperature. 3 final washes in TBST were done before analysing the membranes.

2.7.6 Detection & Densitometry

A 1:1 mix of Super Signal West Femto (Pierce Biotechnologies, UK), an enhanced chemiluminescent (ECL), was mixed in a foil covered falcon tube. 1ml of the ECL was carefully pipetted onto the surface of the membrane, placed on top of a single sheet of acetate, and spread evenly. Using the imaging machine, ChemiDoc XRS (Bio-Rad, UK), the membranes were exposed and chemiluminescence detected. Quantity One software (v4.5.2) was used to analyse the bands detected.

2.7.7 Stripping and Re-Probing

To determine the amount of total protein present on the membranes, membranes were stripped of the previous antibody by boiling in ddH_2O for 5 minutes. The membranes were then blocked again for 1 hour in blocking buffer and further probed with the next desired antibody (Total Akt, ERK or GAPDH) and incubated overnight on an orbital shaker (Section **Error! Reference source not found.** & 2.7.6). The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was used throughout all western blot experiments to ensure equal protein loading and used as a normaliser. The use of housekeeping gene ensures that expression of the gene remains constant and independent of the experimental protocol allowing the date to be normalised against its value .

3 Chapter 3: The role of long and short acting βadrenergic receptor agonists in myocardial ischaemia/reperfusion injury

3.1 Introduction

Asthma is a chronic inflammatory disease that affects the airways leading to airflow obstruction and bronchospasm affecting around 200-300 million people worldwide annually in addition to affecting 10% of the UK adult population (Sharpe et al., 2015, Netuveli et al., 2005). The common symptoms of asthma are shortness of breath, wheezing and coughing and are routinely relieved by the administration of bronchodilators (Apter, 2015). Initially, acute exacerbations of asthma have initially been managed by use of a non-specific β_1 and β_2 adrenergic receptor (βAR) agonist Isoproterenol (Shukla et al., 2015). The adverse cardiac side effects of Isoproterenol resulted in its withdrawal from the market in 1998 due to its use being associated with increased morbidity and mortality in asthmatic patients in particular increase in myocardial infarction (Kurland et al., 1979, Rona et al., 1963), (Pearce and Hensley, 1998, Shukla et al., 2015) .

A number of studies identified that asthma patients with an increased risk of cardiovascular disease, pre-existing heart disease and patients with previous myocardial infarction or heart failure were susceptible to an increased risk of mortality when using βARs such as Isoproterenol to alleviate asthma symptoms (Krenek et al., 2009).

Inhalation therapies of β_2 adrenergic receptor (β_2AR) agonists are the preferred method of administration due to the abundance of β_2 adrenergic receptors available in the smooth

muscle of the trachea (30-40,000 per cell) as well as epithelial and endothelial cells of the lung (Larocca et al., 2011).

Two types of βAR-agonists are available; short acting beta adrenergic receptor agonists (SABA), available since the 1960's or long acting beta agonists (LABA), introduced in the 1990's (Beasley et al., 2009). Each of these categories of βAR-agonists alleviates the symptoms for a different duration of time by acting on β_2ARs located in the bronchioles (Wasilewski et al., 2014).

Acute asthma exacerbations are managed by the use of the short acting β_2 adrenergic receptor agonists (β_2ARs) such as Salbutamol (Ventolin™) that provides an almost instantaneous response that lasts between 1-3 hours (Chen et al., 2002, Giembycz and Newton, 2006). Long acting beta β_2ARs, Salmeterol and Formoterol, are used in long-term management of respiratory symptoms in patients with recurrent moderate to severe asthma or chronic obstructive pulmonary disease (Ball et al., 1991, Beasley et al., 2010, Guhan et al., 2000).

Non-selective βARs like Salbutamol and Isoproterenol are not solely restricted to affecting the respiratory system and are also known to have an affect on the cardiovascular system via activation of β_1AR within the myocardium, leading to an increase in chronotropy and inotropy (Barbato, 2009). Numerous studies have identified that 60% of βARs in the myocardium belong to the β_1 subtype and β_2ARs making up for the majority of the rest of the receptors with a small proportion of the recently identified β_3 and β_4ARs (Gonzalez-Munoz et al., 2011).

Isoproterenol is a non-selective βAR agonist that activates both β_1 and/or β_2 adrenergic receptors, which in turn can affect both the cardiovascular and the respiratory systems (Strauss et al., 1986, Yoo et al., 2009). Salbutamol was marketed as the first β_2AR selective agonist for the treatment of asthma as it has been shown to have a 29 times greater affinity for β_2ARs than β_1ARs (Chong et al., 2003). Furthermore, higher affinity targeted drugs such as Salmeterol and Formoterol and the SABA Salbutamol have also been introduced (Molenaar et al., 2007).

Studies investigating the cardiac safety profile of bronchodilators have recently identified an increased risk in morbidity and mortality with the use of these drugs in patients with underlying cardiovascular diseases such as myocardial ischaemia, myocardial infarction or heart failure (Iribarren et al., 2012, Schanen et al., 2005).

Coronary heart disease (CHD) affects approximately 2.3 million people within the UK and is responsible for over 74,000 deaths annually in addition to ischaemic heart disease (IHD) causing 12.9 million deaths in 2010 (Bellocchia et al., 2013, Lozano et al., 2012). More specifically Onufrak et al., (2009) suggest that adult patients with asthma have a 2-fold increase in the risk of developing CHD (Onufrak et al., 2008).

Progressive atherosclerosis of the coronary arteries leads to narrowing of the coronary arteries leading to myocardial ischaemia where there is an insufficient delivery of oxygen and nutrients to meet demand (Ansley and Wang, 2013). The duration of the ischaemia can be as short as a few minutes or prolonged for several hours to cause sufficient damage (Hearse, 1990). In ischaemic conditions, cardiac contractility is reduced, which in turn can lead to

decreased ventricular blood pressure and loss of diastolic motion of the heart (Mani, 2008, Shine, 1973, Javadov et al., 2014).

Physiological changes have also been observed during ischaemia to the myocardium including ATP depletion, catabolite build up, oxygen depletion and carbon dioxide accumulation (Raedschelders et al., 2012, Ansley and Wang, 2013, Machado et al., 2009). Myocardial ischaemia can be reversed pharmacologically with the use of thrombolytics or mechanically with the use of coronary artery stenting, or in severe cases via coronary artery bypass grafting (CABG) (Garzon et al., 2002, Hoffman et al., 2003).

Restoration of coronary blood flow to the ischaemic region is referred to as reperfusion (Yellon and Hausenloy, 2007). Reperfusion of the ischaemic myocardium is imperative to salvage reversibly damaged tissue but in it self can lead to cardiomyocyte death, a process termed as reperfusion injury (Hausenloy and Yellon, 2013). Reperfusion increases the production of ROS, calcium overload and free radicals, which impair myocardial function and induces cell death via apoptosis and necrosis and have been shown to involve premature opening of the mitochondrial permeability transition pore (mPTP) (Zorov et al., 2014). This process is known as ischaemia reperfusion (IR) injury (Bell and Yellon, 2011, Burniston et al., 2005, Raedschelders et al., 2012). The release of cytochrome c from within the mitochondria initiates a cascade leading to apoptosis, this phenomenon can been seen during reperfusion in contrast to ischaemia, which is thought to cause cell death by necrosis (Buja, 2005, Freude et al., 2000).

In light of recent evidence, the clinical use of β adrenergic receptor agonists has been strongly associated with an increased risk of ischaemic heart disease, heart failure and

myocardial infarction leading to premature death. There is an imperative need to assess the role of long and short acting β adrenergic receptor agonists in cardiac models of ischaemia reperfusion injury (Salpeter et al., 2004, Singh et al., 2008, Au et al., 2000).

3.2 Aims

The aims of the current study were to investigate the effects of long acting β adrenergic receptor agonists Salmeterol/Formoterol and short acting β adrenergic receptor agonists Isoproterenol/Salbutamol in both the isolated perfused Langendorff heart model of ischaemia reperfusion injury and the oxidative stress cardiac myocyte model to determine any detrimental effect they may have on the rat heart.

3.3 Methods

3.3.1 Langendorff protocol

Briefly, Sprague-Dawley rats were sacrificed by cervical dislocation and cannulated to the Langendorff setup and perfused with KHB as described in section 2.3. Hearts were allowed to stabilise for 20 minutes followed by 35 minutes of regional ischaemia and 120 minutes of reperfusion. One minute before the onset of reperfusion hearts were randomly assigned to one of the following groups of drug treatment administered throughout reperfusion: a) Control (KHB) b) Isoproterenol (0.1μM, 0.5μM), c) Salmeterol (0.1μM), d) Formoterol (0.1μM, 0.5μM), e) Salbutamol (0.001μM-1μM), (Fig 1.1). Haemodynamic data were collected throughout the study. At the end of the experiment hearts underwent infarct to risk ratio analysis. Control data collected for infarct to risk ratio was used for all Langendorff experiments throughout this chapter.

3.3.2 Adult rat cardiac myocyte isolation

Briefly, male Sprague Dawley rats were sacrificed by cervical dislocation and the hearts excised and cannulated onto modified Langendorff apparatus and perfused with a constant flow rate of 14ml/min as described in section 2.4. Hearts were perfused for 3-4 minutes with calcium free modified Krebs Heinsleit Buffer. The buffer was then switched and the hearts perfused with digestion buffer for 7 minutes. Isolated ventricular myocytes were used for the oxidative stress model (section 2.6) using confocal microscopy. Myocytes were treated with one of the following drugs: a) Control (KHB) b) Isoproterenol (0.1μM, 0.5μM), c) Salmeterol (0.1μM), d) Formoterol (0.5μM), e) Salbutamol (0.1μM) f) positive control FCCP (1 μM).

3.3.3 Statistical analysis

All haemodynamic data are presented as a mean of the stabilisation period ± SEM. Haemodynamic data was statistically analysed using a two-way analysis of variance ANOVA. Where statistical significance was found between groups a One-Way ANOVA with a Fishers Least Significance Test post hoc was used to determine significance at various time points. A one-way ANOVA with a Fishers Least Significance Test post hoc test was used to determine significance between infarct to risk ratio %, time to depolarisation and hypercontracture. A significance level of $p<0.05$ was considered to be statistically significant.

3.4 Results

3.4.1 The role of Isoproterenol in myocardial ischaemia reperfusion injury.

3.4.1.1 Haemodynamics

Throughout the Langendorff study, left ventricular developed pressure, heart rate and coronary flow were monitored. Hearts were subjected to 35 minutes of ischaemia and 120 minutes of reperfusion with Isoproterenol (0.1µM or 0.5µM) administered throughout the reperfusion period.

A decrease in LVDP was observed after 5minutes of regional ischaemia in non-treated IR control hearts and Isoproterenol (0.1µM or 0.5µM) groups. Overall, there was no significant difference between the groups at any of the time points ($p>0.05$, Figure 3.1).

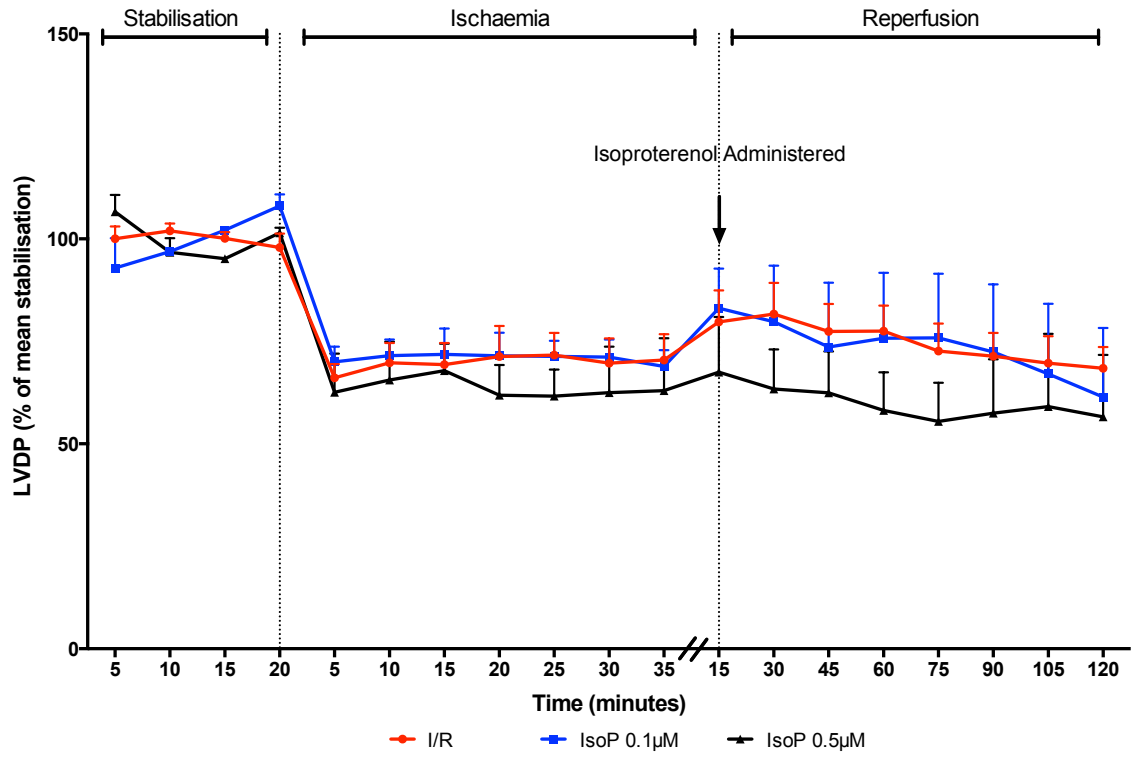


Figure 3.1 The effects of Isoproterenol (IsoP) (0.1µM or 0.5µM) on left ventricular developed pressure in isolated rat hearts subjected to 35 minutes ischaemia and 120 minutes reperfusion. Isoproterenol (0.1µM or 0.5µM) was administered throughout reperfusion. Data presented as mean ± SEM. n=6-8.

Heart rate for IR control values showed minimal fluctuation throughout the protocol with no statistical significance between either of the Isoproterenol groups (0.1µM or 0.5µM) at any time points when compared to time matched controls (Figure 3.2).

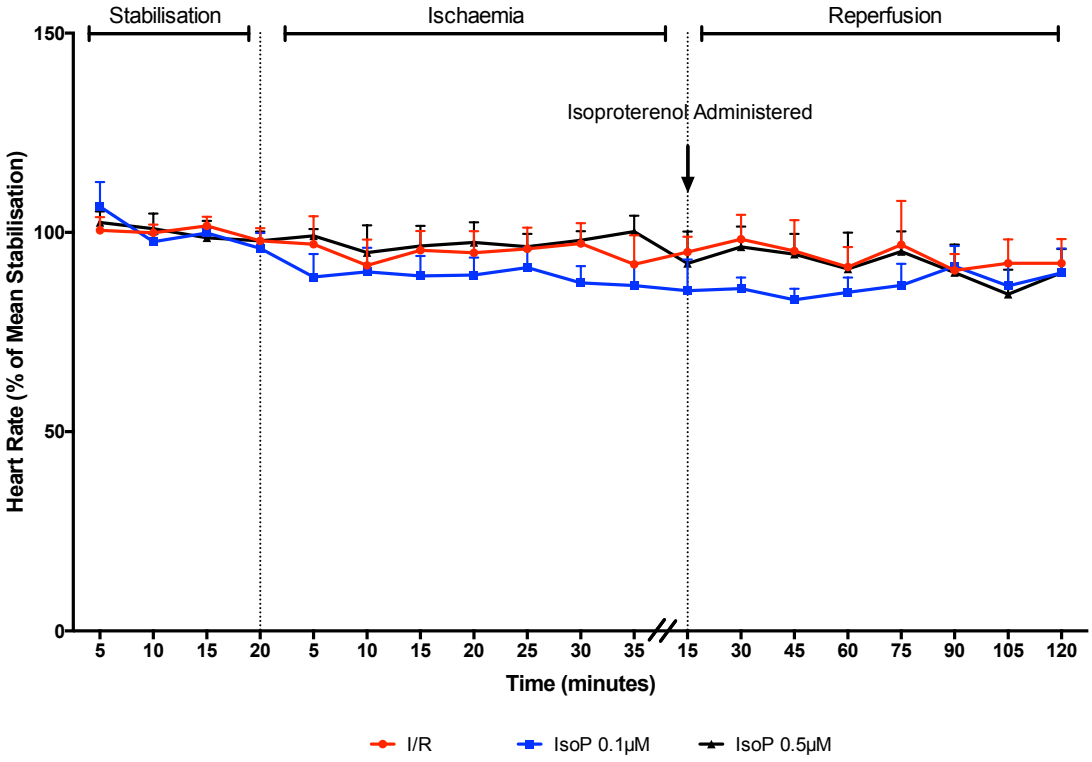


Figure 3.2 The effects of Isoproterenol (IsoP) at 0.1µM or 0.5µM on heart rate in isolated rat hearts subjected to 35 minutes ischaemia and 120 minutes reperfusion. Isoproterenol was administered at the onset and throughout reperfusion. Data presented as mean ±SEM. n=6-8

Occlusion of the left coronary artery significantly reduced coronary flow after 5 minutes post occlusion in all groups. Administration of Isoproterenol (0.1µM or 0.5µM) throughout reperfusion had no significant effect on coronary flow compared to time matched controls ($p>0.05$, Figure 3.3). Interestingly, Isoproterenol (0.1µM or 0.5µM) treated hearts did decrease coronary flow when compared to time matched control hearts.

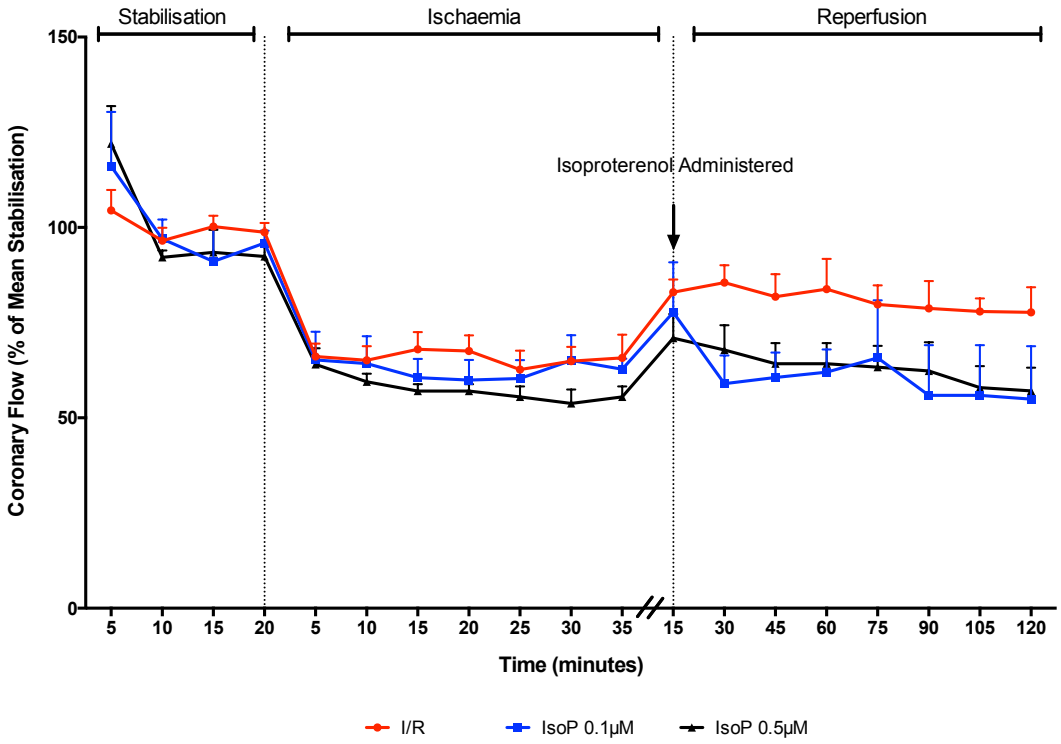


Figure 3.3 The effects of Isoproterenol (IsoP) (0.1µM or 0.5µM) on coronory flow in isolated rat hearts subjected to 35 minutes ischaemia and 120 minutes reperfusion. Isoproterenol (0.1µM or 0.5µM) was administered at the onset and throughout reperfusion. Data presented as mean ±SEM. n=6-8

3.4.1.2 The effect of Isoproterenol on Infarct Size to Risk Ratio in isolated hearts subjected to ischaemia reperfusion injury.

Hearts were subjected to 35 minutes of ischaemia and 120 minutes of reperfusion in the presence and absence of Isoproterenol (0.1µM or 0.5µM) throughout reperfusion followed by TTC staining to determine infarct size to risk ratio (%).

Administration of Isoproterenol (0.5µM) throughout reperfusion significantly increased I/R (%) compared to IR control (62 ± 4% vs. 51 ± 2%, $p<0.001$,). Isoproterenol (0.1µM) when

administered at reperfusion had no significant effect on I/R (%) when compared with IR control (45% ± 3% vs. 51% ± 2%, p>0.05, Figure 3.4).

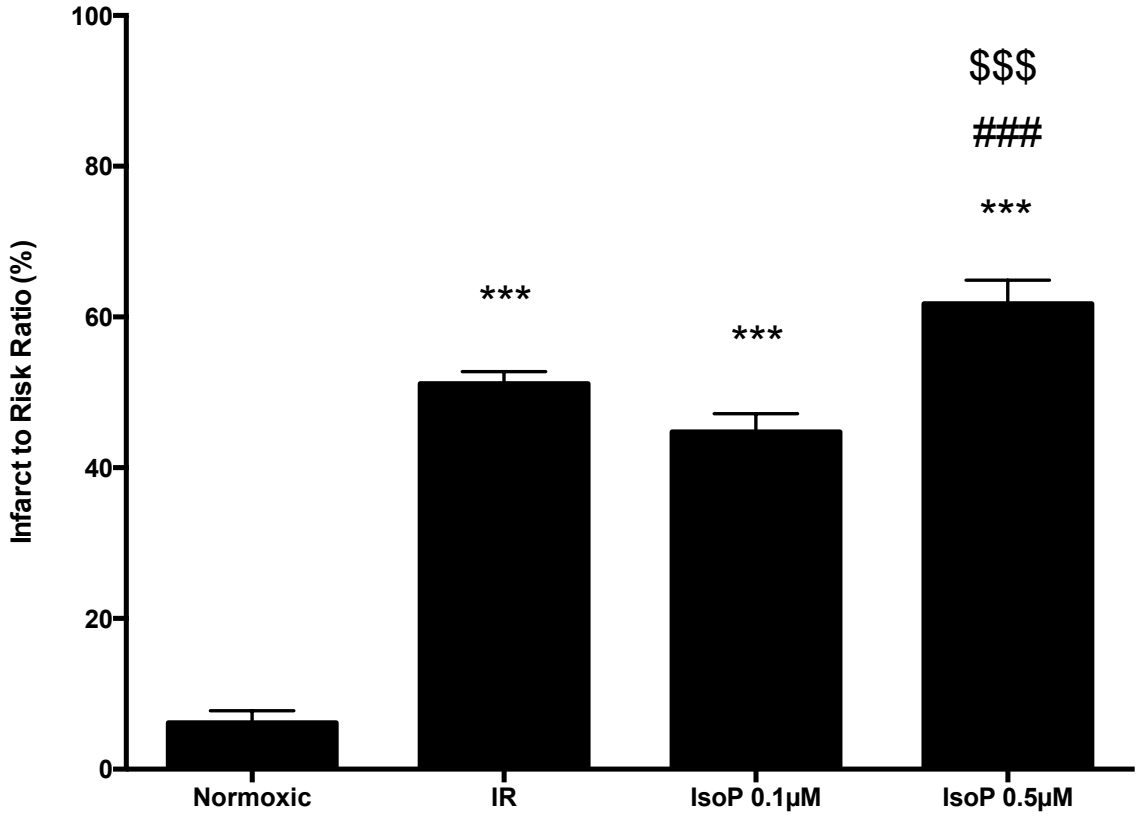


Figure 3.4 Infarct to risk ratio (%) in isolated prefused hearts subjected to 35 minutes of ischaemia and 120 minutes reperfusion in the presence and absences of Isoproterenol (0.1μM or 0.5μM) throughout the reperfusion period. Data presented as ±SEM. n=6-8. ***p<0.001 vs. normoxic, $^{###}$p<0.001 vs. IR, $^{\$\$\$}$p<0.001 vs. IsoP 0.1μM.

3.4.1.3 Effect of Isoproterenol on isolated cardiomyocytes in an Oxidative Stress Model

Continuous oxidative stress to cardiomyocytes via laser stimulation of TMRM loaded cardiomyocytes causes the mitochondrial permeability transition (mPTP) to open (Falchi et al., 2005). Opening of the mPTP pore allows the contents of the mitochondria including the TMRM to leak into the cytosol of the cardiomyocyte causing an increase in fluorescence that can be detected and measured as the point of depolarisation.

Carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP 0.1μM) was used as a positive control for its mitochondrial membrane potential uncoupling properties (Saotome et al., 2005). Administration of FCCP (1μM) significantly decreased the time to depolarisation when compared to control (51 ± 7s vs. 234 ± 18s, $p<0.001$, Figure 3.5). Administration of FCCP (1μM) also significantly reduced the time to hypercontracture compared to the non-treated control group (69 ± 7s vs. 663 ± 40s $p<0.001$, Figure 3.6).

Cardiomyocytes subjected to laser stimulation in the presence of Isoproterenol (0.5μM) significantly decreased the time to depolarisation compared to the non-treated control group (179 ± 18s vs. 234 ± 18s control, $p<0.001$, Figure 3.5) and hypercontracture (442 ± 16s vs. 663 ± 40s control, $p<0.001$, Figure 3.6).

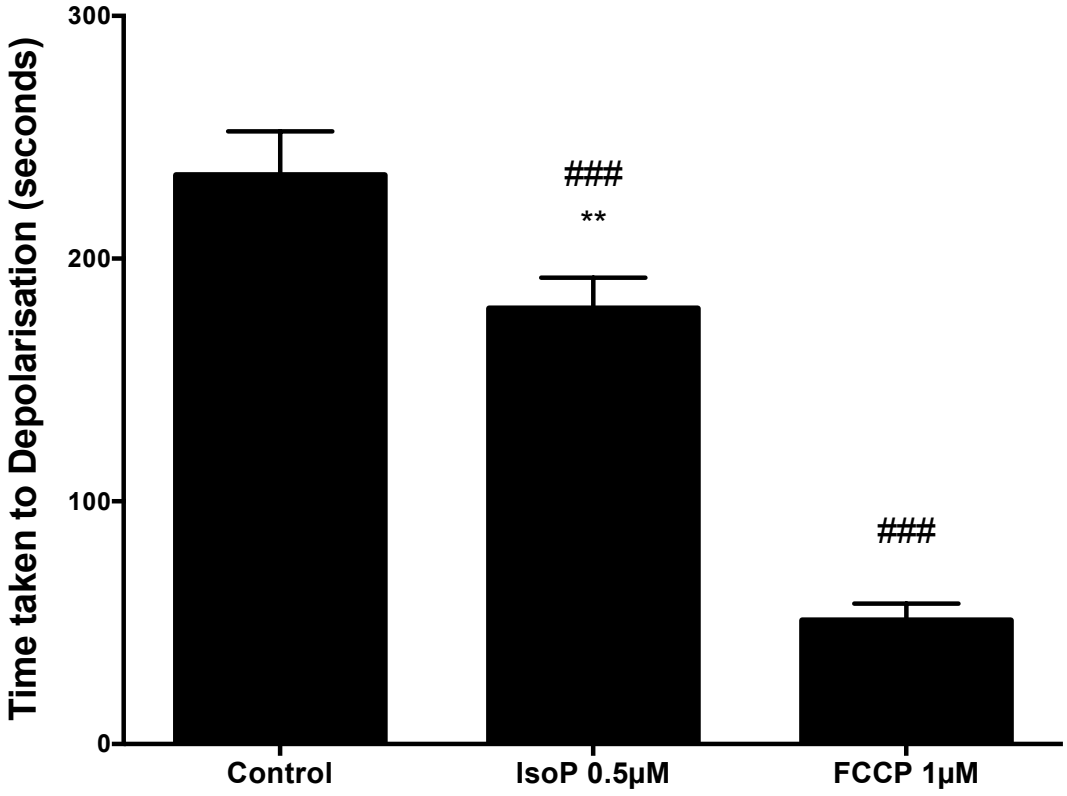


Figure 3.5 The effects of Isoproterenol (0.5µM) or FCCP on time taken to depolarisation in isolated rat cardiomyocytes in a model of oxidative stress. Data presented as mean ±SEM n=6-8. **p<0.01 vs. control, $^{###}$p<0.001 vs. Control.

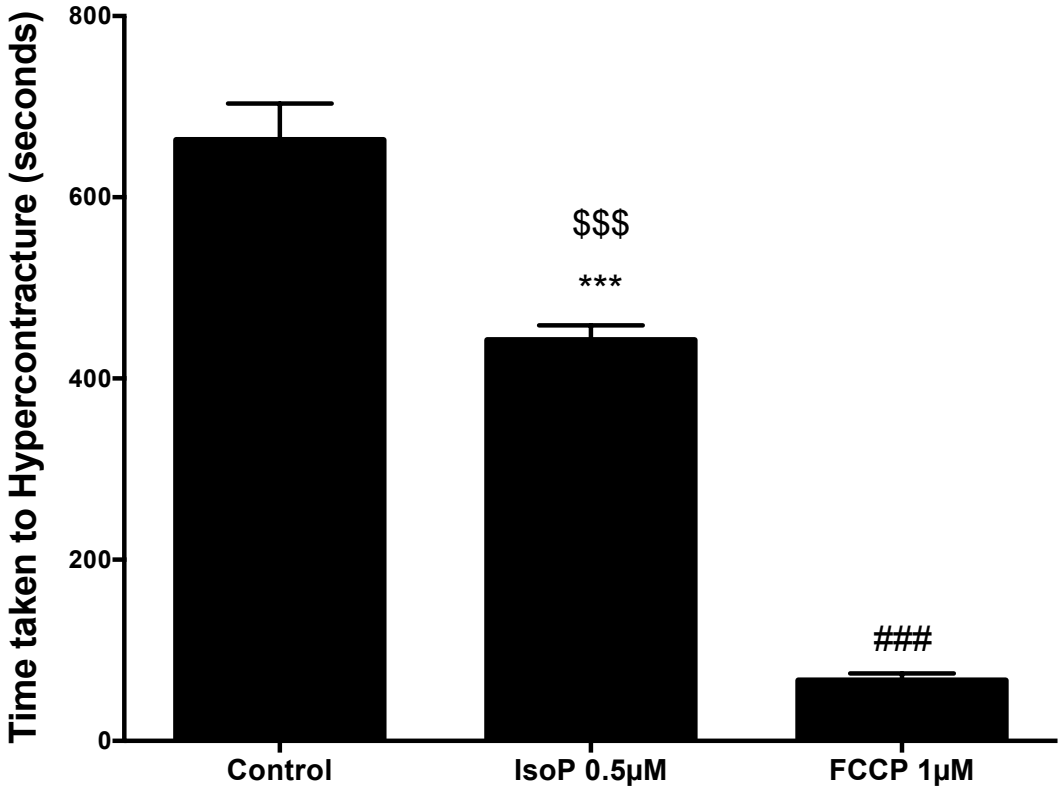


Figure 3.6 The effects of Isoproterenol (0.5µM) or FCCP on time taken to hypercontracture in isolated rat cardiomyocytes in a model of oxidative stress. Data presented as Mean ±SEM n=6-8. ***p<0.001 vs. control, $^{###}$p<0.001 vs. Control.

3.4.2 The role of Salmeterol & Formoterol in myocardial ischaemia reperfusion injury.

3.4.2.1 Haemodynamics

Isolated hearts were subjected to 35 minutes of ischaemia and 120 minutes of reperfusion in the presence and absence of the long acting beta agonists Salmeterol (0.1μM) or Formoterol (0.1μM or 0.5μM) throughout reperfusion. LVDP significantly improved with Formoterol (0.5μM) when compared to time matched control IR hearts 15 minutes post reperfusion (104 ± 17% vs. 79 ± 7%, $p<0.05$, Formoterol 0.1μM, 107 ± 7% vs. 79 ± 7%, $p<0.05$ at 15 minutes reperfusion, Formoterol 0.5μM, Figure 3.7). An elevated LVDP continued throughout reperfusion for both concentrations of Formoterol, with Formoterol (0.5μM) maintaining an elevated LVDP when compared to IR time matched control (100 ± 7% vs. 68 ± 5%, $p<0.01$ at 120 minutes reperfusion, Figure 3.7).

In contrast to Formoterol (0.1μ or 0.5μM), Salmeterol (0.1μM) showed no significant change in LVDP when compared to IR time matched control hearts throughout reperfusion (81.9 ± 7% vs. 79 ± 7%, $p>0.05$ at 15 minutes reperfusion, Figure 3.7).

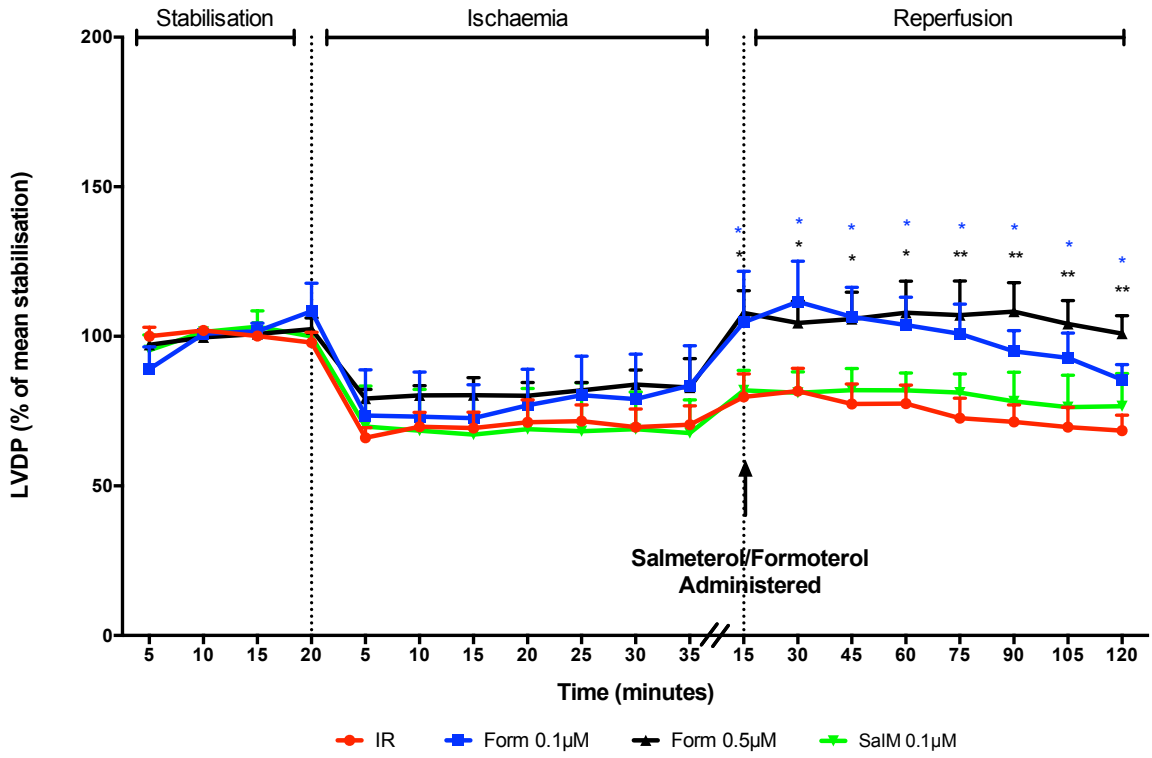


Figure 3.7 The effect of LABA Formoterol (0.1µM or 0.5µM) or Salmeterol (0.1µM) on left ventricular developed pressure in isolated rat hearts subjected to 35 minutes ischaemia and 120 minutes reperfusion. Formoterol (0.1µM or 0.5µM) or Salmeterol (0.1µM) were administered at the onset and throughout reperfusion. Data presented as mean ±SEM. n=6-8 *p<0.05 vs I/R, **p<0.01 vs I/R.

Administration of Salmeterol (0.1µM) or Formoterol (0.1µM or 0.5µM) throughout reperfusion significantly increased heart rate when compared to IR time matched control hearts as shown in Figure 3.8 (156 ± 13% vs. 68 ± 5%, p<0.001, Salmeterol 0.1µM) (142 ± 24% vs. 68 ± 5%, p<0.05, Formoterol 0.1µM) (146 ± 11% vs. 68 ± 5%, p<0.001, Formoterol 0.5µM at 120 minutes reperfusion respectively, Figure 3.8).

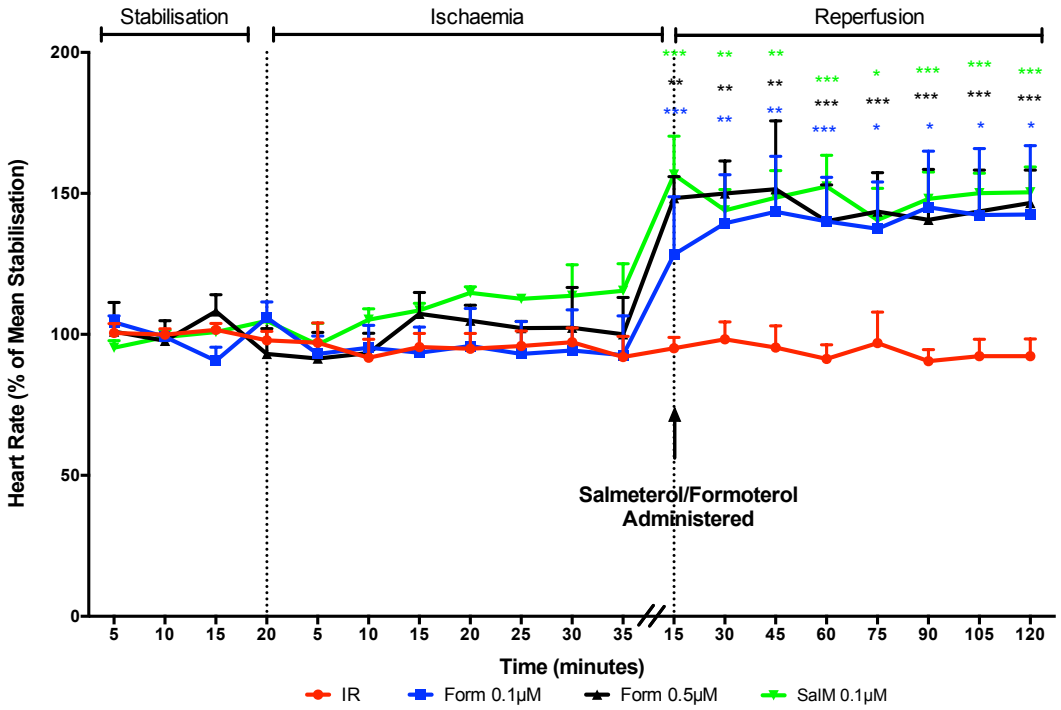


Figure 3.8 The effect of Formoterol (0.1µM or 0.5µM) or Salmeterol (0.1µM) on heart rate in isolated rat hearts subjected to 35 minutes ischaemia and 120 minutes reperfusion. Formoterol (0.1µM or 0.5µM) or Salmeterol (0.1µM) was administered at the onset and throughout reperfusion. Data presented as mean ±SEM. n=6-8. *p<0.05 vs. time matched heart controlp<0.01 vs. time matched heart rate control, ***p<0.001 vs. time matched heart rate control.**

Administration of Formoterol (0.1µM or 0.5µM) at reperfusion significantly increased coronary flow throughout the reperfusion when compared to IR control (115 ± 14% vs. 77 ± 6%, p<0.01, Formoterol (0.1µM) at 120 minutes reperfusion) (112±17% vs. 77 ± 6%, p<0.05, Formoterol (0.5µM) respectively at 120minutes reperfusion, Figure 3.9).

Administration of Salmeterol (0.1µM) showed no statistical change in coronary flow when compared to time matched control IR heart (p>0.05, Figure 3.9).

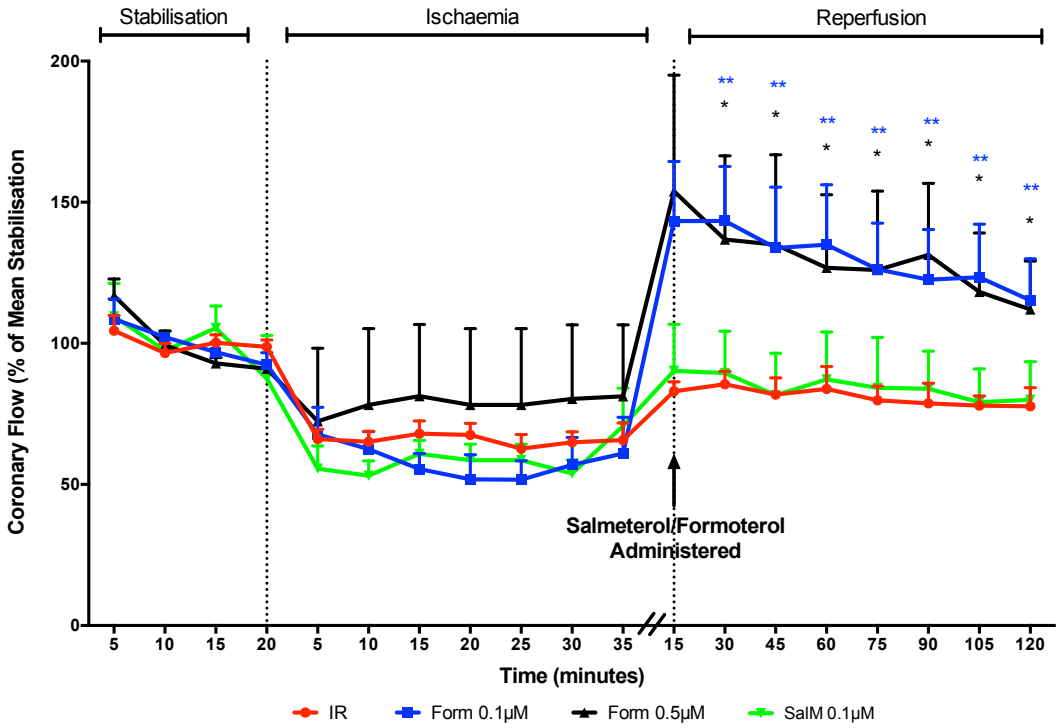


Figure 3.9 The effect of Formoterol (0.1µM or 0.5µM) or Salmeterol (0.1µM) on coronary flow in isolated rat hearts subjected to 35 minutes ischaemia and 120 minutes reperfusion. Formoterol (0.1µM or 0.5µM) or Salmeterol (0.1µM) was administered at the onset and throughout reperfusion. Data presented as mean ±SEM. n=6-8. *p<0.05 vs. time matched controlp<0.01 vs. time matched control.**

3.4.2.2 The effect of Salmeterol or Formoterol on Infarct to Risk Ratio

Hearts were subjected to 35 minutes of ischaemia and 120 minutes of reperfusion. In the presence of Salmeterol (0.1µM or 1µM) or Formoterol (0.1µM or 0.5µM) no significant changes in infarct to risk ratio was observed ($p>0.05$, Figure 3.10).

A significant increase in I/R was recorded in IR hearts when compared to normoxic hearts ($51 \pm 2\%$ vs. $6 \pm 1\%$, $p<0.001$, Figure 3.10).

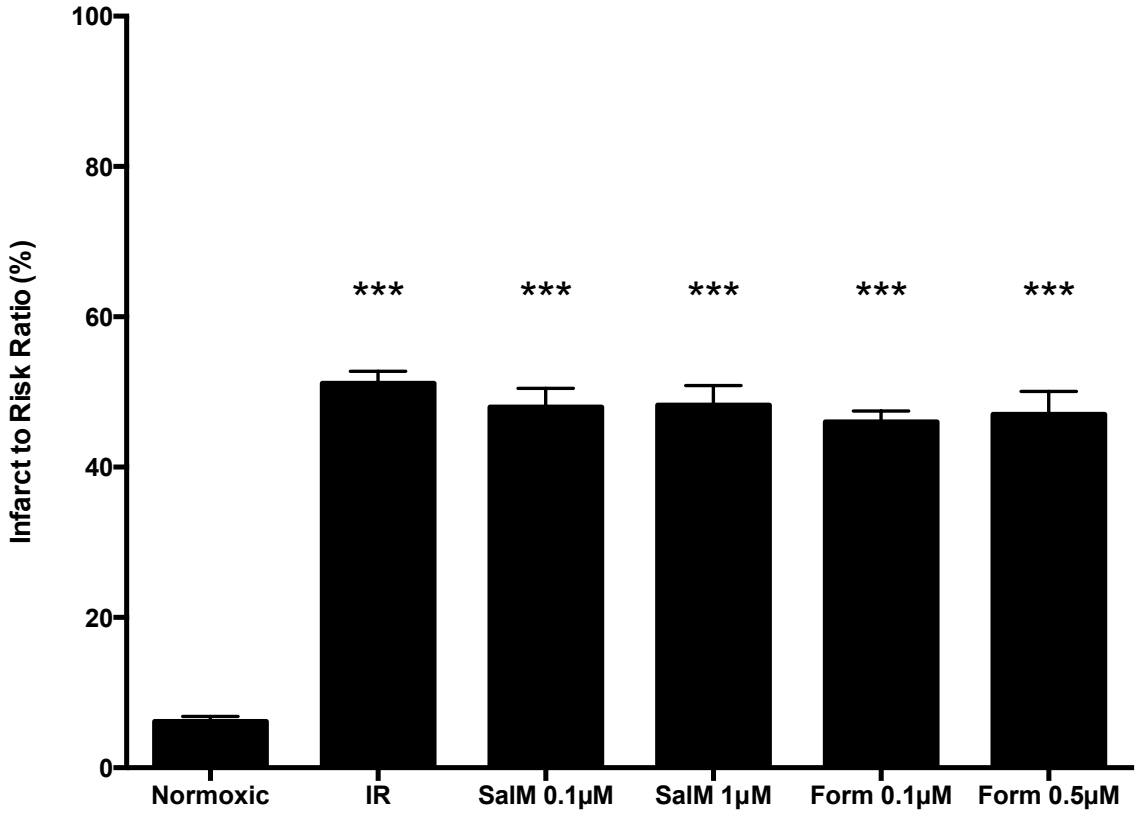


Figure 3.10 Infarct to risk ratio (%) in isolated prefused hearts subjected to 35 minutes of ischaemia and 120 minutes reperfusion in the presence and absences of Salmeterol (0.1µM or 0.5µM) & Formoterol (0.1µM or 0.5µM) throughout the reperfusion period. Data presented as ±SEM. n=6-8. ***p<0.001 vs. normoxic.

3.4.2.3 The effect of Formoterol and Salmeterol on isolated cardiomyocytes in an Oxidative Stress Model

Isolated cardiomyocytes were pre-treated with TMRM and subjected to laser stimulation leading to ROS generation resulting in mitochondrial depolarisation and hypercontracture. Pre-treatment with Salmeterol (0.1µM) had no significant effect on the time to depolarisation (228 ± 5s vs. 255 ± 15s, p>0.05, Figure 3.11).

An effect on time to depolarisation was also shown to be unremarkable in the presence of Formoterol (0.5µM) when compared to non-treated groups (208 ± 10s vs. 255 ± 18s respectively, Figure 3.11). Normoxic and control IR data included has been used from

previous experiment. Normoxic and control IR data included has been used from previous experiment.

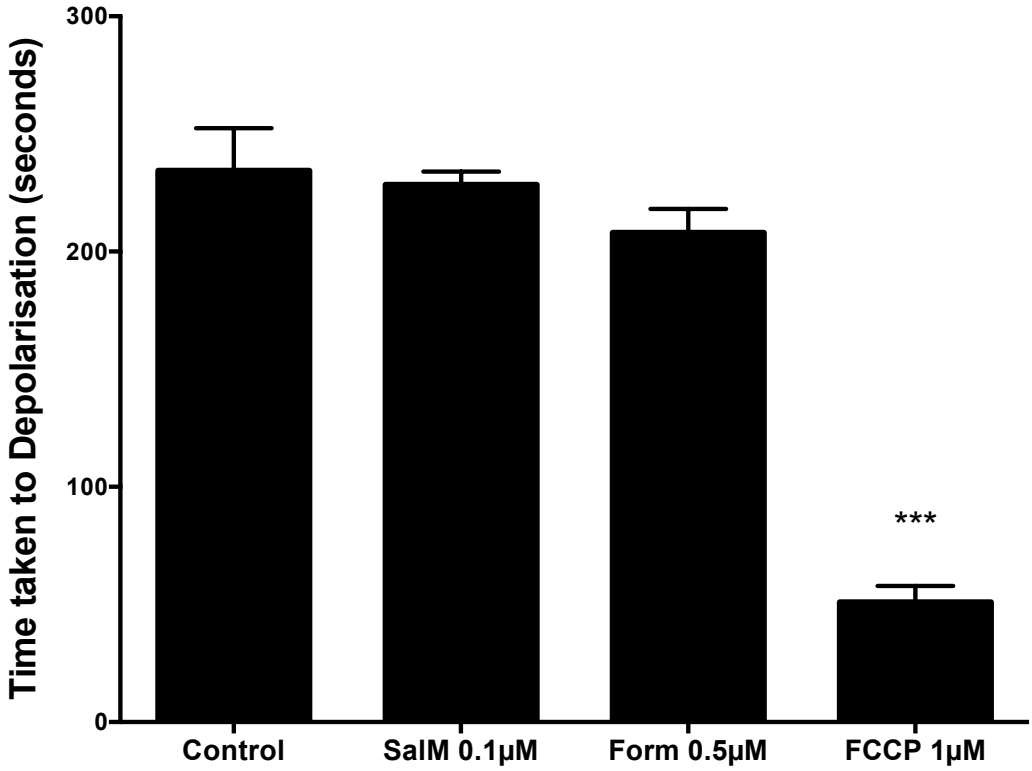


Figure 3.11 The effects of Salmeterol (0.1µM), Formoterol (0.5µM) and FCCP (0.1µM) on time taken to hypercontracture in isolated rat cardiomyocytes in a model of oxidative stress. Data presented as mean ±SEM. n=8. *** p<0.001 vs. control, vs. SalM 0.1µM, vs. Form 0.5µM.

Pre-incubation of cardiomyocytes with Salmeterol (0.1µM) significantly decreased time to hypercontracture compared to non-treated control (544 ± 12 vs. 663 ± 40s, $p<0.001$, Figure 3.12). Administration of Formoterol (0.5µM) also significantly decreased time taken to the onset of hypercontracture when compared to non-treated control (499 ± 20s vs. 663 ±40s, $p<0.001$, Figure 3.12).

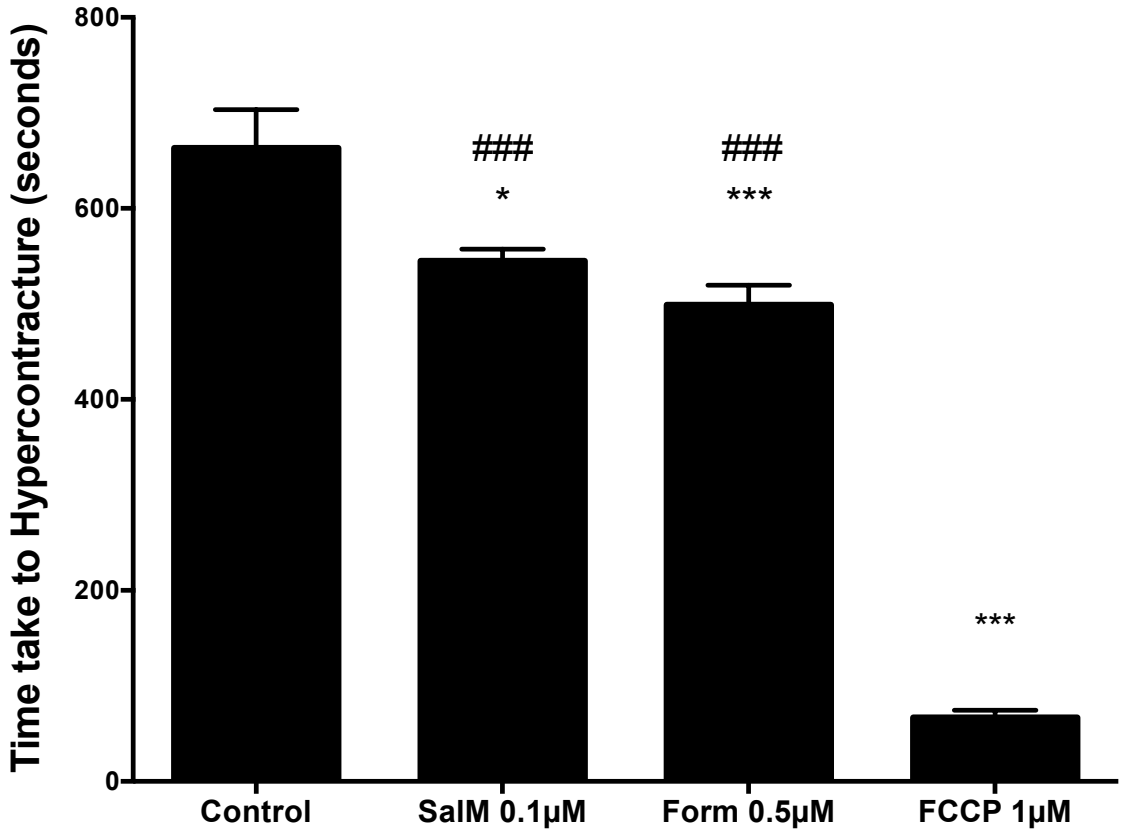


Figure 3.12 The effects of Salmeterol (0.1µM), Formoterol (0.5µM) and FCCP (0.1µM) on time taken to hypercontracture in isolated rat cardiomyocytes in a model of oxidative stress. Data presented as mean ± SEM. n=8. ***p<0.001 vs. control, *p<0.05 vs. control, $^{###}$p<0.001 vs. FCCP.

3.4.3 The role of Salbutamol on myocardial ischaemia reperfusion injury.

3.4.3.1 Haemodynamics

Hearts were subjected to 35 minutes ischaemia and 120 minutes of reperfusion where Salbutamol was administered throughout the reperfusion period. Administration of Salbutamol (0.001µM – 1µM) was shown to have no effect on LVDP at all time points post reperfusion when compared to IR control hearts (p>0.05, **Figure 3.13**).

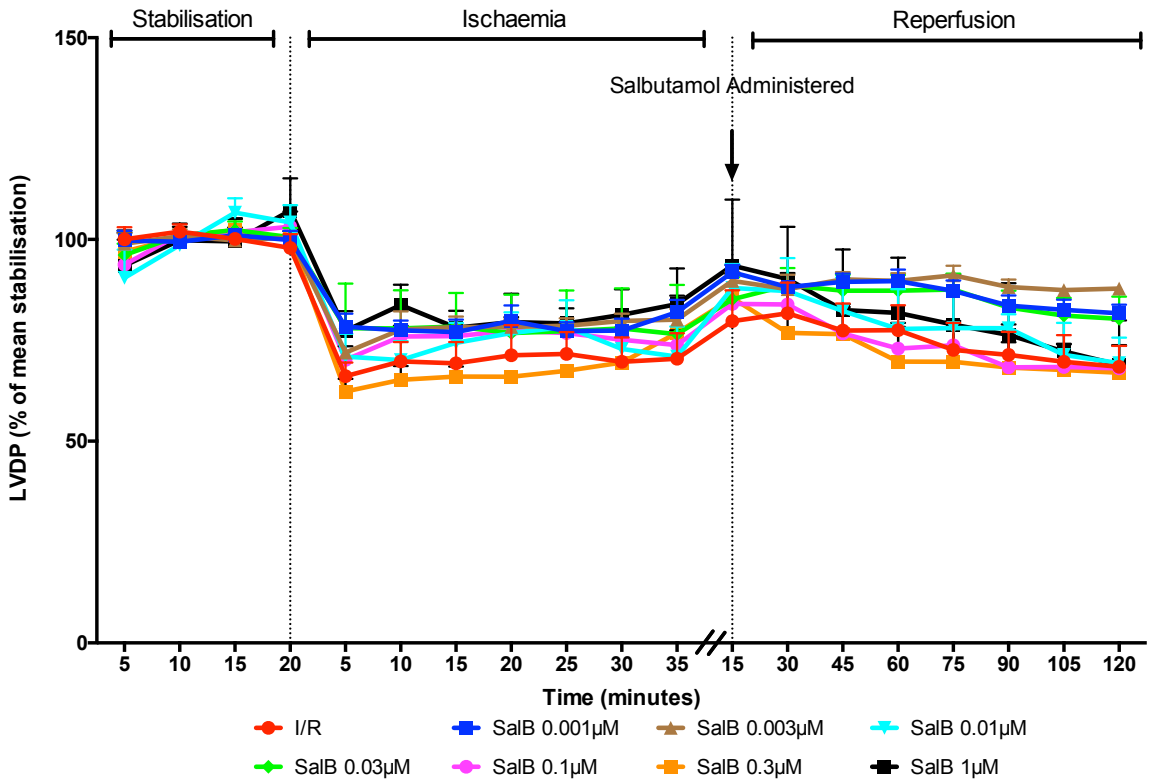


Figure 3.13 The effect of Salbutamol (0.001-1μM) on left ventricular developed pressure in isolated rat hearts subjected to 35 minutes ischaemia and 120 minutes reperfusion. Salbutamol was administered at the onset and throughout reperfusion. Data presented as mean ±SEM. n=6-8.

Administration of Salbutamol (0.3μM and 1μM) throughout reperfusion significantly increased heart rate when compared to IR time matched controls (109±3% vs. 92 ± 6%, $p<0.05$, 0.3μM Salbutamol at 120 minutes reperfusion) (132 ± 7% vs. 92 ± 6%, $p<0.001$, 1μM Salbutamol at 120 minutes reperfusion). Interestingly, there was no statistical significance between 1μM or 0.3μM concentrations of Salbutamol, however at the onset of reperfusion, the highest Salbutamol concentration (1μM) recorded a 30% higher heart rate than that recorded by Salbutamol 0.3μM (132 ± 12% vs. 95 ± 3%, 1μM, 107±7 vs. 95±3%, 0.3μM at 15 minutes reperfusion, $p>0.05$ Figure 3.14).

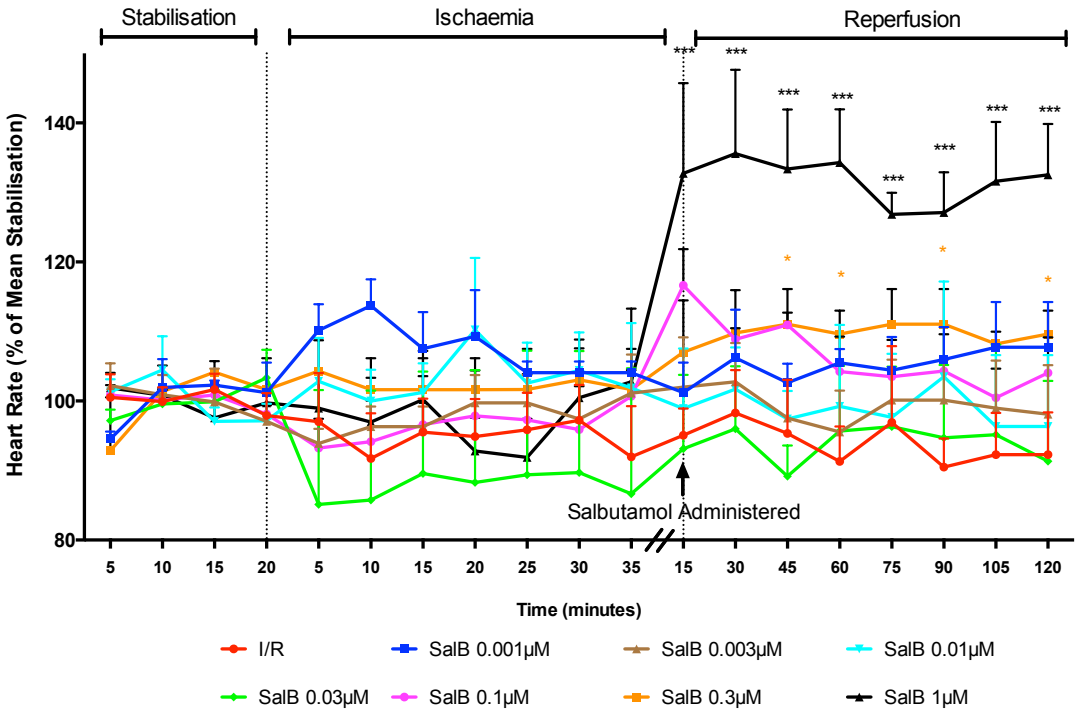


Figure 3.14 The effect of Salbutamol (0.001-1μM) on heart rate in isolated rat hearts subjected to 35 minutes ischaemia and 120 minutes reperfusion. Salbutamol was administered at the onset and throughout reperfusion. Mean ±SEM. n=6-8. *p<0.05 vs. IR control, ***p<0.001 vs. IR control.

Salbutamol (0.001μM- 0.1μM) had no significant effect on coronary flow when compared with IR control hearts (p>0.05, Figure 3.15), interestingly, a noticeable decline in coronary flow was observed with the highest concentration of Salbutamol (1μM) in comparison to all other concentrations and IR control values at 120 min of reperfusion (67 ± 4% vs. 77 ± 4% at 120 minutes reperfusion, Figure 3.15).

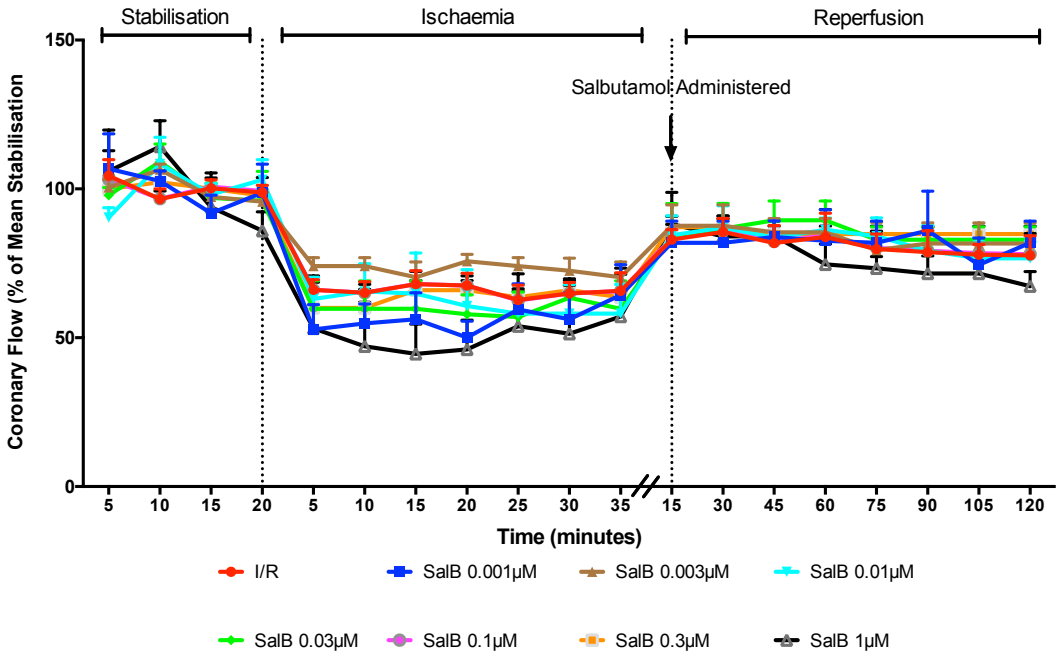


Figure 3.15 The effect of Salbutamol (0.001-1µM) on coronary flow inisolated rat hearts subjected to 35 minutes ischaemia and 120 minutes reperfusion. Salbutamol (0.001-1µM) was administered at the onset and throughout reperfusion. Data presented as mean ±SEM. n=6-8

3.4.3.2 The effect of Salbutamol (0.001-1µM) on Infarct to Risk Ratio in isolated hearts subjected to ischaemia reperfusion injury

Salbutamol (0.001µM, 0.003µM, 0.01µM) showed no statistical change in I/R when compared to IR control hearts (Figure 3.16). In contrast Salbutamol at higher concentrations (0.03µM, 0.1µM, 0.3µM and 1µM) significantly increased I/R ratio when compared to IR control hearts (62 ± 3% vs. 51 ± 2%, $p<0.01$, 0.03µM) (76 ± 4 % vs. 51 ± 2%, $p<0.001$, 0.1µM) (77 ± 2% vs. 51 ± 2%, $p<0.001$, 0.3µM) (78 ± 1% 51 ± 2%, $p<0.001$, 1µM, Figure 3.16).

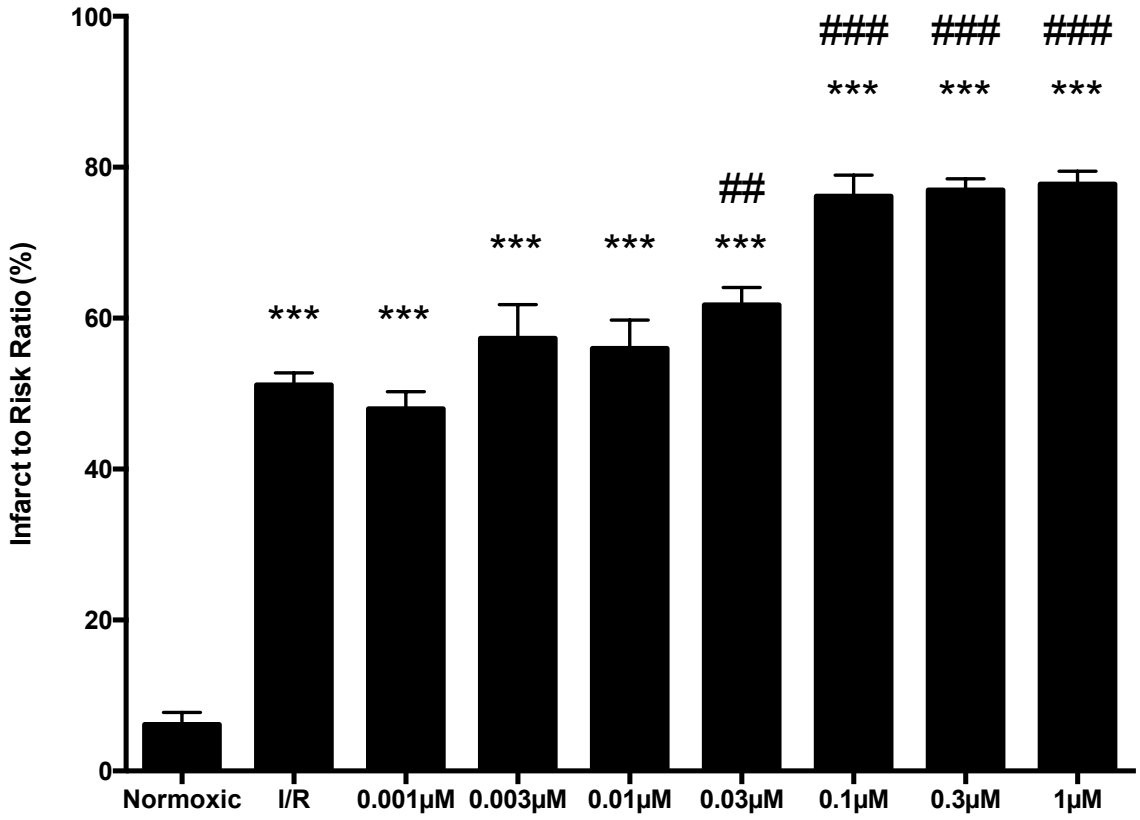


Figure 3.16 Infarct to risk ratio (%) in isolated prefused hearts subjected to 35 minutes of ischaemia and 120 minutes reperfusion in the presence and absences of Salbutamol (0.001µM- 1µM) throughout the reperfusion period. Data presented as ±SEM. n=6-8. ***p<0.001 vs. normoxic, $^{\#\#}$p<0.01 vs. IR control, $^{\#\#\#}$p<0.001 vs. IR control.

3.4.3.3 The effect of Salbutamol on isolated cardiomyocytes in an Oxidative Stress Model

Isolated cardiomyocytes were pre-treated with TMRM and subjected to laser stimulation leading to ROS generation, mitochondrial depolarisation and hypercontracture. Pre-treatment with Salbutamol (0.1µM) had no significant effect on the time to depolarisation (226 ± 14s vs 255 ± 13s, p>0.05, Figure 3.17). Normoxic and control IR data included has been used from previous experiment.

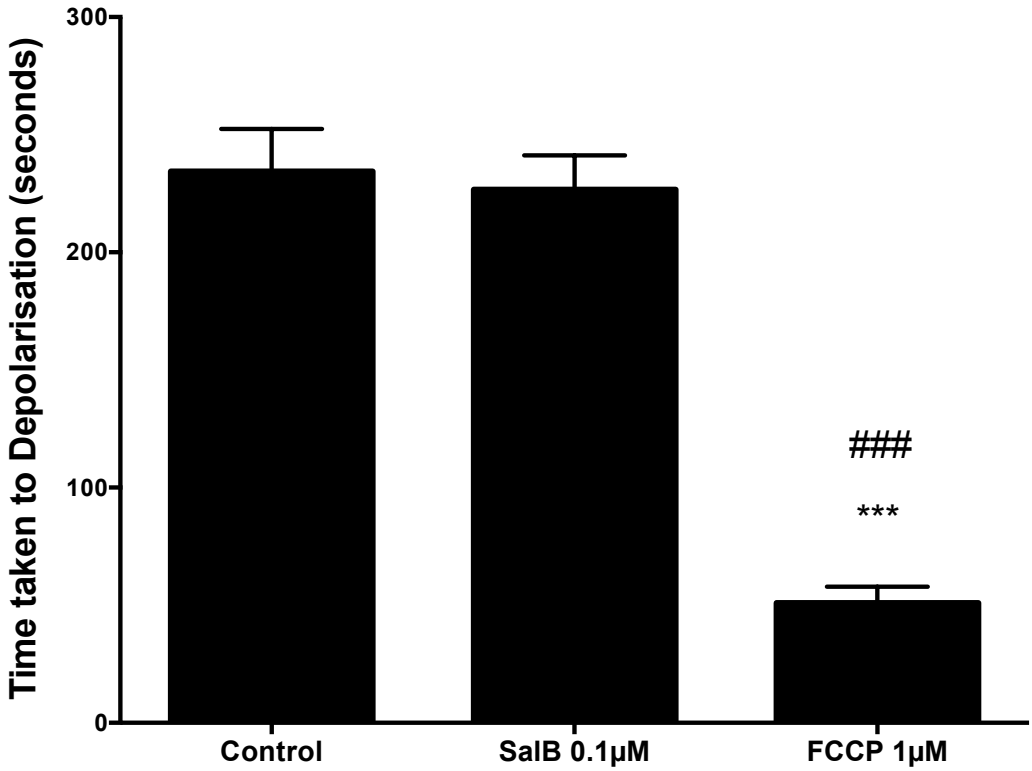


Figure 3.17 The effects of Salbutamol (0.1µM) and FCCP on time taken to depolarisation in isolated rat cardiomyocytes in a model of oxidative stress. Data presented as mean ±SEM. n=6-8. ***p<0.001 vs. Control), $^{###}$p<0.001 vs. Control.

Salbutamol (0.1µM) significantly decreased the time to hypercontracture in isolated cardiomyocytes when compared to non-treated control groups (524 ± 23s vs. 663 ± 40s, p<0.001, Figure 3.18) As a positive control FCCP caused a significant decrease in time to hypercontracture compared with non-treated control cardiomyocytes (67 ± 7s vs. 663 ± 40s, p<0.001, Figure 3.18).

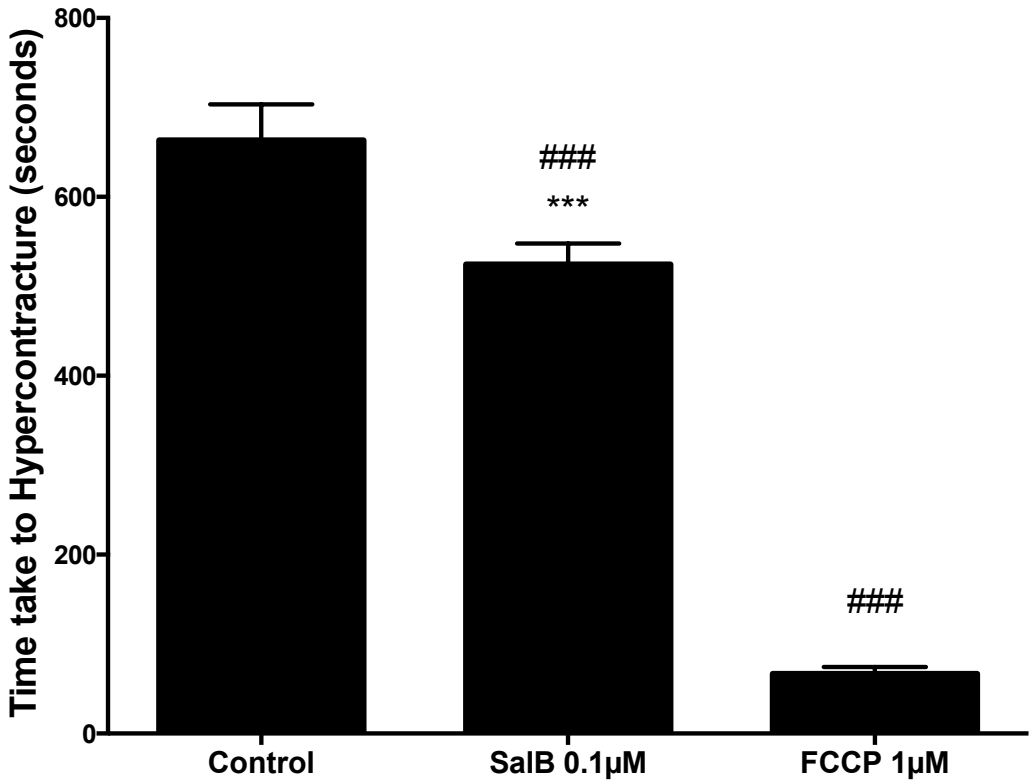


Figure 3.18 The effects of Salbutamol (0.1µM) FCCP (1µM) on time taken to hypercontracture in isolated rat cardiomyocytes in a model of oxidative stress. n=6-8. ***p<0.001 vs. control, $^{###}$p<0.001 vs FCCP. Data presented as mean ± SEM. n=6-8.

3.5 Discussion

The use of β adrenergic receptor agonists has recently been an area of controversy with an increase in morbidity and mortality reported in asthma patients, in particular patients with underlying cardiovascular diseases (Machado et al., 2009). Singh and colleagues (2008) were one of the earliest groups to identify an increase in MI and mortalities with a meta analysis of randomised controlled tests involving patients with underlying heart conditions and severe cases of COPD and asthma (Singh et al., 2008). Conclusions drawn from the meta-analysis were that bronchodilators used for long periods of time (30 days) did increase events of MI, stroke and death (Ortega and Peters, 2010, Wijesinghe et al., 2009, Cates et al., 2013).

The aim of the current chapter was to examine the role of short and long acting β adrenergic receptor agonists in a myocardial model of ischaemia reperfusion injury. This study identifies the chronotropic effects caused by β agonists Salmeterol, Formoterol and Salbutamol at the onset of reperfusion. Further to this, significant increases in infarct to risk ratio with Salbutamol and Isoproterenol were also recorded and a potential link to an effect of βARs on the mPTP pore in an oxidative stress model.

The administration of Isoproterenol was shown by first shown by Bloom and Davies (1972) induce MI and initiate a Ca^{2+} overload in rat hearts resulting in ATP depletion (Krenek et al., 2009, Senthil et al., 2007). Isoproterenol as a partial βAR agonist is non selective to β_1ARs or β_2ARs (Kapel'ko et al., 2014). Previous studies have shown Isoproterenol administration to cause myocardial ischaemia in normoxic hearts when administered intravenously into rats (Wexler and Greenberg, 1978). Administering Isoproterenol (0.5μM) at the onset of reperfusion, a significant increase in infarct to risk ratio was observed (Figure 3.4). With

evidence from previous studies demonstrating the effects of Isoproterenol causing MI and ischaemic damage in normoxic conditions, we can further develop these findings with Isoproterenol exacerbating myocardial injury when administered at reperfusion (Palfi et al., 2005). Communal and colleagues showed that blockade of βARs in rat cardiomyocytes with Propranolol, a non-selective beta adrenergic receptor blocker, or blockade of PKA and voltage dependent calcium channels, abolished the adverse effects of Isoproterenol (Communal et al., 1998). With evidence of abolishing the initiation of Isoproterenol induced apoptosis via βAR blockade, we can purport in our results with Isoproterenol in the reperfusion injury model, exacerbation of myocardial injury is a result of Isoproterenol induced apoptosis via βAR activation.

The activation of β_1ARs in particular has been shown to be pro-apoptotic in mouse and rat heart models further reinforced with specific blocking of the G_i subunit of the βARs with pertussis toxin (Tong et al., 2005). A higher ratio of β_1ARs:β_2AR within the heart allows the high affinity of Isoproterenol to bind more readily to the available β_1ARs. Interestingly, reports of chronic activation of βARs has been linked to pro-apoptotic tendencies (Zhu et al., 2003). Activation of β_1ARs activates the G_s subunit and the cAMP/protein kinase A (PKA) signalling pathway. Activation of this pathway phosphorylates target proteins further down the signalling cascade including L-type calcium channels, phospholamban and troponin I (Steinberg, 1999). The use of the specific PKA inhibitor, KT5720, abolished apoptotic cell death in cardiomyocytes, induced by phenylephrine an Isoproterenol isoform, highlighting an involvement with PKA in cardiomyocyte apoptosis (Perez-Schindler et al., 2011, Iwai-Kanai et al., 1999).

The reduction in time of depolarisation and hypercontracture with Isoproterenol, as seen in Figure 3.5 & Figure 3.6, can be linked to Isoproterenol's metabolites in particular the quinone metabolites (Rathore et al., 2000). Isoproterenol as a catecholamine is oxidated, producing ROS stimulating lipid peroxidation (Ansley and Wang, 2013). This particular increase in lipid peroxidation is another source of ROS production and was demonstrated in rat heart tissue by measurement of malonyldialdehyde in addition to measurement of antioxidant enzymes such as superoxide dismutase (Rathore et al., 1998). Initial dosing of rats with Isoproterenol reduced malonyldildehyde and was observed to cause cardiac hypertrophy, a suggested 'defence' mechanism to increased ROS, however continued Isoproterenol administration caused an increase in malonyldildehyde and decreased antioxidant enzymes, with the failure of the antioxidant system being culpable for the damaging effects of ROS in the heart (Kirshenbaum and Singal, 1992, Kirshenbaum et al., 1995, Rathore et al., 1998).

During IR $O_2^{\bullet-}$ radicals increase damage to the mitochondrial electron transport system in addition to other sources of ROS such as cytochrome p450 and production of xanthine oxidase (Raedschelders et al., 2012). In addition to the degradation of TMRM to produce ROS, a possible further source of ROS may be provided from the electron transport chain as a result of an increase in xanthine oxidase. We observed Isoproterenol to significantly decrease ($p<0.001$) the time taken to depolarisation and hypercontracture in the oxidative stress model. These findings allow us to purport that in the presence of Isoproterenol an increase in oxidative stress is occurring and may be causing further stress on the mitochondria of cardiomyocytes. Previous studies have shown that increased ROS act on the mPTP leading to premature opening and inducing apoptosis (Ansley and Wang, 2013, Andersson et al., 2011, Halestrap and Richardson, 2015). These studies provide future direction to investigate Isoproterenol's involvement directly on the mPTP.

The long acting β_2 adrenergic receptor agonists Formoterol and Salmeterol demonstrated significant changes in some haemodynamic data. Both Formoterol (0.5μM) and Salmeterol (0.1μM) increased heart rate at the onset of reperfusion. Formoterol (0.1μM and 0.5μM) also statistically increased LVDP and coronary flow, however Salmeterol (0.1μM) showed no change in coronary flow (Figure 3.9).

All positive inotropes increase cardiac output but are not correlated to heart rate, however, positive inotropes do increase myocardial oxygen demand and consumption, which could be detrimental to patients with an existing ischaemic heart (Watson et al., 2013). Increase in coronary flow resistance has been shown in human subjects as a result of decreased vasodilatory effects on the smooth muscle surrounding arteries, which may explain the elevated coronary flow readings shown in our studies seen with Formoterol (0.1μM and 0.5μM) (Guhan et al., 2000). Watson and colleagues (2013) experiments in the Langendorff model used a combination of the β_1AR blocker atenolol and β_2AR Formoterol and demonstrated similar findings to ours in addition to increase HR as atenolol wore off (Watson et al., 2013).

In contrast to normoxic experiments carried out (data not shown), in the presence of Formoterol, heart rate was elevated higher at the onset of reperfusion in the Langendorff model in comparison to an elevated heart rate during normoxic naive conditions. This further increase in heart rate still remains unclear as shown in a study in healthy human subjects by Guhan and colleagues (2000) demonstrating elevated heart rates with Formoterol at 4 times the recommended dose (Guhan et al., 2000). With the exception of Levosalbutamol, all other β agonists exist in a racemic mixture (equal R & S enantiomers) (Handley et al., 2002).

Handley and colleagues (2002) through radioligand displacement at β adrenoceptors determined the affinity of Formoterol to be far greater for β_2ARs than β_1ARs and also observed increased heart rate in adult rat hearts (Handley et al., 2002, Bremner et al., 1993)

Administration of β agonists are associated with increases in heart rate with stimulation of β_1ARs, but an increase was observed with Formoterol which acts as a specific β_2AR agonist, due to its 200 fold higher affinity for β_2ARs (Guhan et al., 2000, Handley et al., 2002). Formoterol (0.5μM) demonstrated a significant increase in heart rate linked to elevated coronary flow, which was investigated by Watson and colleagues in Wistar rats in a Langendorff model by administering Atenolol, a β_1AR blocker (Watson et al., 2013). This study supported our findings with Formoterol affecting heart rate and coronary flow with a secondary effect on LVDP and further confirmed the affect was via β_2AR activation with the blockade of β_1ARs (Watson et al., 2013). In support of our findings, other groups have also determined Formoterol (and Salmeterol) to effect the QTc interval in human subjects in a dose dependent manner causing tachycardia (Viskin, 1999a). The observed positive effects we have demonstrated with Salmeterol and Formoterol can be associated strongly with the activation of GPCR's and the release of calcium. Upon β_2AR activation by Formoterol or Salmeterol, activation of the cAMP/PKA signalling cascade results in activation of L-type Calcium channels. Increased levels of cAMP initiate the start of a signalling cascade initiated by Protein Kinase A (PKA). cAMP bound to PKA phosphorylates intracellular proteins such as phospholamban, sarcoplasmic reticulum, calmodulin, ryanodine receptors (RyR), sarcoplasmic reticulum ATPase and L-type calcium channels increasing levels of cytosolic calcium (Yoo et al., 2009, Hudecova et al., 2013, Zhu et al., 2005). Increased concentration of calcium as a result of the depolarisation signal spreading through the t-tubules releasing

calcium into the sarcoplasmic reticulum. This increased calcium initiates cardiomyocyte contraction when bound to troponin.

Like Formoterol, Salmeterol has been shown to be a full agonist, however is slower acting than Formoterol in the Langendorff rat heart model (Watson et al., 2013, Guhan et al., 2000). Salmeterol's effect on heart rate has been shown to affect the QTc interval in humans indicating its interaction with βARs and hearts being susceptible to arrhythmias such as tachycardia (Guhan et al., 2000, Handley et al., 2002). It has been suggested that Salmeterol's (β_2AR agonist) structure makes it less effective on the βARs compared to Formoterol resulting in a much slower effect on haemodynamics due to it being more lipophilic than Formoterol (Anderson, 1993, Smyth et al., 1993).

Normoxic and control IR data were used from the previous experiment and could be considered a limiting factor. Although this can be concerning when using the same control data, the use of control infarct to risk data is widely accepted from other labs within our field (Bell et al., 2011). In addition, as the protocol is examining the area at risk with infarcted tissue, no further cellular activity will occur to affect the results. For this reason, we are able to directly compare hearts treated with drug groups to this data throughout the thesis. We observed no exacerbation of infarct to risk ratio in the Langendorff model in the presence of Salmeterol when compared to IR control hearts. This suggests that activation of the β_2AR does not have a link to apoptotic cell death and that Salmeterol has no damaging effects, which was seen with the specific β_2AR agonist Salbutamol which will be discussed later. Further to this finding, β_2ARs link to the G_i subunit has been suggested to be anti-apoptotic, however there was no further evidence to suggest Salmeterol reduced the I/R injury when compared to IR control hearts (Figure 3.10).

Our studies showed that Salbutamol (1μM) showed a chronotropic effect due to its action on βARs, however clarity is needed to determine whether the damaging effects are due to the activation of β_1 or β_2ARs which will be discussed in Chapter 4. Other studies in horses showed increases in cardiac output with nebulisation of horses with Salbutamol thus supporting Salbutamol's inotropic and chronotropic effects (M. Patschova, 2010).

Interestingly, a clinical study by (Gao Smith et al., 2012) used intravenous Salbutamol to investigate the toxicity, however the trial was terminated due to a high number of mortalities recorded with administration of Salbutamol in the non-placebo groups. The study failed to determine any cellular mechanisms or rationale of Salbutamol induced toxicity resulting in mortalities (Gao Smith et al., 2012). The concentration of Salbutamol administered to patients was 10μM in a group of 161 patients that were randomly assigned the drug. It was at this concentration the study was abandoned due to the high rate of mortalities, with suggestions of Salbutamol toxicity at this particular concentration (Gao Smith et al., 2012). In relation to their findings, we have demonstrated at concentrations lower than 10μM, there is an exacerbation of I/R ratio when compared to IR hearts. Au and colleagues (2000) analysed several studies associated with β-agonists and myocardial infarction and angina. Their findings could not suggest a direct effect of β-agonists to cause myocardial ischaemia, however a link with the use of β-agonists demonstrated to cause a seven fold increase in patients with underlying cardiovascular disease to develop myocardial infarction (Au et al., 2002, Au et al., 2000).

In normoxic conditions, Salbutamol (0.1μM) did not cause any significant damage to the heart in the Langendorff model (data not shown). However, the inotropic effect of Salbutamol was observed and similar to the HR haemodynamic data observed in hearts treated in a reperfusion injury model. In isolated perfused hearts subjected to 35 minutes of ischaemia and 120 minutes of reperfusion, administration of Salbutamol (0.03μM-1μM) throughout reperfusion, significantly increased infarct size to risk ratio ($p<0.001$), with an EC_{50} value of 38.6nM. Our calculated EC_{50} value is below recorded human plasma concentrations of Salbutamol that have been shown to be above 0.1μM when administered via metered dose inhalers (Rodrigo et al., 1996). A Salbutamol concentration of 0.1μM was selected as the standard concentration for the remainder of experiments throughout this thesis as it was the lowest concentration at which the maximum amount of myocardial damage was recorded in the Langendorff model and well within the limits of other studies (Gao Smith et al., 2012, Rodrigo et al., 1996). Patients that have been hospitalised presenting with angina or myocardial infarction were found to be significantly more likely to have previously been administered a meter-dosed inhaler up to 3 months prior to their admission, suggesting a potential link between bronchodilators, angina and myocardial infarction (Au et al., 2000).

Our study demonstrated Salbutamol, as a β_2AR agonist, to increase myocardial injury in the model of ischaemia reperfusion; however, we have also demonstrated that other SABA β_2AR agonists show no increase in infarct to risk ratio. A potential explanation for this varying effect may be linked to the dual pathway signalling capability of β_2ARs switching between G_i and G_s subunits of GPCRs. The involvement of both β_1AR and β_2ARs in the presence of Salbutamol will be discussed in detail in Chapters 5 and 6.

Administration of Salbutamol, Formoterol and Salmeterol were seen to have no effect on the time taken to depolarisation, but were seen to significantly decrease the time taken to hypercontracture in comparison to non-treated control group. This increase in hypercontracture can strongly be linked to increased calcium released as a result of β_2AR agonist activation of the GPCRs as described earlier. It is worth noting that time taken to depolarisation was reduced, but not significantly. The mPTP has been shown to remain closed in ischaemic conditions and open during reperfusion, when levels of reactive oxygen species and calcium are increasing initiating release of pro-apoptotic proteins from within the mitochondria (Husainy et al., 2012). The sarcoplasmic reticulum acts as a main source of calcium required for excitation contraction coupling in cardiac muscle via calcium induced calcium release involving RyR receptors (Baumgartner et al., 2009). The structure of the mPTP still remains an enigma but strong evidence supports that several components are responsible for forming the mPTP; voltage dependant anion channel (VDAC) (Vyssokikh and Brdiczka, 2004)adenine nucleotide translocator (ANT) (Zamzami and Kroemer, 2001) and cyclophilin D (Baines and Molkentin, 2005). Cross talk between β_1AR and β_2AR receptors affecting the mitochondrial death pathway is a potential route for this apoptotic effect (Fajardo et al., 2011) βAR activation has been a target of investigation for cell survival, however in relation to premature opening of the mPTP, this can be linked to the activation of the G_s subunit and increase in calcium release, specifically the phosphorylation of L-type Ca^{2+} channels and phospholamban increasing the SR uptake of Ca^{2+} via SR ATPase (Cros and Brette, 2013). The mode of action of calcium specifically on the mPTP is still not clear, however the effect of calcium is suggested to act specifically on the cyclophilin D and VDAC components of the pore (Basso et al., 2005), (Schlattner et al., 2001).

The distribution of βARs within cardiomyocytes are influential and key to interaction with ligands, with β_1ARs found more readily available at the surface of cardiomyocytes (Nikolaev et al., 2010), upon stimulation they can activate the adenylyl cyclase/cAMP/PKA pathway that in turn can lead to apoptosis (Dakka et al., 1997). In contrast the distribution of β_2ARs, which have been described to be anti-apoptotic, are found deep within the t-tubules of the cardiomyocytes making them less favourable for ligand interaction in the presence of high affinity drugs(Lyon et al., 2009). Cardioprotection has been seen within cardiomyocytes in a scenario of preconditioning and is mediated by β_2AR – G_i activation. This occurs in a similar way to β_1AR activation where by β_2AR couples to the G_s -subunit leading to PKA activation, however in this case PKA activation further phosphorylates the β_2AR causing it to shift its coupling from G_s to G_i (Tong et al., 2005). Nikolaev and colleagues (2010) showed that β_2-ARs in myocytes isolated from rats with a failing heart, redistribute from deep within the t-tubules onto the crest of the cell. They observed cAMP signals in the cell crest and along the t-tubules, which are identical to signals upon β_1AR activation, providing a possible explanation for cell apoptosis via β_2AR activation, when in normal conditions, activation of β_2ARs protects the myocytes from stress/damage such as that of ischaemia/reperfusion injury.

The findings discussed in this chapter demonstrate that the β_2AR agonists Isoproterenol, Formoterol, Salmeterol and Salbutamol can induce stress to the myocardium affecting the cardiomyocytes, however how they influence and affect the mPTP still remains unclear in addition to the activation of specific β receptors. With the specific β_2AR agonists, their signalling pathways are linked to an increase in calcium via G_s stimulation irrespective of the 'favourable' G_i coupled subunit resulting in positive chronotropic and inotropic effects. Little

is known about the detrimental effects of Salbutamol in both an oxidative stress model and Langendorff model in addition to signalling proteins. The suggestion for its non-selective behaviour may be the cause of its toxicity. Further work is needed to investigate the toxic effects of Salbutamol discussed in this chapter through antagonising the β_1 and β_2 adrenergic receptors in addition to investigating the cellular pathways that may be involved causing the increase in injury to the myocardium.

4 Chapter 4: The effect of short acting βadrenergic receptor agonist Salbutamol in myocardial ischaemia/reperfusion injury.

4.1 Introduction

Data presented in Chapter 3 investigated the effects of long acting β-adrenergic receptor agonists Formoterol and Salmeterol, and short long acting β-adrenergic receptor agonists Isoproterenol and Salbutamol on isolated perfused rat hearts exposed to ischaemia reperfusion and isolated cardiomyocytes in the model of oxidative stress. Salbutamol in particular demonstrated a significant increase in infarction and cell death in addition to significantly decreasing the time taken to hypercontracture in the oxidative stress model. In contrast, no significant changes were observed with the other bronchodilators used. In this chapter the effects of Salbutamol on cell signalling proteins and cell viability are investigated by means of MTT analysis, Western blotting and flow cytometry.

Salbutamol is widely used in the treatment of reactive airway disease such as asthma (Gonzalez-Munoz et al., 2011). Salbutamol's structural design allows it as a ligand to specifically target β_2ARs and initiate bronchodilation via activation of the G protein coupled receptor pathway involving adenylyl cyclase/cAMP pathway via the activation of the coupled G_i subunit (Anderson, 2006, Bhattacharya et al., 2010). β_2ARs located on the surface of trachea and bronchioles are easily activated and targeted upon inhalation of Salbutamol thus making it an established treatment for respiratory disease (Selroos, 2014). Distribution of β_2ARs are not restricted to the lining of the trachea and bronchioles but have also been acknowledged in the heart co-existing with β_1ARs with a distribution of 56% β_1ARs to 44%

β_2ARs identified specifically within the rat heart (Xiao and Lakatta, 1993). β_2ARs have shown to be even more localised within caveolae within cardiomyocytes, located within t-tubules of the cardiomyocyte (Cros and Brette, 2013, Calaghan and White, 2006).

During conditions of ischaemia, an increased demand on cardiomyocytes occurs as a result of an insufficient supply of blood and oxygen due to occlusion of the coronary arteries. The development of myocardial ischaemia as a result of the coronary occlusion causes an increased demand on neighbouring cardiomyocytes to compensate for a reduction in cardiac metabolism and a decrease in energy via ATP depletion resulting in cardiomyocyte cell death (Javadov et al., 2014). Triggers such as necrosis of cardiomyocytes initiate tissue repair via leakage of cytokines such as TGF-β1 leading to remodelling of ventricles in order to maintain cardiac output (Desmouliere et al., 1993, Dorn, 2009). During conditions of MI and congestive heart failure (CHF) β_2ARs have been shown to relocate to the surface of cardiomyocytes with a reduction of up to 50% of β_1ARs by interaction with the β_1AR kinase (β_1ARK) causing desensitisation of the β_1ARs via direct phosphorylation of the β_1AR (Cross et al., 1999, Ungerer et al., 1993, Coughlin et al., 1995).

Salbutamol at high concentrations has been linked to cause hypertrophy, a symptom that occurs during (and follows) MI and dilated cardiomyopathy in addition to increased reports of mortality when Salbutamol was administered intravenously. However results were unclear as to how Salbutamol directly contributed to the deaths, the data only shows the increase in mortality in groups administered with Salbutamol. (Rubin et al., 1983, Natale et al., 1999, Giallauria et al., 2008, Spitzer et al., 1992, Au et al., 2000). Although Salbutamol has positive chronotropic effects on the heart via activation of β_1ARs, an understanding as to its effect on

the heart during IR is crucial having already investigated and shown its detrimental effects on infarct to risk ratio, haemodynamics and effects on the opening of the mPTP (Chapter 3).

Several cell signalling pathways have been identified that are activated during IR that are responsible for the onset of cell death in addition to cell survival signalling. These pathways include PI3K/Akt/Bad, MEK1/2/Erk 1/2 and JNK 1/2, the first 2 being linked with cytoprotection and major proteins involved in the Reperfusion Injury Salvage Kinase pathway (RISK) (Armstrong, 2004, Hausenloy and Yellon, 2004). Involvement of phosphorylated Akt can be regulatory of apoptosis, with an up regulation of Akt suppressing apoptosis via inhibition of its pro-apoptotic targets such as Bad. Prolonged activation or over expression of Akt however can also induce apoptosis. Akt activation initiated by stresses such as IR, allows phosphorylation at one of its two phosphorylation sites, serine 473 or threonine 308 (Cross et al., 1995). Mockridge and colleagues (2000) demonstrated that Akt could be dually phosphorylated during IR. With several isoforms of Akt identified, Akt1 is of particular interest due to its involvement with cardiomyocytes and its abundance within the heart (Matsui and Rosenzweig, 2005, Mullonkal and Toledo-Pereyra, 2007). Phosphorylation of Akt1 inhibits apoptosis due to Akt inhibiting pro-apoptotic proteins i.e. Bad, a member of the Bcl-2 family, caspase- 9 and c-Raf (Cardone et al., 1998, Hausenloy et al., 2005, Hussain et al., 2013). Additionally IR has also shown to activate signalling cascades linked to MAPKs such as Erk1/2 and the stress activated proteins JNK/SAPK (Armstrong, 2004, Mockridge et al., 2000) These 2 signalling proteins are initiated with stresses such as ROS produced in conditions of IR. Similar to Akt, Erk and JNK have different isoforms, which can influence different pathways independently. Activation of βARs by bronchodilators as those mentioned in previous chapters has been shown to supress JNK activity, in turn promoting cell survival (Anderson et al., 2014).

The involvement of caspases is an important factor to examine due to their involvement in initiating apoptosis; caspase 3 in particular is shown to be involved with cardiomyocytes during injury and inflammatory responses (Grunenfelder et al., 2001, Hussain et al., 2013). Activation of caspase 3 cleaves Bcl-2 proteins promoting release of cytochrome c, a pro-apopototic protein triggering apoptosis (Kirsch et al., 1999).

Investigating the survival and stress signalling proteins allows an indication of the possible pathways involved in Salbutamol mediated injury during conditions of IR.

4.2 Aims

The aims of this study were to determine the cell signalling pathways associated with Salbutamol induced myocardial injury including the signalling proteins p-Akt (Ser473), p-Erk(Thr202/Tyr204) and cleaved caspase 3. Focussing on the survival proteins, Akt and Erk will indicate initially any stress caused by Salbutamol. The cytotoxic effects of Salbutamol were also investigated in cardiomyocytes by the use the MTT assay.

4.3 Methods

4.3.1 Isolated perfused heart preparation

Briefly, Sprague-Dawley rats were sacrificed by cervical dislocation and cannulated to the Langendorff setup and perfused with KHB as described in section 2.3. Hearts were allowed to stabilise for 20 minutes followed by 35 minutes of regional ischaemia and 120 minutes of reperfusion. One minute before the onset of reperfusion hearts were administered Salbutamol (0.001μM-1μM) in the absence or presence of Wortmannin (0.1μM) or U0126 (10μM). At the end of the experiment hearts underwent infarct to risk ratio analysis. Haemodynamic data were collected throughout the study.

For western tissue collection, hearts were reperfused with Salbutamol (0.001μM-1μM) for either 5, 20 or 120 minutes in the presence or absence of Wortmannin (0.1μM) or U0126 (10μM). After the time elapsed, hearts were removed and the left ventricle removed and snap frozen in liquid nitrogen.

4.3.2 Western blot analysis

Analysis of tissue by western blot was carried out as described in section 2.7. Briefly, following gel electrophoresis, proteins were transferred to a PVDF membrane and probed for the phosphorylated and total forms of the proteins: phospho-Akt (Ser_{473}) (1:1000) and phospho-p44/p42 (Erk 1/2, Thr202/Tyr204) (1:1000).

4.3.3 Adult rat cardiac myocyte isolation

Briefly, male Sprague Dawley rats were sacrificed by cervical dislocation and the hearts excised and cannulated onto modified Langendorff apparatus and perfused with a constant flow rate of 14ml/min as described in section 2.4. Hearts were perfused for 3-4 minutes with calcium free modified Krebs Heinsleit Buffer. The buffer was then switched and the hearts perfused with digestion buffer for 7 minutes. Isolated ventricular myocytes were used for the MTT assay and flow cytometric analysis of cleaved caspase 3 as described previously in sections 2.5.2, 2.5.3. Isolated myocytes were treated with Salbutamol (0.1μM) in the presence or absence of Wortmannin (0.1μM) or U0126 (10μM).

4.3.4 Statistical analysis

All data were presented as a mean of the stabilisation period ± SEM. Infarct size, times taken to depolarisation and hypercontracture, western blot data and flow cytometric data were tested using one way ANOVA with a Fishers Least Significance Test post hoc to determine any significance between groups. Haemodynamic data was statistically analysed using a two-way analysis of variance ANOVA. $p<0.05$ was considered to be significant.

4.4 Results

4.4.1 Cytotoxic effects of Salbutamol on isolated rat cardiomyocytes

The MTT assay was used to investigate the effects Salbutamol (0.001μM-1μM) had on isolated ventricular rat myocytes exposed to 2 hours of hypoxic conditions followed by 2 hours of re-oxygenation, when administered during re-oxygenation. A significant decrease in cell viability when comparing hypoxic re-oxygenated (HR) cells with normoxic cells was observed (100 ± 2.9% vs. 209 ± 8%, $p<0.001$, Figure 4.1). A significant decrease in MTT reductase activity (i.e. decreased cell viability) was observed when Salbutamol (0.1μM or 1μM) was administered during re-oxygenation when compared to HR cardiomyocytes (0.1μM, 76 ± 1%, 1μM, 72 ± 1% vs. 100 ± 2.9%, $p<0.05$, Figure 4.1).

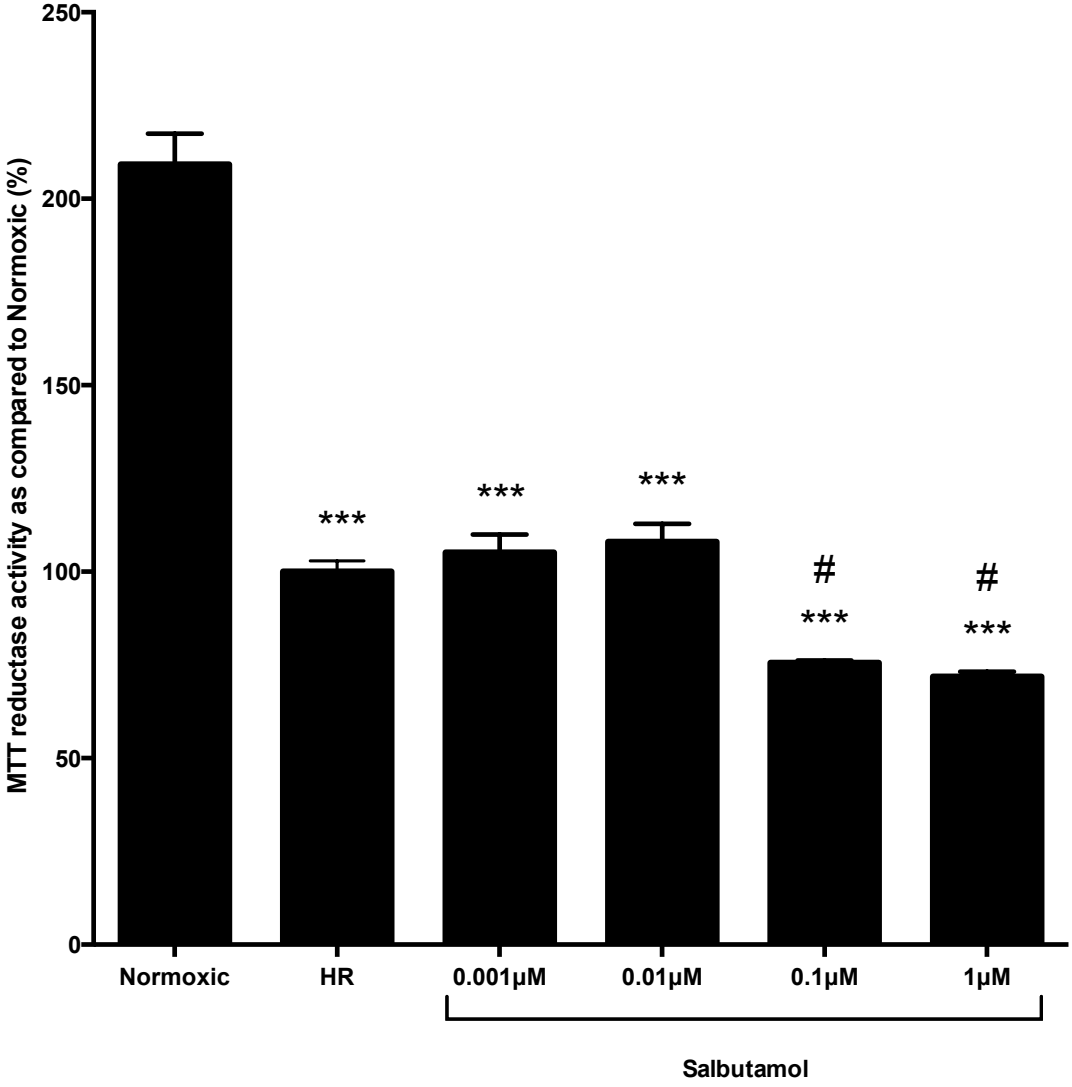


Figure 4.1 MTT reductase activity in cardiomyocytes exposed for 2 hours hypoxia and 4 hours re-oxygenation where Salbutamol (0.001-1µM) was added throughout re-oxygenation. Data presented as mean ± SEM. n=6-8. ***p<0.001 vs. Normoxic, $^{\#}$p<0.05 vs. HR.

4.4.2 The effects of Salbutamol on p-Akt^{473} in a model of ischaemia reperfusion by assessment of western blotting.

To determine the role of the PI3K/Akt cell signalling pathway in Salbutamol mediated cytotoxicity, isolated hearts were subjected to IR where Salbutamol was administered throughout reperfusion and underwent western blot analysis to assess p-Akt status. Investigation into the signalling protein Akt (Ser473) was carried out at 3 separate time periods (5, 20 and 120 minutes) of reperfusion. Hearts administered with Salbutamol (0.001μM-1μM) for 5 minutes at the onset and throughout reperfusion showed no significant change in expression of phosphorylated Akt when compared to time-matched control IR groups (Figure 4.2). Interestingly, a lower expression of p-Akt ($p>0.05$) was observed at 0.1μM and 1μM concentrations when compared to time matched IR control (0.1μM, 85 ± 4%, 1μM, 93 ± 9% vs. 100 ± 12%, $p>0.05$, Figure 4.2).

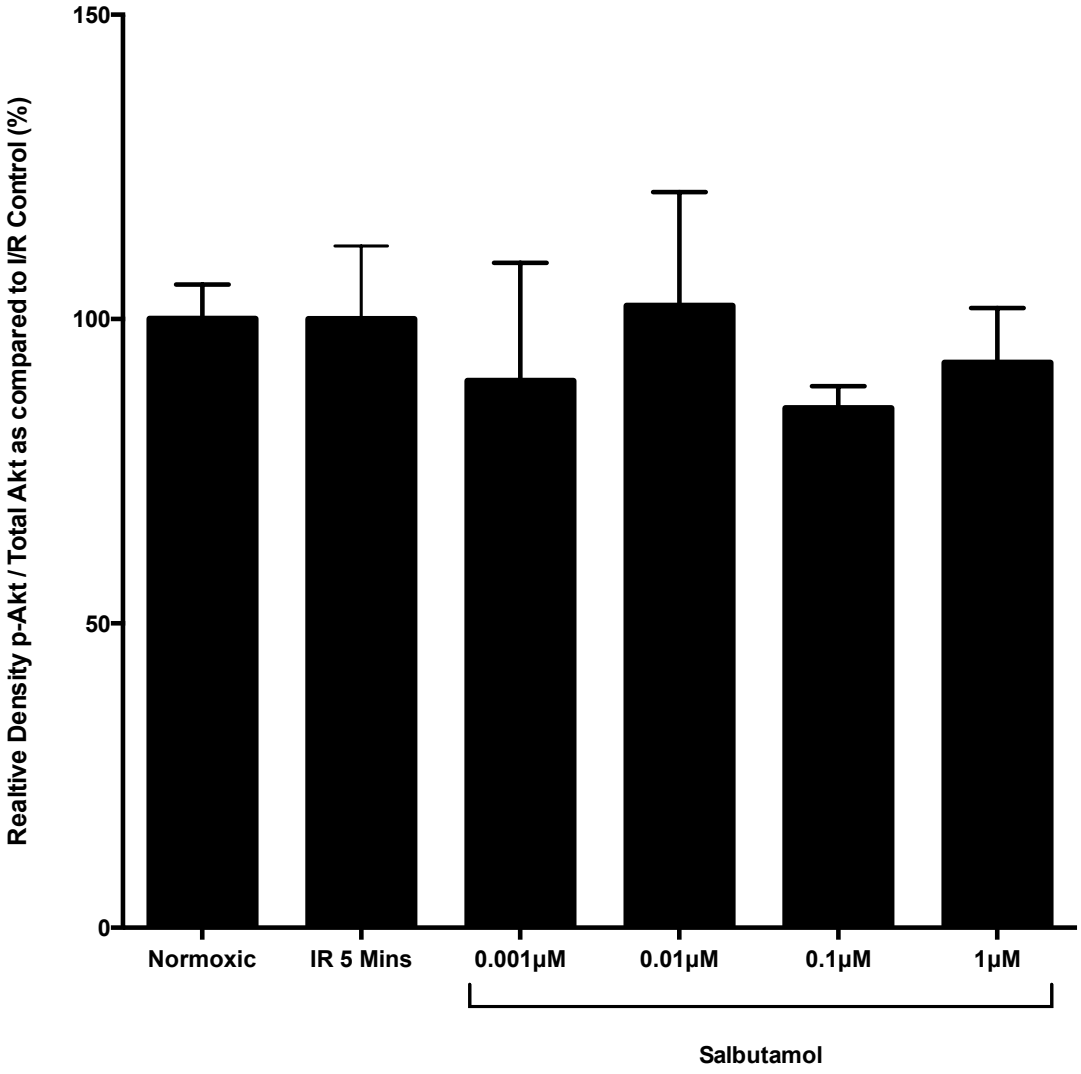


Figure 4.2 The effects of Salbutamol (0.001µM – 1µM) administration at the onset of reperfusion on the expression of phosphorylated Akt (Ser473) after exposure to 35 minutes ischaemia and 5 minutes of reperfusion. Data presented as mean ±SEM. n=3.

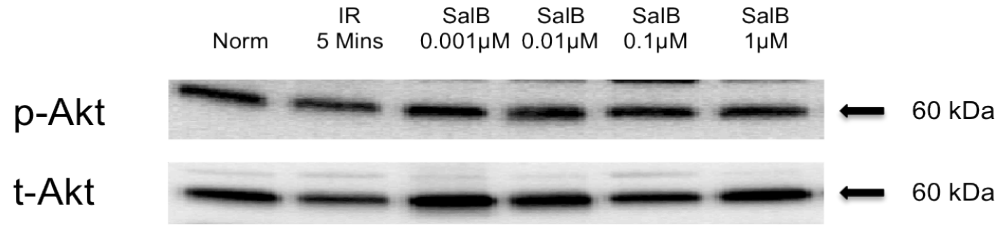


Figure 4.3 Representative blot of p-Akt and t-Akt when Salbutamol (0.001µM-1µM) was administered throughout reperfusion for 5 minutes after 35minutes ischaemia

Hearts were subjected to 35 minutes of ischaemia followed by 20 minutes of reperfusion and were administered with Salbutamol (0001.1μM- 1μM) at the onset and throughout reperfusion for 20 minutes. A significant increase in expression of p-Akt was recorded in hearts administered with Salbutamol (0.1μM, 1μM) when compared to time matched IR control hearts (0.1μM, 240 ± 7%, 1μM, 220 ± 32% vs. 100 ± 19%, $p<0.01$, Figure 4.4).

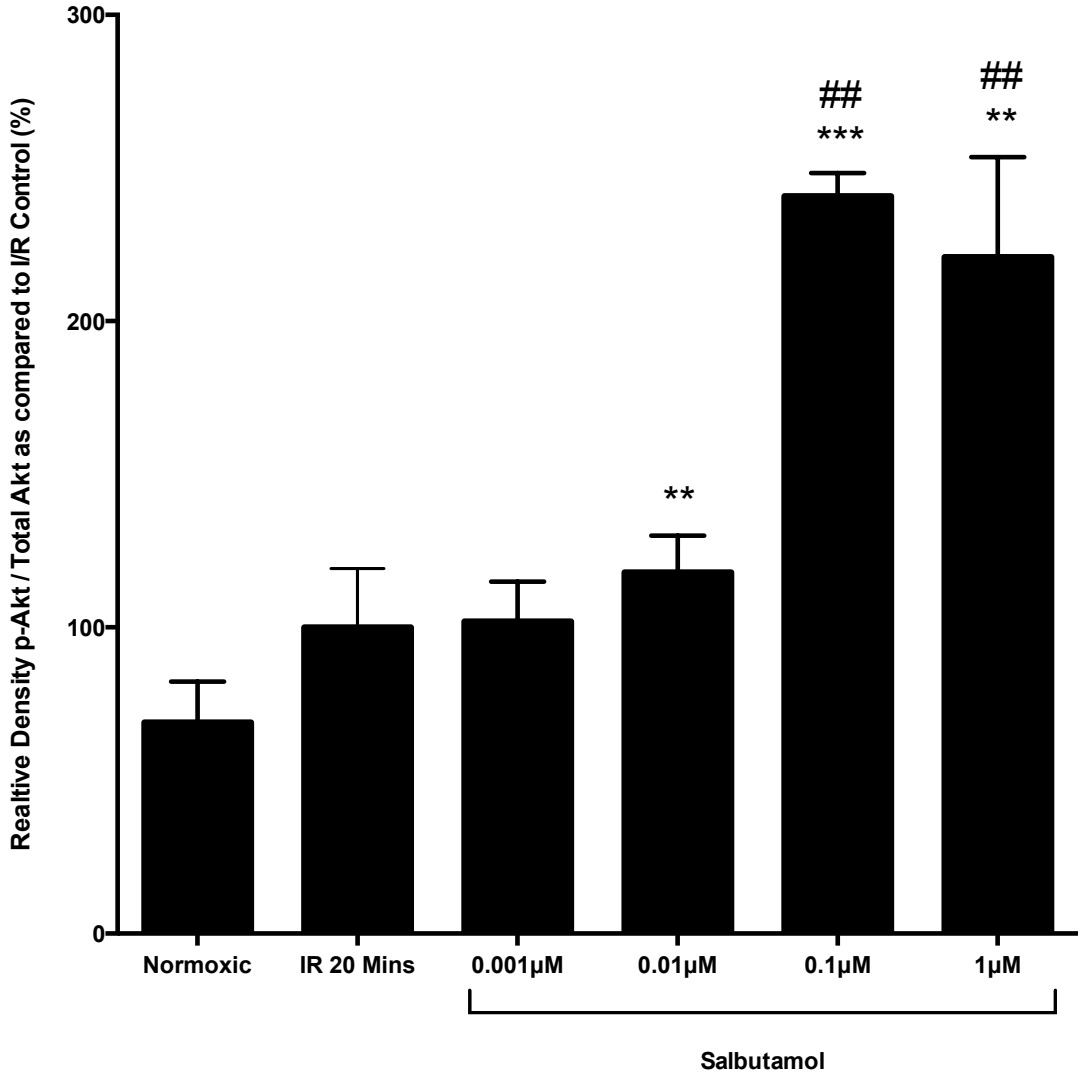


Figure 4.4 The effects of Salbutamol (0.001µM – 1µM) administration at the onset of reperfusion on the expression of phosphorylated Akt (Ser473) after exposure to 35 minutes ischaemia and 20 minutes of reperfusion. Data presented as mean ±SEM. n=3. ***p<0.001 vs. normoxic, **p<0.01 vs. normoxic, $^{\#\#\#}$p<0.001 vs. IR 20 Mins, $^{\#\#}$p<0.01 vs. IR 20 Mins

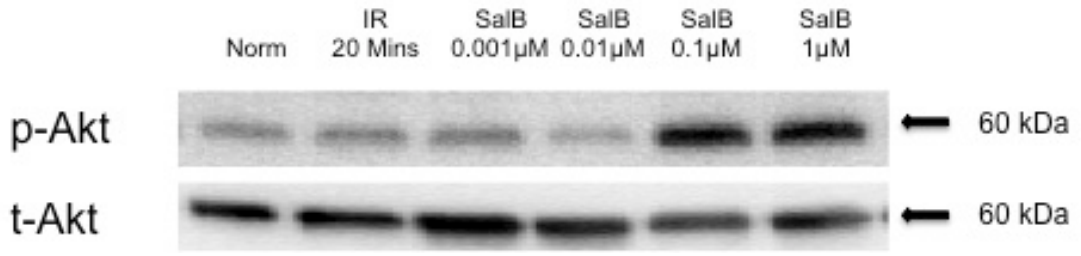


Figure 4.5 Representative blot of p-Akt and t-Akt when Salbutamol (0.001µM-1µM) was administered throughout reperfusion for 20 minutes after 35 minutes ischaemia.

Hearts were subjected to 35 minutes of ischaemia followed by 120 minutes of reperfusion and were administered with Salbutamol (0001.1μM- 1μM) at the onset and throughout reperfusion. Hearts administered with Salbutamol (0.1μM and 1μM) showed a significant increase in p-Akt expression when compared to hearts subjected to normoxic conditions (0.1μM, 192 ± 4%, 1μM, 255 ± 25% vs. 79 ± 6%, $p<0.05$, Figure 4.6).

A significant increase in p-Akt expression was also observed with the same concentrations when compared to IR time matched controls (0.1μM, 192 ± 4%, 1μM, 255 ± 25% vs. 100 ± 11, $p<0.05$, Figure 4.6).

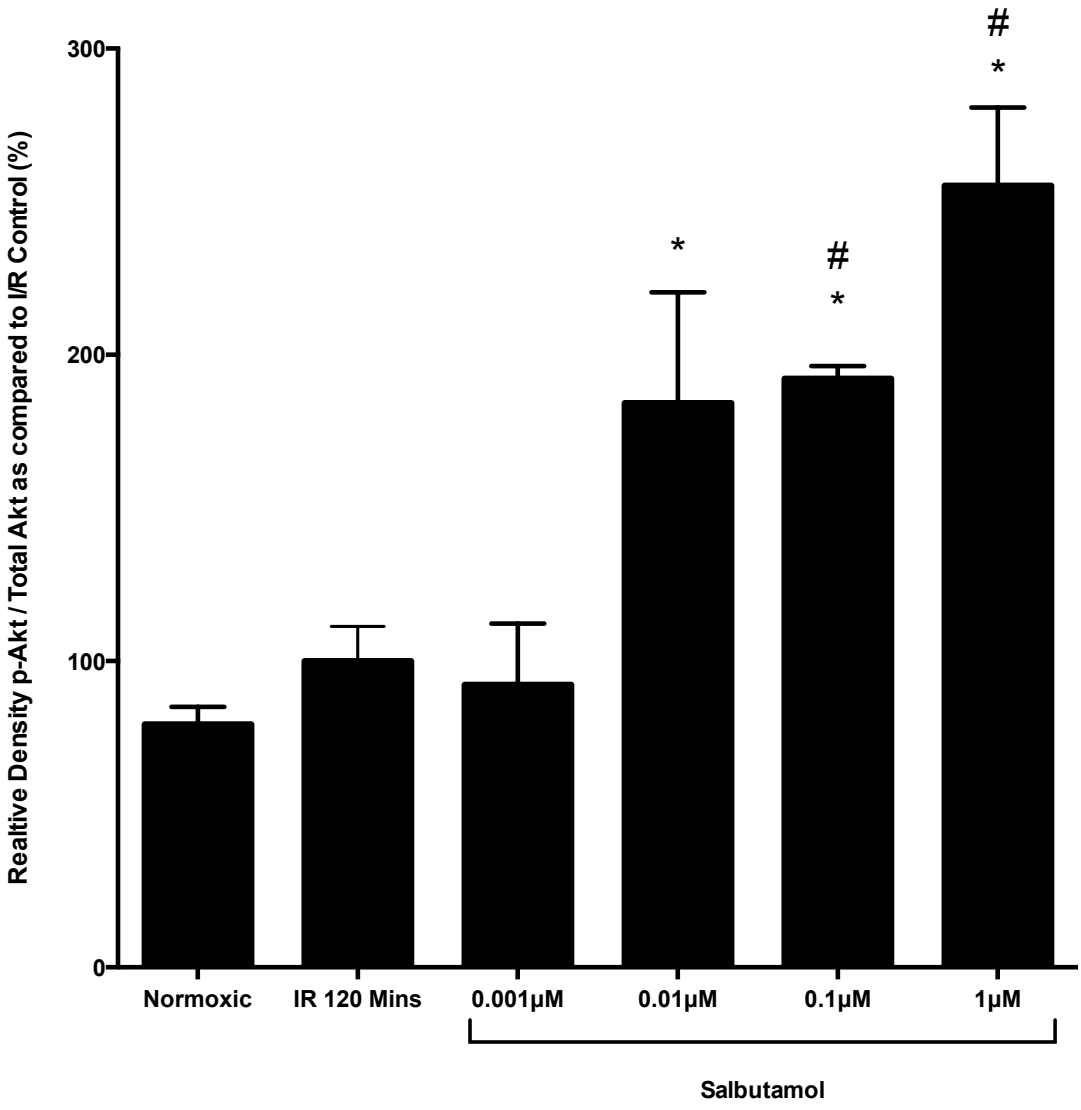


Figure 4.6 The effects of Salbutamol (0.001µM – 1µM) administration at the onset of reperfusion on the expression of phosphorylated Akt (Ser473) after exposure to 35 minutes ischaemia and 120 minutes of reperfusion. Data presented as mean ±SEM. n=3. $^{*}p<0.05$ vs. normoxic, $^{\#}p<0.05$ vs. IR 120 Mins

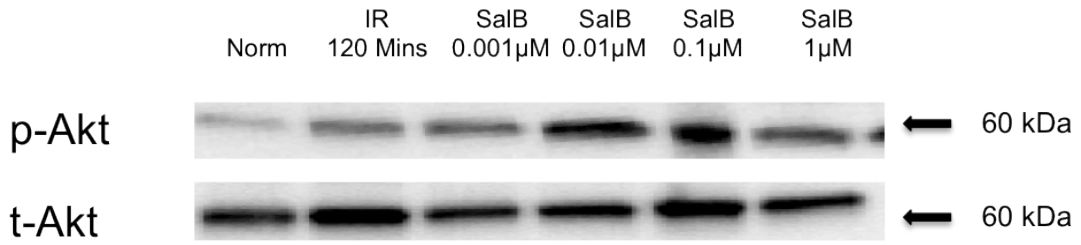


Figure 4.7 Representative blot of p-Akt and t-Akt when Salbutamol (0.001µM-1µM) was administered throughout reperfusion for 120 minutes after 35 minutes ischaemia.

4.4.3 The effects of Salbutamol on signaling protein p-Erk 1/2 (p44/p42) in a model of ischaemia reperfusion by assessment of western blotting.

Expression of the Mitogen Activating Protein Kinase (MAPK) Erk1/2 (p44/42) (Thr_{202}/Tyr_{204}) was investigated to determine the role of intracellular p-Erk. Three separate time periods (5, 20, 120 minutes) of reperfusion were used after 35 minutes of ischaemia to measure the level of activity of the specific signaling protein in the presence and absence of Salbutamol (0.001μM-1μM).

Hearts were subjected to 35 minutes of ischaemia followed by 5 minutes of reperfusion and were administered with Salbutamol (0001.1μM- 1μM) at the onset and throughout reperfusion.

No significant decrease in expression of p-Erk was observed when IR control hearts were compared to normoxic hearts, however a significant decrease in p-Erk was found between concentrations of Salbutamol (0.001μM-0.1μM) when compared to time matched IR control hearts (0.001μM, 40 ± 10%, 0.01μM, 36 ± 3%, 0.1μM, 33 ± 10%, vs. 100 ± 33%, $p<0.05$, Figure 4.8). Interestingly, an increase in p-Erk expression was observed at the highest concentration of Salbutamol (1μM) when compared to time matched control hearts (63 ± 28% vs. 100 ± 33%, $p>0.05$, Figure 4.8).

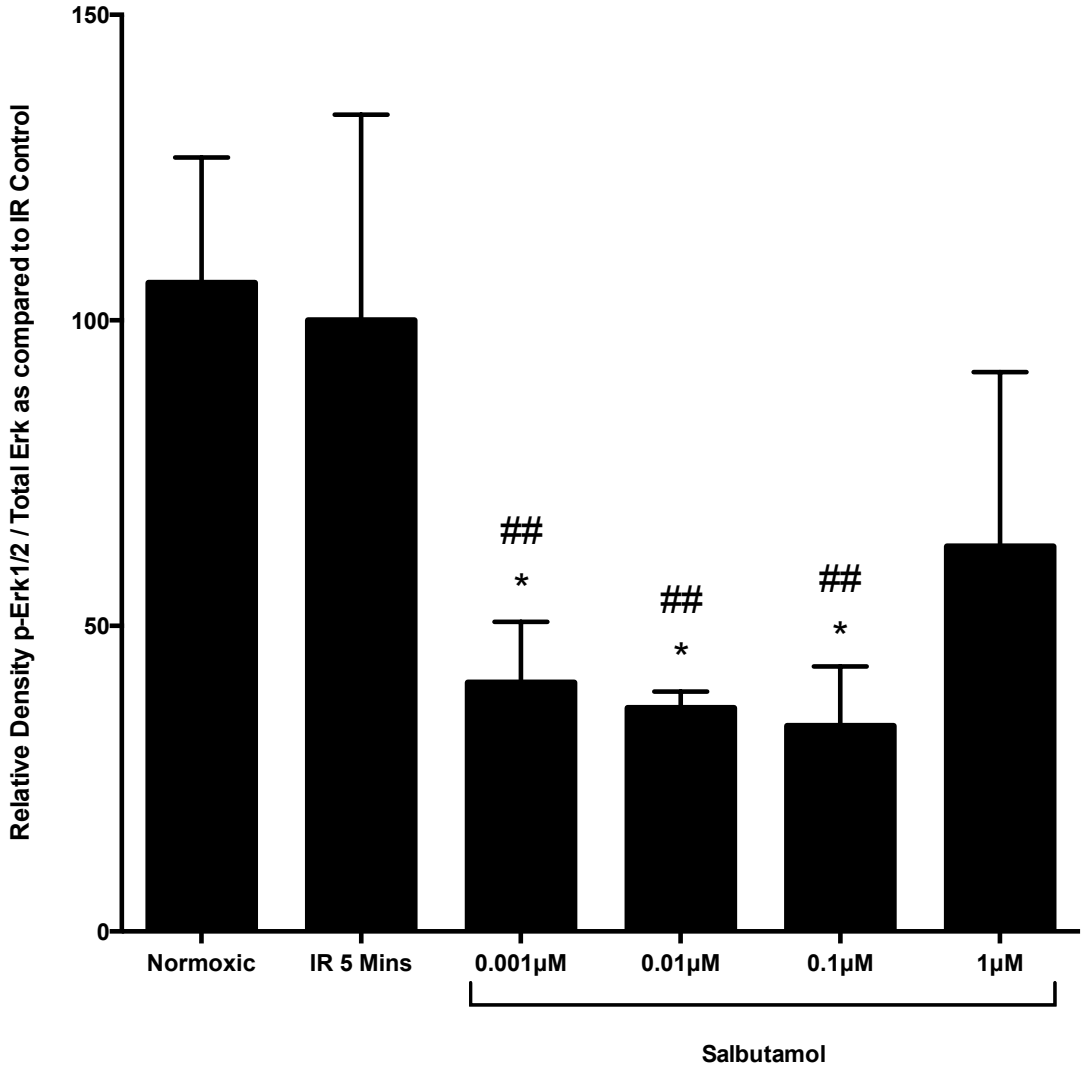


Figure 4.8 The effects of Salbutamol (0.001µM – 1µM) administration at the onset of reperfusion on the expression of phosphorylated Erk (p44/p42) after exposure to 35 minutes ischaemia and 5 minutes of reperfusion. Data presented as mean ±SEM. n=3. *p<0.05 vs. normoxic, $^{\#\#}$p<0.01 vs. IR 5 Mins.

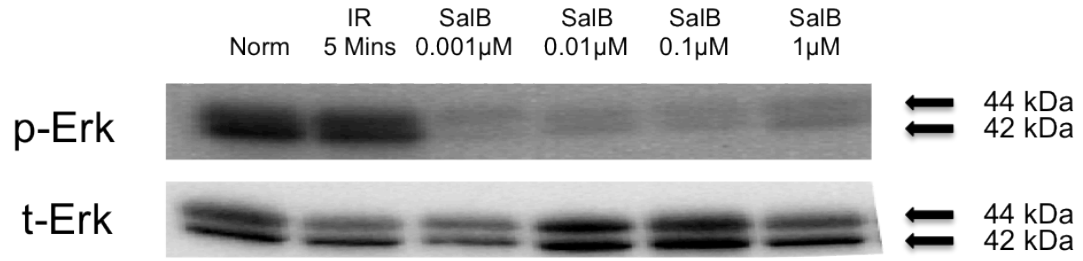


Figure 4.9 Representative blot of p-Erk and t-Erk when Salbutamol (0.001µM-1µM) was administered throughout reperfusion for 5minutes after 35 minutes ischaemia

Hearts were subjected to 35 minutes of ischaemia followed by 20 minutes of reperfusion and were administered with Salbutamol (0001.1μM- 1μM) at the onset and throughout reperfusion. Hearts administered with Salbutamol (0.001μM-1μM) demonstrated a significant decrease in Erk expression when compared to time matched IR control hearts (0.001μM, 34 ± 11, 0.01μM, 44 ± 4%, 0.1μM, 39 ± 6%, 1μM, 39 ± 15% vs. 100 ± 28%, $p<0.01$, Figure 4.10).

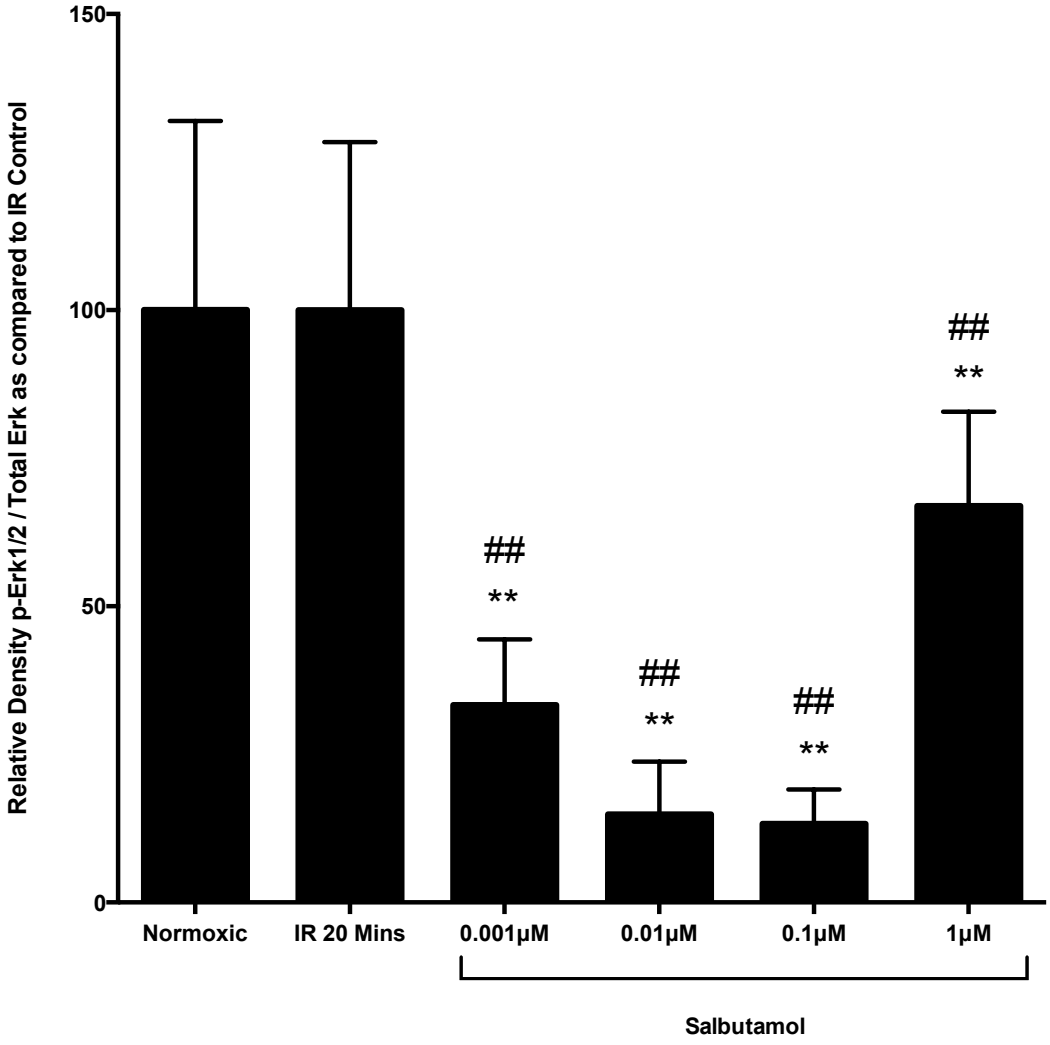


Figure 4.10 The effects of Salbutamol (0.001µM – 1µM) administration at the onset of reperfusion on the expression of phosphorylated Erk (p44/p42) after exposure to 35 minutes ischaemia and 20 minutes of reperfusion. Data presented as mean ±SEM. n=3. **p<0.01 vs. normoxic, $^{\#\#}$p<0.01 vs. IR 20 Mins

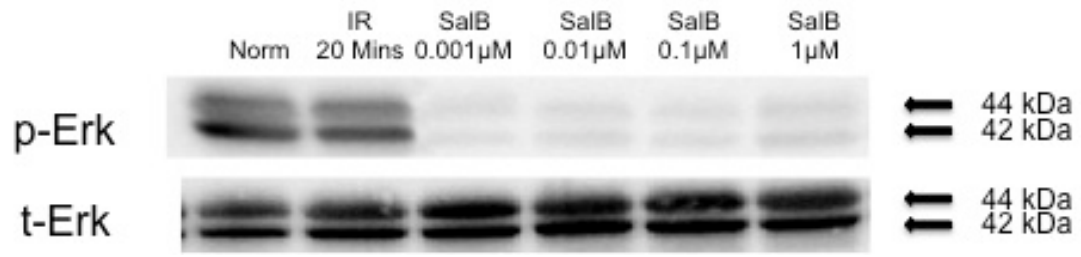


Figure 4.11 Representative blot of p-Erk and t-Erk when Salbutamol (0.001µM-1µM) was administered throughout reperfusion for 20 minutes after 35 minutes ischaemia

Hearts were subjected to 35 minutes of ischaemia followed by 120 minutes of reperfusion and were administered with Salbutamol (0001.1μM- 1μM) at the onset and throughout reperfusion.

In the presence of Salbutamol (0.001μM-1μM) a significant decrease in p-Erk expression was recorded when comparing time matched controls to Normoxic hearts (100 ± 23% vs. 205 ± 20%, $p<0.001$, Figure 4.12). No significance in p-Erk expression was observed with any concentration of Salbutamol (0.001μM-1μM) when compared to time matched IR control hearts ($p>0.05$, Figure 4.12).

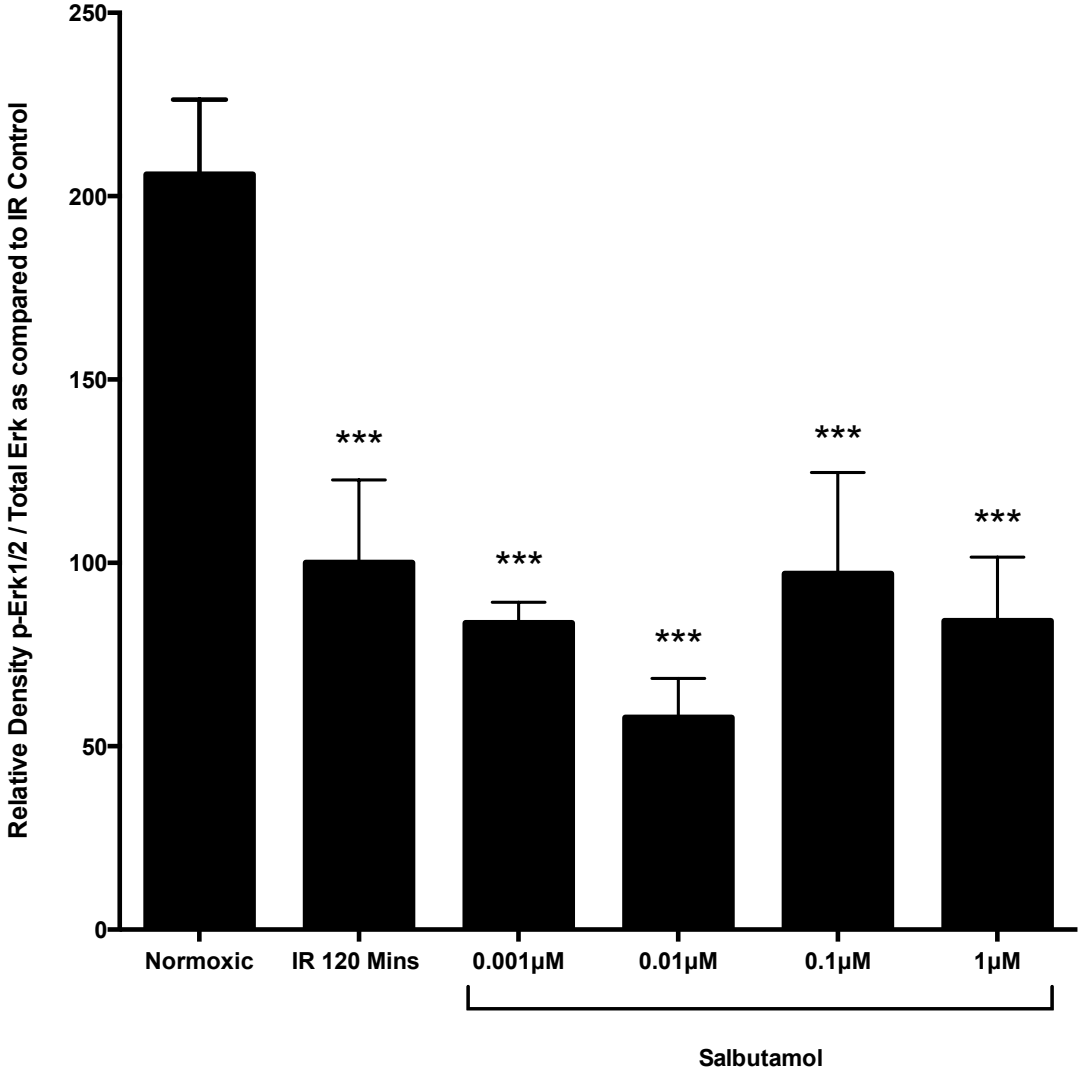


Figure 4.12 The effects of Salbutamol administration at the onset of reperfusion (0.001µM – 1µM) on the expression of phosphorylated Erk (p44/p42) after exposure to 35 minutes ischaemia and 120 minutes of reperfusion. Data presented as mean ±SEM. n=3. *** $p<0.01$ vs. normoxic.

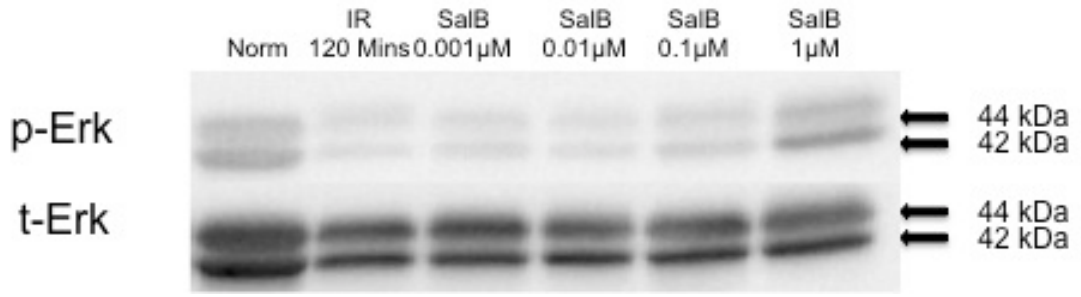


Figure 4.13 Representative blot of p-Erk and t-Erk when Salbutamol (0.001µM-1µM) was administered throughout reperfusion for 120 minutes after 35 minutes ischaemia

4.4.4 The effects of Salbutamol on cleaved caspase-3 expression in cardiomyocytes subjected to hypoxia/re-oxygenation injury

Isolated cardiomyocytes were exposed to hypoxia for 2 hours followed by 4 hours re-oxygenation in the absence or presence of Salbutamol (0.001μM-1μM) throughout the re-oxygenation period. Assessment of expression of cleaved caspase 3 was done by flow cytometry analysis. HR control cardiomyocytes expressed significantly higher levels of cleaved caspase 3 when compared to normoxic cardiomyocytes (100 ± 3% vs. 23 ± 13%, $p<0.01$, Figure 4.14).

Cardiomyocytes treated with Salbutamol (0.001μM – 1μM) significantly increased expression of cleaved caspase 3 when compared to HR control cardiomyocytes (0.001μM, 190 ± 14%, 0.01μM, 213 ± 15%, 0.1μM, 190 ± 22%, 1μM, 200 ± 19% vs. 100 ± 52%, $p<0.001$, Figure 4.14).

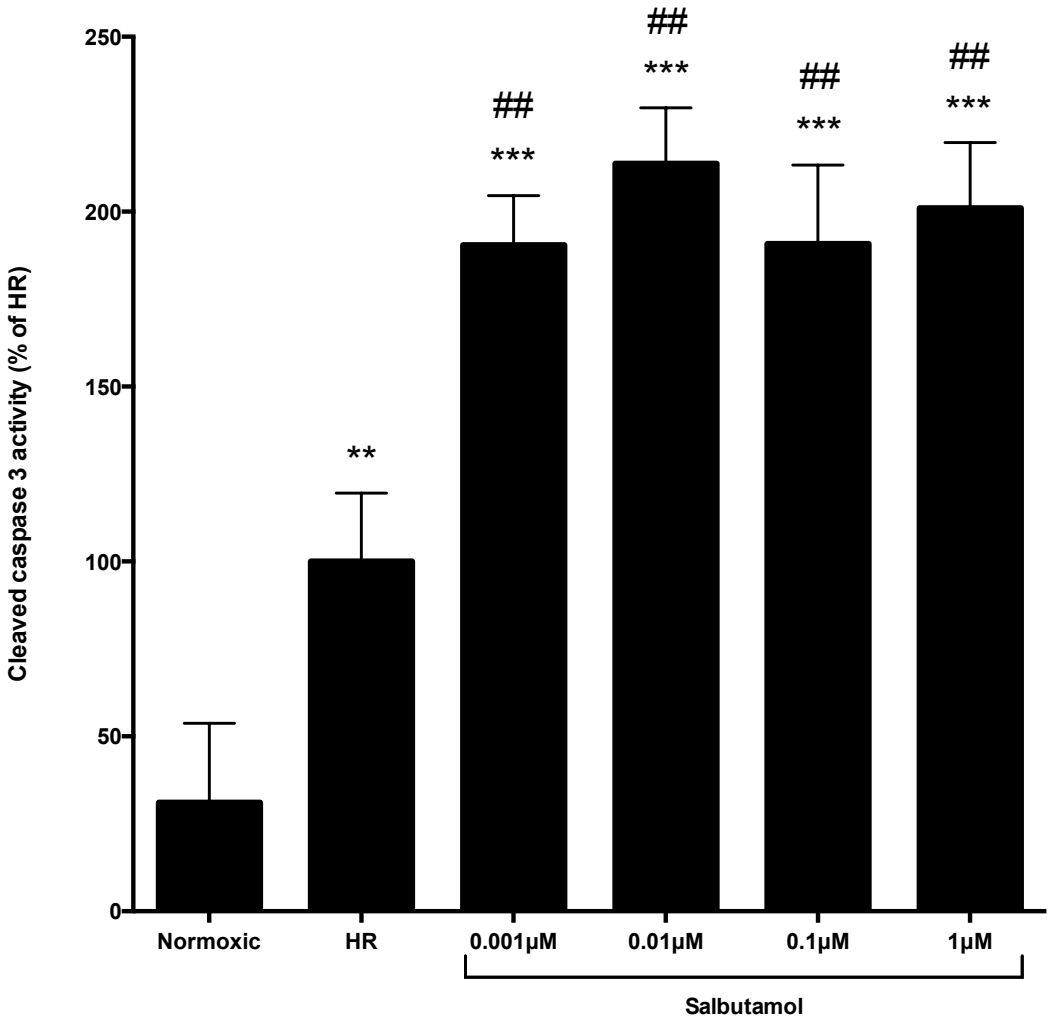


Figure 4.14 The effects of Salbutamol (0.001µM – 1µM) administered throughout re-oxygenation on expression of cleaved caspase 3 in cardiomyocytes treated for 2 hours in hypoxia and 4 hours of re-oxygenation. Data presented as mean ±SEM. n=6-8. ***p<0.001 vs. Normoxic, **p<0.01 vs. Normoxic, $^{\#\#}$p<0.01 vs. HR.

4.4.5 Cytometric effects of Salbutamol on isolated cardiomyocytes with co-administration of Salbutamol with PI3K inhibitor Wortmannin

Isolated cardiomyocytes were exposed to hypoxia for 2 hours followed by 4 hours re-oxygenation in the absence or presence of Salbutamol (0.1μM) co-administered with the PI3K inhibitor Wortmannin (0.1μM) throughout the re-oxygenation period. Assessment of cell viability was undertaken and a significant decrease in MTT reductase activity was observed in cardiomyocytes in the presence of Salbutamol (0.1μM) when compared to normoxic and HR cardiomyocytes (Normoxic, 209 ± 8% $p<0.001$, HR, 100 ± 2% vs. 75 ± 1%, $p<0.05$, Figure 4.15). Cardiomyocytes in the presence of the PI3K inhibitor Wortmannin and Salbutamol recorded a significant increase in MTT reductase activity when compared to Salbutamol alone (SalB + Wort, 96 ± 2% vs. 75 ±1%, $p<0.01$, Figure 4.15)

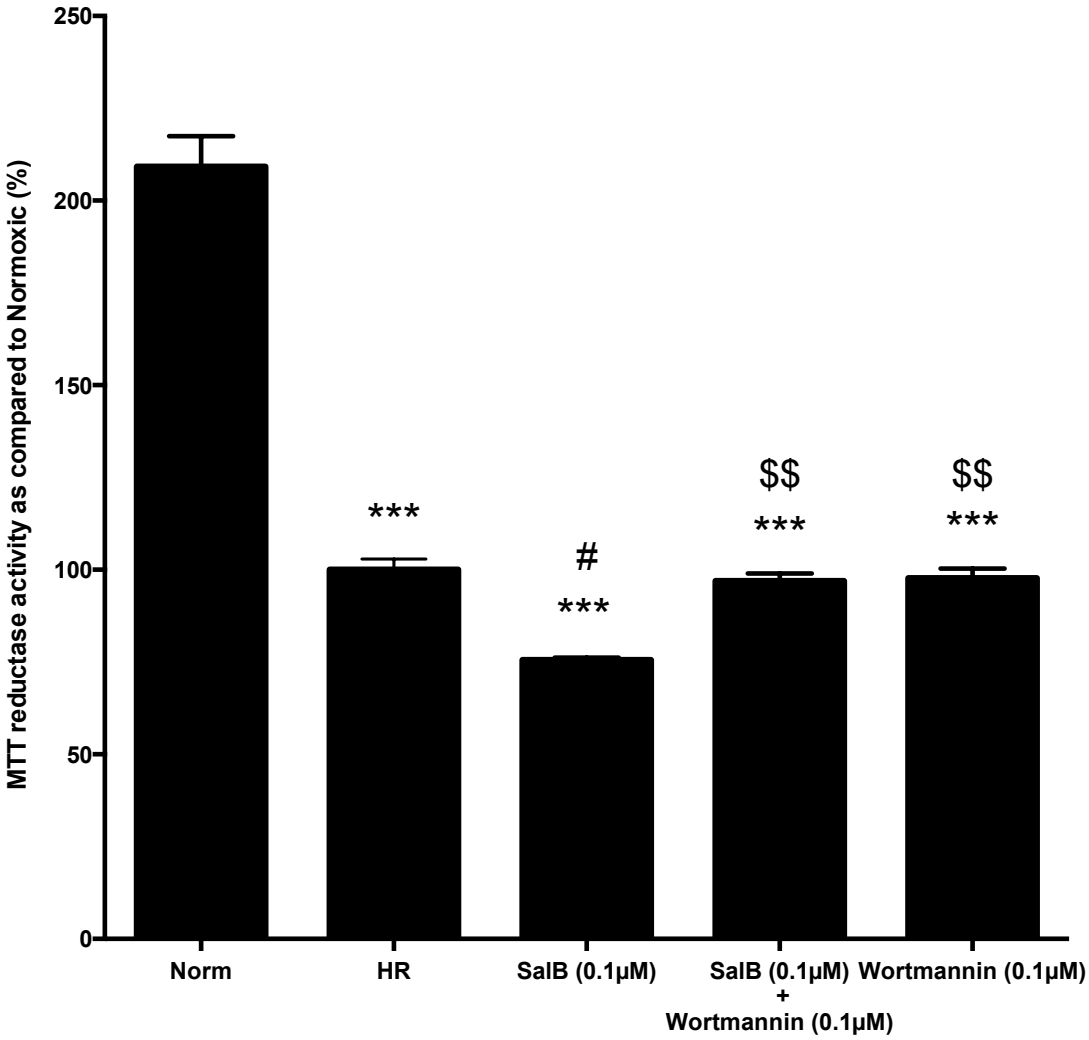


Figure 4.15 MTT reductase activity in cardiomyocytes exposed for 2 hours hypoxia and 4 hours re-oxygenation where Salbutamol (0.1) was added throughout re-oxygenation to cardiomyocytes treated with Wortmannin (0.1µM). Data presented as mean ± SEM. n=6-8. ***p<0.001 vs. Normoxic, $^{\#}$p<0.05 vs. HR, $^{\$\$}$p<0.01 vs. 0.1µM.

4.4.5.1 The effects of Salbutamol co-administered with PI3K inhibitor Wortmannin on the signaling protein p-Akt in a model of ischaemia reperfusion by assessment of western blotting

Hearts were subjected to 35 minutes of ischaemia followed by 20 minutes of reperfusion and were administered with Salbutamol (0.1μM) at the onset and throughout reperfusion. The effect of Wortmannin (0.1μM) alone on the expression of Akt significantly decreased when compared to IR control hearts (41 ± 4% vs. 100 ± 12%, $p<0.05$, Figure 4.16). Interestingly, p-Akt expression in hearts treated with Salbutamol (0.1μM) alone was significantly higher when compared to hearts administered with a combination of Salbutamol (0.1μM) and Wortmannin (0.1μM) (240 ± 7% vs. 114 ± 18%, Figure 4.16). Normoxic, control IR 20 minute data included has been used from previous experiment.

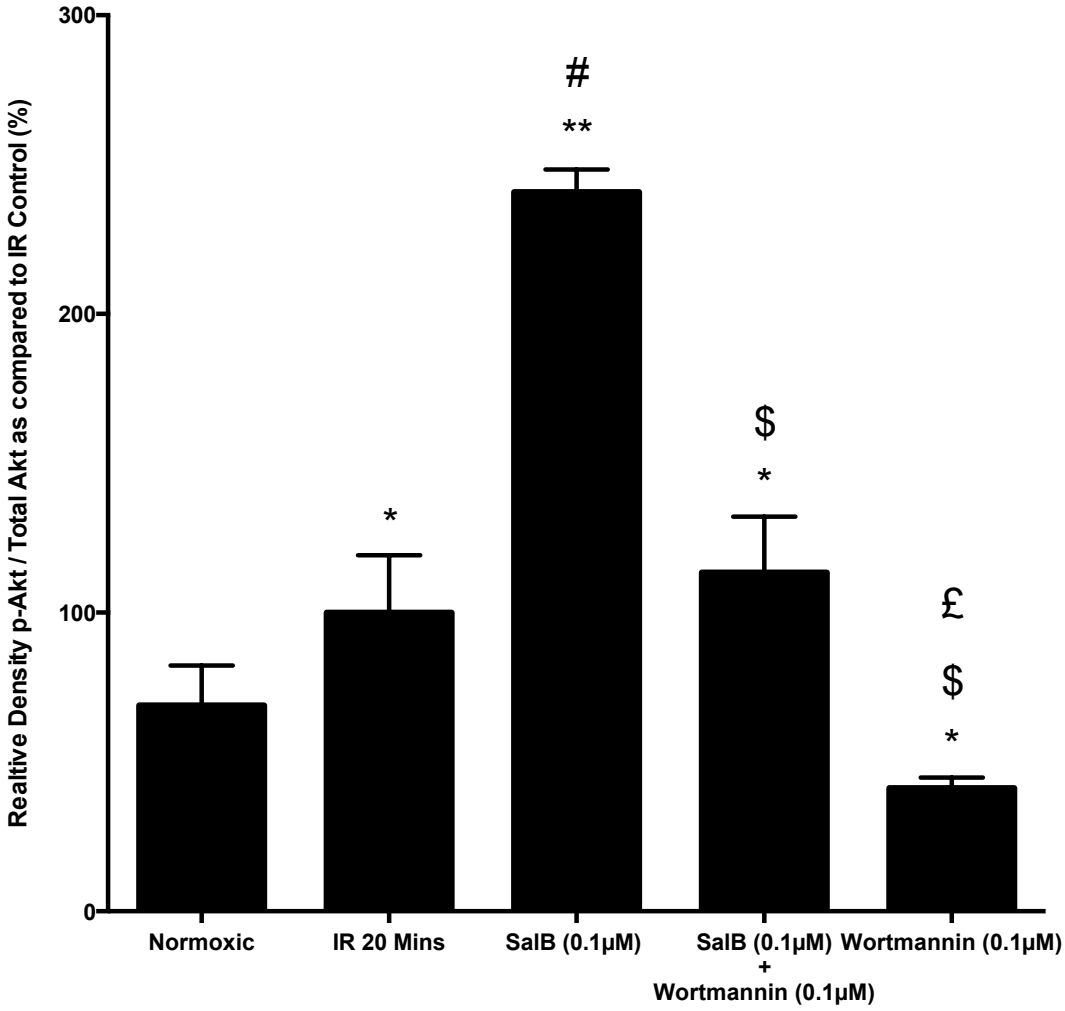


Figure 4.16 The effects of Salbutamol (0.1µM) administration at the onset of reperfusion on the expression of phosphorylated Akt (Ser473) after exposure to 35 minutes ischaemia and 20 minutes of reperfusion in hearts treated in the presence or absence of Wortmannin (0.1µM). Data presented as mean ±SEM. n=3. **p<0.01 vs. Normoxic, *p<0.05 vs. Normoxic, $^{\#}$p<0.05 vs. IR 20 Mins, $^{\$}$p<0.05 vs. 0.1µM, $^{£}$p<0.05 vs SalB + Wort.

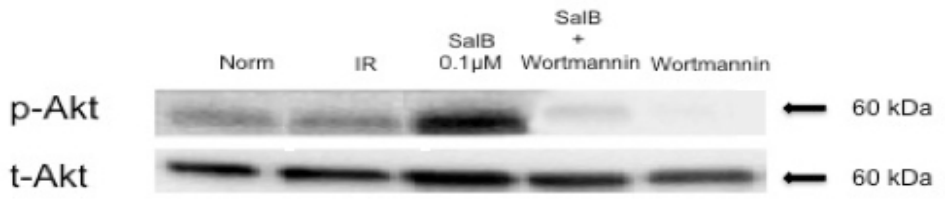


Figure 4.17 Representative blot of p-Akt and t-Akt when Salbutamol (0.1µM) was administered throughout reperfusion for 20 minutes after 35 minutes ischaemia in the presence and absence of Wortmannin (0.1µM).

4.4.5.2 The effect of co administration Salbutamol and PI3K inhibitor Wortmannin on signalling protein cleaved caspase 3 by assessment of flow cytometry

Isolated cardiomyocytes were exposed to hypoxia for 2 hours followed by 4 hours re-oxygenation in the absence and presence of Salbutamol (0.1μM) throughout the re-oxygenation period. Cardiomyocytes were also treated in the presence or absence of the PI3K inhibitor Wortmannin (0.1μM). Assessment of expression of cleaved caspase 3 was done by flow cytometric analysis.

A significant increase in cleaved caspase 3 levels was recorded in HR control groups when compared to the normoxic group (100 ± 3% vs. 23 ± 13%, $p<0.05$).

Cleaved caspase 3 expression in cardiomyocytes co-administered with Wortmannin (0.1μM) and Salbutamol (0.1μM) showed a significant increase when compared to normoxic cardiomyocytes (102 ± 23% vs. 23 ± 13%, $p<0.05$). A significant increase in levels of caspase 3 was recorded when comparing the adjunct administration of Salbutamol (0.1μM) and Wortmannin (0.1μM) with HR control cardiomyocytes (102 ± 23% vs. 136 ± 24%, $p<0.05$). Interestingly cardiomyocytes treated with Wortmannin (0.1μM) alone showed a significant increase in expression of cleaved caspase when compared to HR control cardiomyocytes (136 ± 24% vs. 100 ± 3%, $p<0.01$, Figure 4.18).

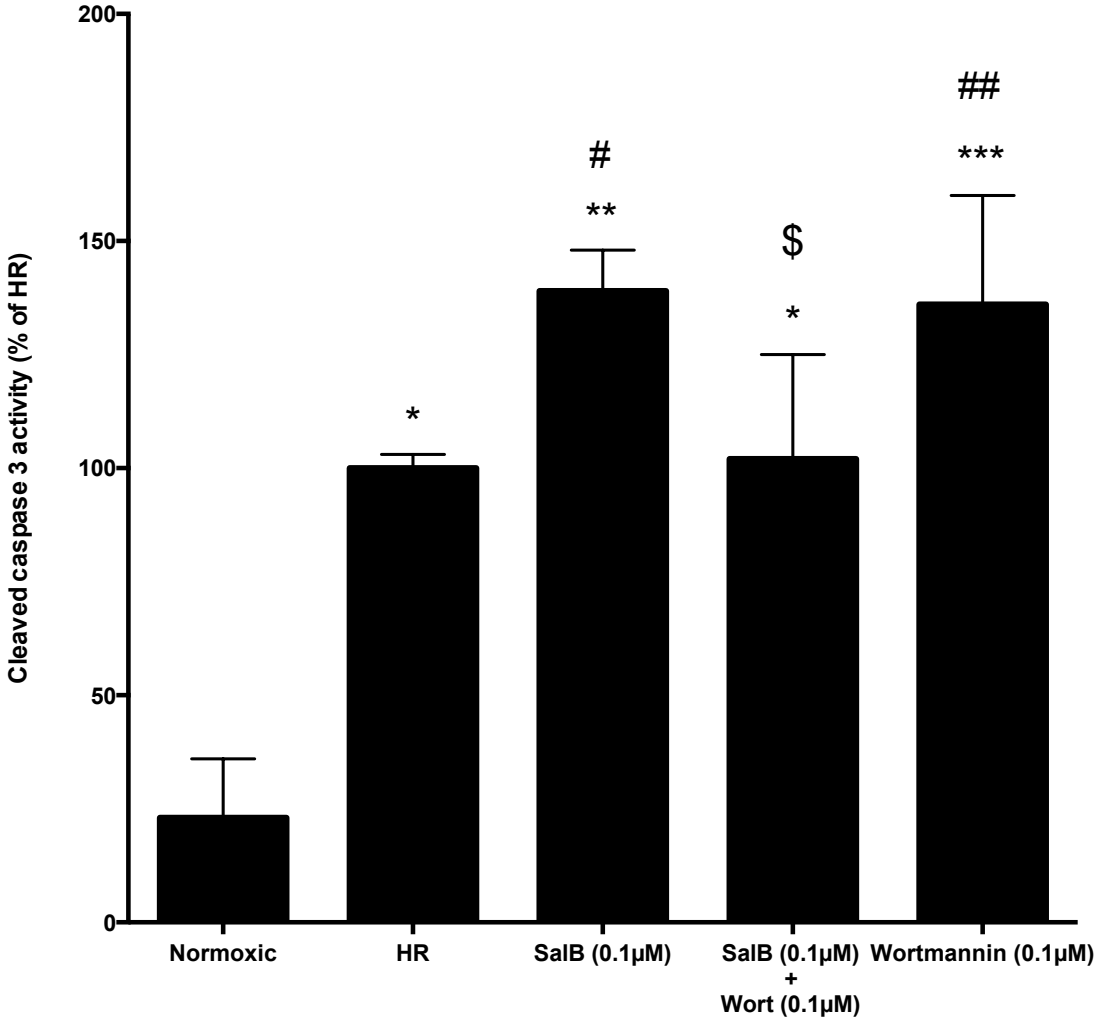


Figure 4.18 The effects of a Salbutamol dose response (0.1μM) on expression of cleaved caspase 3 in the presence of PI3K inhibitor Wortmannin (0.1μM). Data presented as mean ±SEM. n=6-8. ***p<0.001 vs. Normoxic **p<0.01 vs. Normoxic, *p<0.05 vs. Normoxic, $^{\#\#}$p<0.01 vs. HR, $^{\#}$p<0.05 vs. HR, $^{\$}$p<0.05 vs. Wortmannin (0.1μM).

4.4.6 Cytometric effects of Salbutamol on isolated cardiomyocytes with co-administration of Salbutamol with MAP Kinase Kinase inhibitor U0126

A significant increase in MTT reductase activity was observed with co-administration of U0126 and Salbutamol (0.1µM) when compared to cardiomyocytes administered with Salbutamol (0.1µM) alone (SalB + U0126, 96 ±2% vs. 75 ±1%, p<0.01, Figure 4.19).

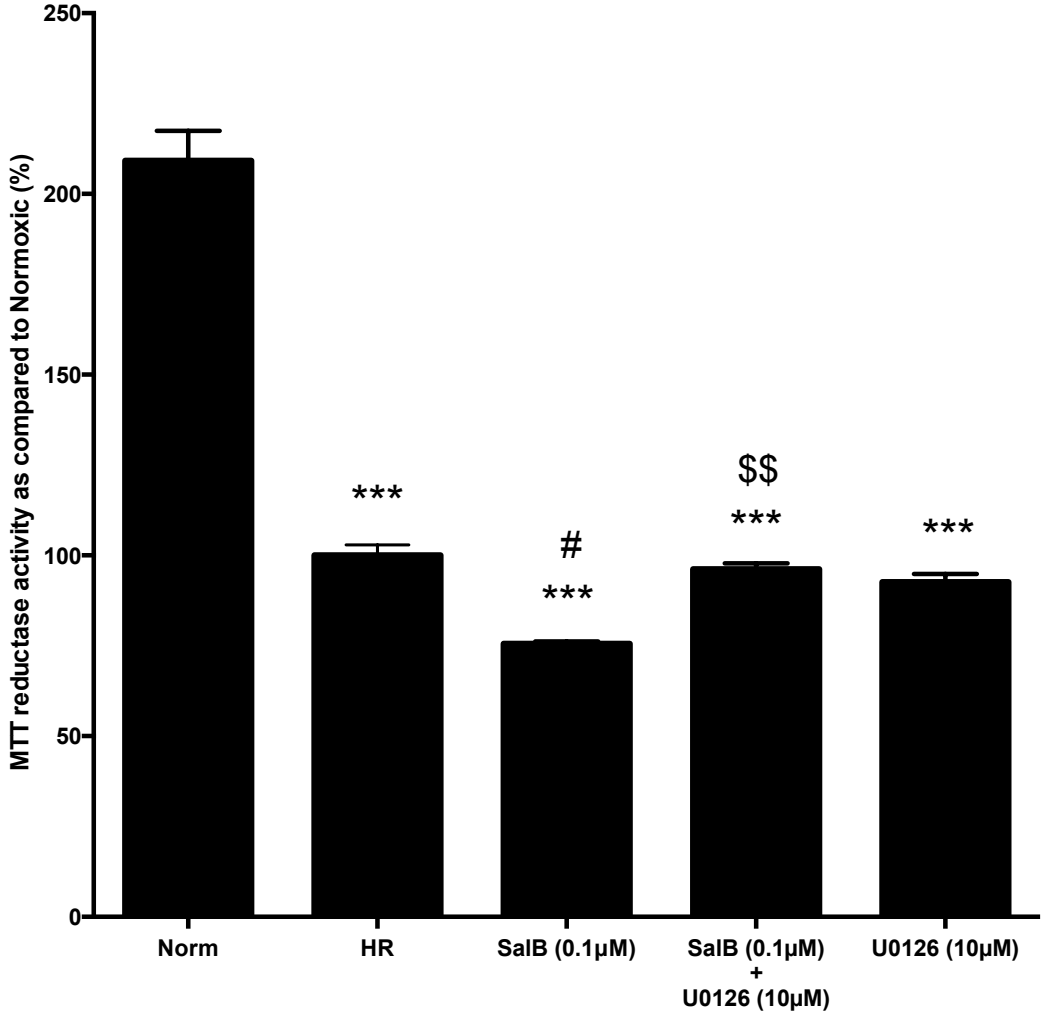


Figure 4.19 The MTT cytotoxic effect of Salbutamol (0.1µM) on the viability of cardiomyocytes in the presence of U0126 (10µM). Data presented as mean ± SEM. n=6-8. ***p<0.001 vs. Normoxic, $^{\#}$p<0.05 vs. HR, $^{\$\$}$p<0.01 vs. 0.1µM.

4.4.6.1 The effects of Salbutamol co-administered with MAP Kinase Kinase inhibitor U0126 on the signaling protein p-Erk in a model of ischaemia reperfusion by assessment of western blotting

Hearts were subjected to 35minutes of ischaemia followed by 20 minutes of reperfusion in the presence of Salbutamol (0.1μM) at the onset and throughout reperfusion. Hearts treated alone with the Erk inhibitor U0126 (10μM) showed a significant decrease in expression of p-Erk when compared to both IR time matched control hearts (U0126, 13 ±8% vs. 100 ±25%, $p<0.05$). Interestingly, p-Erk expression significantly increased with co administration of U0126 (10μM) with Salbutamol (0.1μM) when compared to hearts administered with Salbutamol alone (0.1μM) (SalB + U0126, 77 ±11% vs. 13 ±6%, $p<0.001$, Figure 4.20). Normoxic and control IR 20 data included has been used from previous western blots.

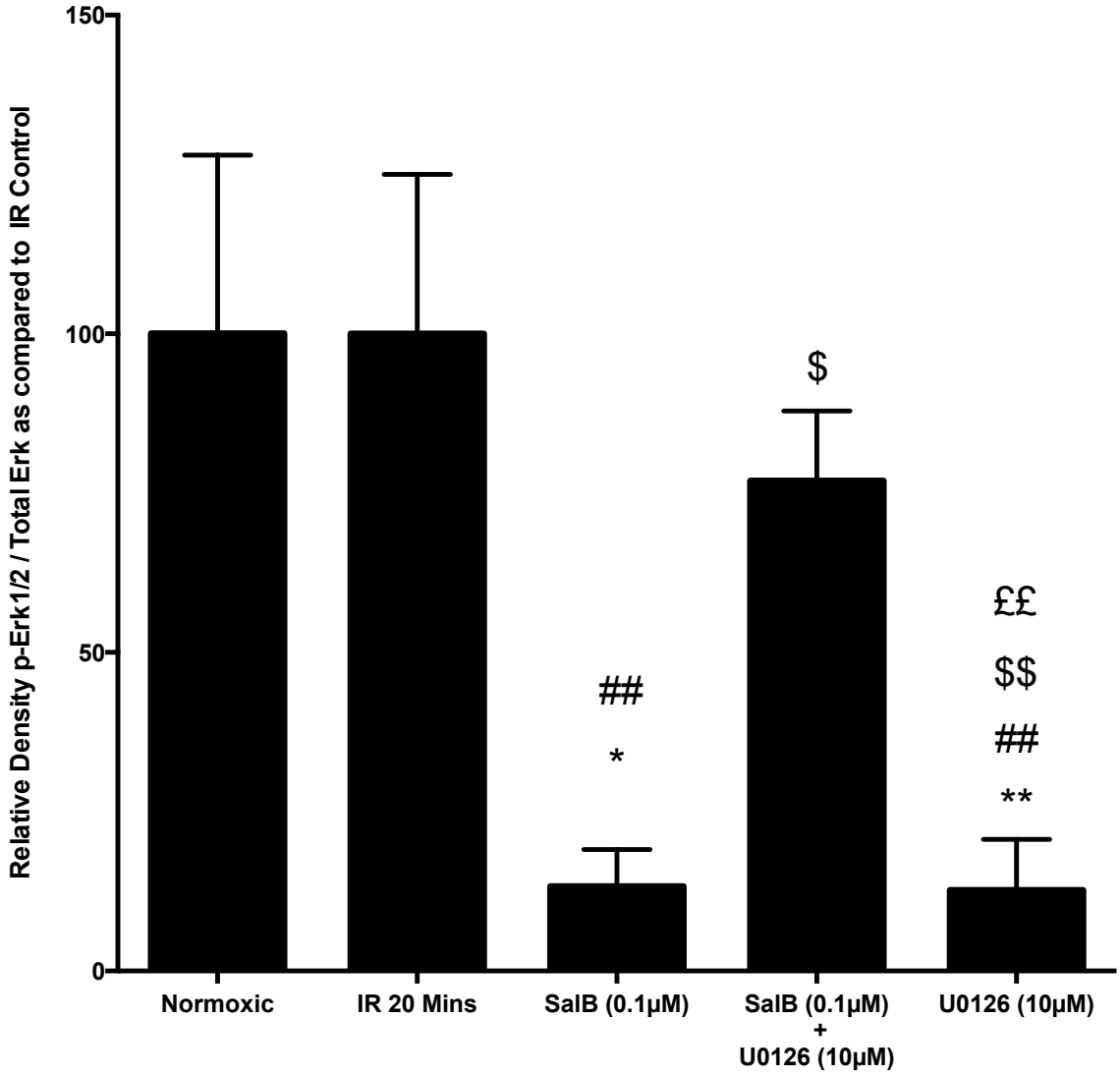


Figure 4.20 The effects of Salbutamol (0.1µM)) on the expression of phosphorylated Erk 1/2 (p44/p42) after exposure to 35 minutes ischaemia and 20 minutes of reperfusion in the absence and presence of Erk inhibitor U0126 (10µM). Data presented as mean ±SEM. n=3. ***p<0.01 vs. Normoxic, **p<0.01 vs. Normoxic, $^{###}$p<0.001 vs. IR 20 Mins, $^{#}$p<0.05 vs. IR 20 Mins, $^{$$$}$p<0.01 vs. 0.1µM, $^{£££}$p<0.05 vs. U0126.

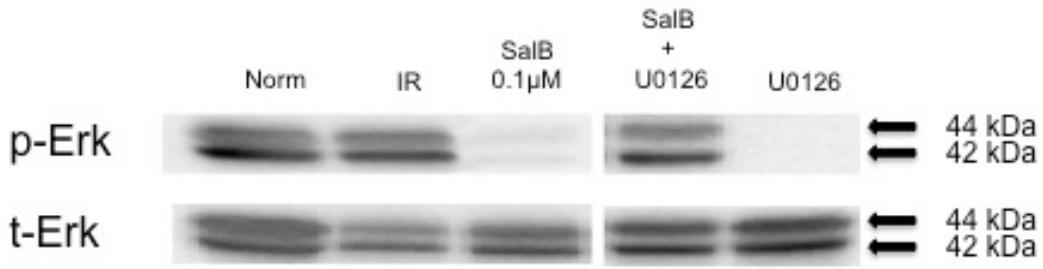


Figure 4.21 Representative blot of p-Erk and t-Erk in the presence of Salbutamol (0.1µM) when administered with or without U0126 (10µM)

4.4.6.2 The effect of co administration of Salbutamol and MAP Kinase Kinase inhibitor U0126 on signalling protein cleaved caspase 3 by assessment of flow cytometry

Isolated cardiomyocytes were exposed to hypoxia for 2 hours followed by 4 hours re-oxygenation in the absence and presence of Salbutamol (0.1μM) throughout the re-oxygenation period. Cardiomyocytes were also treated in the presence or absence of the Erk inhibitor U0126 (10μM). Assessment of expression of cleaved caspase 3 was done by flow cytometry analysis.

Cleaved caspase 3 expressions were significantly affected by co-administration of Salbutamol (0.1μM) and U0126 (10μM) in comparison to HR cardiomyocytes (SalB + U0126, 190 ±15% vs. 100 ±19%, $p<0.05$). However, no significant change was observed when comparing cardiomyocytes administered with Salbutamol (0.1μM) alone with cardiomyocytes co-administered with U0126 (10μM) (Figure 4.22).

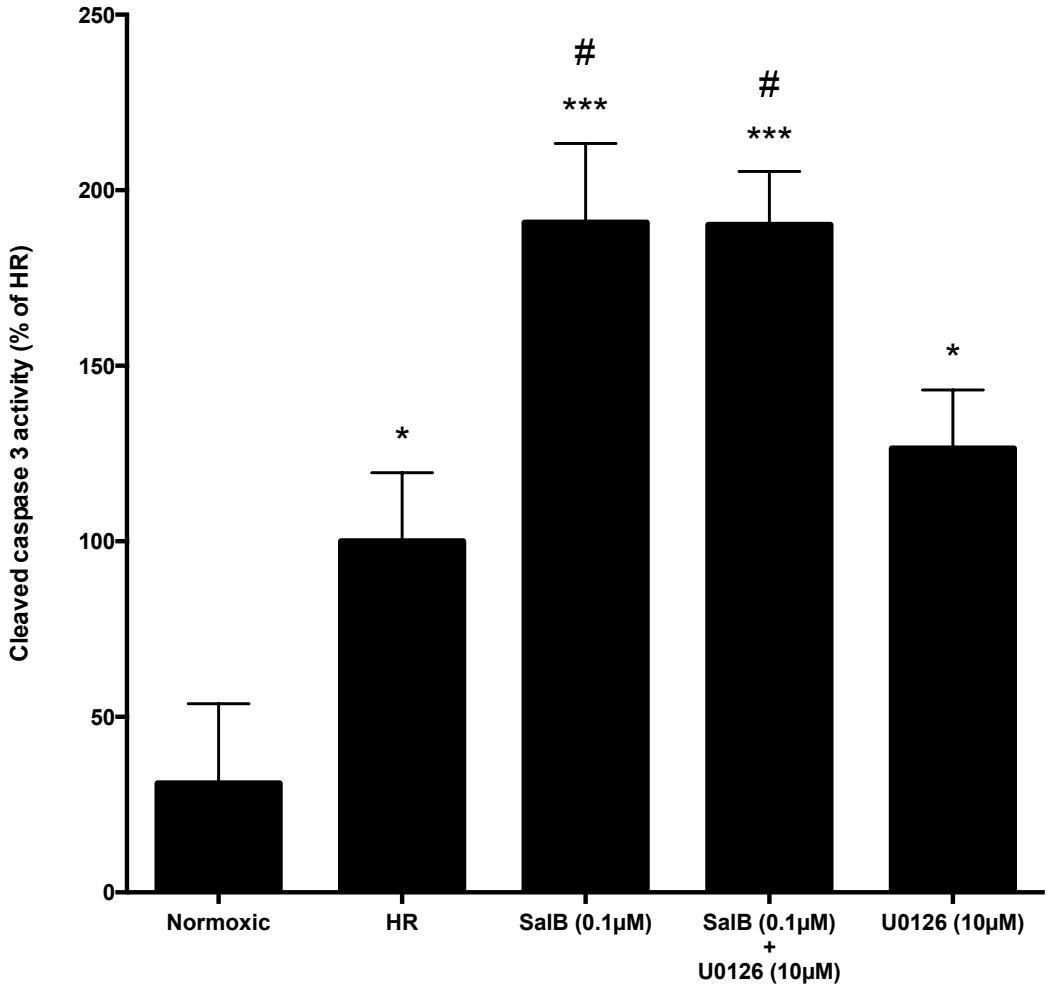


Figure 4.22 The effects of a Salbutamol dose response (0.1μM) on expression of cleaved caspase 3 in the presence of Erk inhibitor U0126 (10μM). Isolated myocytes were exposed to 2 hours hypoxia followed by 4 hours reoxygenation in the presence or abscene of the drug. Data presented as mean ±SEM. n=6-8. $^{***}p<0.01$ vs. Normoxic, $^{*}p<0.05$ vs. Normoxic, $^{\#}p<0.05$ vs. HR.

4.5 Discussion

In Chapter 3 the effects of long and short acting beta agonists on the whole heart and their effects on the mPTP were investigated. From Chapter 3, Salbutamol as a short acting beta agonist was identified to exacerbate myocardial injury in the model of ischaemia reperfusion injury. In this chapter we investigated the signalling pathways associated with Salbutamol mediated injury in ischaemia reperfusion.

In this chapter we demonstrate Salbutamol administered to isolated adult cardiomyocytes in conditions of hypoxia and re-oxygenation caused a reduction in the viability of cardiomyocytes. We also demonstrate the varying effects that Salbutamol had on the signalling proteins p-Akt, p-Erk and cleaved caspase 3 in the presence and absence of the Akt and Erk pathway inhibitors, Wortmannin and U0126.

4.5.1 The effect of Salbutamol on cardiomyocytes and the PI3K/Akt signalling pathway

Cardiomyocytes are dependent on oxidative phosphorylation, which provides up to 95% of the required energy for contraction and metabolism (Chiong et al., 2011). During hypoxic conditions cardiomyocyte function is hindered with a dramatic decrease in ATP levels due to an increase in anaerobic respiration and are countered by an increase in AMP levels (Matsui et al., 2007). In such conditions, cardiomyocytes undergo cell death via apoptosis or necrosis, however the clarity between the choices of type of cell death during early stages of HR is still unclear (Hausenloy and Yellon, 2004, Yan et al., 2005, Zhao et al., 2001).

Cardiomyocytes exposed to HR conditions in the presence of Salbutamol were shown to have a cytotoxic effect reducing the viability of cardiomyocytes when compared to control HR cardiomyocytes (Figure 4.1). A range of Salbutamol doses (0.001μM-1μM) were used in these experiments. As mentioned previously, 0.1μM Salbutamol was recorded in our studies

to induce the most significant myocardial injury at the lowest concentration of Salbutamol administered (Figure 3.16). For this reason, Salbutamol 0.1μM was used as the standard concentration for all experiments. Increasing concentrations of Salbutamol up to 1μM is well within previous studies that have administered Salbutamol before observing any detrimental effects (Gao Smith et al., 2012). Although the use of the MTT assay enables detection of cell viability, it is unable to detect what stage of cellular injury is occurring or determine the type of cell death i.e. apoptosis or necrosis (Gomez et al., 1997, Piper et al., 1984). As a result we were unable to determine a more accurate measure of cells that may have been in early phases of apoptosis or necrosis to include in the results.

Investigation into levels of p-Akt expression showed that Salbutamol at higher concentrations (0.01μM, 0.1μM & 1μM), significantly elevated expression of p-Akt in hearts reperfused for 20 and 120 minutes, but no elevation was observed in hearts reperfused for 5 minutes. Acute activation of the PI3K/Akt pathway in some studies has been shown to protect against IR injury by recruitment of Akt, an anti-apoptotic/ pro survival protein (Hausenloy and Yellon, 2004, Fujio et al., 2000). A variety of factors are capable of inducing cardioprotection against IR injury such as growth hormones and cellular stresses, which have all been shown to activate the common downstream target Akt (Matsui and Rosenzweig, 2005). Previous in vitro studies with adenoviral expression of PI3K in rat cardiomyocytes showed a reduction in HR induced apoptosis (Dhanasekaran et al., 2008). Downstream targets of Akt phosphorylation, such as Bad, have been shown to locate subcellularly and bind with 14-3-3 proteins causing inhibition and restricting them within the cytoplasm of the cell. Inhibition of Bad by phosphorylation at the serine^{136} site prevents dephosphorylation of Bad to occur and not to activate downward cascade protein targets such as Bax or Bak (Mullonkal and Toledo-Pereyra, 2007, Kim et al., 2001). Isolated rat hearts excised are immediately submerged in

ice-cold KH buffer to slow down metabolic rate. Although time was minimised to attach the heart to the Langendorff apparatus, whilst submerged in the KH buffer, global ischaemia may occur (Bell et al., 2011). The recorded elevated expression levels of p-Akt and p-Erk observed in our studies at 5 minutes perfusion may be explained by this phenomenon as shown by previous groups (Schwartz and Lagranha, 2006, Yellon and Hausenloy, 2007). These groups demonstrated with short spells of ischaemia followed by reperfusion expression of p-Akt and p-Erk elevated. Initial trauma to the heart after excision may also activate pro-survival proteins. Our time matched controlled normoxic hearts exposed to 20 and 120 perfusion showed decreasing expression levels of p-Akt (Figure 4.4, Figure 4.6). As hearts were perfused for longer periods of time i.e. 20 and 120 minutes, levels of p-Akt were reduced. This was also observed in expression levels of p-Erk (Figure 4.8, Figure 4.10, Figure 4.12). Studies in transgenic mouse hearts have demonstrated initial elevated levels of p-Erk and p-Akt, which then declined over time. The consequence of such down regulation of both these proteins lead to increased myocyte apoptosis (Li et al., 2009). The integrity of normoxic hearts will naturally degrade as a result of increasing levels of necrotic cell death over the period of perfusion (Bell et al., 2011).

Interestingly, expression of p-Akt in IR time matched control hearts compared to Salbutamol (0.1μM) at 20 and 120 minutes showed a significant increase. Our findings support previous work presenting elevated expression of p-Akt in models of IR injury/HR conditions that cause no protection against injury such as studies carried out by Gharanei and colleagues with the use of the anti cancer drug Doxorubicin. Here the authors showed an increase in reperfusion injury in the Langendorff model in addition to elevated p-Akt levels with Doxorubicin (1μM) (Gharanei et al., 2013). Other studies involving the non-selective βAR

Isoproterenol also have been shown to elevate p-Akt expression in addition to causing hypertrophy of mouse cardiomyocytes (Condorelli et al., 2002).

As shown previously in Chapter 3, an increase in infarct to risk ratio occurs in the presence of Salbutamol (0.1μM) exacerbating injury to the rat heart. Further to this we now have demonstrated in the presence of Salbutamol (0.1μM) expression of p-Akt increases. This provides an alternative concept of the perception that p-Akt can solely be an anti-apoptotic signalling protein but may in fact contribute to the exacerbation of myocardial injury as observed in the previous chapter.

Nagoshi and colleagues (2005) showed that prolonged or repetitive activation of Akt could lead to increased IR injury mainly through feedback inhibition of upstream pathways such as PI3K (Nagoshi et al., 2005). They further showed with the use of transgenic mice overexpressing Akt demonstrated increased LVDP, coronary flow (also recorded in Chapter 3) in addition to increased reperfusion injury.

Other studies have used transgenic mice with specific cardiac activation of Akt causing an increase in mortalities. One of these studies by Matsui and colleagues (2005) established an overexpression of active Akt increased mortalities in mice as a result of cardiac enlargement in the form of hypertrophy (Matsui and Rosenzweig, 2005). Other detrimental cardiac dysfunctions resulting from chronic p-Akt expression included increase in I/R ratios (Matsui and Rosenzweig, 2005, O'Neill and Abel, 2005).

Salbutamol's effect on haemodynamics (Chapter 3) showed a dose dependent increase in LVDP, which can cause an increased pressure overload on cardiac function, specifically

within the left ventricle. Similar results have been shown to encourage hypertrophy in both a maladaptive and adaptive manner (Nagoshi et al., 2005, Shiojima et al., 2005). The activation of p-Akt by Salbutamol directly occurs via activation of βARs that are coupled to GPCRs, specifically Salbutamol's selective activation of β_2ARs is linked to the G_i subunit which is linked to the regulation and activation of Akt. (Larocca et al., 2011).

Wortmannin was used as an irreversible non-selective PI3K inhibitor. Previous studies have successfully blocked the PI3K pathway in rat hearts with Wortmannin (0.1μM) (Ravingerova et al., 2009). In keeping with this, a 0.1μM concentration of Wortmannin was used in all experimental protocols. With the inhibition of the PI3K pathway by Wortmannin (0.1μM) in the presence of Salbutamol (0.1μM), expression of p-Akt was significantly reduced close to expression levels observed in IR time matched control hearts. Hearts administered with Wortmannin (0.1μM) alone decreased expression of p-Akt significantly more than hearts co-administered with Salbutamol and Wortmannin, confirming that activation of βARs with Salbutamol (0.1μM) is linked to the increased expression of p-Akt via the PI3K pathway (Figure 4.16). Normal activation of the PI3K has been shown to promote cell survival by inhibiting apoptosis by increased expression of p-Akt (Jeong et al., 2012). Hearts treated alone with Wortmannin showed a significant decrease ($p<0.05$) in p-Akt expression when compared to normoxic hearts. This supports previous findings of the inhibitory properties of Wortmannin and demonstrates in our experiments that inhibition of the PI3K pathway is successful.

Interestingly, hearts treated with Wortmannin (0.1μM) and Salbutamol (0.1μM) had p-Akt expression elevated higher than compared to hearts treated alone with Wortmannin (0.1μM) indicating an alternative pathway may be involved in the activation of p-Akt, independent of

the PI3K pathway. This mechanism of elevating p-Akt indirectly may be that of a completely independent pathway or via cross-talk.

To establish a potential link of cross-talk between p-Akt and another signalling pathway, hearts were treated in the presence of the Erk inhibitor U0126 (10μM) and Salbutamol (0.1μM). Previous studies have used U0126 successfully to inhibit Erk activation in the Langendorff model (Hussain et al., 2014). From these studies the concentration of 10μM was used throughout the experimental protocol.

4.5.2 The effect of Salbutamol on phosphorylated Erk 1/2 (p44/p42) MAPK pathway

Activation of p-Erk is also linked to prompting cell survival via the RISK pathway and has been shown to activate through growth hormone receptors via activation of the Ras/Raf pathway and GPCRs (Armstrong, 2004, Mendoza et al., 2011). The Erk 1/2 signalling pathway has been shown to be an anti-apoptotic pathway and linked to cardioprotection of the heart (Lu and Xu, 2006, Hausenloy et al., 2005). We demonstrated a varying effect of Salbutamol (0.001-1μM) on expression of phosphorylated Erk. Interestingly, expression of p-Erk in the presence of Salbutamol (0.1μM) significantly decreased when compared to IR time matched controls, implying Salbutamol's activation of the β_2ARs does not recruit p-Erk signalling.

p-Erk's role is similar to that of p-Akt, inhibiting downstream targets such as the phosphorylation of Bad, the difference being p-Erk phosphorylates Bad at Serine^{112} in contrast to p-Akt's phosphorylation of Bad at Serine^{136} (Datta et al., 2000, Tan et al., 1999). Interestingly, p-Erk expression in the presence of Wortmannin (0.1μM) increased significantly when compared to time matched IR control hearts (data not shown). We can

purport that upon administration of Salbutamol (0.1μM) in the absence of PI3K pathway, increase in p-Erk expression compensates for the inhibition of p-Akt expression to promote cardiomyocyte survival. We can postulate this shift from p-Akt activation to increased Erk activation may be a deliberate protection response of the heart in order to minimise the stresses observed by IR injury in order to continue the phosphorylation of Bad.

Contrasting effects have been shown for Erk 1 and Erk 2 pathways, each identified having a different part to play on cell survival. In vivo studies expressing only the Erk2 pathway increased infarct to risk ratio in mice after IR, in addition, Erk1 null mice showed similar levels of infarct to risk ratio in wild type mice (Lips et al., 2004, Pearson et al., 2001). Interestingly, we have seen in both p-Akt and p-Erk studies carried out, a delay in phosphorylation of both proteins. Ischaemia alone has previously been shown not to be enough of an insult to the heart to elevate p-Akt or p-Erk expression significantly, however introduction of a reperfusion period greater than 10 minutes increases these proteins expression (Armstrong, 2004, Omura et al., 1999). One study in an in vivo rat model of IR, showed levels of p44 Erk to decrease during ischaemia, followed by an increase in Erk after reperfusion for 30 minutes (Omura et al., 1999).

Further investigation into the involvement of p38 inducing apoptosis in rat cardiomyocytes may suggest a reason for the observed reduction in Erk expression in the presence of Salbutamol in a cross-talk dependent mechanism as elevated levels of p38 expression has been shown to inhibit p-Erk via serine-threonine protein phosphatase 2A (PPA2)(Liu and Hofmann, 2004, Zhou et al., 2002).

Having established both p-Akt and p-Erk to be active in the presence of Salbutamol (0.1μM) and IR conditions, previous studies have suggested a favourable response upon activation of these proteins via the RISK pathway to initiate cardioprotection (Hausenloy and Yellon, 2004, Hausenloy et al., 2005, Maddock et al., 2002). In contrast to these findings, we have demonstrated a detrimental effect on the heart with activation of these proteins from our studies with cardiomyocyte cytotoxicity, mPTP and infarct to risk ratio (Chapter 3).

A further similarity between p-Akt and p-Erk is a link to hypertrophy, which we have already discussed in response to elevated Akt expression. βARs stimulation has shown to lead to p-Erk induced hypertrophy in rodent models in vitro and in cultured cardiomyocytes, brought about by the Ras-Erk signalling cascade (Bueno and Molkentin, 2002, Kim et al., 2008, Yamazaki et al., 1993).

During IR conditions, hypertrophy may manifest due to the similarity of conditions such as calcium overload as was investigated by Allard and colleagues using the calcium channel blocker Verapamil (Allard et al., 1994). They found in rodent hypertrophied hearts, an increase in ventricular events occurred with IR conditions. In contrast, when administering calcium antagonists such as Verapamil, a reduction in reperfusion injury events was observed highlighting a role of Calcium in a model of IR injury. (Allard et al., 1994, Baxter and Yellon, 1992, Baxter and Yellon, 1993).

4.5.3 Cross-talk between the signalling cascades of p-Akt, and p-Erk

We have observed behaviours of signalling proteins to act differently from what has been considered to be the 'norm' based on previous studies, such as cardioprotection. Signalling

cascades are still not fully understood, however evidence from our studies propose an interaction between the PI3K pathway and p-Erk pathway influencing each other in a positive or negative manner, which is referred to as cross-talk (Mendoza et al., 2011, Yang et al., 2011).

Increased levels of p-Akt in addition to decreased expression of p-Erk in the presence of Salbutamol (0.1μM) were recorded, which confirms that Salbutamol's interaction at β_2ARs has the ability to increase p-Akt more readily than p-Erk. There is evidence of suppression of p-Erk by overexpression of p-Akt, which was also seen by Moelling and collegaues (Moelling et al., 2002). Moelling and colleagues showed Raf-Akt cross-talk can be regulated in a concentration dependent manner, and found rapid activation of p-Akt with insulin growth factor 1 supressed Raf kinase activity via phosphorylation of serine^{259}. A potential cross-talk mechanism between these cascades has been linked to the ability of Akt to negatively regulate Erk by the abrogation of the Raf-Erk cascade (Mendoza et al., 2011, Suire et al., 2002).

Collectively, our data confirms upon administration of Salbutamol in IR conditions, an increase in p-Akt is observed. Through p-Akt's high level of expression, cross talk exists between the Akt-Erk pathways in a suppression manner as seen by their respective protein expression levels. A common link in mechanisms involving coronary heart failure, ischaemia reperfusion injury, hypertrophy and cardiomyopathy involve Akt in an over expressive manner. However, the selectivity of Salbutamol needs to be investigated to clarify through which GPCR subunit the discussed signalling proteins are activated as contrasting literature indicates specific recruitment of these signalling proteins via the G_s or G_i subunits, both of

which are linked to β_2ARs in addition to activation of the G_s subunit solely through β_1AR stimulation.

5 Chapter 5: Role of β_1 Adrenergic Receptor Signalling in Salbutamol Mediated Injury In The Presence of β_1 Adrenergic Receptor Antagonist CGP 20712

5.1 Introduction

Data from the previous chapter (Chapter 4) demonstrated Salbutamol mediated exacerbation of I/R injury and associated with cell signalling mechanisms and potential crosstalk between these signalling mechanisms in isolated rat heart that may contribute to the toxic effects of Salbutamol. However, how these signalling proteins are activated in the presence of Salbutamol still remains unclear due to the complexities of β adrenergic receptor activation, coupling and signalling. In this chapter we specifically determine the role of the β_1 adrenergic receptor (β_1ARs) in Salbutamol mediated injury using the β_1AR antagonist CGP 20712 to investigate the selectivity of Salbutamol and how it affects signalling proteins and cytotoxicity via β_2 adrenergic receptor (β_2ARs) activation.

The structure of β_1ARs has been closely linked to the structure of β_2ARs due to the latter being the first GPCR to be successfully cloned (Dixon et al., 1986, Steinberg, 1999). More recent crystallography studies have determined a 67% identical similarity between β_1 and β_2 receptors in the human heart (Warne et al., 2008). Differences between βAR structures are mainly determined by their extracellular cytoplasmic loops (Scarselli et al., 2007). Cytoplasmic loops (CL) 2 and 3 of GPCRs are responsible for ligand interaction, selection and activation of the receptor (Warne et al., 2008). The CL2 within β_1ARs forms a short α helix that allows the formation of hydrogen bonds between adjacent amino acids Tyr149 and the α helix3 located close to the membrane surface and more readily available in

cardiomyocytes than β_2ARs (Warne et al., 2008). Although similarities exist between the βARs there is still a specificity of agonists and antagonists for a particular βAR subtype, such as the antagonist CGP 20712 having over 500 times greater in affinity for β_1ARs than β_2ARs and the β_2 antagonist ICI 118,551 (Cherezov et al., 2007, Warne et al., 2008).

Acute or chronic activation of β_1ARs can bring about a variety of effects such as vasodilation, increase in heart rate and have been linked to necrotic and apoptotic cell death in cardiomyocytes (Communal et al., 1998, Communal et al., 1999, Zaugg et al., 2000). βAR activation is not restricted solely to selective β agonists, but can also be activated by non-selective β agonists such as Isoproterenol (Ruzsnavszky et al., 2014). Upon activation of β_1ARs by an agonist, such as Salbutamol, the G_s subunit of the GPCRs is activated initiating the cAMP/PKA pathway (Iwai-Kanai et al., 1999, Zhu et al., 2003).

Activation of this pathway promotes the increase of cytosolic calcium mediating an increase in heart rate and force of contraction (Zornoff et al., 2009). This pathway has been suggested to be responsible for cardiac apoptosis within the myocardium and cardiomyocytes. Zhu and colleagues (2003) with the use of β_2AR knockout mice, demonstrated that independent of the cAMP/PKA pathway, the activation of calmodulin kinase II (CaMKII) also initiates ventricular myocyte cell apoptosis in the presence of Isoproterenol (Zhu et al., 2003).

β_1ARs offer a range of therapeutic attributes whether it is through targeting by agonists such as Isoproterenol, Dobutamine or β-blocker drugs for heart failure, angina or hypertension (Wang et al., 2014). Overstimulation/activation of βARs can cause the receptors to be compromised and desensitise leading to a maladaptive response (Penn et al., 1999, Zhu et al., 2003). Activation of β_1ARs longer than 30 minutes has been shown to cause desensitisation

of the cAMP/PKA pathway, resulting in cardiac apoptosis via a cAMP/PKA independent pathway (Zhu et al., 2003).

Distribution of β_1ARs has been investigated through various techniques of radio ligand binding, immunohistochemistry and activation of cAMP (Cros and Brette, 2013, He et al., 2005). These studies have identified the majority of β_1ARs are located toward the cell crest namely the sarcolemma of ventricular myocytes and within t-tubules (Cros and Brette, 2013). The location and distribution of β_1ARs are important within cardiomyocytes for activation for chronotropic and inotropic effects, however as discussed, overstimulation can cause desensitisation of these receptors (Esposito et al., 2002). Conditions such as ischaemia reperfusion injury, congestive heart failure and myocardial infarction can also contribute to effecting β_1ARs on cardiac cell surfaces via remodelling (Lyon et al., 2009, Nikolaev et al., 2010). Cardiac remodelling covers a range of aspects such as coronary vessel remodelling and specific βAR remodelling that can be affected by stresses such as IR injury and myocardial infarction (Heusch, 2013, Yellon and Hausenloy, 2007). Remodelling of cardiomyocytes involves the redistribution of βARs previously located in 'normal' healthy locations to new positions. Although this remodelling process does not directly affect the function of β_1ARs, a linked effect is the redistribution of β_2ARs from deep within t-tubules to the cell crest shifting the ratio of β_1:β_2 in favour of an increased β_2AR expression (Heusch, 2013, Lyon et al., 2009).

Previously, we have seen the effect of Salbutamol on the mitochondrial permeability transition pore (mPTP) in a model of oxidative stress (Chapter 3). It has been established that the mPTP plays an important role in managing the contents of the mitochondria in both

normal and stress conditions such as increased ROS and increase in Ca^{2+} ions and is linked to cardiac apoptosis and necrosis (Baines, 2009, Machado et al., 2009). With the structure of the mPTP still unclear, a common postulation of its structure includes a voltage dependent anion channel, adenine nucleotide translocator and a matrix protein cyclophilin D (Baines, 2009, Javadov et al., 2009). The combination of a β_1AR antagonist CGP 20712 with Salbutamol will help identify if activation of β_2ARs plays a significant role in the detrimental effects of Salbutamol. Cellular stresses such as ROS and calcium overload have been shown to effect the expression levels of p-Akt and p-Erk, in particular during reperfusion injury (Hausenloy and Yellon, 2004, Schwartz and Lagranha, 2006). With the antagonist CGP 20712 present in hearts administrated with Salbutamol, the expression of these signalling proteins can be determined to see what, if any, effect they have on the Salbutamol administration in a model of ischaemia reperfusion injury.

5.2 Aims

The aims of the current study were to investigate the effects of the short acting β adrenergic receptor agonist Salbutamol by using the isolated perfused Langendorff heart model of ischaemia reperfusion injury, the oxidative stress cardiac myocyte model and the MTT assay. Salbutamol was administered in the presence and absence of the β_1AR antagonist CGP 20712 to determine the role of β_1AR signalling in Salbutamol induced myocardial injury.

5.3 Methods

5.3.1 Langendorff protocol

Briefly, Sprague-Dawley rats were sacrificed by cervical dislocation and cannulated to the Langendorff setup and perfused with KHB as described in section 2.3. Hearts were allowed to stabilise for 20 minutes followed by 35 minutes of regional ischaemia and 120 minutes of reperfusion. One minute before the onset of reperfusion hearts were administered Salbutamol (0.1μM) in the absence or presence of β Adrenergic Receptor antagonists CGP 20712 (0.0014μM). At the end of the experiment hearts underwent infarct to risk ratio analysis. Haemodynamic data were collected throughout the study.

For western tissue collection, hearts were reperfused with Salbutamol (0.1μM) for either 5, 20 or 120 minutes in the presence or absence of CGP 20712 (0.0014μM). After the time elapsed, hearts were removed and the left ventricle removed and snap frozen in liquid nitrogen.

5.3.2 Western blot analysis

Analysis of tissue by western blot was carried out as described in section 2.7. Briefly, following gel electrophoresis, proteins were transferred to a PVDF membrane and probed for the phosphorylated and total forms of the proteins: phospho-Akt (Ser_{473}) (1:1000) and phospho-p44/p42 (Erk 1/2, Thr202/Tyr204) (1:1000).

5.3.3 Adult rat cardiac myocyte isolation

Briefly, male Sprague Dawley rats were sacrificed by cervical dislocation and the hearts excised and cannulated onto modified Langendorff apparatus and perfused with a constant flow rate of 14ml/min as described in section 2.4. Hearts were perfused for 3-4 minutes with calcium free modified Krebs Heinsleit Buffer. The buffer was then switched and the hearts perfused with digestion buffer for 7 minutes. Isolated ventricular myocytes were used for the oxidative stress model, MTT assay and flow cytometric analysis of cleaved caspase 3 as described previously in sections 2.5.2, 2.5.3, 2.6. Myocytes were assigned to one of the following groups: a) Control (KHB) b) Salbutamol (0.1μM) c) CPG 20712 + Salbutamol d) CGP 20712 (0.0014μM).

5.3.4 Statistical analysis

All data were presented as a mean of the stabilisation period ± SEM. Infarct size, times taken to depolarisation and hypercontracture and western blot data were tested using one way ANOVA with a Fishers Least Significance Test post hoc test to determine any significance between groups. $p<0.05$ was considered to be significant.

5.4 Results

5.4.1 Effect of Salbutamol co-administered with CGP 20712 on cardiomyocytes assessed by MTT

Cardiomyocytes administered with Salbutamol (0.1μM) and CGP 20712 (0.0014μM) were treated in hypoxic conditions and re-oxygenated (HR). A significant decrease in MTT reductase activity was recorded via spectrophotometry analysis when HR control cardiomyocytes were compared to normoxic cardiomyocytes (HR, 100 ± 3% vs. 209 ± 8%, $p<0.01$, Figure 5.1). Salbutamol (0.1μM) significantly decreased MTT reductase activity when compared to HR cardiomyocytes (HR, 100 ± 3% vs. 76 ± 1%, $p<0.05$, Figure 5.1). Interestingly, Salbutamol in the presence of CGP 20712 caused a significant increase in reductase activity when compared to cardiomyocytes treated alone with Salbutamol (SalB + CGP 20712, 94 ± 4% vs. 76 ± 1%, Figure 5.1). Cardiomyocytes treated with CGP 20712 alone showed no significance when compared to HR cardiomyocytes.

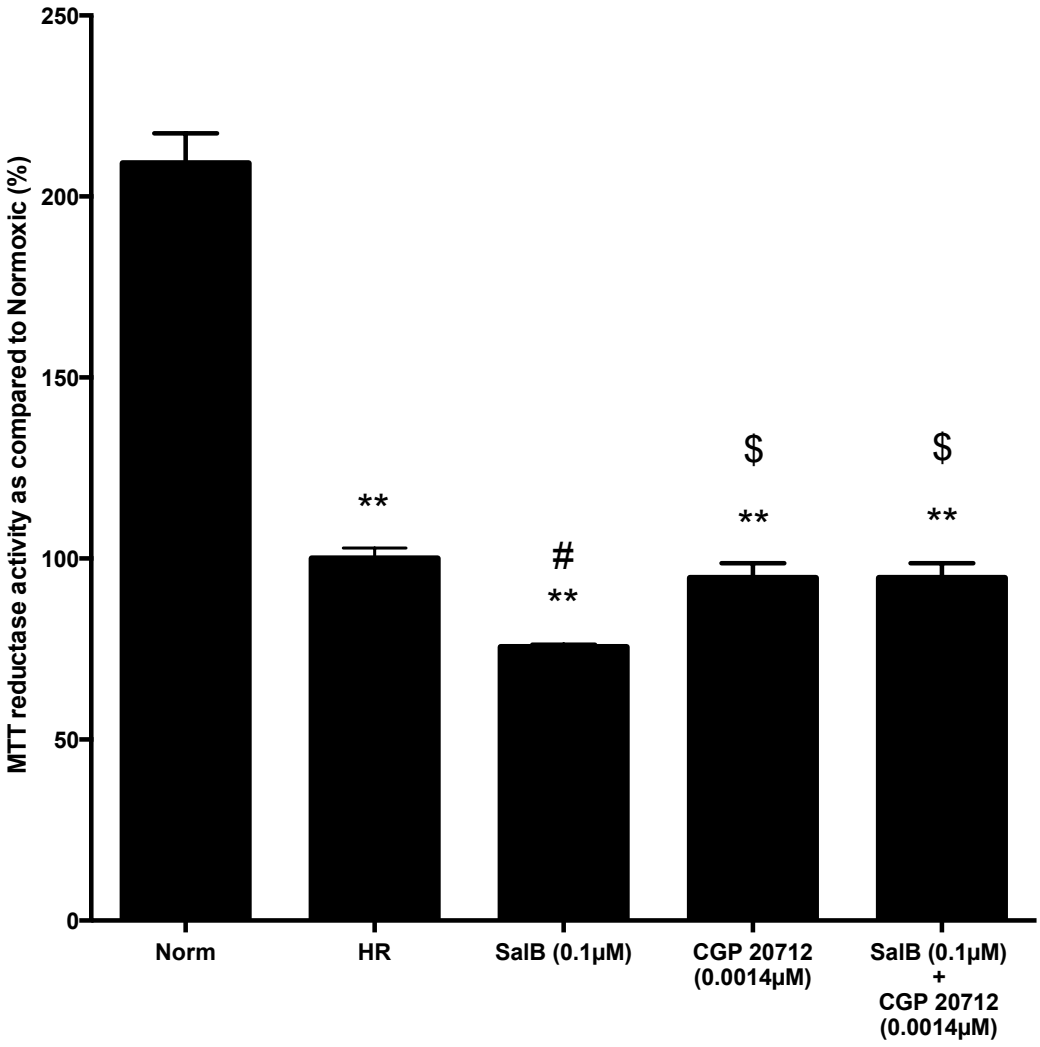


Figure 5.1 The MTT cytotoxic effect of Salbutamol (0.1μM) on the viability of cardiomyocytes in the presence and absence of β_1 adrenergic receptor antagonist CGP 20712 (0.0014μM). Data presented as mean ± SEM. n=6-8. $^{**}p<0.01$ vs. Normoxic, $^{\#}p<0.05$ vs. HR, $^{\$}p<0.05$ vs. SalB 0.1μM

5.4.1.1 The effect of Salbutamol with co-administration of CGP 20712 on Infarct to Risk Ratio in isolated hearts subjected to ischaemia reperfusion injury

Hearts were administered with Salbutamol (0.1μM) in the presence and absence of β_1AR antagonist CGP 20712 (0.0014μM) and underwent 35 minutes ischaemia followed by 120 minutes reperfusion. Salbutamol (0.1μM) significantly increased I/R ratio when compared to IR control hearts (SalB 0.1μM, 76 ± 3% vs. 51 ± 2%, $p<0.001$).

Interestingly, Salbutamol in the presence of CGP 20712 (0.0014μM) abrogated the IR ratio effect caused by hearts treated alone with Salbutamol (63 ± 4% vs. 76 ± 3%, $p<0.01$, Figure 5.2). Control IR data included has been used from previous experiment.

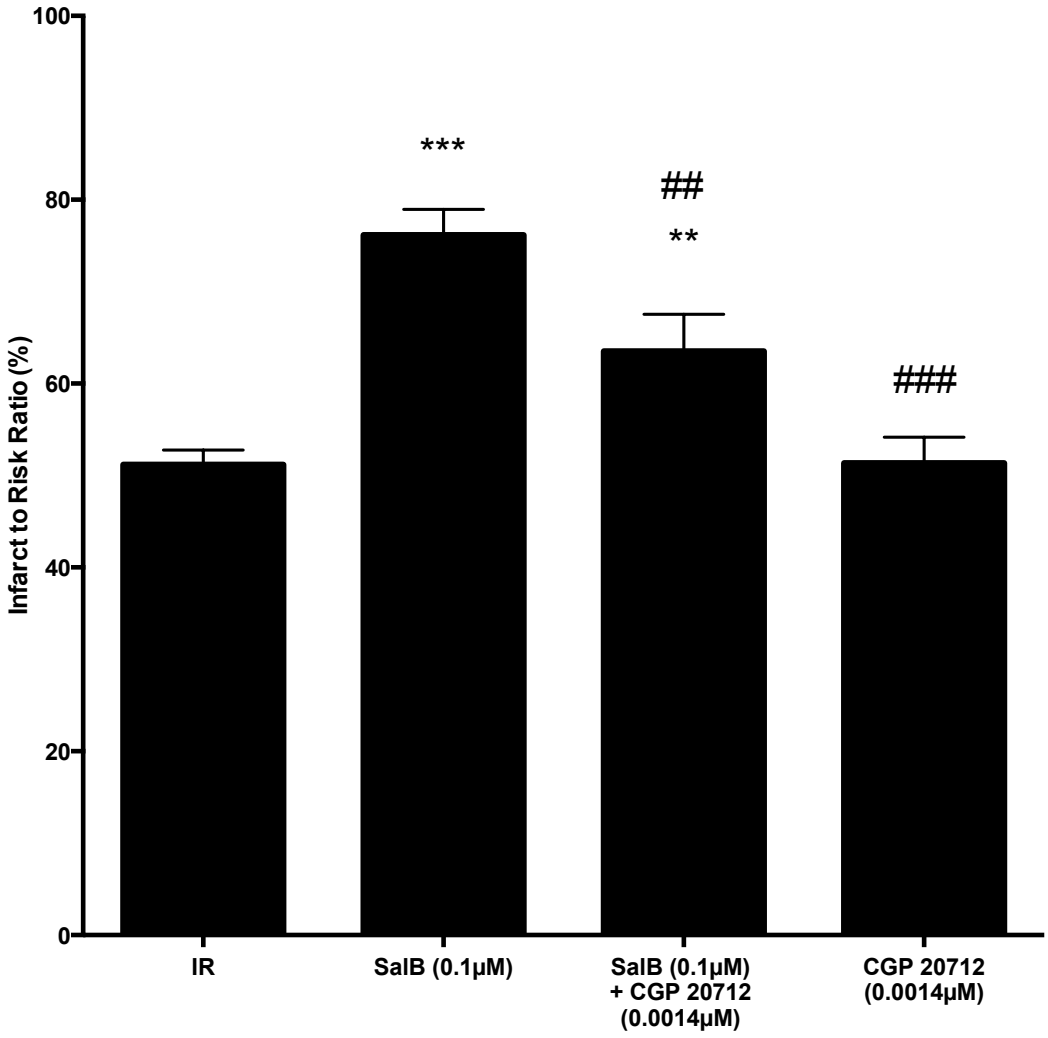


Figure 5.2 Infarct size to risk ratio (%) in isolated perfused hearts subjected to 35 minutes of ischaemia and 120 minutes reperfusion in the presence and absence of Salbutamol (0.1μM) and with co-administration of β_1AR antagonist CGP 20712 (0.0014μM) throughout the reperfusion period. Data presented as mean ±SEM. n=6-8. ***p<0.001 vs. IR, $^{###}$p<0.001 vs. SalB 0.1μM, $^{##}$p<0.01 vs. SalB 0.1μM.

5.4.1.2 Effect of Salbutamol in the presence or absence of the β_1AR antagonist CGP 20712 in a model of Oxidative Stress

Cardiomyocytes were subjected to laser stimulation in the presence of Salbutamol (0.1μM) in addition to the presence and absence of CGP 20712 (0.0014μM). Cardiomyocytes subjected to administration with Salbutamol (0.1μM) alone decreased time to the onset of depolarisation however it did not reach significance when compared to control (218 ± 20s vs. 234 ± 18s). No significant change in time to the onset of depolarisation was observed in cardiomyocytes administered with Salbutamol and CGP 20712 when compared to control cardiomyocytes (221 ± 17s vs. 234 ± 18s, Figure 5.3).

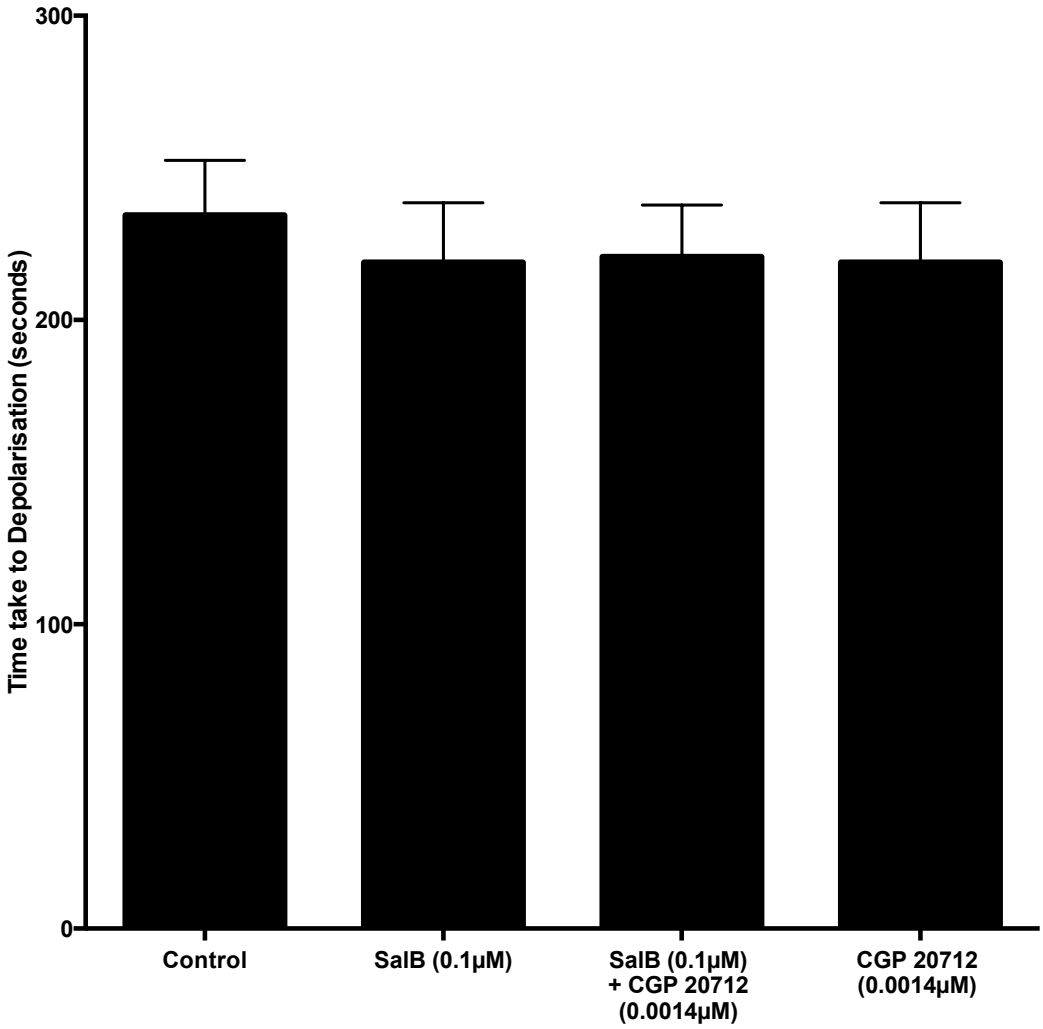


Figure 5.3 The effects of Salbutamol (0.1μM) on time taken to depolarisation in isolated rat cardiac myocytes in a model of oxidative stress in the presence or absence of β_1AR antagonist CGP 20712 (0.0014μM). Data presented as mean ±SEM. n=6-8.

Salbutamol (0.1μM) significantly decreased the time to hypercontracture in isolated cardiomyocytes when compared to non-treated control groups (524 ± 23s vs. 663 ± 40s, $p<0.001$, Figure 5.4). Co-administration of Salbutamol (0.1μM) and CGP 20712 (0.0014μM) significantly decreased time taken to hypercontracture when compared to non-treated control groups also (528 ± 8s vs. 663 ± 40s, $p<0.01$). Interestingly, cardiomyocytes treated with β_1-AR antagonist CGP 20712 alone, significantly increased the time taken to hypercontracture when compared to Salbutamol treated groups (626 ± 18s vs. 528 ± 8s, $p<0.05$, Figure 5.4).

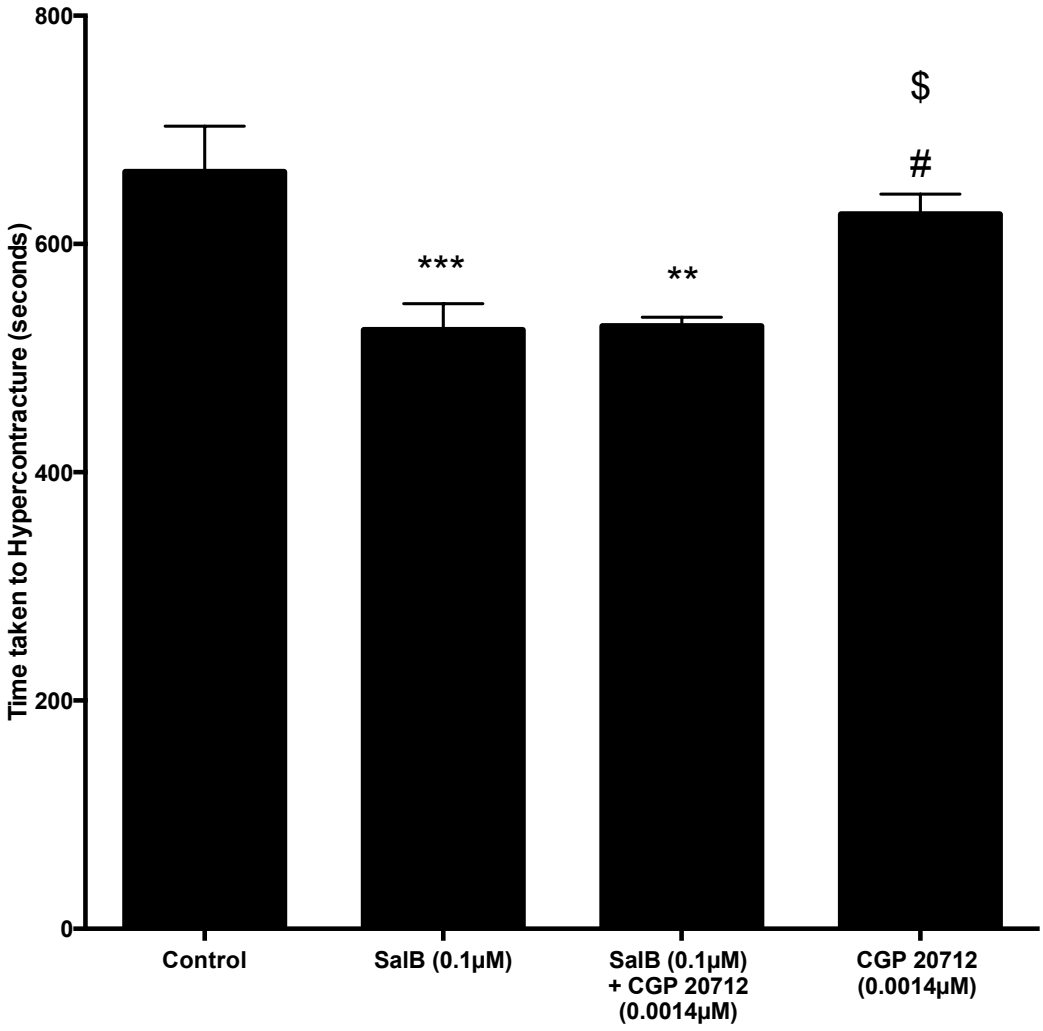


Figure 5.4 The effects of Salbutamol (0.1µM on time taken to hypercontracture in isolated rat cardiac myocytes in a model of oxidative stress in the presence or absence of β_1AR antagonist CGP 20712 (0.0012µM). n=6-8. Data presented as mean ±SEM. n=6-8.***p<0.001 vs. control, **p<0.01 vs. control, $^{\#}$p<0.05 vs. SalB (0.1µM), $^{\$}$p<0.05 vs. SalB + CGP 20712.

5.4.1.3 The effect of Salbutamol on signalling protein p-Akt by assessment of Western blotting with co-administration of β_1AR antagonist CGP 20712

Investigation into the signalling protein p-Akt (Ser^{473}), was carried out in the presence of Salbutamol (0.1μM) in the absence or presence of CGP 20712 (0.0014μM). Hearts were reperfused with Salbutamol in combination with CGP 20712 for 120 minutes throughout reperfusion after 35 minutes ischaemia. IR control hearts showed a significant increase in levels of p-Akt when compared to normoxic hearts (100 ± 14% vs. 69 ± 20%, $p<0.01$). Hearts treated with Salbutamol (0.1μM) significantly increased levels of p-Akt when compared to IR control hearts (240 ± 10% vs. 100 ± 14%, $p<0.001$, Figure 5.5).

Interestingly, co-administration of Salbutamol (0.1μM) and CGP 20712 (0.0014μM) significantly decreased levels of p-Akt when compared to Salbutamol treated hearts, suggesting presence of CGP 20712 abrogates the effect of Salbutamol on p-Akt activation (42 ± 10% vs. 240 ± 10%, $p<0.01$, Figure 5.5). Co-administration of Salbutamol (0.1μM) and CGP 20712 (0.0014μM) also significantly decreased levels of p-Akt when compared to IR control hearts (42 ± 10% vs. 100 ± 14%, $p<0.01$)

Levels of p-Akt in hearts administered with CGP 20712 (0.0014μM) alone showed a significant decrease when compared to time matched IR control hearts however a decrease was observed (47 ± 1% vs. 100 ± 14%, $p<0.05$). Normoxic, control IR and Salbutamol data included has been used from previous experiment.

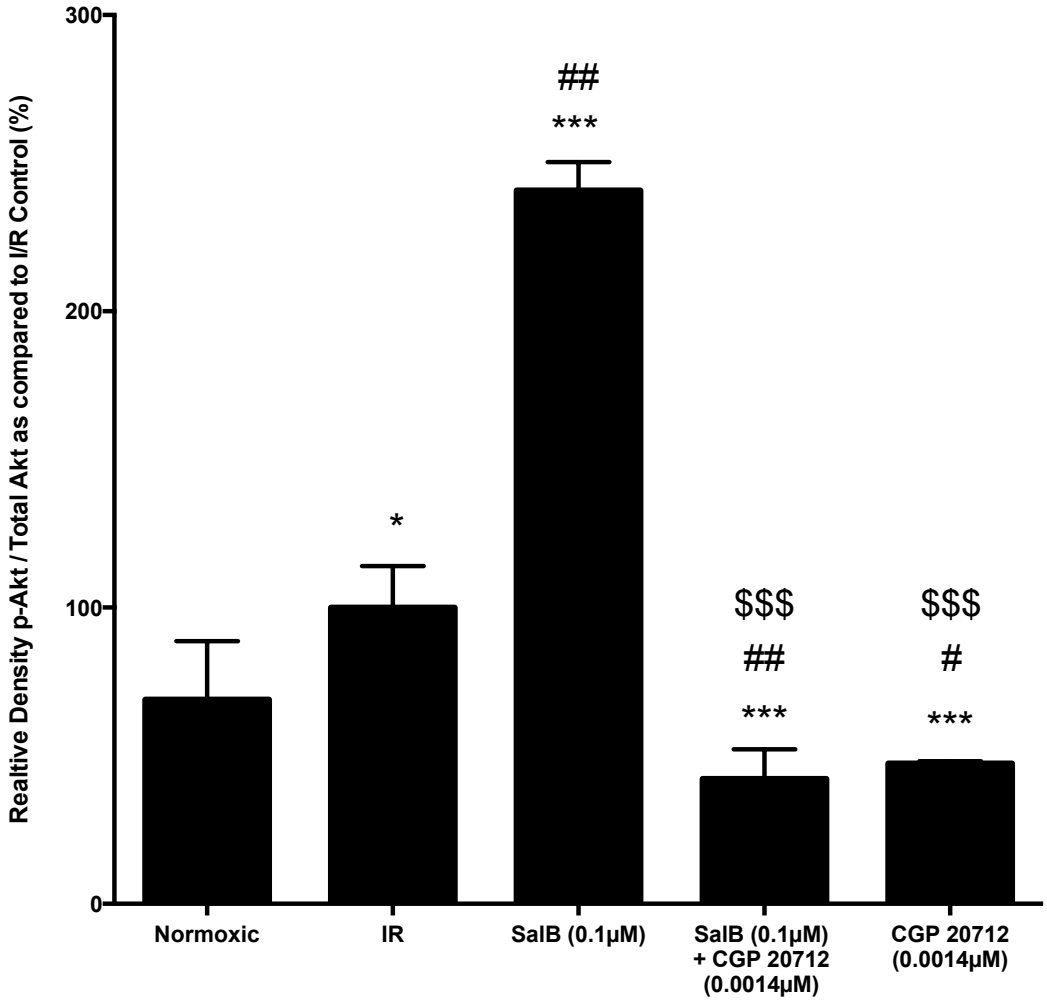


Figure 5.5 The effects of Salbutamol (0.1μM) on the levels of phosphorylated p-Akt after exposure to 35 minutes ischaemia and 20 minutes of reperfusion in the presence and absence of CGP 20712 (0.0014μM). Data presented as mean ±SEM. n=3. ***p<0.001 vs. Normoxic, *p<0.05 vs. Normoxic, $^{\#\#}$p<0.001 vs. IR, $^{\#}$p<0.05 vs. IR, $^{\$\$\$}$p<0.001 vs. SalB (0.1μM).

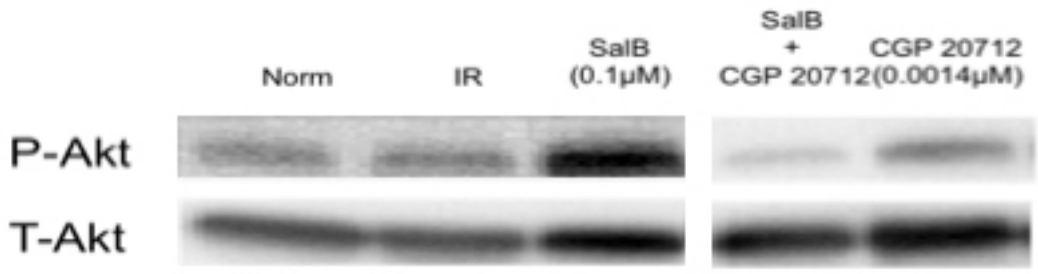


Figure 5.6 Representative blot of p-Akt and t-Akt when Salbutamol (0.1μM) was administered throughout reperfusion for 120 minutes after 35 minutes ischaemia in the presence and absence of CGP 20712 (0.0014μM)

5.4.1.4 The effect of Salbutamol on signalling protein p-Erk by assessment of Western blotting with co-administration of β_1AR antagonist CGP 20712

Administration of Salbutamol (0.1μM) in the presence and absence of CGP 20712 (0.0014μM) had a no significant change on levels of p-Erk when compared to control IR control hearts. Interestingly, a significant decrease in p-Erk was observed when hearts were co-administered with Salbutamol(0.1μM) and CGP 20712 (0.0014μM) when compared to Salbutamol alone (43 ± 14% vs. 75 ± 26%, $p<0.05$, Figure 5.7). Normoxic, control IR and Salbutamol data included has been used from previous experiment.

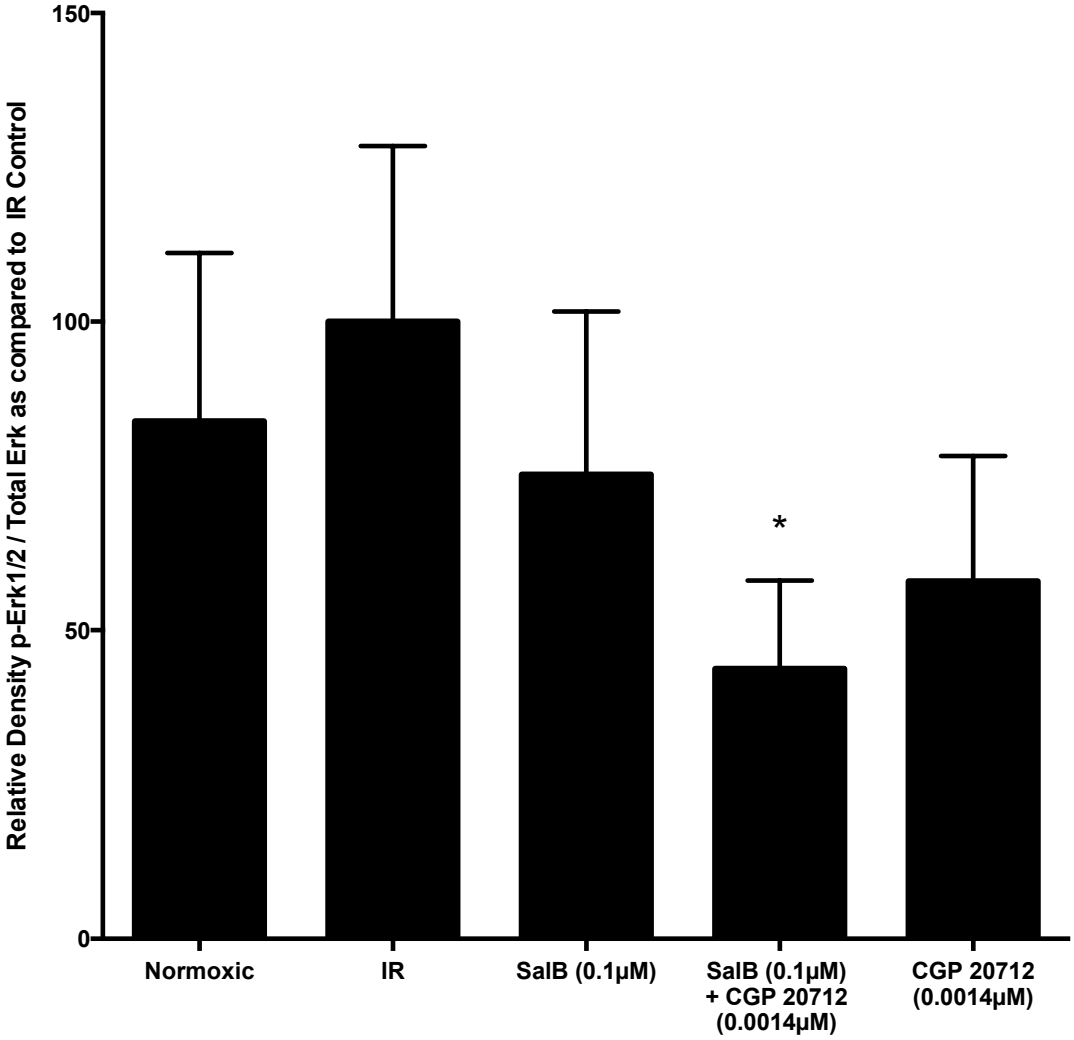


Figure 5.7 The effects of Salbutamol (0.1μM) on the levels of phosphorylated Erk after exposure to 35 minutes ischaemia and 120 minutes of reperfusion in the presence and absence of CGP 20712 (0.0014μM). Data presented as mean ±SEM. n=3. *p<0.05 vs. IR.

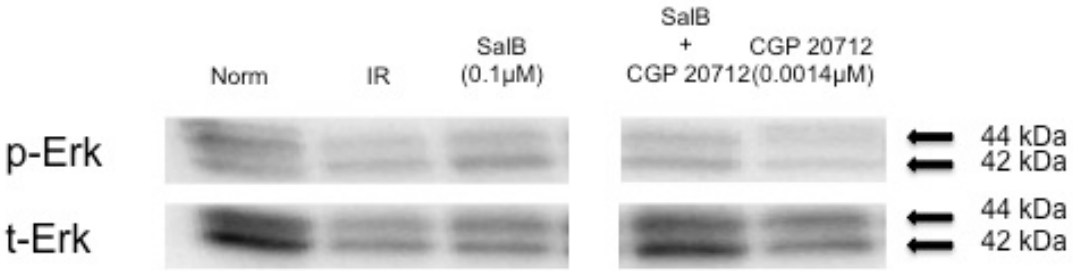


Figure 5.8 Representative blot of p-Erk and t-Erk when Salbutamol (0.1μM) was administered throughout reperfusion for 120 minutes after 35 minutes ischaemia in the presence and absence of CGP 20712 (0.0014μM)

5.4.1.5 The effect of Salbutamol on signalling proteins cleaved caspase 3 with co-administration of the β_1AR antagonist CGP 20712 by assessment of flow cytometry

Hearts treated with the co-administration of Salbutamol (0.1μM) and CGP 20712 (0.0014μM) significantly increased ($p<0.01$) levels of activated caspase 3 when compared to HR cardiomyocytes (214 ± 73% vs. 100 ± 20%, $p<0.01$) (Figure 5.9).

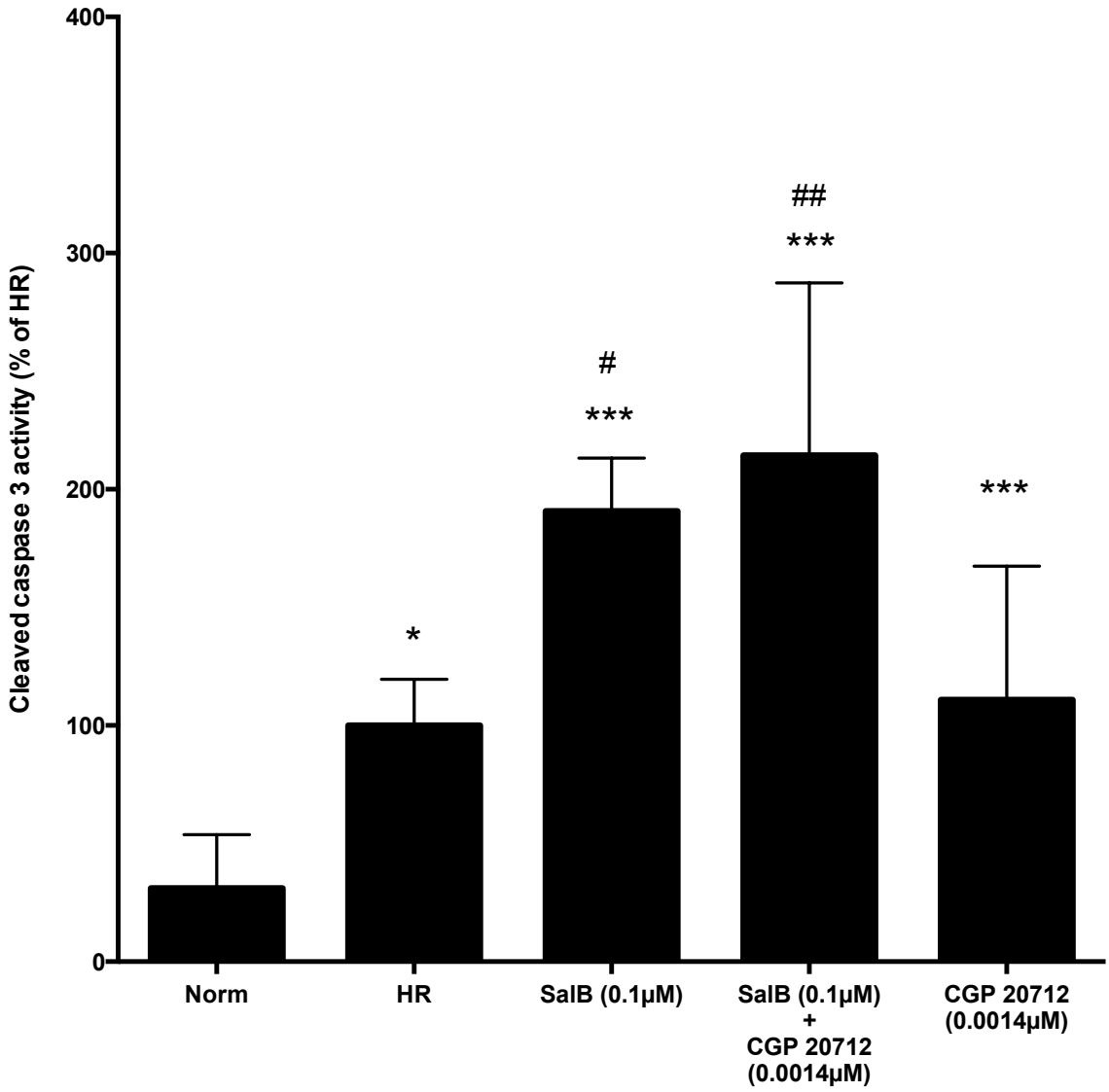


Figure 5.9 The effects of a Salbutamol (0.1μM) on cleaved caspase 3 in the absence and presence of CGP 20712 (0.0014μM). Isolated myocytes were exposed to 2 hours hypoxia followed by 4 hours reoxygenation in the presence or abscene of the drug. Data presented as mean ±SEM. n=6-8. ***p<0.001 vs. Normoxic, *p<0.05 vs. Normoxic, $^{\#\#}$p<0.01 vs. HR, $^{\#}$p<0.05 vs. HR.

5.5 Discussion

It has been reported that Salbutamol can cause and exacerbate injury on the heart in conditions of myocardial ischaemia and in patients with underlying ischaemic heart disease, hypertrophy or cardiomyopathy (Odigie-Okon et al., 2010). Salbutamol as a specific β_2AR agonist allows it to target these receptors specifically due to its high affinity (Bandaru et al., 2015, Dougall et al., 1991). Any toxic effect we have observed from previous studies, we assumed to be due to the activation of the β_2AR based on affinity values alone. However, it is possible that partial agonists, such as Salbutamol, in higher concentrations are capable of acting non-specifically on other receptors. With this attribute, we must investigate Salbutamol's effect by the sole activation of the β_2ARs. As an experimental model, the use of Isoproterenol to induce myocardial ischaemia and infarction is widely accepted in addition to developing models of heart failure via ventricular hypertrophy (Upaganlawar and Balaraman, 2011).

In this study, we antagonised the β_1ARs with the specific antagonist CGP 20712 in the presence of Salbutamol and investigated the effect on cardiomyocytes, cytotoxicity, signalling proteins and infarct to risk ratio. Normoxic and control IR data for this protocol were used from previous experiments as discussed in section 3.5.

We have previously demonstrated that administration of Salbutamol (0.1μM-1μM) exacerbates myocardial ischaemia reperfusion injury with an increase in infarct size and have also observed a decrease in MTT reductase activity in cardiomyocytes exposed to HR conditions (Figure 5.1 & Figure 5.2). The current study investigated the effects of Salbutamol

(0.1μM) co-administered with CGP 20712 (0.0012μM) in the model of cytotoxicity and in the model of isolated perfused rat heart model and myocardial ischaemia reperfusion injury.

Co-administration of CGP 20712 with Salbutamol increased MTT reductase activity similar to levels observed in HR control cardiomyocytes; whilst Salbutamol treated cardiomyocytes reduced MTT reductase activity. This increase in reductase activity with CGP 20712 and Salbutamol implies a decrease of injury to cardiomyocytes. A similar finding was reflected in the model of ischaemia reperfusion. In the presence of Salbutamol and CGP 20712, a decrease in infarct to risk (I/R) ratio was observed when compared to hearts administered with Salbutamol (0.1μM) alone, however the size of the I/R ratio was not reduced to IR control levels and was still significantly higher.

The MTT assay is sensitive in detecting cell viability, as a result some results may not reflect similar results seen in other models such as the ischaemia reperfusion injury model (Steenbergen et al., 1978, Gomez et al., 1997). MTT assay sensitivity is unable to determine between apoptosis or necrosis nor the early stages of cardiomyocyte cell death and therefore can determine that 'healthy rod shaped' cardiomyocytes in addition to those cardiomyocytes undergoing cell death rather than cardiomyocytes that had completed the cell death process, explaining a stronger protective effect of CGP 20712 on cardiomyocytes (Gomez et al., 1997, Piper et al., 1984).

A reduction in the I/R ratio in hearts co-administered with Salbutamol and CGP 20712, when compared to hearts treated with Salbutamol alone, indicates that there is some activation of β_1ARs, however Salbutamol's exacerbation of injury indicate an involvement of the β_2ARs. All agonists and antagonists have an efficacy and affinity for receptors (Strange, 2008). It is

the level of efficacy and/or affinity that determines how an agonist (full or partial) is subtype selective not only to receptor type, but also sub-receptor type i.e. G_S or G_i (Baker, 2010). Salbutamol has a 29 times greater affinity for β_2ARs than β_1AR's in the racemic version of Salbutamol (Cockcroft and Swystun, 1997), interestingly the 'R' enantiomer of Salbutamol has 150 times greater affinity for β_2AR's than S-Salbutamol(Ameredes and Calhoun, 2006). Salbutamol administered in clonal CHO-K1 cell lines transfected with a range of beta-adrenergic receptors (β_1, β_2 and β_3), showed to have a very high affinity and efficacy for β_2ARs over other any other type β-adrenoceptors such as Formoterol and Salmeterol (Baker, 2010). With a high affinity of CGP 20712 for β_1ARs, we can be conclude in our experiments that β_1ARs were antagonised allowing Salbutamol to solely act on β_2ARs thus confirming that the toxicity of Salbutamol is occurring via activation of the β_2ARs in the Langendorff model of ischaemia reperfusion injury, however some injury is caused by activation of β_1-ARs.

All β-adrenergic receptors are capable of binding to G_S pathway, however β_2ARs are unique and capable of binding to the G_i pathway (Baker, 2010, Brodde and Michel, 1999). Cardiomyocyte apoptosis via activation of βARs has been strongly linked with specific activation of the β_1AR, which is only able to activate the G_S pathway (Spear et al., 2007). Either β_1AR or β_2AR-G_S activation initiates coupling adenylyl cyclase to increase cAMP levels, which further activates Protein Kinase A (PKA) (Desantiago et al., 2008). The activation of PKA is key to activating signalling proteins shown to initiate cell apoptosis and necrosis in a dependent and independent manner (Spear et al., 2007). Active PKA phosphorylates several of the sarcolemma signalling proteins including L-type Ca^{2+} channels, phospholamban and ryanodine receptors, all of which contribute to the influx and efflux of

Ca^{2+} ions, which at high concentrations can cause apoptosis by altering the permeability of mitochondria (Kamp and Hell, 2000, Kaumann and Molenaar, 1997). In contrast, G_i pathway activation is able to inhibit the G_S pathway cascade by inhibition of the production of adenylyl cyclase thus reducing cAMP production resulting in a decrease in levels of calcium levels and also an increase in pro-survival proteins such as Akt and MAPK (Hill and Baker, 2003).

PKA is also involved in the switching of β_2ARs from G_S to G_i by direct phosphorylation of β_2ARs by uncoupling them from G_S and enhancing binding to G_i (Martin et al., 2004). In addition to PKA, the specialist GPCR kinase (GRK) GRK2 has been shown to directly phosphorylate the β_2AR-G_S pathway to uncouple G_S and encourage G_i coupling, especially on agonist activated β_2ARs (Pavoine and Defer, 2005). Elevated GRKs levels recruit β-arrestins to bind to G_S to prevent any further binding by β_2ARs thus encouraging binding to G_i (Zhu et al., 2012). The influence and importance of GRKs role has been shown in mouse and rat cardiomyocytes by inhibition of GRK directly via G_iCT (specific G_i inhibitor peptide) and mutations at sites of GRK phosphorylation whereby PKA phosphorylation levels alone were not sufficient enough to cause β_2AR-G_i activation (DeGeorge et al., 2008, Liu et al., 2009, Wang et al., 2008). Liu and colleagues, in addition to demonstrating the influence of GRKs on G_i coupling, showed that the concentration of GRKs is dose dependent on an agonist i.e. a higher concentration of agonist activates GRKs (Liu et al., 2009).

In the presence of Isoproterenol (non selective βAR agonist), concentrations <0.1μM showed minute activity of GRKs however concentrations >0.1μM, GRKs were active and an increase in G_i coupling was recorded (Liu et al., 2009). The importance of GRK in pro-survival

signalling has been demonstrated in studies in myocardial ischaemia and hypertension, as with the inhibition of GRK, an increase in apoptotic cardiomyocyte was recorded (Hata and Koch, 2003, Zhu et al., 2012). With the activation of G_S pathway as the primary link to the induction of cardiomyocyte apoptosis and the activation of G_i linked to being anti-apoptotic, we propose that Salbutamol's toxic effect is via activation of β_2AR-G_S pathway.

In the model of oxidative stress a significant decrease in time taken to the onset of hypercontracture was recorded in cardiomyocytes administered with Salbutamol and also with the co-administration of CGP 20712 when compared to control cardiomyocytes. Interestingly, a decrease in time taken to the onset of depolarisation (no significance) was observed similarly in cardiomyocytes administered with Salbutamol and CGP 20712 when compared to control. This decrease can be linked to the change in the mitochondria permeability of the cardiomyocytes, in particular to ROS and Ca^{2+} (Szalai et al., 1999).

Isoproterenol's ability to induce myocardial ischaemia and infarction in rat hearts has been investigated extensively. As discussed in Chapter 3, the production of catecholamines by Isoproterenol is a factor contributing to damaging of the myocardium and can induce myocardial ischaemia and/or infarction in hearts via production of ROS(Navarro-Sobrino et al., 2010, Lobo Filho et al., 2011). The use of Isoproterenol on rat myocardium demonstrated a significant reduction in Superoxide Dismutase (SOD), an antioxidant as a protective feature of the myocardium against free radicals (Dhalla et al., 2000, Halestrap et al., 1997b). Isoproterenol's maladaptive effect on the sodium/calcium exchanger (NCX) has been shown in pancreatic beta cells to increase levels of calcium ions which has lead to calcium dependent cell apoptosis (Hudecova et al., 2013). In relation to our findings with Salbutamol, we can postulate that upon Salbutamol induced calcium release a similar effect may occur on

the NCX resulting in the premature opening of the mPTP as seen in our experiments. The calcium release as a result of a maladaptive NCX has been localised to the endoplasmic reticulum causing complete depletion of calcium stores increasing stress on the mPTP (Herchuelz et al., 2002). In Chapter 3 we demonstrated the chronotropic effects of both Isoproterenol and Salbutamol. With increase in heart rate, the number of cardiac cycles increases thus resulting in more cardiomyocyte contractions. Work by Bell and colleagues (2006) in rat cardiomyocytes used Isoproterenol to demonstrate a correlation between intracellular calcium release and ATP synthesis (Bell et al., 2006). Their findings showed that with increasing cardiac cycles, an increase in ATP occurs. However, in respect to our findings with Salbutamol inducing premature opening of the mPTP resulting in faster times to the onset of hypercontracture, we can apply this logic that with increased Salbutamol induced calcium release as a stressor to the mPTP, an increase in ATP demand would be required. At the point of reperfusion the sarcoplasmic reticulum experiences a calcium overload triggering Sarcoplasmic Reticulum Ca^{2+}-ATPase (SERCA), which increases uptake of calcium, however due to the increased calcium from reperfusion, calcium levels exceed those that can be handled thus initiating release from ryanodine receptors (Ruiz-Meana and Garcia-Dorado, 2009). The 'window of opportunity' has been highlighted in respect of the time immediately after reperfusion as a very detrimental time to mitochondria (Hausenloy et al., 2005).

With the activation of β_2AR-G_S pathway in the presence of Salbutamol, reported elevated release of Ca^{2+} from the sarcoplasmic reticulum occurs thus elevating intracellular Ca^{2+} within cardiomyocytes through the mPTP (Keller et al., 2014). The elevated release of Ca^{2+} is regulated with an influx into mitochondria (Giorgi et al., 2012). This increase in cytosol Ca^{2+} can causes a calcium overload initiating pro-apoptotic proteins in particular cytochrome c

(Joza et al., 2001). The increased concentration of Ca^{2+} in conjunction with the elevated ROS from laser stimulation of TMRM causes increased stress within the mitochondria, which initiates the opening of the mPTP (Giorgi et al., 2012). The time to the onset of hypercontracture seen with the co-administration of Salbutamol and CGP on cardiomyocyte hypercontracture is no different when compared to cardiomyocytes treated with Salbutamol alone. This further support Salbutamol's toxic effect is via activation of β_2ARs.

The mPTP is still debated in regards to its structure and specific functionality, however the inclusion of the Voltage Dependent Anion Channel (VDAC) as a part of the mPTP, has been described to be responsible in particular for the movement of Ca^{2+} across the outer mitochondrial membrane. The location of VDAC between the cytosol and mitochondria are located in close proximity between mitochondria and the sarcoplasmic reticulum (Shoshan-Barmatz et al., 2006, Szabadkai et al., 2006). Further high-resolution 3D electron topography identified that up to 20% of the mitochondria surface is in contact with the sarcoplasmic reticulum making Ca^{2+} available a lot quicker for uptake directly by mitochondria rather than previously thought vesicular transport of Ca^{2+} (Marsh et al., 2001). With such a close proximity between the sarcoplasmic reticulum and mitochondria, Salbutamol β_2AR-G_S activation would allow a more rapid release of Ca^{2+} thus causing a Ca^{2+} overload leading to a faster depolarisation time and faster time to the onset of hypercontracture that we consider the initiation of cell death via apoptosis. In addition to Ca^{2+} overload, which is present during myocardial ischaemia and reperfusion, in conjunction with ROS, further damage to the mitochondria initiates cardiomyocyte death. ROS initiation alone is sufficient enough to cause mitochondrial damage, however a combination of both these stresses would be 'overwhelming' and detrimental to the composition and functioning of the mPTP in any cardiomyocyte. This can be linked to the scenario we observed in the oxidative stress model

that both Ca^{2+} overload and ROS initiate the rapid onset of hypercontracture due to the delay's observed in depolarisation. Activation of Protein Kinase C (PKC), another regulatory kinase of Ca^{2+}, is also activated upon βAR stimulation. In doing so, direct phosphorylation by PKC on p66Shc has shown to cause an increase in free radicals via oxidation of cytochrome c once p66Shc has translocated into the mitochondria (Giorgi et al., 2012, Pinton et al., 2007, Pinton et al., 2008). This action of p66Shc may give further reason to the toxic effect of Salbutamol by production of ROS causing premature opening of the mPTP.

Control and Salbutamol data obtained from other western blots have been used throughout the results section. A limiting factor of such practice can be the degradation of the collected tissue samples due to the time elapsed between running the gels. To minimise this, raw tissue samples were snap frozen in liquid nitrogen (as described in section 2.7.1) in order to freeze or cellular activity. This sample can be kept for a long period of time and homogenised when required. Further to this, to prevent any further phosphorylation of samples during the homogenisation stage, a phosphorylation inhibitor (Phos-STOP) was added. The time between running gels was kept to a minimum with all gels being run and probed for each study in this thesis within 1-2 months to minimize any parameters of the experiment that may affect the data. To further reduce this as a limiting factor, a housekeeping gene, GAPDH, was used in all western experiments to ensure equal loading due to its high expression in rat tissue (Mahmood and Yang, 2012).

Elevated levels of p-Akt were observed in hearts treated with Salbutamol (0.1μM). These findings have been recorded in previous studies that also identified elevated activity of the PI3K/Akt pathway and p-Erk levels in neonatal rat cardiomyocytes in response to βAR stimulation (Pavoine and Defer, 2005, Steinberg, 2004,, Steinberg, 2004, Zhang et al., 2011).

We have previously discussed in detail the detrimental effects of chronic activation of p-Akt (Chapter 4) as a mechanism that may contribute to the increased I/R ratios we have recorded with Salbutamol treated hearts. Interestingly, the combination of β_1AR antagonist CGP 20712 with Salbutamol, significantly decreased expression of p-Akt when compared to Salbutamol treated hearts (Figure 5.5). However, with elevated I/R ratios recorded with Salbutamol and CGP 20712 when compared to IR control hearts, and a recorded decrease in p-Akt expression, another signalling pathway must be recruited in order to initiate cardiomyocyte cell death. One such pathway we propose is the p-Erk pathway. Previous studies have identified cross talk between Akt and Erk, specifically at the Akt Raf 1 level. In these circumstances, increased levels of p-Akt inhibit Raf 1 at serine^{259} (Moelling et al., 2002). CGP 20712 administration to Salbutamol treated hearts showed, although not significant, a decrease in expression of p-Erk. Recruitment of this particular MAPK is a key protein in the reperfusion injury salvage kinase pathway (RISK) (Hausenloy et al., 2005). Our recorded levels of p-Erk were lower than time matched control IR hearts suggesting that the reduced recruitment of p-Erk is not able to inhibit downstream pro-apoptotic proteins such as BAD by phosphorylation at serine^{112} by ERK activated p90RSK (Lu and Xu, 2006).

A significant elevation in levels of cleaved caspase 3 activity was recorded in hearts treated alone with Salbutamol (0.1μM)(Figure 5.9). Activation of cleaved caspase 3 has been linked to the release of cytochrome c from mitochondria initiating the caspase 9- caspase 3 cascade leading to cell apoptosis (Li et al., 2010). The formation of an apoptosome with cytochrome c, Apaf-1 and initiator caspase 9 allows caspase 9 to cleave further effector caspases as part of the intrinsic cell death pathway (Parsons and Green, 2010). Emphasis on the importance of caspase 3 activation, linked to reperfusion injury, has been demonstrated by Hussain and colleagues (2014) by inhibition of caspase 3 activation through adenosine receptors in rat

hearts (Hussain et al., 2014). Further to this, βARs, as mentioned earlier, have been linked to inducing apoptosis via the mitochondrial death pathway, in particular via activation of the β_1AR/G_s pathway (Communal and Colucci, 2005). Increased cleaved caspase 3 with Salbutamol treated hearts compared to IR control hearts identifies that cardiomyocytes are undergoing an additional source of stress other than that caused by reperfusion. With the administration of β_1AR antagonist CGP 20712, an increase was in cleaved caspase 3 was recorded when compared to Salbutamol treated hearts. This increase may be explained with a link to the selective activation of cardiomyocyte β_2ARs and the observed detrimental effects in the Langendorff model and oxidative stress model that has been observed in this chapter. In contrast to previous studies of that β_2AR activation results in anti-apoptotic signalling pathways, we demonstrate the opposite effect of activation of the β_2AR receptor. The resulting effects of antagonising the β_2AR will be discussed in the next chapter (Chapter 6).

This study confirms that the β_2AR receptor is responsible for some toxicity observed with Salbutamol in the model of ischaemia reperfusion and in cardiomyocytes. Investigation of signalling proteins suggest that the mechanism by which Salbutamol causes its toxicity is via dual activation of both the β_2AR-Gs and Gi pathways, with suggestions of desensitisation of βARs and ventricular remodelling. Chronic activation of p-Akt has been shown to increase maladaptive effects of pro-survival signalling leading to increased myocardial infarction, hypertrophy and heart failure. Further to this, rapid activation of p-Akt has also been shown to inhibit expression of p-Erk at by inhibition of Raf 1 of the Ras/Raf/Erk signalling pathway. Activation of such pathways has shown to increase ROS and a suggestion of Salbutamol's effect influencing Calcium release by the sarcoplasmic reticulum, with both these stresses

being detrimental to the cardiomyocytes due to the opening of the mPTP causing premature cardiomyocyte death.

Further investigations into the possibility of non-selective behaviour of Salbutamol must be carried out to determine if activation of β_1ARs contribute to the toxicity.

6 Chapter 6: The Effect Of The Short Acting β Adrenergic Receptor Agonist Salbutamol in Myocardial Ischaemia Reperfusion Injury In The Presence of β_2 Adrenergic Receptor Antagonist ICI 118, 551

6.1 Introduction

With the use of the β_1AR antagonist CGP 20712, it was established that Salbutamol's detrimental effect on the heart was via activation of the β_2AR receptor cell signalling pathway (Chapter 5). Activation of the β_2ARs was shown to increase in levels of Akt and reduced levels of Erk. In this chapter we focus on using the β_2AR antagonist ICI 118, 551 to establish if there may be a non-selective capability of Salbutamol to activate β_1ARs that may contribute to the toxicity caused by Salbutamol. In addition to using the β_2AR antagonist, co-administration of ICI 118, 551 and CGP 20712 in the presence of Salbutamol was also investigated to observe if the toxic effect of Salbutamol could be abolished.

Partial agonists, such as Salbutamol, must occupy a higher proportion of receptors than full agonists to cause a therapeutic effect (Johnson, 2001). β_2ARs have a 67% identical homology to β_1ARs and other GPCRs (Warne et al., 2008, Johnson, 2006). Typically the β_2AR structure includes a 7 transmembrane region made up of α helices and 3 extracellular loops with a carboxy-terminus (Johnson, 2006, Mialet-Perez et al., 2004). β_2ARs exist in an equilibrium state between inactive and active. Activation of β_2ARs is similar to that of β_1ARs as both are bound to Gs subunits, shifting the equilibrium to a high-energy active state (Rasmussen et al., 2011, Warne et al., 2008).

The Gs subunit attached with a guanosine diphosphate (GDP) is replaced by guanosine triphosphate (GTP) upon activation by a ligand, such as Salbutamol. Rather than eliciting a conformational change of the receptor, Salbutamol, as well as other partial and full agonists, has shown to stabilise the receptor in their active states (Johnson, 2006, Onaran et al., 1993). Swaminath and colleagues (2005) suggested that the aromatic ring of Salbutamol interacts with the second extracellular loop of the β_2AR and the carboxy-terminal end of transmembrane 6 (Swaminath et al., 2005).

Stimulation of G_s subunits activates the adenylyl cyclase (AC)-cAMP-PKA pathway, which results in the release of calcium ions from the sarcoplasmic reticulum in addition to other sources (Zaugg et al., 2000, Zhu et al., 2003). Unlike β_1ARs, which only have the capability of binding to G_s subunits, β_2ARs can also bind and activate the G_i subunit causing an inhibitory effect (Woo and Xiao, 2012). The inhibitory effects of β_2AR-G_i stimulation has been shown to reduce levels of AC, cAMP and PKA (Duarte et al., 2012).

Distribution of β_2ARs are widespread on a variety of tissues such as the lining of the trachea, smooth muscle lining in the lungs and coronary endothelial, vascular smooth muscle and cardiomyocytes in the heart (Barbato et al., 2005). In normal healthy hearts the distribution of β_1:β_2 adrenergic receptors is in favour of β_1 with an approximate ratio of 80%:20% located at the cell crest of cardiomyocytes (Cros and Brette, 2013). The specific distribution of β_2ARs are focussed deep within the t-tubules, which are deep sarcolemmal invaginations found at the Z line (Cros and Brette, 2013). Cros and Brette (2013) also described that the compartmentalization of β_2ARs was also confined specifically within the caveolae, which are located along the surface of the sarcolemma and t-tubules (Cros and Brette, 2013, Balijepalli

and Kamp, 2008). During detrimental conditions to the heart such as heart failure, cardiomyopathy, ischaemic heart disease and myocardial infarction, the re-distribution of βARs has been observed, in particular the redistribution and number of βARs in favour of β_2ARs (Nikolaev et al., 2010). Using a combination of scanning ion conductance microscopy (SICM) with the detection of cAMP production, Nicolaev and colleagues (2010) confirmed the redistribution of β_2ARs in the failing myocardium after inducing heart failure via myocardial infarction in the rat heart. Specifically, the β_2ARs were shown to redistribute from the t-tubules to the cell crest (Nikolaev et al., 2010).

Overexpression of β_2ARs in rat hearts was shown to cause an increase in ischaemic injury, contradictory to the suggestion of the β_2AR-G_i pathway promoting cell survival via activation of Erk MAPK (Cross et al., 1999).

Desensitization and cardiac remodelling are common attributes in the failing heart effecting βARs (Heusch, 2013). The effect of desensitization on β_2ARs is to protect the receptor itself during short-term stimulation, however during overstimulation for long periods of time, the receptor can become maladaptive and cause further damage via internalisation (Lipsky et al., 2008). An example of a maladaptive response of β_2ARs has been noted in the coronary arteries. Normal β_2AR stimulation within coronary arteries induces vasodilation, whereas in hearts with mild atherosclerotic coronary arteries, the vasodilation response was reduced, further still, in stenotic coronary arteries treated with Salbutamol, a vasoconstrictive response was recorded leading to some patients presenting with symptoms of angina (Barbato et al., 2005).

The use of antagonists on βARs can be therapeutic to the heart to treat conditions including angina, arrhythmias, cardiomyopathy, hypertension and patients who have suffered from acute myocardial infarction (Frishman, 2013). The use of the antagonist ICI 118, 551 has a 550-fold specificity for β_2ARs over β_1ARs (Baker, 2005a, Warne et al., 2008). The mechanism by which ICI 118, 551 antagonises the receptor is through its high affinity for the receptor and shifting the activation state of the β_2AR towards the inactive state. β-antagonists do not compete with full or partial agonists for receptor activation at the same site of the βAR, this is a result of antagonists and agonists interacting with the βARs at different sites (Johnson, 2001, Johnson, 2006).

6.2 Aims

The aims of the current study were to investigate the effects of the short acting β adrenergic receptor agonist Salbutamol by using the isolated perfused Langendorff heart model of ischaemia reperfusion injury, the oxidative stress cardiac myocyte model, the MTT assay. Salbutamol was administered in the presence and absence of the β_2AR antagonist ICI 118, 551 to determine the role of β_2AR signalling in Salbutamol induced myocardial injury.

6.3 Methods

6.3.1 Langendorff protocol

Briefly, Sprague-Dawley rats were sacrificed by cervical dislocation and cannulated to the Langendorff setup and perfused with KHB as described in section 2.3. Hearts were allowed to stabilise for 20 minutes followed by 35 minutes of regional ischaemia and 120 minutes of reperfusion. One minute before the onset of reperfusion hearts were administered Salbutamol (0.1μM) in the absence or presence of β Adrenergic Receptor antagonists CGP 20712 (0.0014μM). At the end of the experiment hearts underwent infarct to risk ratio analysis. Haemodynamic data were collected throughout the study.

For western tissue collection, hearts were reperfused with Salbutamol (0.1μM) for either 5, 20 or 120 minutes in the presence or absence of ICI 118, 551 (0.0012μM). After the time elapsed, hearts were removed and the left ventricle removed and snap frozen in liquid nitrogen.

6.3.2 Western blot analysis

Analysis of tissue by western blot was carried out as described in section 2.7. Briefly, following gel electrophoresis, proteins were transferred to a PVDF membrane and probed for the phosphorylated and total forms of the proteins: phospho-Akt (Ser_{473}) (1:1000) and phospho-p44/p42 (Erk 1/2, Thr202/Tyr204) (1:1000).

6.3.3 Adult rat cardiac myocyte isolation

Briefly, male Sprague Dawley rats were sacrificed by cervical dislocation and the hearts excised and cannulated onto modified Langendorff apparatus and perfused with a constant flow rate of 14ml/min as described in section 2.4. Hearts were perfused for 3-4 minutes with calcium free modified Krebs Heinsleit Buffer. The buffer was then switched and the hearts perfused with digestion buffer for 7 minutes. Isolated ventricular myocytes were used for the oxidative stress model, MTT assay and flow cytometric analysis of cleaved caspase 3 as described previously in sections 2.5.2, 2.5.3, 2.6. Myocytes were assigned to one of the following groups: a) Control (KHB) b) Salbutamol (0.1μM) c) ICI 118, 551 + Salbutamol d) ICI 118, 551 (0.0012μM).

6.3.4 Statistical analysis

All data were presented as a mean of the stabilisation period ± SEM. Infacrt size, times taken to depolarisation and hypercontracture and western blot data were tested using one way ANOVA with a Fishers Least Significance Test post hoc test to determine any significance between groups. $p<0.05$ was considered to be significant.

6.4 Results

6.4.1 Effect of Salbutamol co-administered with ICI 118 551 on cardiomyocytes assessed by MTT

Cardiomyocytes underwent 2 hours hypoxia followed by administered with Salbutamol (0.1μM) and ICI 118, 551 (0.0012μM) for 2 hours re-oxygenation (HR). A significant decrease in MTT reductase activity was recorded via spectrophotometry analysis when HR control cardiomyocytes were compared to Normoxic cardiomyocytes (100 ± 3% vs. 209 ± 8%, $p<0.01$, Figure 6.1) Salbutamol (0.1μM) significantly decreased MTT reductase activity when compared to HR cardiomyocytes (76 ± 1% vs. 100 ± 3%, $p<0.05$, Figure 6.2) Interestingly, Salbutamol (0.1μM) in the presence of ICI 118, 551 (0.0012μM) caused a significant increase in reductase activity when compared to cardiomyocytes treated alone with Salbutamol (0.1μM) (129 ± 2% vs. 76 ± 1%, Figure 6.1).

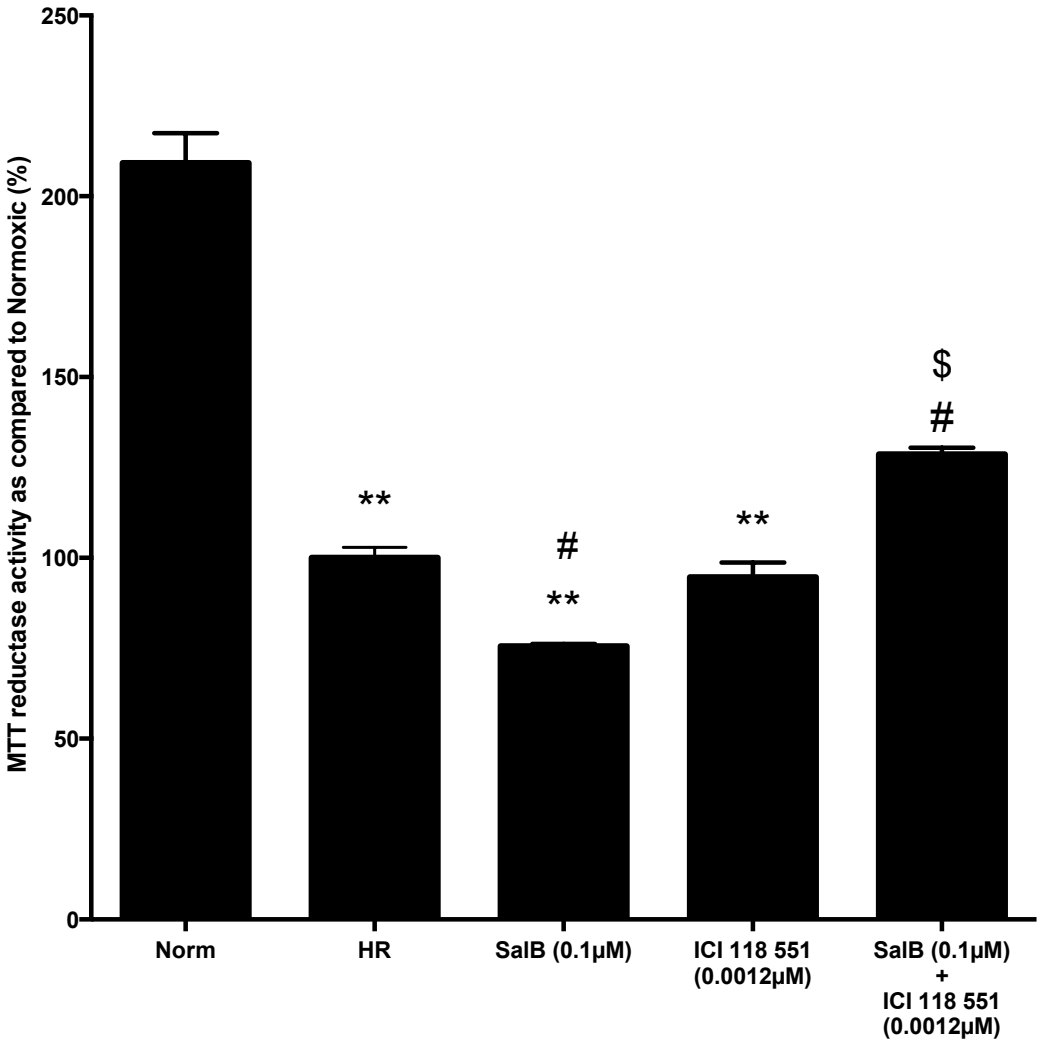


Figure 6.1 The MTT cytotoxic effect of Salbutamol (0.1μM) on the viability of cardiomyocytes in the presence and absence of β_2 adrenergic receptor antagonist ICI 118, 551 (0.0012μM). Data presented as mean ± SEM. n=6-8. $^{**}p<0.01$ vs. Normoxic, $^{\#}p<0.05$ vs. HR, $^{\$}p<0.05$ vs. SalB 0.1μM

6.4.1.1 The effect of Salbutamol with co-administration of ICI 118, 551 on Infarct to Risk Ratio in isolated hearts subjected to ischaemia reperfusion injury

Hearts administered with Salbutamol (0.1μM) in the presence and absence of β_2AR antagonist ICI 118, 551 (0.0012μM) underwent 35 minutes ischaemia followed by 120 minutes reperfusion. Salbutamol significantly increased I/R ratio when compared to IR control hearts (76 ± 3% vs. 51 ± 2%, $p<0.001$, Figure 6.2). Interestingly, Salbutamol in the presence of ICI 118, 551 abrogated the I/R ratio effect caused by hearts treated alone with Salbutamol (51 ± 3% vs. 76 ± 3%, $p<0.001$, Figure 6.2). A significant decrease in I/R ratio was observed in hearts administered with a combination of both βAR antagonists ICI 118, 551 (0.0012μM), CGP 20712 (0.0014μM) and Salbutamol (0.1μM) when compared to hearts treated with Salbutamol (0.1μM) alone (52 ± 3% vs. 76 ± 3%, $p<0.001$, Figure 6.2).

Hearts treated with ICI 118, 551 alone had no significance on I/R ratio when compared to control hearts. Control IR and Salbutamol data has been used from previous experiment.

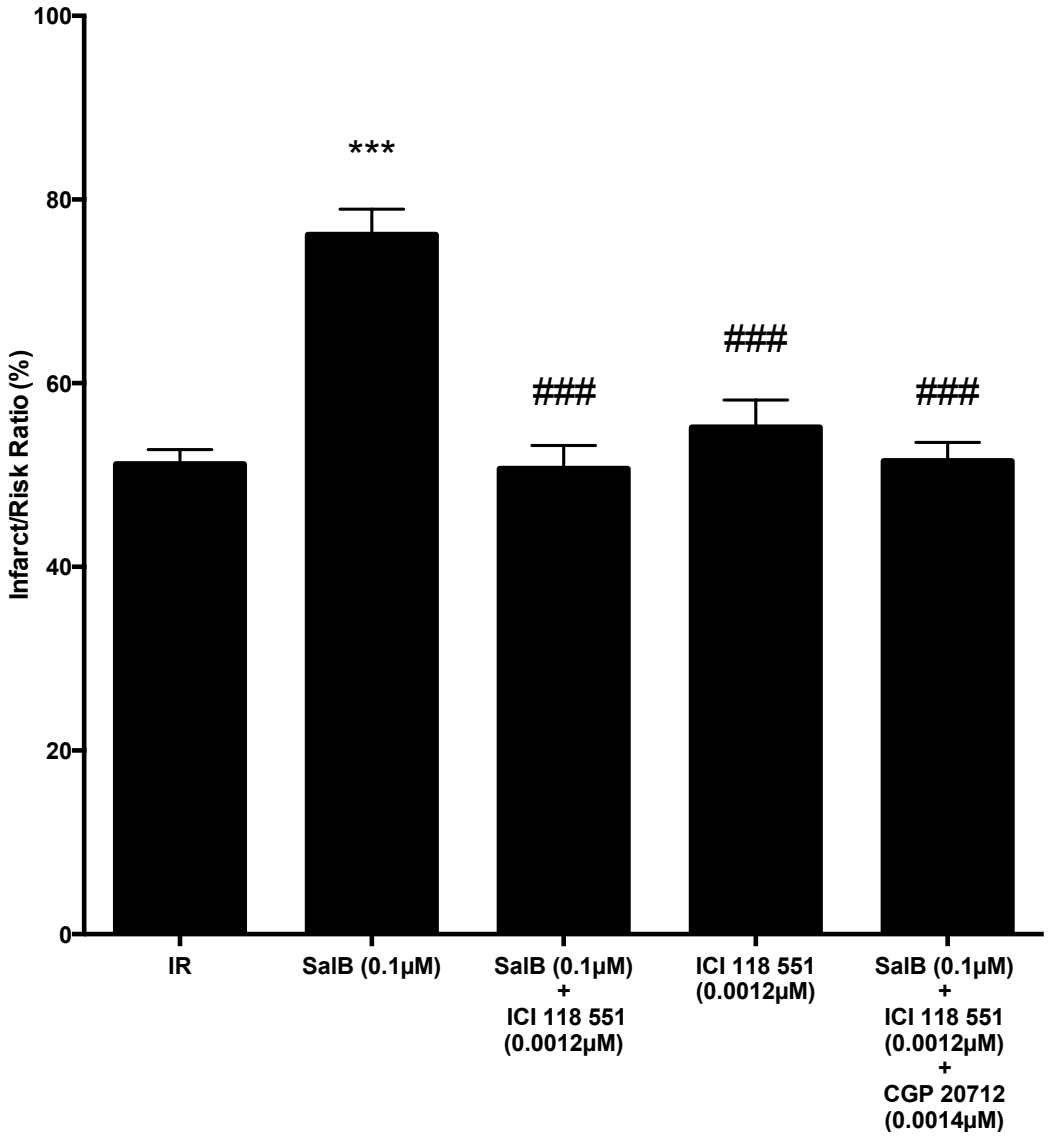


Figure 6.2 Infarct size to risk ratio (%) in isolated perfused hearts subjected to 35 minutes of ischaemia and 120 minutes reperfusion. Hearts were reperfused in the presence of Salbutamol (0.1μM) with co-administration of β_2AR antagonist ICI 118, 551 (0.0012μM) alone or in the presence of both β_1AR antagonists CGP 20712 (0.0014μM). Data presented as mean ±SEM. n=6-8. ***p<0.001 vs. IR, $^{###}$p<0.001 vs. SalB 0.1μM

6.4.1.2 Effect of Salbutamol in the presence or absence of the β_1AR antagonist ICI 118, 551 in a model of Oxidative Stress

Cardiomyocytes were subjected to laser stimulation in the presence of Salbutamol (0.1μM) in addition to the presence and absence of ICI 118, 551 (0.0012μM). Cardiomyocytes subjected to administration with Salbutamol (0.1μM) alone had no significant change on time to the onset of depolarisation when compared to control cardiomyocytes (226 ± 15s vs. 234 ± 18s, $p>0.05$, Figure 6.3). No significant change was observed in cardiomyocytes administered with the combination of Salbutamol (0.1μM) and ICI 118, 551 (0.0012μM) when compared to cardiomyocytes treated alone with Salbutamol (0.1μM) (230 ± 16s vs. 226 ± 15s, $p>0.05$, Figure 6.3). Cardiomyocytes were also treated with the combination of both βAR antagonists, ICI 118, 551 (0.0012μM), CGP 20712 (0.0014μM) and Salbutamol (0.1μM) however, no significant change in the time taken to the onset of depolarisation when compared to Salbutamol (0.1μM) treated cardiomyocytes (237 ± 15s vs. 226 ± 15s, $p>0.005$, Figure 6.3).

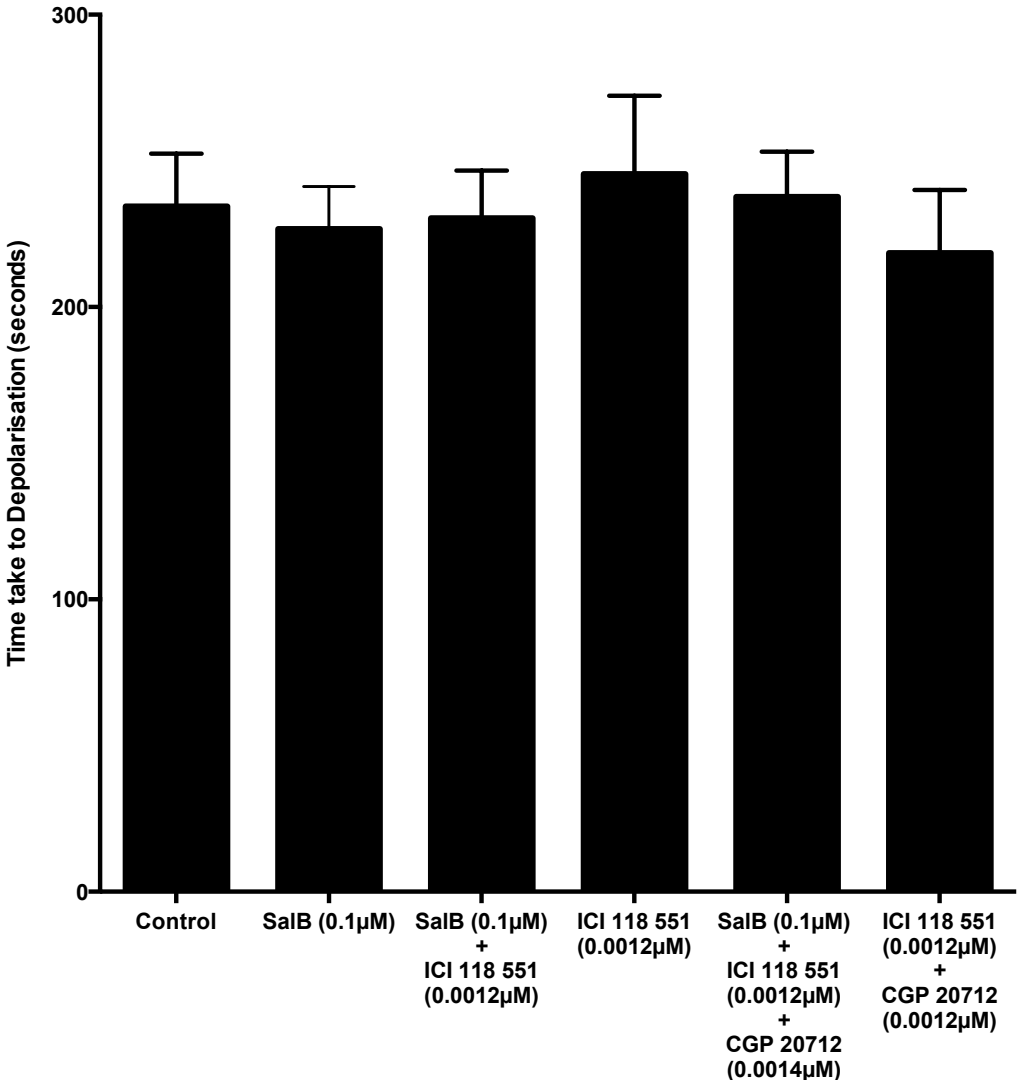


Figure 6.3 The effects of Salbutamol (0.1µM) on time taken to depolarisation in isolated rat cardiac myocytes in a model of oxidative stress in the presence or absence β_sAR antagonist ICI 118,551 (0.0012µM). Data presented as mean ±SEM. n=6-8.

Salbutamol (0.1μM) significantly decreased the time to hypercontracture in isolated cardiomyocytes when compared to non-treated control groups (524 ± 23s vs. 663 ± 40s, $p<0.001$, Figure 6.4). Co-administration of Salbutamol (0.1μM) and ICI 118, 551 (0.0012μM) significantly increased time taken to hypercontracture when compared to Salbutamol treated groups (601 ± 30s vs. 663 ± 40s, $p<0.05$, Figure 6.4). Interestingly, cardiomyocytes treated with both β_1AR antagonist CGP 20712 (0.0014μM) and β_2AR antagonist ICI 118, 551 (0.0012μM) in the presence of Salbutamol (0.1μM), significantly increased time taken to hypercontracture when compared to Salbutamol treated groups (592 ± 10s vs. 528 ± 8s, $p<0.05$, Figure 6.4). Cardiomyocytes treated with both antagonists showed no significant effect in hypercontracture when compared to non-treated control groups.

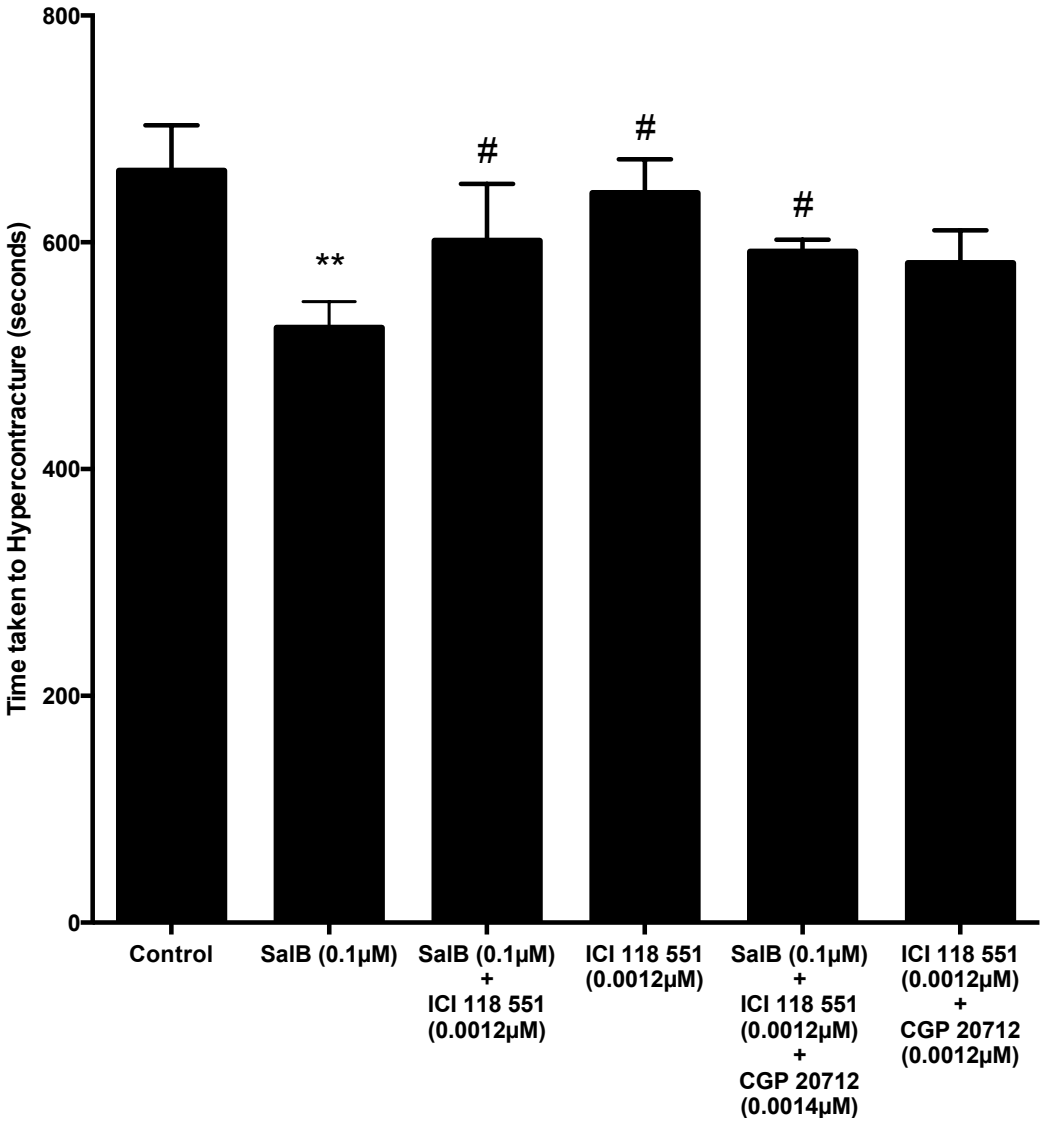


Figure 6.4 Effects of Salbutamol (0.1μM) on time taken to hypercontracture in isolated rat cardiac myocytes in a model of oxidative stress. Salbutamol (0.1μM) treated cardiomyocytes were co-administered with β_2AR antagonist ICI 118, 551 (0.0012mM) alone or a combination with β_1AR antagonist CGP 20712 (0.0014μM). n=6-8. Data presented as mean ±SEM. n=6-8.**p<0.01 vs. control, $^{\#}$p<0.05 vs. SalB (0.1μM).

6.4.1.3 The effect of Salbutamol on signalling protein p-Akt by assessment of Western blotting with co-administration of β_2AR antagonist ICI 118, 551

Investigation into the signalling protein p-Akt (Ser^{473}), was carried out in the presence of Salbutamol (0.1μM) in the absence or presence of the β_2AR antagonist, ICI 118, 551 (0.0012μM) and in the absence or presence of β_1AR antagonist CGP 20712 (0.0014μM). Hearts were reperfused with Salbutamol (0.1μM) in combination with ICI 118, 551 (0.0012μM) for 120 minutes throughout reperfusion after 35 minutes ischaemia.

IR control hearts showed a significant increase in levels of p-Akt when compared to normoxic hearts (100 ± 14% vs. 69 ± 20%, $p<0.01$). Hearts treated with Salbutamol (0.1μM) significantly increased levels of p-Akt when compared to IR control hearts (240 ± 10% vs. 100 ± 14%, $p<0.001$, Figure 6.5).

Interestingly, co-administration of Salbutamol (0.1μM) and ICI 118, 551 (0.0012μM) significantly decreased levels of p-Akt when compared to Salbutamol treated hearts, suggesting the presence of ICI 118, 551 abrogates the effect of Salbutamol on p-Akt activation (85 ± 8% vs. 240 ± 10%, $p<0.001$, Figure 6.5).

Hearts treated with both antagonists ICI 118, 551 (0.0012μM) and CGP 20712 (0.0014μM) in the presence of Salbutamol (0.1μM), significantly decreased p-Akt expression when compared to hearts treated alone with Salbutamol (0.1μM) (33 ± 4% vs. 240 ± 10%, $p<0.001$, Figure 6.5). Interestingly, this combination significantly decreased p-Akt expression levels below levels seen in control IR hearts (33 ± 4% vs. 100 ± 14%, $p<0.01$, Figure 6.5). Control, IR time matched control and Salbutamol data has been obtained from previous western blot.

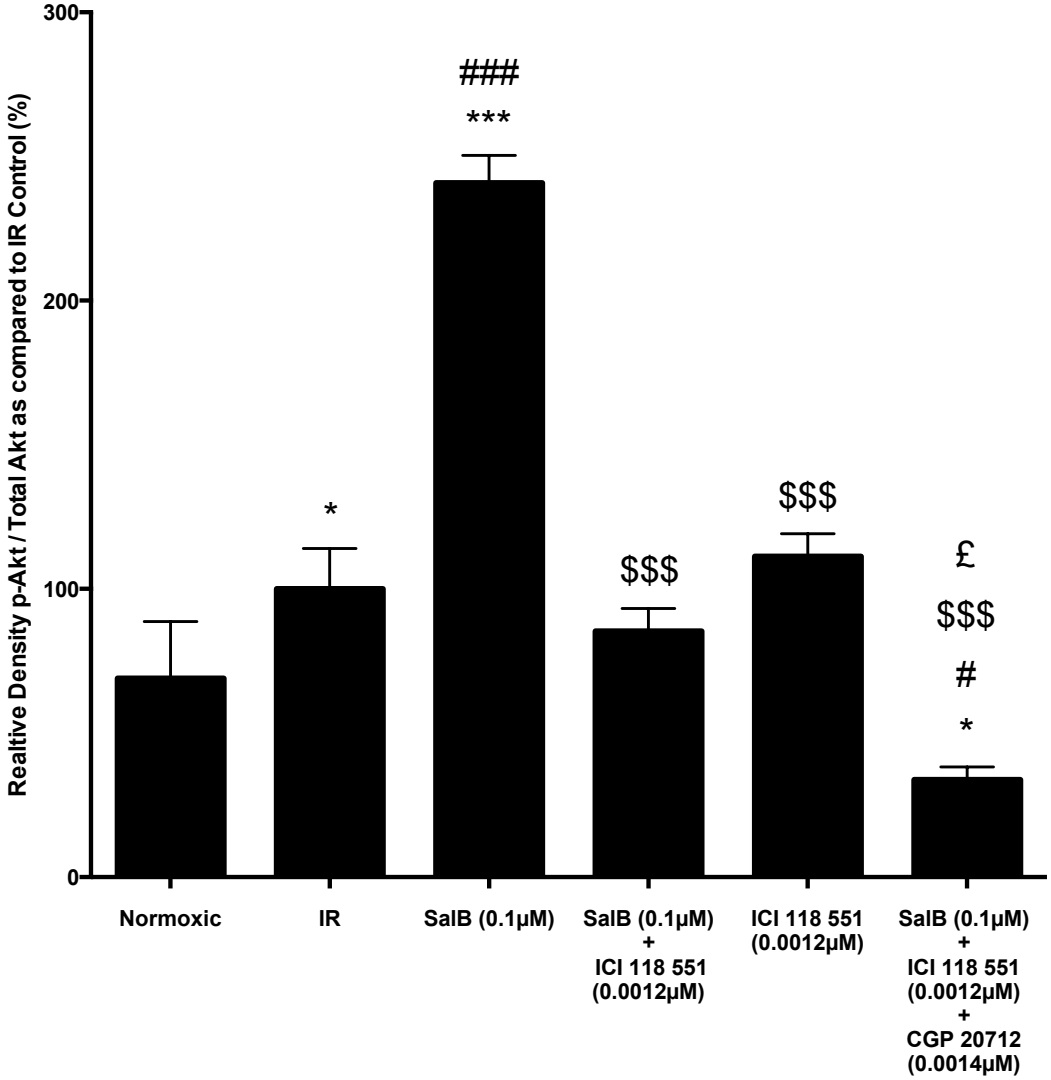


Figure 6.5 The effects of Salbutamol (0.1μM) on the levels of phosphorylated Akt after exposure to 35 minutes ischaemia and 20 minutes of reperfusion in the presence of ICI 118, 551 (0.0012μM) or combination of both β_1AR antagonist CGP 20712 (0.0014μM) and β_2AR antagonist ICI 118, 551. Data presented as mean ±SEM. n=6-8. $^{*}p<0.05$ vs. Normoxic, $^{###}p<0.001$ vs. IR, $^{\#}p<0.05$ vs. IR, $^{\$\$\$}p<0.001$ vs. SalB (0.1μM), $^{£}p<0.05$ ICI 118, 551 (0.0012μM).

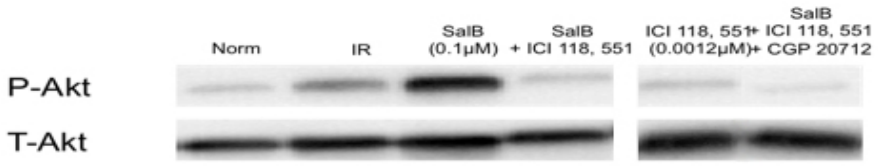


Figure 6.6 Representative blot of p-Akt and t-Akt when Salbutamol (0.1μM) was administered throughout reperfusion for 120 minutes after 35 minutes ischaemia in the presence and absence of ICI 118, 551 (0.0012μM) or a combination of both β_1AR antagonist CGP 20712 and β_2AR antagonist ICI 118, 551

6.4.1.4 The effect of Salbutamol on signalling protein p-Erk by assessment of Western blotting with co-administration of β_2AR antagonist ICI 118,551

Administration of Salbutamol (0.1μM) had no significant effect on expression of p-Erk. Administration of Salbutamol (0.1μM) in the presence and absence of ICI 118, 551 (0.0012μM) significantly decreased levels of p-Erk when compared to IR control hearts (75 ± 16% vs. 100 ± 18%, $p<0.05$, Figure 6.7).

Hearts administered with Salbutamol (0.1μM) in the presence of ICI 118, 551 (0.0012μM), had a significant decrease on levels of p-Erk when compared to IR control hearts (75 ± 16% vs. 100 ± 18%, $p<0.05$, Figure 6.7). Interestingly this same combination did not abrogate the effects of hearts treated alone with Salbutamol (0.1μM) (71 ± 16% vs. 75 ± 16%, $p>0.05$, Figure 6.7).

Hearts that were treated with both β receptor antagonists CGP 20712 (0.0014μM) and ICI 118, 551 (0.0012μM) in the presence of Salbutamol (0.1μM), significantly decreased levels of p-Erk when compared to hearts treated alone with Salbutamol (0.1μM) (41 ± 4% vs. 75 ± 16%, $p<0.05$, Figure 6.7). In addition to this comparison, a statistical decrease was also observed in levels of p-Erk when both antagonists were present with Salbutamol treated hearts when compared to Salbutamol treated hearts with β_2AR antagonists ICI 118, 551 alone (41 ± 4% vs. 71 ± 16%, $p<0.05$, Figure 6.7). Control, IR time matched control and Salbutamol data has been obtained from previous western blot.

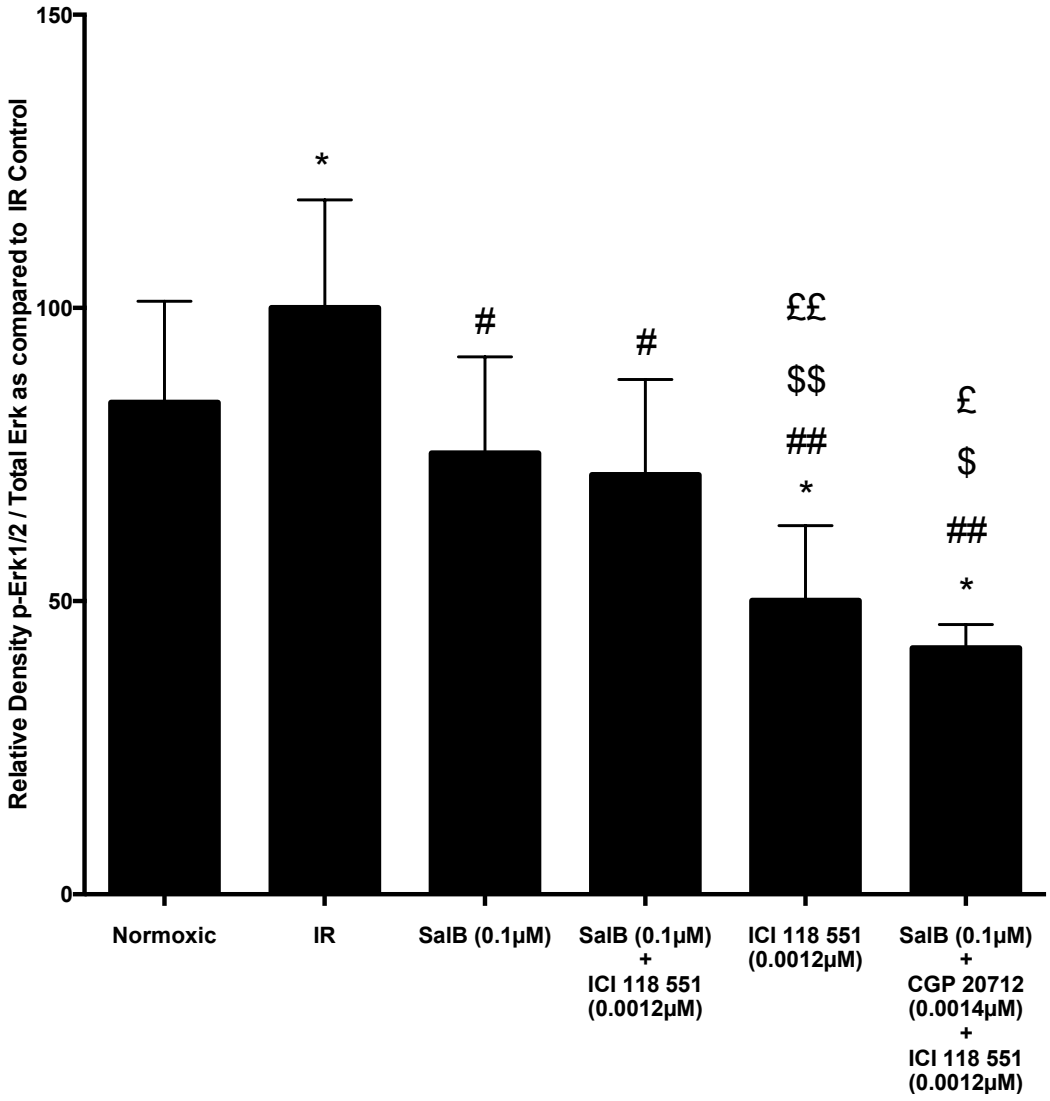


Figure 6.7 The effects of Salbutamol (0.1µM) on the levels of phosphorylated Erk after exposure to 35 minutes ischaemia and 120 minutes of reperfusion in the presence of ICI 118, 551 (0.0012µM) or combination of both β_1AR antagonist CGP 20712 (0.0014µM) and β_2AR antagonist ICI 118, 551. Data presented as mean ±SEM. n=6-8. *p<0.05 vs. Normoxic, $^{\#\#}$p<0.01 vs. IR, $^{\#}$p<0.05 vs. IR, $^{\$\$}$p<0.01 vs. SalB (0.1µM), $^{\$}$p<0.05 vs. SalB (0.1µM), $^{££}$p<0.01 vs. SalB + ICI 118, 551, $^{£}$p<0.05 vs. SalB + ICI 118, 551.

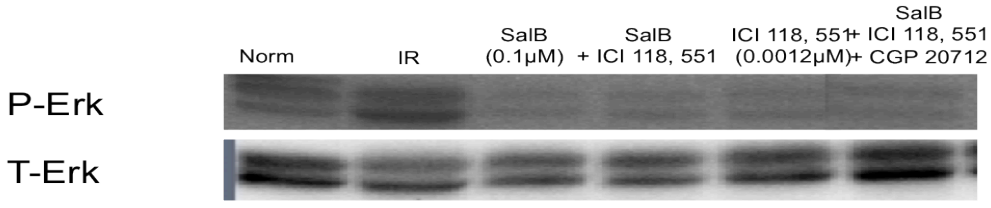


Figure 6.8 Representative blot of p-Erk and t-Erk when Salbutamol (0.1µM) was administered throughout reperfusion for 120 minutes after 35 minutes ischaemia in the presence and absence of ICI 118, 551 (0.0012µM) or a combination of both β_1AR antagonist CGP 20712 and β_2AR antagonist ICI 118, 551

6.4.1.5 The effect of Salbutamol on signalling proteins cleaved caspase 3 with co-administration of the β_2AR antagonist ICI 118, 551 by assessment of flow cytometry

Cardiomyocytes were treated in the presence and absence of the βAR antagonists CGP 20712 (0.0014μM) and ICI 118, 551 (0.0012μM) in the presence of Salbutamol (0.1μM). Cardiomyocytes treated with Salbutamol (0.1μM) alone showed a significant increase in cleaved caspase 3 activity when compared to non treated control HR cardiomyocytes (190 ± 23% vs. 100 ± 20%, $p<0.05$, Figure 6.9).

Cardiomyocytes treated with the co-administration of Salbutamol (0.1μM) and ICI 118, 551 (0.0012μM) significantly decreased levels of activated cleaved caspase 3 when compared to HR cardiomyocytes (71 ± 12% vs. 100 ± 20%, $p<0.05$, Figure 6.9). Interestingly, the co-administration of ICI 118, 551 and Salbutamol also significantly abrogated levels of activated caspase 3 when compared to Salbutamol (0.1μM) treated cardiomyocytes (19 ± 23% vs. 71 ± 12%, $p<0.01$, Figure 6.9). Cardiomyocytes treated with both antagonists significantly increased activated caspase 3 levels when compared to HR control myocytes (185 ± 35% vs. 100 ± 20%, Figure 6.9).

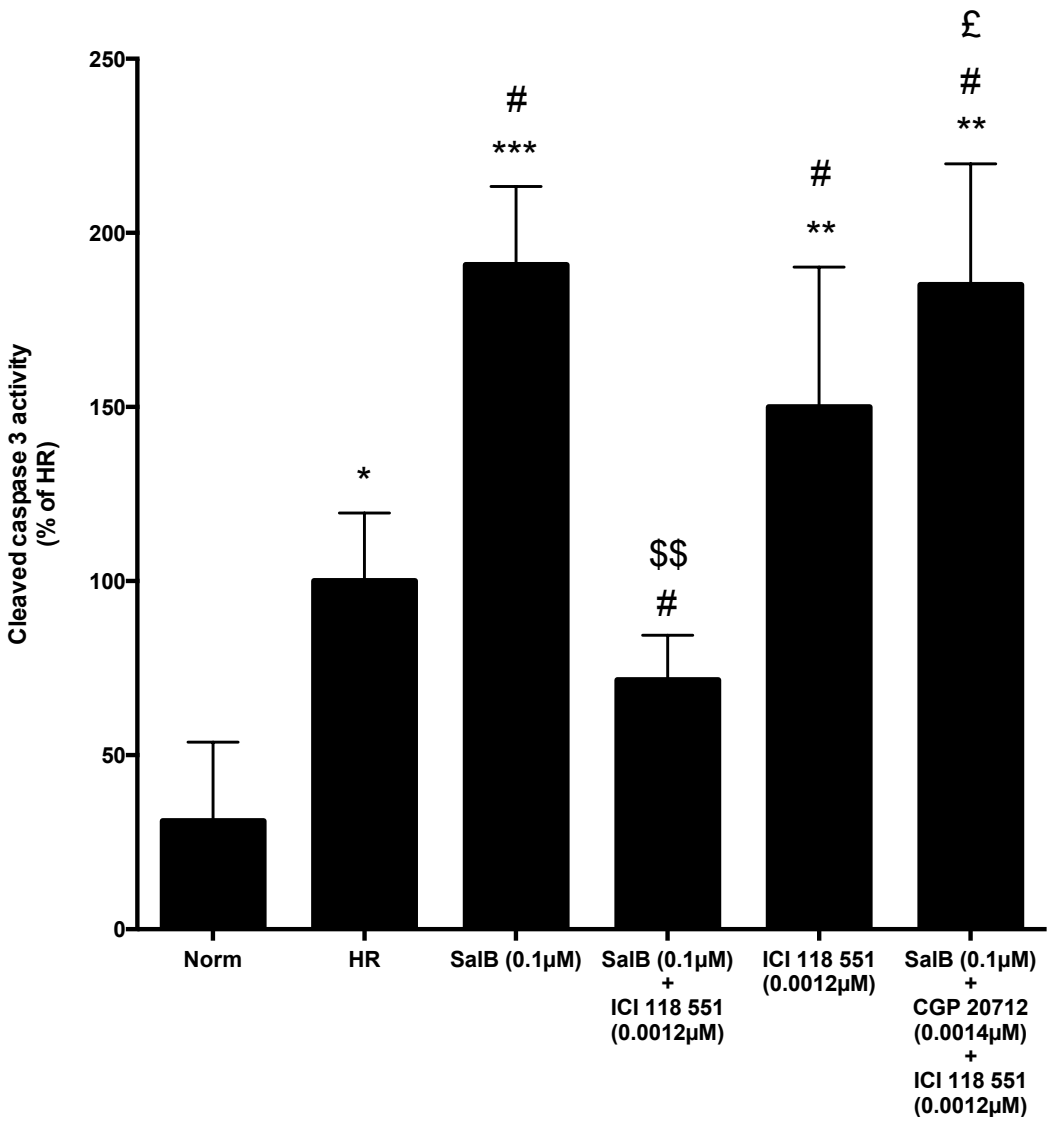


Figure 6.9 The effects of a Salbutamol (0.1μM) on levels of cleaved caspase in the presence of ICI 118, 551 (0.0012μM) or combination of both β_1AR antagonist CGP 20712 (0.0014μM) and β_2AR antagonist ICI 118, 551. Isolated myocytes were exposed to 2 hours hypoxia followed by 4 hours reoxygenation in the presence or abscene of the drug. Data presented as mean ±SEM. n=6-8. ***p<0.001 vs. Normoxic, **p<0.01 vs. Normoxic, *p<0.05 vs. Normoxic, $^{\#}$p<0.05 vs. HR, $^{\$\$}$p<0.01 vs. SalB (0.1μM), $^{£}$p<0.05 vs. SalB + ICI 118, 551.

6.5 Discussion

In the previous chapter (Chapter 5) we investigated the role of the β_1AR, in Salbutamol induced toxicity using the specific antagonist CGP 20712. We established that Salbutamol's partially mediated injury could be linked to the activation of the β_1AR. Salbutamol as an adrenergic receptor agonist has been shown to have a higher affinity for β_2ARs compared with β_1ARs, approximately 150 times greater (Bandaru et al., 2015, Dougall et al., 1991). To determine the role of the β_2ARs in Salbutamol mediated injury, we used the β_2AR specific antagonist ICI 118, 551 in the presence of Salbutamol and investigated its effects on cardiomyocytes, cytotoxicity, signalling proteins and infarct to risk ratio.

6.5.1 The effect of the co-administration of ICI 118, 551 and Salbutamol in a model of ischaemia reperfusion and cytotoxicity

In previous chapters (Chapters 3, 4 & 5) we have demonstrated that administration of Salbutamol at higher concentrations (0.1μM-1μM) exacerbates myocardial ischaemia reperfusion injury with an increase in infarct size. In addition, we have also seen an increase in cytotoxicity in cardiomyocytes treated with Salbutamol.

Figure 6.1 shows a significant decrease in reductase activity in cardiomyocytes treated with Salbutamol (0.1μM) alone when compared to HR control cardiomyocytes. Co-administration of ICI 118, 551 (0.0012μM) and Salbutamol (0.1μM) significantly increased the reductase activity when compared to cardiomyocytes treated alone with Salbutamol (0.1μM). Interestingly, reductase activity in cardiomyocytes treated with Salbutamol and ICI 118,551 increased to levels higher than the reductase activity recorded in control HR cardiomyocytes.

This observation suggests the cytotoxic effect observed in Salbutamol (0.1μM) treated cardiomyocytes can be linked activation of β_2ARs.

Control IR and Salbutamol data have been used from previous Langendorff ischaemia reperfusion experiments as discussed in 3.5. With a significant increase of infarction recorded in hearts treated alone with Salbutamol (0.1μM), administration of the β_1AR antagonist was shown to reduce infarct size (Chapter 5) but not significantly. In contrast to this finding, hearts administered with Salbutamol (0.1μM) and the β_2AR antagonist ICI 118, 551 (0.0012μM), infarction size was significantly reduced to IR control levels. This confirms that the majority of Salbutamol mediated injury is linked specifically to the activation of the β_2AR. Interestingly, when both βARs were antagonised in the presence of Salbutamol; infarction size was also significantly reduced to levels similar to those found in IR control hearts (Figure 6.2).

β_2ARs are able to couple to both the G_s and G_i subunits of GPCRs. With a majority of injury recorded via activation of the β_2ARs by Salbutamol we must first establish any link that may exist with either the G_s or G_i subunits to increased heart abnormalities. Such abnormalities that have been linked to the activation of β_2ARs are cardiomyopathies (in particular Takotsubo cardiomyopathy), tachycardia, heart failure and with our studies increase in reperfusion injury in presence of Salbutamol (Nikolaev et al., 2010, Salpeter et al., 2004, Zeb et al., 2011).

Studies that have treated cardiomyocytes with pertussis toxin (as a G_i inhibitor) showed increased inotropic activity, which are features associated with G_s stimulation (Xiao et al.,

1995, Xiao et al., 2003). Further to this, mice overexpressed with specific human β_2ARs confirmed this increase in inotropic activity (Bisognano et al., 2000).

Interestingly, studies by Foerster and colleagues (2003) with pertussis toxin in rat cardiomyocytes concluded that 2 types of pertussis toxin sensitive G_i proteins existed known as Gαi-2 and Gαi-3 (Foerster et al., 2003, Pavoine and Defer, 2005). Each of these subtypes of the G_i subunit isoforms demonstrated to have different roles that hindered or promoted survival of the transgenic mice used during their studies. Ultimately their findings indicated the activation of Gαi-2 isoform promoted survival in the mice whereas Gαi-3 activation effected regulation of Calcium, which may be one plausible link to Salbutamol mediated injury during β_2AR activation (Foerster et al., 2003). Should there be a strong regulatory effect of Calcium by Gαi-3 this may give rise to an additional source of stress to cardiomyocytes in addition to increased ROS produced as a result of reperfusion (Braunersreuther and Jaquet, 2012).

Direct phosphorylation of the β_2ARs by either PKA or PKC uncouples β_2ARs from the G_s subunit and switches coupling from the G_s subunit to the G_i subunit (Daaka et al., 1997). It is important to understand that upon phosphorylation of either of the βARs in cardiomyocytes, activation of the adenylyl cyclase pathway increases levels of cAMP ultimately resulting in increased PKA phosphorylation of several proteins to initiate L-type Ca^{2+} channels for cardiac function (Gerhardstein et al., 1999, Kamp and Hell, 2000). Other suggestions of how the G_s/G_i switch occurs is the involvement of G protein coupled receptor kinases (GRKs), discussed previously in Chapter 3 (Wang et al., 2008). Research has shown that activation of GRKs directly phosphorylates the βAR-G_s pathway and increases β-arrestin binding

It has been advocated that predominant activation of the β_1-G_s pathway promotes cell death whilst activation of the β_2-G_i pathway predominantly promotes cell survival (Amin et al., 2011, Communal et al., 1999). However, such studies suggesting cell survival through activation via β_2ARs and cell death via β_1ARs has been challenged by our findings of Salbutamol mediated injury in a cytotoxic model and ischaemia reperfusion model. Further to this, the use of Isoproterenol, a non-selective βAR agonist, has also been shown to increase myocardial injury through activation of both β_1 and β_2ARs, also seen in Chapter 3 (Homburger et al., 1981, Shin et al., 2014). Explanation for this dual promotion of cell death from activation of either of the βARs has been linked to the ability of βARs activating the β_2-G_s pathway. How exactly the β_2-G_s subunit is activated over the G_i subunit is still unclear in addition to how stressors of Salbutamol and reperfusion injury are involved in mechanisms to promote cardiomyocyte death.

Suggestions of receptor desensitisation and receptor internalisation have been described previously, especially as a result of pathophysiological events causing redistribution β_2ARs to the cell crest of cardiomyocytes whilst internalising β_1ARs (Madamanchi, 2007, Nikolaev et al., 2010). With evidence of redistribution of β_2ARs during detrimental myocardial events such as heart failure, myocardial ischaemia and myocardial infarction, reduction in the ratio of β_1ARs: β_2ARs is shifted significantly in β_2ARs favour (Lyon et al., 2009, Nikolaev et al., 2010).

Extensive research has been carried out in relation to the stressors of reperfusion injury such as Calcium overload and increased ROS activity and their direct effects on the Mitochondrial Permeability Transition Pore (mPTP) (Shimoke et al., 2003).

Our studies have indicated a delay in the time taken to the onset of depolarisation in cardiomyocytes loaded with TMRM when stimulated with a laser when compared to control cardiomyocytes in the presence of Salbutamol (0.1μM). A significant decrease in time taken to the onset of hypercontracture was observed in cardiomyocytes treated with Salbutamol (0.1μM) alone. Interestingly, a significant increase in time taken to hypercontracture was recorded in cardiomyocytes treated with Salbutamol and ICI 118, 551. This demonstrates that antagonising the β_2AR does in fact inhibit Salbutamol mediated injury via the mPTP pore. How this is achieved can still not be clearly defined. Upon laser stimulation, TMRM, which specifically quenches within mitochondria of the cardiomyocyte, degrades, increasing the production of ROS, which can act directly on the mPTP pore (Joshi and Bakowska, 2011). During ischaemia ROS can increase from <50% to 90% within the mitochondrial matrix, as was demonstrated by Loor and colleagues (2011). The recorded effect of cardiomyocytes treated alone with Salbutamol (0.1μM) compared to control cardiomyocytes in the model of oxidative stress, shows that there is an increase in stress causing a significant decrease in time to hypercontracture, however it can be purported that this reduction is not due to an increase in ROS. In other studies, Doxorubicin induced a significant decrease in time to depolarisation and hypercontracture in a model of oxidative stress as a result of further increase in ROS caused by Doxorubicin (Gharanei et al., 2013).

Another possible source of stress must be considered to explain this reduction in time taken to hypercontracture in the presence of Salbutamol and how it is affecting the mPTP. One such source related to opening of the mPTP is Calcium overload and ATP depletion (Baumgartner et al., 2009).

Activation of the β_2AR-G_S pathway increases Ca^{2+} release from the sarcoplasmic reticulum thus elevating intracellular Ca^{2+} within cardiomyocytes through the mPTP as shown in previous studies (Giorgi et al., 2012). We can suggest from previous literature that elevated Ca^{2+} may be linked to Salbutamol when activating β_2ARs. An increase in cytosolic Ca^{2+} can cause a calcium overload activating pro-apoptotic proteins such as cytochrome c (Joza et al., 2001). Premature opening of the mPTP has already been linked to increased levels of ROS in conjunction with increased Ca^{2+} concentration (Giorgi et al., 2012). Studies by Baumgartner and colleagues (2009) used mitochondria from pancreatic cells to determine the role of Calcium in inducing apoptosis via the mPTP. Their findings emphasised the importance of a relationship between ROS and increased Ca^{2+} released from stores such as the endoplasmic reticulum to initiate apoptosis. Only when Ca^{2+} ions were elevated in addition to elevated ROS, due to ischaemia followed by reperfusion, was apoptosis induced (Baumgartner et al., 2009).

Activation of β_2ARs have been shown to cause an increase in intracellular Calcium, from our studies we have shown Salbutamol can bind to and activate these receptors in the rat heart. Such a response from activated β_2ARs can suggest an increase in Calcium in addition to the increased ROS as a result of ischaemia reperfusion. Such conditions, in particular increased ROS, we have observed in the oxidative stress and Langendorff models in the presence of Salbutamol.

The delay in onset of depolarisation in cardiomyocytes with Salbutamol may be explained by the time taken for Calcium to be released upon activation of β_2ARs that are located on the cardiomyocyte cell crest (Nikolaev et al., 2010, Wright et al., 2014). Salbutamol in the

presence of ICI 118,551, significantly reduced the time taken to the onset of hypercontracture due to β_2ARs being antagonised. We can propose, Salbutamol in the presence of the β_2AR antagonist, reduces activation of the receptors, and with support from other studies, this may reduce any further release of Calcium via the β_2AR-Gs pathway thus reducing calcium overload that may lead to premature opening of the mPTP.

Control, IR time matched control and Salbutamol data has been included from other western blots. The limiting factors and consequences of this have been discussed previously in section 5.5. The significant increase in expression of p-Akt observed in hearts treated with Salbutamol (0.1μM) has been discussed in detail in Chapter 4, however it is worth re-iterating that some studies have indicated that prolonged or repetitive activation of p-Akt has been linked to increased IR injury (Nagoshi et al., 2005). This increase in p-Akt has also been shown to increase mortalities in transgenic mice with specific cardiac activation of Akt, in addition to increased I/R ratios, as seen in hearts treated Salbutamol (Matsui and Rosenzweig, 2005, O'Neill and Abel, 2005). Having recorded a significant increase in expression of p-Akt with Salbutamol, we can purport a potential link to increased Calcium levels having identified Salbutamol mediated injury is predominately via β_2AR activation via G_s subunit and/or G_i in particular the Gαi-3 isoform (Pavoine and Defer, 2005). Interestingly, hearts treated with Salbutamol in the presence of the β_2AR antagonist, ICI 118, 551, significantly reduced expression of p-Akt to control IR levels. Interestingly, in comparison to β_1ARs antagonised by CGP 20712 (Chapter 5), expression of p-Akt was seen to be much lower in hearts treated with Salbutamol in the presence of CGP 20712. This observation can link the affinity of Salbutamol for β_2AR-G_i pathway and also link the inhibitory effects caused by activation of this particular pathway (Salazar et al., 2013).

Akt has been identified to have 3 separate isoforms, of particular interest is the Akt1 isoform which is linked to the heart (Yu et al., 2015). A possible link in our experiments regarding increased p-Akt expression is an increase in intracellular calcium. Salbutamol as a positive inotrope increases calcium release from the endoplasmic reticulum via ryanodine receptors, having activated the β_2AR (Prakash et al., 1997). This increase in calcium can further release calcium via calcium-induced calcium release from the sarcoplasmic reticulum in cardiomyocytes (Chaanine and Hajjar, 2011). With this response initiated by Salbutamol, the sarcoendoplasmic reticulum calcium ATPase (SERCA) replenishes calcium stores in the sarcoplasmic reticulum by pumping calcium ions from the cytoplasm leading to increased cardiomyocyte contractions (Chaanine and Hajjar, 2011).

Salbutamol mediated toxicity via activation of β_2ARs was abolished in the presence of β_2AR antagonist ICI 118, 551 in addition to decreasing expression levels of p-Akt. Interestingly, although not significant ($p>0.05$), p-Erk expression in hearts treated with ICI 118, 551 and Salbutamol remained similar to hearts treated with Salbutamol alone. This expression of p-Erk may act as a compensatory mechanism for the significant loss of p-Akt expression to promote cell survival. As discussed in Chapter 5, p-Erk expression is linked to the inhibition of pro-apoptotic signalling proteins (Gao Smith et al., 2012). However, in Chapter 5, hearts treated with Salbutamol and β_1AR antagonist CGP 20712 demonstrated elevated p-Akt levels and supressed p-Erk as a result of Akt/Raf 1 cross-talk (Moelling et al., 2002, Zhou et al., 2015).

Cleaved caspase 3 activity, as discussed in Chapter 5, are strongly associated to mitochondrial cell death due to the release of pro-apoptotic signalling proteins (McIlwain et al., 2013). Cardiomyocytes treated with Salbutamol and ICI 118, 551 significantly decreased

caspase 3 levels when compared to Salbutamol treated cardiomyocytes (Figure 6.9) and returned caspase 3 levels to those observed in control HR cardiomyocytes. With such a significant decrease in caspase 3 activity, this further suggests and highlights the activation of β_2ARs mediates Salbutamol induced toxicity in contrast to previous work suggesting the anti-apoptotic effects of β_2AR/G_i signalling pathway (Shin et al., 2014).

This study confirms that activation of β_2ARs is predominately responsible for Salbutamol mediated injury in the model of ischaemia reperfusion and in cardiomyocytes. Antagonising the β_2AR receptor with ICI 118, 551 abolished the toxic effects of Salbutamol in the model of reperfusion injury.

Investigation of signalling proteins highlight that increased activation of p-Akt in rat hearts treated alone with Salbutamol contributes to myocardial injury. Explanations for the observed increase in p-Akt expression remain limited, however some links from other research indicate the involvement of calcium in addition to ROS and chronic activation of p-Akt. In our studies of oxidative stress, an emphasis can be made that ROS is affecting the mPTP pore directly as seen in the decrease in time to the onset of hypercontracture, however the time taken to the onset of depolarisation is not significantly reduced as expected in the presence of Salbutamol. This contributes to Salbutamol not degrading or increasing levels of ROS.

Activation of the Gαi-3 isoform of the β_2-G_i subunit could be proposed as the favoured pathway resulting in increased myocardial injury, however this requires further investigation. Other explanations for Salbutamol mediated injury via β_2AR activation can again be linked to

β_1AR receptor internalisation and desensitisation in addition to ventricular cardiomyocyte remodelling involving the migration of β_2ARs to the cardiomyocyte cell crest.

Further investigation into the role of the mPTP and ROS should be carried out. Once identifying a potential link between the two, the reversal or reduction of Salbutamol mediated injury in a model of ischaemia reperfusion and oxidative stress can be investigated.

7 Chapter 5: The role of Cyclosporin A in Preventing Salbutamol Mediated Myocardial Injury

7.1 Introduction

In previous chapters we have identified that the bronchodilator Salbutamol exacerbates myocardial injury in a model of reperfusion injury. How exactly this occurs, has been linked to the activation the β_1AR-G_s pathway and the β_2AR-G_s and G_i pathway (Chapters 5 & 6). In the model of oxidative stress, a decrease in time taken to the onset of depolarisation and hypercontracture was observed in the presence of Salbutamol. Previous studies had shown that the use of Isoproterenol is capable of inducing myocardial ischaemia and infarction in addition to maladaptive responses with the Na/Ca exchanger (Herchuelz et al., 2002). As a result, we could apply this information and purport a similar scenario with the use of Salbutamol in rat hearts.

Studies in chapters 3-6 have discussed an involvement of the mitochondrial permeability transition pore (mPTP) in βAR agonist mediated exacerbation of ischaemia reperfusion injury. Having reinforced the involvement of the mPTP in stress conditions similar to those found in reperfusion injury, investigation into the prevention/reversal of such injury would be the next logical step. Further to this, we could investigate if Salbutamol mediated injury could be reduced by means of targeting the mPTP.

The mPTP is a non-selective pore and capable of opening in normal conditions allowing movement of small molecules across the mitochondrial membrane (Halestrap and Pasdois, 2009). The mPTP has been identified as a key component maintaining mitochondrial integrity especially when involved in the events of reperfusion injury (Lemasters et al.,

2012). In normal circumstances the mPTP regulates the passing of molecules less than 1.5kDa in size from the outer mitochondrial membrane to the inner mitochondrial membrane (Halestrap and Pasdois, 2009).

Increase in stress factors such as oxidative stress or increased calcium concentration can cause the mPTP to open resulting in cell death in two different manners. The first is the lack of ability to synthesise Adenosine Triphosphate (ATP) via oxidative phosphorylation (Halestrap and Pasdois, 2009). A further detriment to cardiomyocytes is ATPase breaking down ATP produced by glycolysis leading to necrotic cell death (Dedkova and Blatter, 2012, Maddock et al., 2002). The second method in response to the same stressors involves mitochondrial swelling, initiating apoptosis (Dedkova and Blatter, 2012, Halestrap, 1982, Green and Kroemer, 2004). This phenomenon is a result of increased permeability of the inner mitochondrial membrane (Halestrap and Pasdois, 2009). With increased permeability allowing more molecules into the mitochondria causing swelling of the mitochondrial matrix and eventually rupturing of the outer mitochondrial membrane, releasing pro-apoptotic proteins into the cardiomyocyte cytoplasm such as cytochrome c (Dedkova and Blatter, 2012, Sesso et al., 2012).

The components making up the mPTP are continuously debated in regards to its specific composition and functions of the proposed components. One of the most recent proposals for the formation of the mPTP includes adenine nucleotide translocase (ANT), voltage-dependent anion channel (VDAC), phosphate carrier (PiC) and most importantly for our studies cyclophilin D (Cyp D) (Green and Kroemer, 2004, Karch and Molkentin, 2014, Halestrap and Davidson, 1990, Szabo et al., 1993, Lemasters et al., 2012, Lopez-Erauskin et al., 2012). In respect of Cyp D as a “known mitochondrial localized cyclophilin protein”, this

has been targeted for therapeutic reasons to prevent premature opening of the mPTP to prevent cardiomyocyte death (Karch and Molkentin, 2014). The use of the immunosuppressant cyclosporin A (CsA) as early as 1997 was found to inhibit the opening of the mPTP by specifically inhibiting Cyp D its activity (Halestrap et al., 1997a). The initial clinical use of CsA has been in the surgical field as a potent immunosuppressant post successful transplantation of an organ (Rezzani, 2006). Inhibition of Cyp D was found to protect hearts against ischaemia reperfusion injury via delaying the opening if the mPTP (Hausenloy et al., 2003, Song et al., 2015). Loss of mitochondrial function as a result of poor calcium handling and increased ROS leads to mitochondrial dysfunction ultimately resulting in prolonged opening of the mPTP leading to cardiomyocyte apoptosis (Song et al., 2015).

Simulating ischaemia in the model of reperfusion injury by the physical tightening of the ligature around the left descending coronary artery reduces oxygen and nutrient supply to left ventricle (Bell et al., 2011). This reduction in nutrient hinders the left ventricle increasing ischaemic tissue which if not reperfused will become infarcted. However, as paramount it is to reperfuse the ischaemic area of the heart, an accumulation of calcium and ROS occurs, and upon reperfusion introduces these stressors to the previously ischaemic tissue resulting in further damage and further increase in infarcted tissue (Bell et al., 2011, Hussain et al., 2014). In conditions such as these, increase in ROS and calcium act as stressors affecting the function of cardiomyocytes and ultimately lead to the phenomenon known as ischaemia reperfusion injury (Bell et al., 2011, Ong et al., 2015b).

To highlight the importance of Cyp D involvement in opening of the mPTP, Cyp D knockout mice showed to be highly resistant to mPTP opening when subjected to calcium overload, similar to wild type mice treated with CsA (Nakagawa et al., 2005).

By targeting the Cyp D component of the mPTP with a drug such as CsA, we can determine if hearts and cardiomyocytes treated with Salbutamol does in fact initiate cell death via premature opening of the mPTP or via another pathway that we may have eluded.

7.2 Aims

The aims of the current study were to investigate the effects of the short acting β adrenergic receptor agonist Salbutamol by using the isolated perfused Langendorff heart model of ischaemia reperfusion injury, the oxidative stress cardiac myocyte model and the MTT assay. Salbutamol was administered in the presence and absence of Cyclosporin A to determine whether Salbutamol mediated injury can be reduced or prevented with inhibition of cyclophilin D in the mitochondrial permeability transition pore.

7.3 Methods

7.3.1 Langendorff protocol

Briefly, Sprague-Dawley rats were sacrificed by cervical dislocation and cannulated to the Langendorff setup and perfused with KHB as described in section 2.3. Hearts were allowed to stabilise for 20 minutes followed by 35 minutes of regional ischaemia and 120 minutes of reperfusion. One minute before the onset of reperfusion hearts were administered Salbutamol (0.1μM) in the absence or presence of β Adrenergic Receptor antagonists CGP 20712 (0.0014μM). At the end of the experiment hearts underwent infarct to risk ratio analysis. Haemodynamic data were collected throughout the study.

For western tissue collection, hearts were reperfused with Salbutamol (0.1μM) for either 5, 20 or 120 minutes in the presence or absence of CsA (0.2μM). After the time elapsed, hearts

were removed and the left ventricle removed and snap frozen in liquid nitrogen. Control IR data have been used from previous experiments.

7.3.2 Western blot analysis

Analysis of tissue by western blot was carried out as described in section 2.7. Briefly, following gel electrophoresis, proteins were transferred to a PVDF membrane and probed for the phosphorylated and total forms of the proteins: phospho-Akt (Ser_{473}) (1:1000) and phospho-p44/p42 (Erk 1/2, Thr202/Tyr204) (1:1000).

7.3.3 Adult rat cardiac myocyte isolation

Briefly, male Sprague Dawley rats were sacrificed by cervical dislocation and the hearts excised and cannulated onto modified Langendorff apparatus and perfused with a constant flow rate of 14ml/min as described in section 2.4. Hearts were perfused for 3-4 minutes with calcium free modified Krebs Heinsleit Buffer. The buffer was then switched and the hearts perfused with digestion buffer for 7 minutes. Isolated ventricular myocytes were used for the oxidative stress model, MTT assay and flow cytometric analysis of cleaved caspase 3 as described previously in sections 2.5.2, 2.5.3, 2.6. Myocytes were assigned to one of the following groups: a) Control (KHB) b) Salbutamol (0.1μM) c) CsA + Salbutamol d) CsA (0.2μM).

7.3.4 Statistical analysis

All data were presented as a mean of the stabilisation period ± SEM. Infarct size, times taken to depolarisation and hypercontracture and western blot data were tested using one way ANOVA with a Fishers Least Significance Test post hoc test to determine any significance between groups. $p<0.05$ was considered to be significant.

7.4 Results

7.4.1 Effect of Salbutamol co-administered with Cyclosporin A on cardiomyocytes assessed by MTT

Isolated cardiomyocytes were subjected to 2 hours of hypoxia and 4 hours of reoxygenation where Salbutamol (0.1μM) was administered throughout the reoxygenation period in the presence and absence of CsA (0.2μM). Cardiomyocytes treated with Salbutamol (0.1μM) significantly decreased reductase activity when compared to HR control cardiomyocytes (75 ± 1% vs. 209 ± 8%, $p<0.001$, Figure 7.1). Cardiomyocytes treated alone with CsA (0.2μM) when compared to control HR cardiomyocytes significantly increased reductase activity (141 ± 2% vs. 100 ± 3%, $p<0.01$, Figure 7.1).

Interestingly, cardiomyocytes administered with both Salbutamol (0.1μM) and CsA (0.2μM) when compared to cardiomyocytes treated with Salbutamol (0.1μM) recorded a significant increase in reductase activity (162 ± 2% vs. 75 ± 1%, $p<0.05$, Figure 7.1). This indicates that CsA (0.2μM) in the presence of Salbutamol (0.1μM) abrogates the cytotoxic effect Salbutamol (0.1μM) alone has on cardiomyocytes during hypoxia/reoxygenation injury.

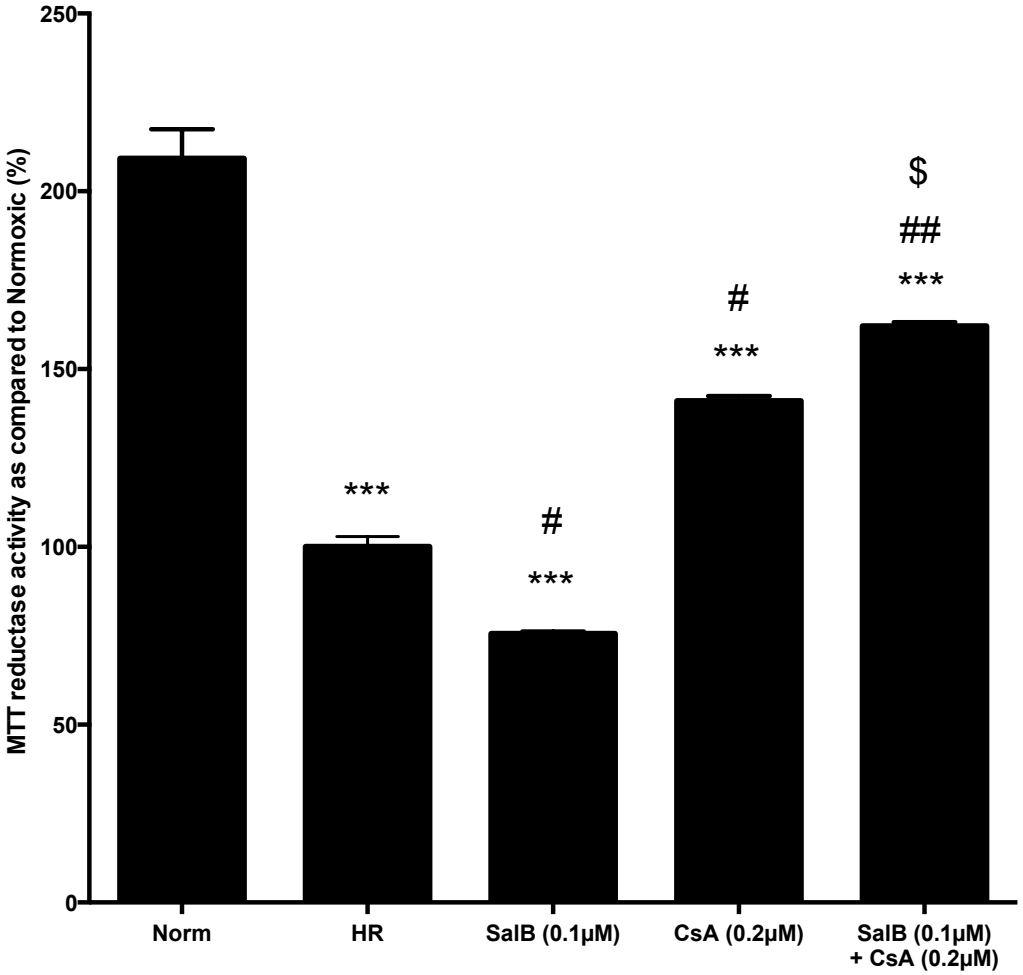


Figure 7.1 The MTT cytotoxic effect of Salbutamol (0.1μM) on the viability of cardiomyocytes in the presence and absence of Cyclosporin A (0.2μM). Data presented as mean ± SEM. n=6-8. ***p<0.001 vs. Normoxic, $^{\#\#}$p<0.01 vs. HR, $^{\#}$p<0.05 vs. HR, $^{\$}$p<0.05 vs. SalB (0.1μM)

7.4.1.1 The effect of Salbutamol with co-administration of Cyclosporin A on Infarct to Risk Ratio in isolated hearts subjected to ischaemia reperfusion injury

Isolated perfused hearts were subjected to 35min ischaemia and 120 minutes of reperfusion where Salbutamol (0.1μM) was administered in the presence and absence of CsA (0.2μM) throughout the reperfusion period. Salbutamol (0.1μM) significantly increased I/R ratio when compared to IR control hearts (SalB 0.1μM, 76 ± 3% vs. 51 ± 2%, p<0.001, Figure 7.2).

Interestingly, hearts treated alone with CsA (0.2μM) significantly decreased I/R ratio when compared to IR control hearts (39 ± 2% vs. 51 ± 2%, $p<0.001$, Figure 7.2).

Hearts treated with Salbutamol (0.1μM) and CsA (0.2μM) when compared to hearts treated with Salbutamol (0.1μM) alone, abrogated the damaging effect of Salbutamol (0.1μM) (46 ± 2% vs. 76 ± 3%, $p<0.001$, Figure 7.2). Control IR and Salbutamol data have been used from previous experiments.

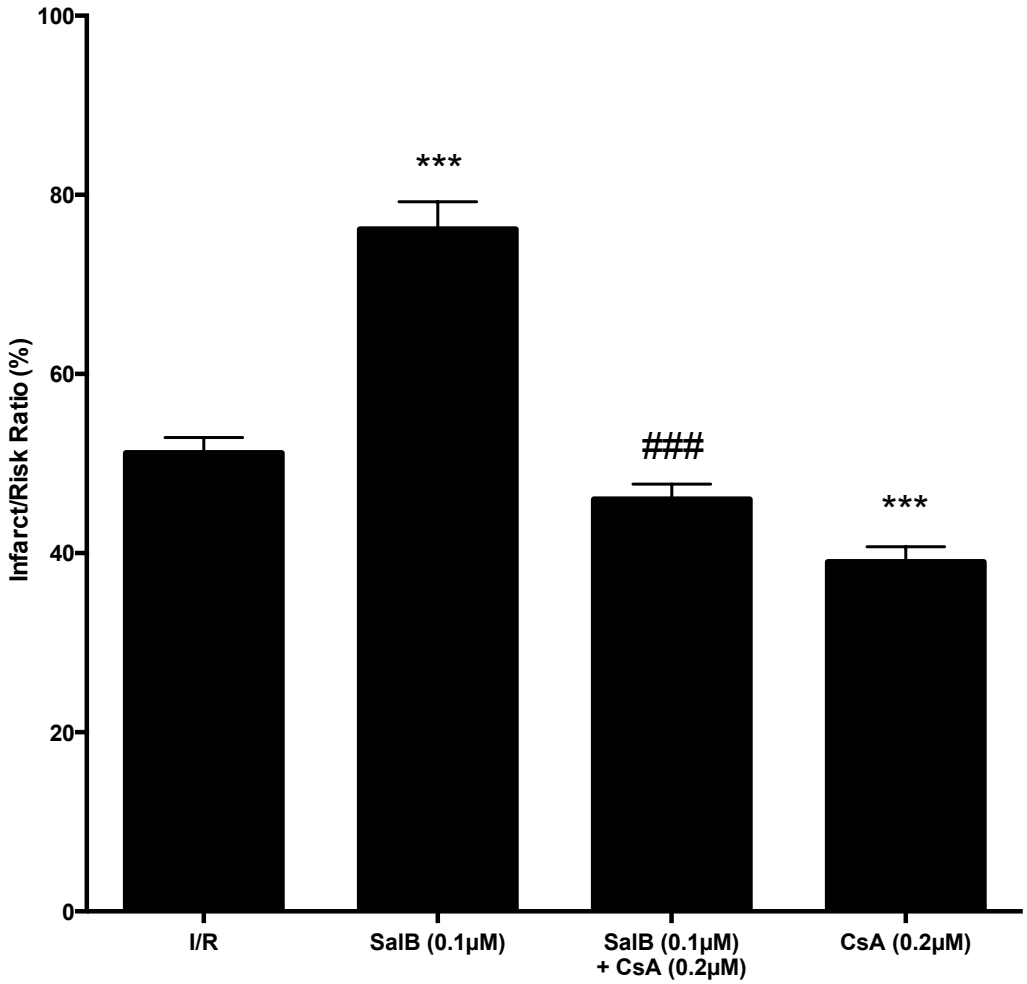


Figure 7.2 Infarct size to risk ratio (%) in isolated perfused hearts subjected to 35 minutes of ischaemia and 120 minutes reperfusion in the presence and absence of Salbutamol (0.1µM) and with co-administration of Cyclosporin A (0.2µM) throughout the reperfusion period. Data presented as mean ±SEM. n=6-8. ***p<0.001 vs. IR, $^{###}$p<0.001 vs. SalB 0.1µM, $^{##}$p<0.01 vs. SalB 0.1µM.

7.4.1.2 Effect of Salbutamol in the presence or absence Cyclosporin A in a model of Oxidative Stress

Cardiomyocytes were subjected to laser stimulation in the presence of Salbutamol (0.1µM) in addition to the presence and absence of Cyclosporin A (0.2µM). Cardiomyocytes subjected to administration with Salbutamol (0.1µM) alone decreased time to the onset of depolarisation however it did not reach significance when compared to control (226 ± 15s vs. 255 ± 13s,

p>0.05, Figure 7.3). Cardiomyocytes administered alone with Cyclosporin A (0.2μM) significantly increased time taken to the onset of depolarisation when compared to non-treated control cardiomyocytes (325 ± 12s vs. 255 ± 13s, p<0.01, Figure 7.3).

Interestingly, the co-administration of Salbutamol (0.1μM) and Cyclosporin A (0.2μM) significantly abrogated the effect observed in cardiomyocytes treated alone with Salbutamol (0.1μM) (325 ± 22s vs. 226 ± 15s, p<0.001, Figure 7.3).

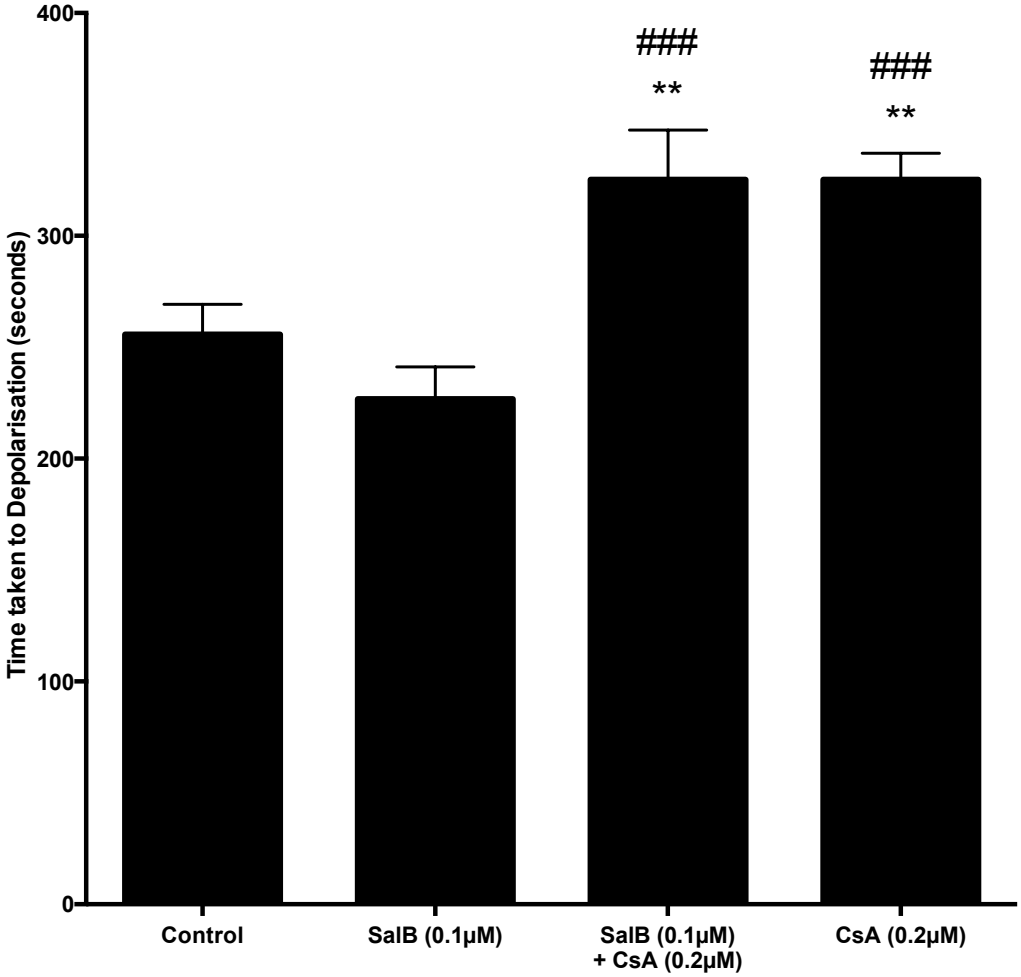


Figure 7.3 The effects of Salbutamol (0.1μM) on time taken to depolarisation in isolated rat cardiac myocytes in a model of oxidative stress in the presence or absence of Cyclosporin A (0.2μM). Data presented as mean ±SEM. n=6-8. **p<0.01 vs. Control, $^{###}$p<0.001 vs. SalB (0.1μM), $^{##}$p<0.01 vs. SalB 0.1μM.

Salbutamol (0.1μM) significantly decreased the time to hypercontracture in isolated cardiomyocytes when compared to non-treated control groups (524 ± 23s vs. 663 ± 40s, $p<0.01$, Figure 7.4). Cardiomyocytes treated alone with Cyclosporin A (0.2μM) significantly increased time taken to the onset of hypercontracture when compared to non-treated control cardiomyocytes (741 ± 17s vs. 663 ± 40s, $p<0.001$).

Interestingly, the co-administration of Salbutamol (0.1μM) and Cyclosporin A (0.2μM) significantly increased the time taken to the onset of hypercontracture when compared to cardiomyocytes treated alone with Salbutamol (0.1μM)(662 ± 17s vs. 525 ± 23s, $p<0.01$, Figure 7.4).

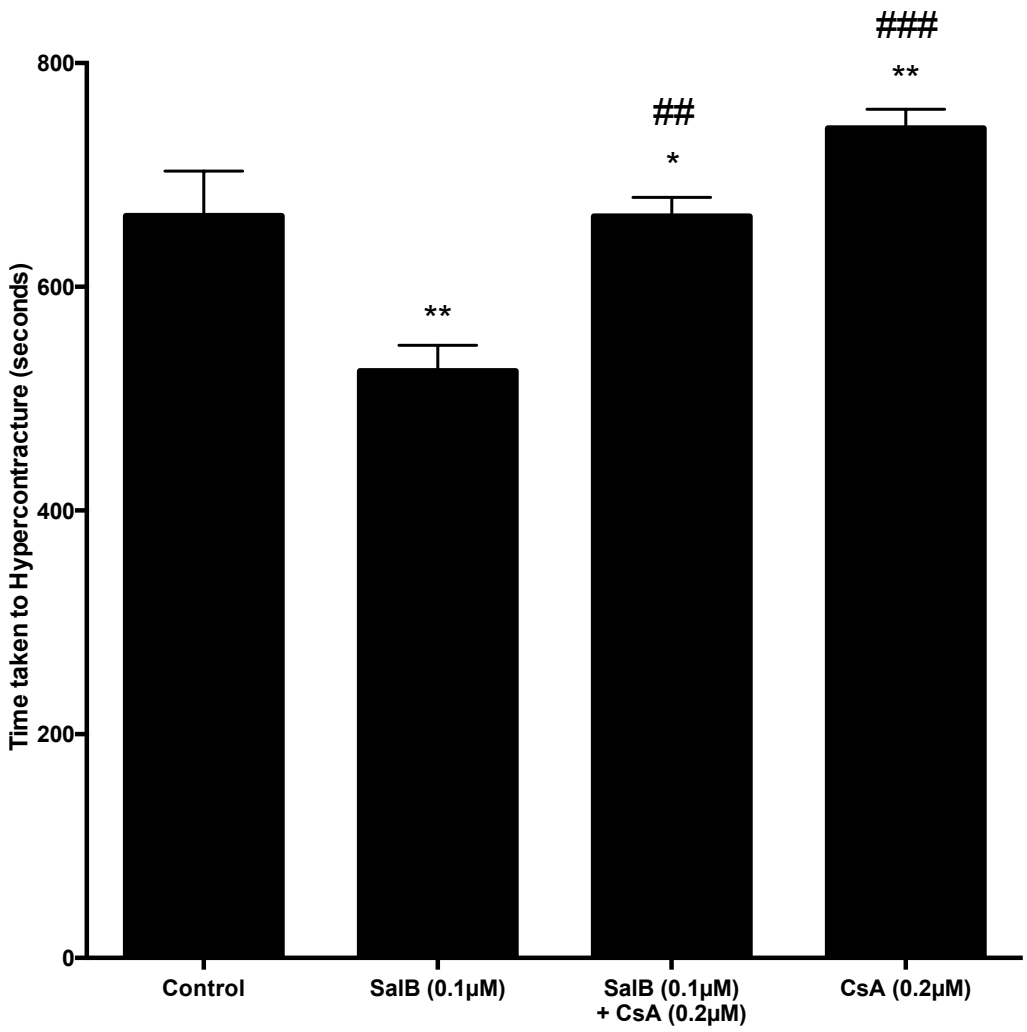


Figure 7.4 The effects of Salbutamol (0.1µM) on time taken to hypercontracture in isolated rat cardiac myocytes in a model of oxidative stress in the presence or absence of Cyclosporin A (0.2µM). n=6-8. Data presented as mean ±SEM. n=6-8. ****p<0.01 vs. control,** ***p<0.05 vs. control,** $^{###}$**p<0.01 vs. SalB (0.1µM),** $^{##}$**p<0.01 vs. SalB (0.1µM).**

7.4.1.3 The effect of Salbutamol on signalling protein p-Akt by assessment of Western blotting with co-administration of Cyclosporin A

Investigations into the signalling protein p-Akt (Ser^{473}), was carried out in the presence of Salbutamol (0.1μM) in the absence or presence of Cyclosproin A (0.2μM). Hearts were reperfused with Salbutamol (0.1μM) in combination with Cyclosproin A (0.2μM) for 120 minutes throughout reperfusion after 35 minutes ischaemia.

IR control hearts showed a significant increase in levels of p-Akt when compared to normoxic hearts (100 ± 14% vs. 69 ± 20%, $p<0.01$, Figure 7.5). Hearts treated with Salbutamol (0.1μM) significantly increased levels of p-Akt when compared to IR control hearts (240 ± 10% vs. 100 ± 14%, $p<0.001$, Figure 7.5).

Hearts treated alone with Cyclosporin A (0.2μm) significantly decreased expression of p-Akt when compared to IR control hearts (28 ± 4% vs. 100 ± 20%, $p<0.05$). Interestingly, hearts treated with the co-administration of Salbutamol (0.1μM) and Cyclosporin A (0.2μM) abrogated the expression of p-Akt seen in hearts treated with Salbutamol (0.1μM) alone (35 ± 4% vs. 240 ± 8%, $p<0.001$, Figure 7.5). Control, IR time matched control and Salbutamol data have been used from previous western blots run.

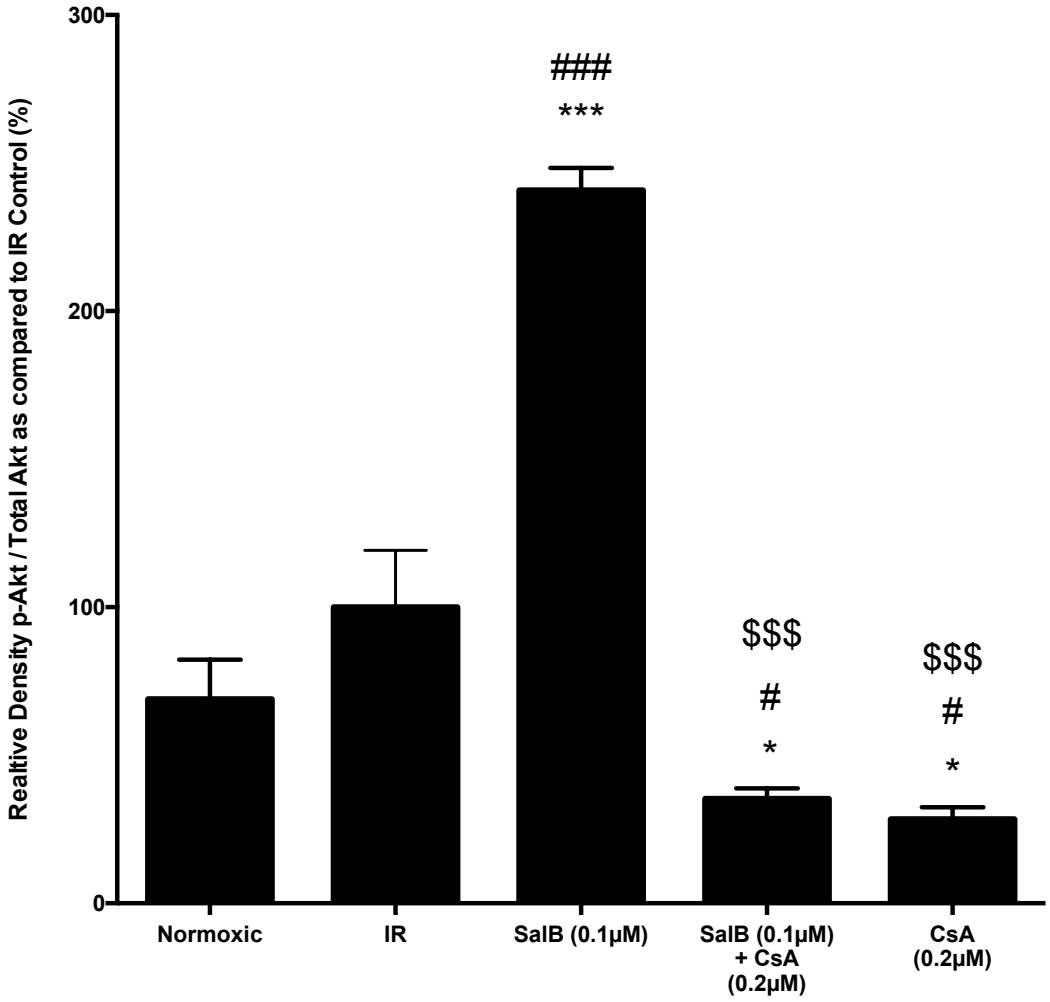


Figure 7.5 The effects of Salbutamol (0.1µM) on the levels of phosphorylated p-Akt after exposure to 35 minutes ischaemia and 120 minutes of reperfusion in the presence and absence of Cyclosporin A (0.2µM). Data presented as mean ±SEM. n=3. ***p<0.001 vs. Normoxic, *p<0.05 vs. Normoxic, $^{###}$p<0.001 vs. IR, $^{#}$p<0.05 vs. IR, $^{\$\$\$}$p<0.001 vs. SalB (0.1µM).

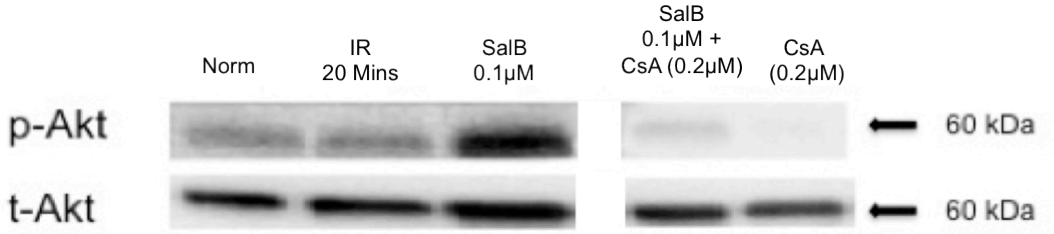


Figure 7.6 Representative blot of p-Akt and t-Akt when Salbutamol (0.1µM) was administered throughout reperfusion for 120 minutes after 35 minutes ischaemia in the presence and absence of Cyclosporin A (0.2µM).

7.4.1.4 The effect of Salbutamol on signalling protein p-Erk by assessment of Western blotting with co-administration of Cyclosporin A

Investigation into the signalling protein p-Erk was carried out in the presence of Salbutamol (0.1μM) in the absence or presence of Cyclosproin A (0.2μM). Hearts treated with Salbutamol (0.1μM) alone had no significant change on expression of p-Erk when compared to IR control hearts (75 ± 11% vs. 100 ± 28%, $p>0.05$, Figure 7.5).

Hearts treated in the presence of Salbutamol (0.1μM) and Cyclosporin A (0.2μM) significantly decreased levels of p-Erk when compared to control IR control hearts (18 ± 5% vs. 100 ± 28%, $p<0.05$, Figure 7.7) Hearts treated with Cyclosporin A (0.2μM) alone significantly abrogated with increase in levels p-Erk recorded in IR control hearts (43 ± 5% vs. 100 ± 28%, $p<0.05$, Figure 7.7). Control, IR time matched control and Salbutamol data have been used from previous western blots run.

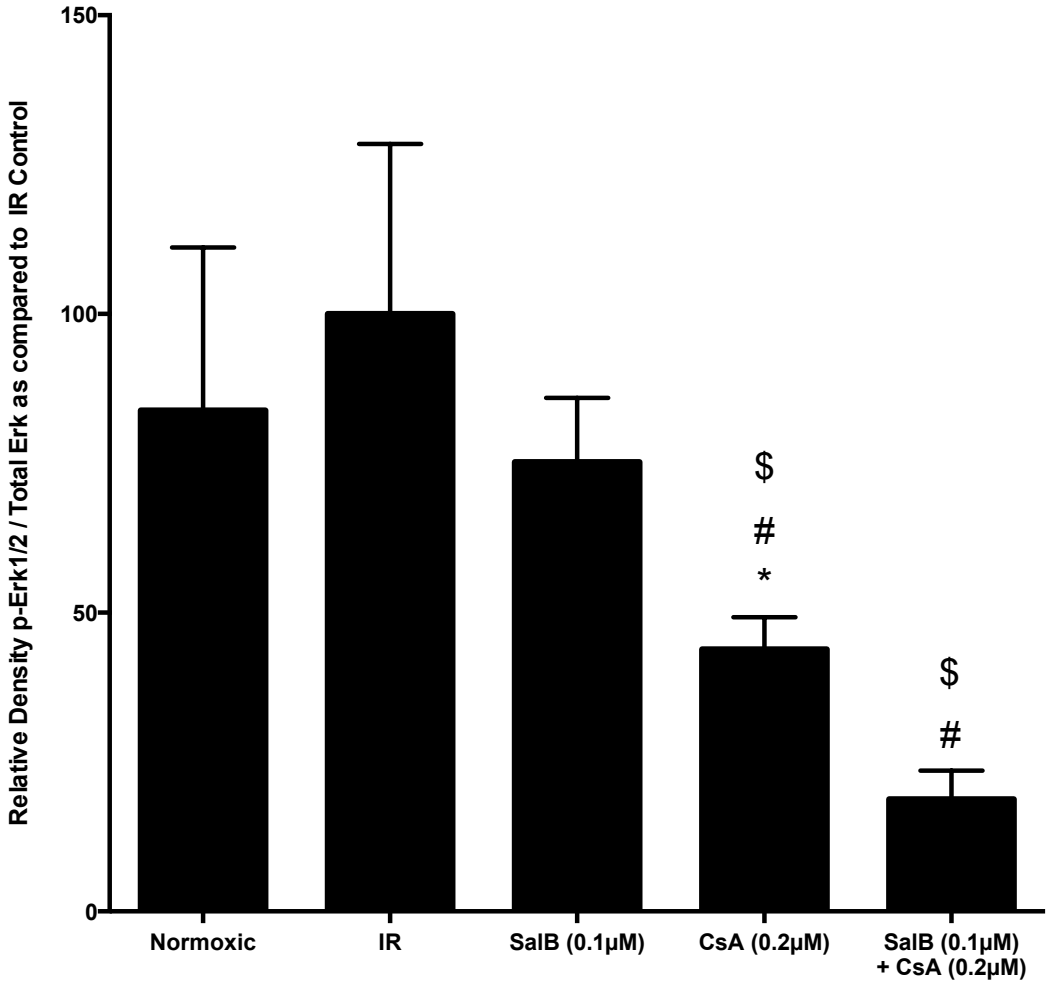


Figure 7.7 The effects of Salbutamol (0.1µM) on the levels of phosphorylated Erk after exposure to 35 minutes ischaemia and 120 minutes of reperfusion in the presence and absence of Cyclosporin A (0.2µM). Data presented as mean ±SEM. n=3. *p<0.05 vs. Normoxic, $^{\#}$p<0.05 vs. IR, $^{\$}$p<0.05 vs. SalB (0.1µM).

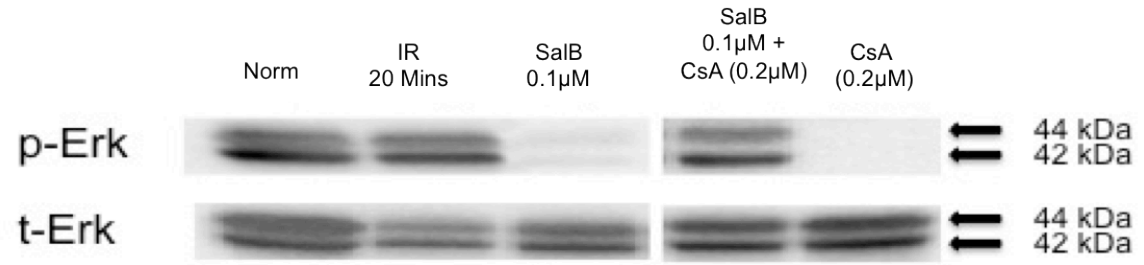


Figure 7.8 Representative blot of p-Erk and t-Erk when Salbutamol (0.1µM) was administered throughout reperfusion for 120 minutes after 35 minutes ischaemia in the presence and absence of Cyclosporin A (0.2µM).

7.4.1.5 The effect of Salbutamol on signalling proteins cleaved caspase 3 activity with co-administration of Cyclosporin A

Control HR cardiomyocytes were seen to have significantly higher levels of cleaved caspase 3 when compared to non-treated normoxic cardiomyocytes (100 ± 20% vs. 31 ± 22%, $p<0.05$, Figure 7.7). Interestingly, cardiomyocytes treated with Salbutamol (0.1μM) alone expressed higher levels of cleaved caspase 3 activity when compared to HR control cardiomyocytes (190 ± 23% vs. 100 ± 20%, $p<0.001$, Figure 7.7).

Hearts co-administration with Salbutamol (0.1μM) and Cyclosporin A (0.2μM) did not significantly abrogate the effect observed in cardiomyocytes treated alone with Salbutamol (0.1μM) (143 ± 20 % vs. 190 ± 23%, $p>0.05$, Figure 7.7).

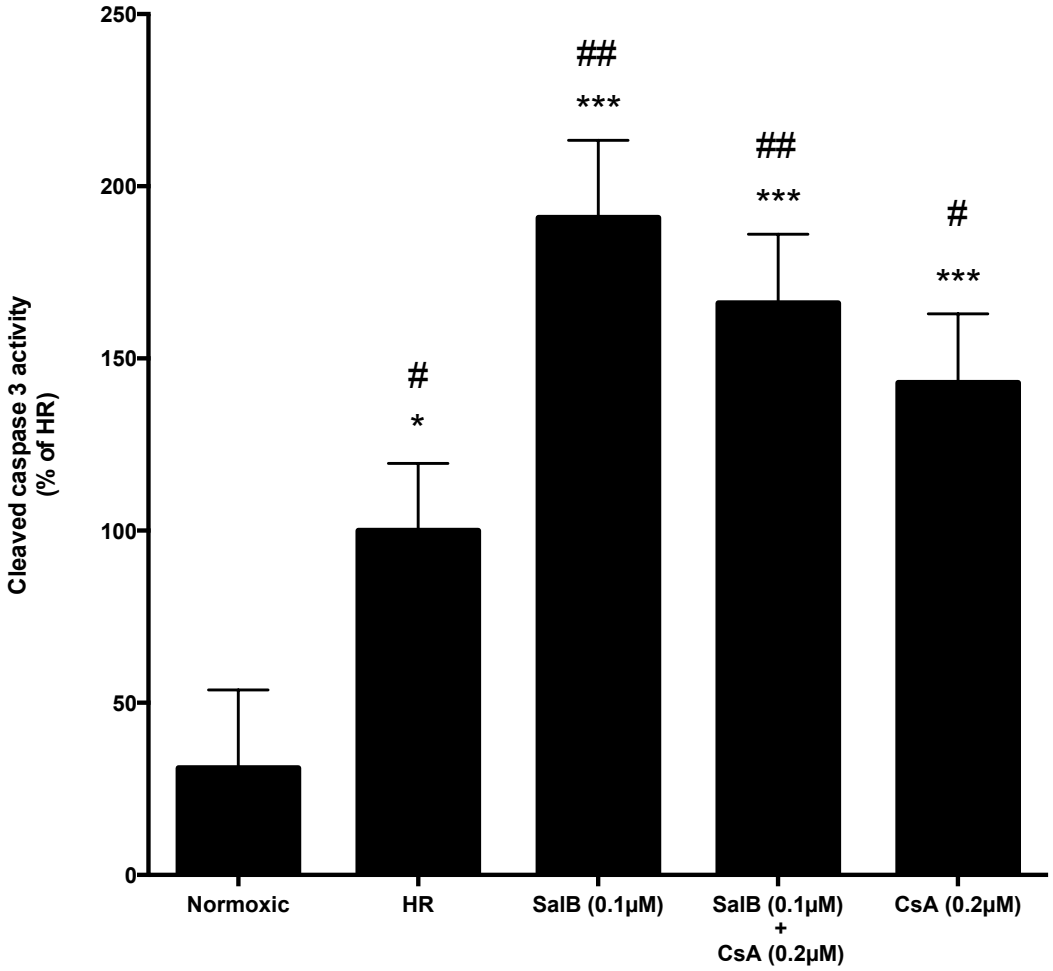


Figure 7.9 The effects of a Salbutamol (0.1μM) on cleaved caspase 3 in the absence and presence of CsA (0.2μM). Data presented as mean ±SEM. n=6-8. ***p<0.001 vs. Normoxic, *p<0.05 vs. Normoxic, $^{\#\#}$p<0.01 vs. HR, $^{\#}$p<0.05 vs. HR.

7.5 Discussion

In the previous chapter (Chapter 6), we investigated the role of the β_2AR in Salbutamol induced toxicity using the specific antagonist ICI 118, 551, establishing that Salbutamol mediated injury could strongly be linked to the activation of the β_2AR. A decrease in the time taken to the onset of depolarisation and hypercontracture was recorded in addition to a significant increase in these times by antagonising the βAR receptors as seen in Chapters 5 & 6. Our findings from these studies identified Salbutamol to cause increased stress and to cardiomyocytes and the mPTP. It remains unclear from our studies which stressor (ROS or calcium) is specifically responsible for the premature opening of the mPTP.

Previous literature has highlighted Salbutamol's involvement with calcium signalling due to its positive inotropic and chronotropic nature, however the assessment of Salbutamol's involvement with an increase in ROS is limited. Previous work by Zhang and colleague (2005) have used the non-selective βAR Isoproterenol in rat hearts and showed that this βAR induced oxidative stress via increase in ROS in addition to cardiac remodelling (Zhang et al., 2005, Hudecova et al., 2013, Capozza et al., 1992)

To determine the role of oxidative stress in Salbutamol mediated injury, we used the Cyp D inhibitor CsA in the presence of Salbutamol and investigated its effects on cardiomyocytes, cytotoxicity, signalling proteins and infarct to risk ratio.

Having identified in previous chapters the exacerbation of I/R ratio in hearts treated with Salbutamol (0.1μM-1μM) and increase in cytotoxicity, the co-administration of CsA (0.2μM) with Salbutamol (0.1μM) was recorded. Figure 7.1 shows a significant decrease in reductase

activity in cardiomyocytes treated with Salbutamol (0.1μM) alone when compared to HR control cardiomyocytes. Cardiomyocytes treated with Salbutamol (0.1μM) and CsA (0.2μM) showed a significant increase in reductase activity when compared to cardiomyocytes treated alone with Salbutamol (0.1μM). This significant increase illustrates CsA is capable of inhibiting the cytotoxic effects of Salbutamol and interestingly able to increase the reductase activity higher than control HR cardiomyocytes. This increase was also mimicked in cardiomyocytes treated alone with CsA. This effect of CsA initially suggests the importance of Cyp D in response to the stressors produced by Salbutamol in cardiomyocytes, and the affinity of CsA able to inhibit Cyp D.

A variety of pharmacological agents have previously been shown to induce cardiotoxicity and exacerbate I/R ratio in rat hearts such as Isoproterenol, Doxorubicin and Ipratropium Bromide (Gharanei et al., 2013, Harvey et al., 2014, Lobo Filho et al., 2011). Interestingly, the co-administration of CsA and Isoproterenol has recently been carried out and was shown to prevent Isoproterenol mediated injury in rat hearts, highlighting the importance of a role for the mPTP in cardiomyocyte apoptosis (Khaliulin et al., 2014).

IR control data from previous Langendorff ischaemia reperfusion experiments have been included as discussed in section 3.5. The significant decrease in I/R ratio in hearts treated with Salbutamol in the presence of either the β_1AR antagonist CGP 20712 or β_2AR antagonist ICI 118, 551, highlighted Salbutamol can mediate myocardial injury via activation of βARs, particularly β_2ARs (Chapter 5 & 6). Interestingly, using CsA as an adjunct therapy with Salbutamol significantly decreased I/R ratio in rat hearts to levels similar to control IR hearts. Hearts treated alone with CsA also showed a significant decrease in I/R ratio. This

confirms not only that CsA is capable of preventing Salbutamol mediated injury, but also CsA treatment alone is capable of preventing I/R ratio in hearts exposed to reperfusion injury conditions.

CsA has been identified as an inhibitor of Cyp D as early as 1988, however the manner and importance of this component has been continuously debated (Crompton et al., 1988), (Halestrap et al., 1997a, Halestrap, 2010, Ong et al., 2015b). Adding to the complexities of understanding the mPTP is the general understanding of the components that contribute to the structure of the pore. These components have been scrutinised and extensively researched and it is now widely accepted that the mPTP comprises of a VDAC, ANT, Cyp D and PiC component (Green and Kroemer, 2004, Karch and Molkentin, 2014, Halestrap and Davidson, 1990, Szabo et al., 1993, Lemasters et al., 2012, Lopez-Erauskin et al., 2012). However, these components are continuously contested in regards to their exact functions and contributions to formation of the mPTP. In respect of Cyp D's interaction with CsA, two suggestions have been made as to how inhibition can occur, the first being the direct interaction of CsA with the Cyp D component (Waldmeier et al., 2003). The second involves the inhibition of calcineurin-mediated dephosphorylation of Bad, which ultimately inhibits apoptosis (Waldmeier et al., 2003).

Cardiomyocytes treated with Salbutamol alone showed to significantly decrease time taken to the onset of hypercontracture and also a decrease in time taken to the onset of depolarisation (Figure 7.3). Salbutamol in the presence of CsA (0.2μM) significantly increased the time taken to the onset of depolarisation and hypercontracture reiterating the inhibitory effects of CsA on premature opening of the mPTP. Interestingly, CsA nullifies the effect seen in cardiomyocytes treated with Salbutamol alone, returning the hypercontracture time back to

control levels, thus preventing the cardiotoxic effects of Salbutamol. Further to this, CsA treatment alone in cardiomyocytes significantly increased time to depolarisation and hypercontracture highlighting the abrogating effect of CsA in a model of oxidative stress. Several clinical trials with the use of CsA to investigate its reversible effects of ischaemia reperfusion injury have been successful but not conclusive. Song and colleagues (2015) meta-analysis of several of these trials concluded that sample sizes of each of the trials were too small to determine a true beneficial effect of CsA reducing reperfusion injury (Song et al., 2015). Interestingly, like the rodent model of inhibition of reperfusion injury with CsA, pig hearts were also protected with administration of CsA, emphasising the importance of the mPTP regulating cardiomyocyte apoptosis (Skyschally et al., 2010).

A unique feature of the mPTP is the suggestion of the critical timing in which the pore remains closed and conditions under which it opens. In particular, Hausenloy and colleagues (2003) characterised such timings. With the use of the Cyp D inhibitor Sangliferhrin A, they identified during ischaemic conditions that the mPTP remained closed in rat cardiomyocytes. Interestingly, the mPTP was shown to open during the first several minutes of reperfusion, emphasising the importance of ROS and calcium as triggers of mPTP opening (Hausenloy et al., 2003, Ong et al., 2015a). This group also linked inhibition of the mPTP to a reduction in infarct size with Sangliferhrin A, similar to the observations we have made with the adjunct therapy of Salbutamol and CsA.

More recent studies have purported an alternate composition of the mPTP but still include the above-mentioned components. Of particular interest are the proposed inclusion of the Bcl-2 family members Bax and Bak contributing as part of outer mitochondrial membrane part of the mPTP (Karch and Molkentin, 2014). The physical link between the outer mitochondrial

membrane and inner mitochondrial membrane has been considered to be directly via the mPTP, however, Karch and colleagues (2013) have proposed that the formation of the outer mitochondrial membrane is solely due to a pore formation formed between Bax and Bak, which then allow passage of larger molecules across the outer mitochondrial membrane onto the inner mitochondrial membrane where the widely accepted components of the mPTP are present (Karch et al., 2013). They do stress that the Bcl-2 family proteins still remain part of the mPTP formation however; they act separately to the inner mitochondrial membrane components of the mPTP (Karch et al., 2013). The groups' experiments using Bax/Bak knockout mice concluded that necrotic cell death and apoptosis were unable to occur highlighting the importance of Bax and Bak in mPTP pore formation.

As discussed previously (Chapter 5), depolarisation time was reduced but not significantly in cardiomyocytes treated with Salbutamol. Salbutamol's affinity for βARs and release of calcium stores has been discussed in detail previously (Chapter 3). With a proposed increase in calcium upon Salbutamol activation, these excess levels of calcium, in addition to ROS production as a result of reperfusion/TMRM degradation, may also be a major contributing factor to the premature opening of the mPTP (Joshi and Bakowska, 2011). Experiments with Isoproterenol induced calcium release question whether the calcium efflux mechanisms can handle the increased rate at which influx of calcium into the mitochondria occurs (Bell et al., 2006). In normal conditions of cardiomyocyte at rest, elevated calcium concentration within the inner mitochondria membrane will increase the mitochondrial permeability transition allowing the mitochondria to lose solutes via the mPTP (Wong et al., 2012). The rate at which calcium is able to be transported has been reported to be very slow, in particular the Na^{+}/Ca^{2+} exchanger (Bell et al., 2011). However, during reperfusion, the mPTP is subjected to a spike in calcium concentration in addition to frurther elevated calcium levels induced by

Salbutamol. With extremely high levels of calcium, simultaneous opening of mPTP's result in release of cytochrome c initiating the caspase cascade, supported by our findings showing elevated caspase 3 levels (Figure 7.7) (Murphy and Steenbergen, 2008, McIlwain et al., 2015).

With Salbutamol's chronotropic effect (Chapter 3) and evidence of increased inotropy, the energetics of cardiomyocytes will alter dramatically to compensate for Salbutamol induced calcium release (Casoni et al., 2014, Prakash et al., 1997). Bell and colleagues (2011) in rat cardiomyocytes demonstrated when cardiomyocytes are stimulated to beat rapidly from rest, ATP levels deplete in order for contraction (Bell et al., 2006). We can propose from Bell and colleagues work in addition to our findings that Salbutamol may induce calcium release within cardiomyocytes causing premature opening of the mPTP resulting in more rapid ATP depletion. With this theory, this may give an explanation for the observed decreased time to the onset of hypercontracture in the presence of Salbutamol.

Our proposal can also be linked with studies that found administration of Isoproterenol induced calcium release, which has been directly linked to increased infarct size and premature opening of the mPTP (Xiong et al., 1994, Bell et al., 2006, Mukherjee et al., 2015)

Control, IR time matched control and Salbutamol data have been included from other western blots that have been run. Limiting factors and consequences of this have been discussed previously in section 5.5. The Expression of signalling protein p-Erk was not effected significantly with the administration of Salbutamol and CsA. Increased expression of p-Akt has been shown to promote cell survival in combination with elevated levels of p-Erk. Our findings show that Salbutamol with CsA significantly reduced p-Akt expression when compared to hearts treated with Salbutamol alone (Miyamoto et al., 2009). This emphasises a

link between p-Akt expression and Salbutamol mediated injury, i.e. elevated levels of p-Akt has a role for Salbutamol induced cardiotoxicity. Studies by Gharanei and colleagues (2013) showed a similar finding with Doxorubicin. In rat hearts treated with the adjunct therapy of CsA and Doxorubicin, p-Akt expression was reduced significantly (Gharanei et al., 2013). They suggested that the reduction on p-Akt expression was a factor of the reperfusion injury salvage kinase (RISK) pathway to bring about some protection of the heart to the insult caused by Doxorubicin. We can postulate that reduction of p-Akt expression by CsA administration in the presence of Salbutamol is a method by which the heart can initiate the RISK pathway in order to prevent reperfusion injury (Davidson et al., 2006, Gharanei et al., 2013, Hausenloy and Yellon, 2007). Interestingly, both expression levels of p-Akt and p-Erk were significantly reduced in the presence of CsA alone. This is in contrast to findings by other groups that have shown increases in these particular survival proteins as they are a part of the RISK pathway (Halestrap and Pasdois, 2009). A possible elucidation for our observed results is activation of p-Akt by phosphorylation at threonine 308 instead of serine473. Interestingly, Kwiatkowska and colleagues (2011) demonstrated a down regulation of p-Akt with CsA that did not cause a detrimental effect to cellular apoptosis. Further studies into the investigation of CsA activation of Akt^{Thr308} by western blotting would clarify this.

In conclusion, we have identified that Salbutamol mediated injury can be prevented in a model of oxidative stress and myocardial reperfusion injury with administration of the cyclophilin D inhibitor CsA. We can also emphasise from this study the importance of p-Akt expression in inducing Salbutamol mediated injury. Future studies may look at different components of the mPTP and specifically target other components such as the use of Bonkergic acid to lock the ANT in a confirmation (Wong et al., 2012).

8 General Discussion

8.1 Rationale

The primary therapy for alleviating symptoms associated with respiratory disorders has been the use of bronchodilators, in particular the treatment and management of asthma and Chronic Obstructive Pulmonary Disorder (COPD). The most recent published material from the Global Asthma Report (2014) identified an approximate 334 million sufferers of asthma worldwide in addition to an estimated 65 million people suffering from COPD. 3 million people worldwide were reported to have died as a result of COPD in 2005 with an estimated increase of 30% to the present date (WHO, 2005). Wide and diverse ranges of bronchodilators are available on the market including steroid inhalers, anti-cholinergic drugs and β_2 adrenergic receptor agonists (β_2ARs).

Previous clinical studies have shown effectiveness of these different classes of bronchodilators and have been shown to alleviate symptoms rapidly and improve quality of life in patients over the past 60 years (Castle et al., 1993, Guyatt et al., 1997, Ellepola and Samaranayake, 2001, Smith and Parry-Billings, 2003, Rodrigo and Castro-Rodriguez, 2005).

More recently, a number of clinical studies have associated with the use of bronchodilators with an increase in morbidity and morality in patients, especially those with underlying cardiovascular conditions (Singh et al., 2008, Macie et al., 2008). Such underlying cardiovascular conditions include myocardial ischaemia, myocardial infarction, cardiomyopathies, hypertrophy and heat failure (Au et al., 2000, Suissa et al., 2003).

Of particular interest in relation to our studies were the increasing number of reported cardiovascular events linked with β adrenergic receptor agonists (βARs), in particular the short acting βAR agonist Salbutamol (Burggraaf et al., 2001, Suissa et al., 2003,).
Salbutamol was one of the first selective β_2AR agonists launched and has been widely used since its launch in 1968 to alleviate symptoms of asthma (Icha, 2007).

The primary aim of our work was to characterise the cardiovascular effects of currently prescribed βARs agonists (excluding Isoproterenol). In the study we assessed the role of short and long acting βARs agonists in a relevant cardiovascular model of ischaemia reperfusion injury using isolated perfused rat hearts (Bell et al., 2011, Hudecova et al., 2013, Fajardo et al., 2011). Having characterised the adverse cardiovascular effects of βAR agonists we further investigated the associated cell signalling pathways and their effects on the mitochondrial permeability transition pore. Figures 8.2-Figure 8.6 illustrate a summary of some of the key findings involved with treatment with Salbutamol in the presence of various types of inhibitors in the Langendorff model (Figure 8.2), oxidative stress model (Figure 8.3 & Figure 8.4) and western blot analysis (Figure 8.5, Figure 8.6).

8.2 Role of β_2AR agonists on Myocardial Ischaemia Reperfusion injury and the Mitochondrial Permeability Transition Pore

From our studies using the Langendorff model in Chapter 3, we identified the haemodynamic effects of the βAR agonists in addition to investigating the effects they had on myocardial infarction during ischaemia reperfusion injury. Isoproterenol, a non-selective βAR agonist, had no significant effect on Left Ventricular Developed Pressure (LVDP), Heart Rate (HR), or Coronary Flow (CF) (Figure 3.1, Figure 3.2, Figure 3.3). Interestingly, a decline ($p>0.05$)

in LVDP was observed in hearts administered with Isoproterenol (0.5μM). This decline in LVDP can be linked to previous studies that have identified Isoproterenol to induce myocardial ischaemia, hypertrophy and heart failure (Leenen et al., 2001). Left ventricular pressure overload has been reported to induce ventricular hypertrophy that can lead to heart failure in hearts administered with Isoproterenol thus reducing the LVDP (Chen et al., 2014).

An increase in LVDP, HR and CF was seen with the administration of selective long acting β_2AR agonists Formoterol and Salmeterol (Figure 3.7, Figure 3.8, Figure 3.9). This observation was consistent with previous work by other groups in regards to the positive inotropic and chronotropic effects of these particular β_2AR agonists, Watson and colleagues (2013) showing a 20% increase in coronary flow in rat hearts (Guhan et al., 2000, Watson et al., 2013). These particular agonists activate the β_2AR GPCR G_s pathway initiating the signalling cascade involving calcium release via the cAMP/PKA pathway. Opening of L-type calcium channels in response to either of the β_2AR agonists increases cytosolic calcium concentrations leading to further calcium induced calcium release via ryanodine receptors on the sarcoplasmic reticulum. This increase in calcium concentration is then available to increase excitation contraction coupling of cardiomyocytes (Diaz et al., 2005, Louch et al., 2012).

In isolated hearts and cardiomyocytes subjected to ischaemia and reperfusion injury, β_2AR agonists Salmeterol and Formoterol were shown to have no significant effects when compared to non-treated controls in both myocardial infarction and on isolated cardiomyocytes (Figure 3.10, Figure 3.11, Figure 3.12). Previous studies have suggested activation of βAR can initiate contrasting effects of apoptosis. Specifically, activation of

β_1ARs initiates pro-apoptotic signalling in contrast to β_2AR activation promoting anti-apoptotic signals (Communal and Colucci, 2005, Zhu et al., 2005). Its has been suggested by Zhu and colleagues (2005) that the ability of β_2ARs to dually bind to both G_s and G_i subunits allows it to inhibit the anti apoptotic effects of β_1AR-G_s signalling (Zhu, et al., 2005).

In contrast, administration of the non-selective βAR agonists Isoproterenol and Salbutamol demonstrated significant increased size in infarct to risk ratio and decreased cardiac myocyte viability, with Salbutamol also initiating an increase in cleaved caspase 3 activity (Figure 3.16, Figure 4.1). A number of previous studies have shown that Isoproterenol induces myocardial injury as a result of the quinine metabolite produced by Isoproterenol increasing reactive oxygen species (ROS) thus causing damage to mitochondria as seen in a number of different models and species including canine, rat and humans (Andersson et al., 2011), (Hunt and Ross, 1990, Krenek et al., 2009, Herrmann et al., 2014).

Previous clinical trials in patients administered with Salbutamol have been abandoned due to a high mortality rate (Gao Smith et al., 2012). In this particular study, a multicentre double blind placebo-controlled in the UK was carried out with 162 patients assigned to the Salbutamol group. Intravenous administration of Salbutamol (15μg/kg) increased the number of mortalities in the measured 28-day period patients were monitored. Another clinical trial involving nebulization of Salbutamol in patients suffering from Acute Lung Injury (ALI) was also abruptly stopped due to the high incidence of mortality in patients. In this study, 20.5% of the 282 patients that participated died as a result of treatment with Salbutamol (Matthay et al., 2011).

Salbutamol mediated myocardial injury is a phenomenon that has not previously been investigated especially in a pre-clinical model of ischaemia reperfusion injury. We demonstrated the administration of Salbutamol increased infarct size in a concentration dependent manner, especially at higher concentrations of Salbutamol (0.01μM-1μM) in addition to an increase in cytotoxicity (Figure 3.16, Figure 4.1).

Activation of βARs has been shown to activate the cAMP-PKA dependent pathway, which in turn results in an increased release of calcium ions from the sarcoplasmic reticulum as a plausible link to increased myocardial injury via calcium overload (Zhu et al., 2003). Interestingly, Isoproterenol mediated myocardial injury has also been associated with calcium overload, where the co-administration of calcium channel blockers prevented this injury (Setaro et al., 1990). Having identified Salbutamol as unique short acting β agonist we continued our investigations focusing on the mPTP.

Isolated ventricular rat cardiomyocytes were administered with βAR agonists to observe the time taken to the onset of depolarisation and hypercontracture to determine the role of the mPTP in βAR agonist mediated myocardial injury. Interestingly, Isoproterenol was the only βAR agonist to record a significant decrease in time to depolarisation compared to non-treated controls in addition to a significant decrease in time to hypercontracture (Figure 3.5, Figure 3.6). The mechanism of hypercontracture results in irreversible cardiomyocyte shortening and is initiated by several factors including increased ROS and increased calcium concentrations. In respect to the β_2AR Salbutamol, previous studies have indicated significant increase in calcium concentration in response to βAR activation (Halestrap, 2010). This significant rise in calcium concentration in addition to the increased ROS as a result of

reperfusion, are detrimental to the mPTP causing premature opening releasing the contents of the mitochondria into the cytosol (see section 8.5). The release of cytochrome c as a result of mPTP opening initiates several caspases including caspase 3, which were elevated in the presence of Salbutamol treated hearts (Figure 4.14). This suggested that Salbutamol has a role inducing cardiomyocyte cell death (Shin et al., 2014).

The administration of Salbutamol, Salmeterol or Formoterol was seen to decrease the time to depolarisation and significantly reduce the time to hypercontracture (Figure 3.11, Figure 3.12). The time taken to depolarisation represents opening of the mPTP, which can be initiated with increased ROS or increased calcium ions (Andrews et al., 2012). Isoproterenol induced myocardial injury and has previously been demonstrated to be associated with increased ROS and calcium overload by non-selective activation of either β_1AR or β_2AR (Andersson et al., 2011, Hudecova et al., 2013). The rate at which calcium is able to be transported in relation to release from the sarcoplasmic reticulum through ryanodine receptors or removed into the cytosol, has been reported to be very slow, in particular when controlled by the Na^+/Ca^{2+} exchanger (Bell et al., 2011). During reperfusion, cardiomyocyte mitochondria are subjected to a spike in calcium concentration in addition to further elevated calcium levels induced by Salbutamol resulting in the premature opening of the mPTP.

With the observed chronotropic effects of Salbutamol, cardiac myocyte energetics will alter to compensate the additional Salbutamol induced calcium release (Casoni et al., 2014, Prakash et al., 1997). We propose Salbutamol induced calcium release causes premature opening of the mPTP inducing rapid ATP depletion similar to the findings by Mukherjee and Colleagues (2015) who demonstrated the change in mitochondrial energetics in rat myocytes.

8.3 Identifying Salbutamol β Adrenergic Receptor selectivity in mediating myocardial injury

Having identified Salbutamol's effect in a model of ischaemia reperfusion injury and oxidative stress, identification of the specific βAR subtype that mediates this injury was further investigated. To date, four βAR subtypes have been identified as β_1, β_2, β_3 and β_4 with a large proportion of research focussed on the activity of β_1ARs and β_2ARs (Zaugg et al., 2000). Antagonising β_1 or β_2ARs independently with ICI, 118 551 and CGP 20712 respectively, we identified that Salbutamol mediated injury was predominantly via activation of the β_2ARs. Both β_1 and β_2ARs are able capable of activating the βAR-G_s-cAMP pathway resulting in increased calcium release from the sarcoplasmic reticulum and may explain calcium mediated myocardial injury with Salbutamol (Communal et al., 1999, Heubach et al., 2004,). A summary of the involvement of ICI 118, 551 and CGP 20712 can be seen in Figure 8.2-Figure 8.4 with respect to the Langendorff and oxidative stress model.

To determine the specific role of β_2ARs in Salbutamol medicated injury we used the selective β_2AR antagonist ICI 118,551 (Beer et al., 1988). Our claims can be concluded from myocardial injury being significantly reduced in the presence of the β_2AR antagonist ICI 118, 551, only allowing activation of the β_1ARs (Figure 6.2). Suggestions of contrasting effects of activation of βARs has been suggested with β_1ARs promoting cell apoptosis and β_2ARs promoting cell survival (Communal and Colucci, 2005). Contrary to these groups, we propose that Salbutamol mediated cardiomyocyte apoptosis is via β_2AR activation due to its capability of binding not only to the G_s subunit, but also the G_i subunit. Activation of the β_2AR-G_i pathway has inhibitory effects on adenylyl cyclase thus reducing calcium release (Zhu et al., 2005). We also demonstrated with the β_1AR antagonist CGP 20712 that

Salbutamol mediated injury was significantly reduced compared to control hearts however not to levels as seen with ICI 118, 551 (Figure 5.2).

The involvement of G-protein couples receptor kinases (GRKs) have been linked to directly phosphorylate and uncouple the β_2AR-G_s pathway to encourage G_i pathway activation, however this also has been suggested to contribute to cell apoptosis via different isoforms of the G_i subunit existing (Violin et al., 2006). In particular, the Gαi-3 isoform has been linked to regulation of calcium, which may be the pathway in which Salbutamol β_2AR-G_i pathway activation may continue to contribute to cellular apoptosis (Foerster et al., 2003).

Further to βAR subtypes, distribution of βARs in the heart must also be considered. The ratio of βARs is in favour of β_1ARs compared to β_2ARs (Lyon et al., 2009). During cardiovascular events, this redistribution has been shown to shift in favor of β_2ARs from deep within t-tubules of cardiomycoytes to the cell crest (Nikolaev et al., 2010). As a result of internalisation, re-distribution of β_2ARs to the cell crest of cardiomyocytes enable more interaction with Salbutamol that is present and compensate for the loss of β_1ARs. Such redistribution of β_2ARs could explain, in addition to Salbutamol's high affinity for β_2ARs, our proposals of Salbutamol mediated injury as concentration of the β_2AR subtype would be significnatly higher in hearts with underlying heart conditions such as myocardial ischaemia. With diminished expression and distribution of β_1ARs in such conditions, β_2AR-G_s activation may be promoted over β_2AR-G_i activation to compensate for the loss of adenyly cyclase activation (Nikolaev et al., 2010).

The distribution of βARs also potentially may play an influential role in Salbutamol mediated injury. In conditions of ischaemia, receptor internalisation and desensitisation of β_1ARs have been suggested. Further to this re-distribution, the effect of Salbutamol increasing intracellular calcium concentrations may give rise to detrimental concentrations that effect the mPTP pore in conjunction with increased ROS production as a result of reperfusion. The combination of these particular conditions gives strong arguments as to how Salbutamol may mediate its cardiotoxic effects.

8.4 Cell Signalling

Our studies investigated the cell signalling proteins p-Akt and p-Erk and identified that in the model of ischaemia reperfusion, Salbutamol (0.1μM) significantly increased levels of p-Akt with no significant change observed in p-Erk (Figure 4.6, Figure 4.8). The activation of the PI3K/Akt pathway has been linked to activation of the βARs in particular via β_2AR/G_i pathway in circumstances of promoting cell survival (Xu et al., 2010). Such survival has been shown to be mediated with increased recruitment of the PI3K pathway thus increasing the levels of p-Akt promoting anti-apoptotic effects (Hausenloy and Yellon, 2004). However, our studies up to this point have demonstrated that Salbutamol mediates injury in a model of ischaemia reperfusion increasing myocardial infarction (Figure 3.16). Elevated levels of p-Akt and prolonged activation have been shown to cause deleterious effects to hearts such as increase in infarction size, hypertrophy and heart failure (Matsui and Rosenzweig, 2005, O'Neill and Abel, 2005). Nagoshi and colleagues (2005) suggested that increase expression of p-Akt might activate the cardioprotective properties of the heart, however activation of the PI3K may be dependent and a link to inducing cardiomyocyte apoptosis (Nagoshi et al., 2005). Elevated levels of cleaved caspase 3 were recorded with cardiomyocytes treated with

Salbutamol (Figure 4.14). The activation of the PI3K/Akt pathway has been shown to inhibit activation of caspase 3 thus inhibiting cell apoptosis (Wu et al., 2000). Against these findings, we have demonstrated that caspase 3 levels still remain elevated significantly with elevated expression of p-Akt suggesting that cardiomyocyte apoptosis is still occurring in the presence of Salbutamol.

The different isoforms of Akt have varying effects and regulatory properties, in particular the Akt1 isoform has been shown to regulate calcium levels in the heart directly (Yu et al., 2015). In combination with previous work identifying Salbutamol induced calcium release and in addition to our findings of elevated p-Akt we can purport a link to this particular isoform of Akt that may give reasoning to a mechanism by which Salbutamol mediated myocardial injury can occur (Santi and Lee, 2010). Figure 8.5 illustrates an overview of the levels of p-Akt expression in rat hearts administered with Salbutamol in the presence of various inhibitors used throughout the thesis.

Although shown not to be significant, levels of p-Erk still need to be considered as a potential link to Salbutamol mediated injury (Figure 4.8). A proposal of crosstalk between elevated p-Akt levels inhibiting the levels of p-Erk has been put forward, similar to our findings (Moelling et al., 2002). Moelling et al., (2002) showed Raf-Akt cross talk in a concentration dependent manner, suggesting prolonged rapid activation of p-Akt suppressed the Raf-Erk pathway activation thus diminishing levels of p-Erk as seen in our studies (Mendoza et al., 2011, Suire et al., 2002). Figure 8.6 illustrates the non-significant effect of hearts reperfused for 20 minutes with Salbutamol. Interestingly, the combination of inhibitors did have some significant effects, most notably Salbutmaol + Wortmannin, further supporting our postulation of a cross talk link between p-Erk and p-Akt.

Overall, from our findings we can postulate that p-Akt, via the PI3K/Akt signalling cascade, is recruited and responsible for Salbutamol mediated injury in a model of ischaemia reperfusion in addition to direct activation of this pathway by ROS (Takano et al., 2003). Details of how this particular study could be elaborated are discussed later. Interestingly, levels of p-Akt were inhibited in the presence of the β_2AR antagonist ICI 118, 551 confirming that p-Akt is recruited in the presence of Salbutamol (Figure 6.5). Further to this, linking this same adjunct treatment in the Langendorff model, ICI 118, 551 inhibited Salbutamol mediated injury, thus reinforcing our proposal that p-Akt is a key signalling protein linked to Salbutamol mediated injury.

8.5 Role of mPTP in Salbutamol mediated myocardial injury

With previous evidence of the mPTP playing a detrimental role in mitochondrial integrity during ischaemia-reperfusion injury and our evidence of Salbutamol mediated injury via premature opening of the mPTP, a focus on abolishing this injury by targeting the mPTP was evaluated. The use of Cyclosporin A (CsA) has been documented to inhibit the opening of the mPTP, more specifically inhibiting the cyclophilin D (Cyp D) component of the mPTP (Halestrap et al., 1997a, Halestrap, 2010, Ong et al., 2015b).

Cyp D has been identified to be one of the components to make up the mPTP however this its structure and role is still debated (Karch and Molkentin, 2014). The adjunct therapy of Salbutamol and CsA significantly increased the time taken to the onset of depolarisation and hypercontracture compared to hearts treated alone with Salbutamol (Figure 7.3, Figure 7.4). Previously, we have discussed Salbutamol induced calcium release in addition to increase in ROS as a result of ischaemia reperfusion. The protective manner of CsA in our studies has been shown to inhibit these effects both in the model of oxidative stress and in cell signalling.

Interestingly, similar to expression levels of p-Akt with hearts treated with Salbutamol antagonised with ICI 118, 551, CsA significantly decreased p-Akt expression (Figure 7.6). CsA treated hearts also abrogated the expression of p-Akt. These findings reaffirm our previous proposal that p-Akt plays a key role in Salbutamol mediated injury. Although Salbutamol and CsA adjunct therapy demonstrated to nullify Salbutamol mediated injury in our studies, application of this particular adjunct therapy in patients is highly unlikely. CsA in a clinical setting is a successful immunosuppressant, however complications have been shown with CsA post surgery nephrotoxicity and hepatotoxicity (Rezzani, 2006). Further to this, meta-analysis of several small sample sized clinical trials with administration of CsA in patients undergoing cardiac surgery showed inconclusive reduction of infarct size (Song et al., 2015).

Salbutamol as a chronotropic drug, increases intracellular calcium via activation of β_2AR activation of L-type calcium channels through the cAMP/PKA signalling pathway, which affects cellular energetics including ATP consumption (Casoni et al., 2014). With such increase in calcium, increased contraction of cardiomyocytes can cause rapid depletion of ATP causing premature opening of the mPTP as we seen in our studies (Bell et al., 2006). Our findings confirm that of previous studies that premature opening of the mPTP can be inhibited with the Cyp D inhibitor, CsA. In addition to this we identified that CsA treated hearts can abolish Salbutamol mediated injury as seen in the Langendorff and cardiomyocytes. Further to this, we propose that premature opening of the mPTP by Salbutamol is a contribution of the increased stress of calcium release as observed by previous studies using the non selective βAR Isoproterenol (Xiong et al., 1994, Bell et al., 2006, Mukherjee et al., 2015) Prolonged activation of βARs as a result of calcium overload causes an increase in ATP depletion within cardiomyocytes due to the inhibition of the

N^+/K^+ATPase resulting in increased Na^+ concentration that inhibits the NCX leading to maladaptive calcium homeostasis (Garcia-Dorado et al., 2012).

8.6 Future Directions & Limitations

Our studies identified the cardiotoxic effects of Salbutamol in a model of ischaemia reperfusion injury (Figure 8.2). These findings can be further elaborated on via investigation into further specific cell signalling mechanisms by which Salbutamol exacerbates myocardial infarction.

Firstly, further studies into the activation of the β_2AR-G_s pathway could be carried out with use of the G_i inhibitor Pertussis Toxin (PTX) (Rybin et al., 2003). With inhibition of the G_i pathway, further clarification could be made as to whether the sole cardiotoxic effect of Salbutamol is via the $\beta_{1/2}$AR-G_s pathway. Further to this, the G_s pathway inhibitor Cholera Toxin (CTX) could also be used to identify whether activation of the β_2AR-G_i pathway can also induce cell apoptosis. Further to the use of CTX, transgenic mice specifically expressing the Gαi-3 isoform of the G_i subunit in combination with CTX could help clarify further the pathway of Salbutamol toxicity and further knowledge in understanding the various isoforms of the G_i subunit (Foerster et al., 2003).

Elaborative work on signalling proteins may prove to be useful, in particular investigating a relationship of p-Akt with the stress activated MAPK p-Jnk. Having previously proposed a crosstalk link between p-Akt and p-Erk, further cross talk links may exist with other signalling cascades. Inhibition of the PI3K/Akt pathway with Wortmannin may clarify the signalling cascade by which Akt expression occurs. Our preliminary studies with

Wortmannin showed no significant decrease in expression of p-Akt. This suggests that activation of p-Akt could be a result of another signalling cascade via cross talk (data not shown). Increased expression of p-Erk in the presence of Wortmannin (data not shown) can further link to a potential crosstalk between p-Akt and p-Erk in contrast to findings of p-Akt inhibition of p-Erk (Mendoza et al., 2011, Suire et al., 2002). Further investigation into these particular pathways may elaborate the complexities of intracellular pathway signalling in addition to the use of the p44/p42 pathway inhibitor U0126 (Zhu et al., 2003).

Our studies reaffirmed previous studies in relation to haemodynamics with the observed increase inotropic and chronotropic effects of Salbutamol (Mettauer et al., 1985, Tzoufi et al., 2005). Previous studies by other groups have looked at in detail the relationship of Salbutamol and calcium release, however investigation into the relationship of Salbutamol and calcium and its effects directly on the mPTP should be further investigated (Woo and Xiao, 2012). The use of the FURA-2 fluorescent dye in isolated cardiomyocytes may give clarification on the movement of intra-cellular calcium in the mitochondria and its direct effects on the mPTP pore with potential to identify if Salbutamol induced calcium release is as potent as we proposed (Lipp and Niggli, 1993). Further work with calcium spark detection may also contribute to a broader understanding of the movement of Salbutamol mediated intracellular calcium movement from the sarcoplasmic reticulum via ryanodine receptors (Lindner et al., 2002).

A rudimentary approach in the Langendorff model may be the use of a calcium blocker such as verapamil and note the effect on I/R ratio in the presence of Salbutamol, or alternatively the use of a calcium-chelating agent to highlight the importance of Salbutamol induced calcium release in premature opening of the mPTP. The use of the Langendorff model as an ex-vivo model of ischaemia reperfusion injury has enabled some interesting findings in

relation to Salbutamol mediated injury. An in-vivo model of our studies with surgical ligation of the coronary arteries in rats followed by pro-longed treatment with Salbutamol may further our knowledge and understanding as to the mechanisms by which Salbutamol exacerbates ischaemia reperfusion injury.

8.7 Overall Conclusion

To conclude, the data presented from these studies demonstrate for the first time the exacerbation of infarct size in rat hearts, signalling proteins and ROS in a model of ischaemia reperfusion in the presence of the βAR agonist Salbutamol. In a model of oxidative stress, Salbutamol does not induce premature opening of the mPTP however it does cause premature hypercontracture and subsequent cardiac myocyte apoptosis. The mechanism by which this occurs remains unclear but we can propose the involvement of calcium in addition to ROS is a contributing factor.

We propose that the signalling protein p-Akt via the PI3K pathways plays a key role in Salbutamol mediated injury via prolonged activation as shown by other groups (Nagoshi et al., 2005). We can confirm that the majority of Salbutamol mediated injury is via activation of the β_2ARs with some toxicity occurring as a result of activation of β_1ARs. A potential therapeutic target for the future administration of Salbutamol in patients with underlying heart disease could be the use of the Cyp D inhibitor CsA, as we are the first group to demonstrate the adjunct therapy of CsA and Salbutamol reduces myocardial infarction. A summary diagram in Figure 8.1 depicts the proposed signalling cascade of Salbutamol mediated injury from data that was collected throughout this project.

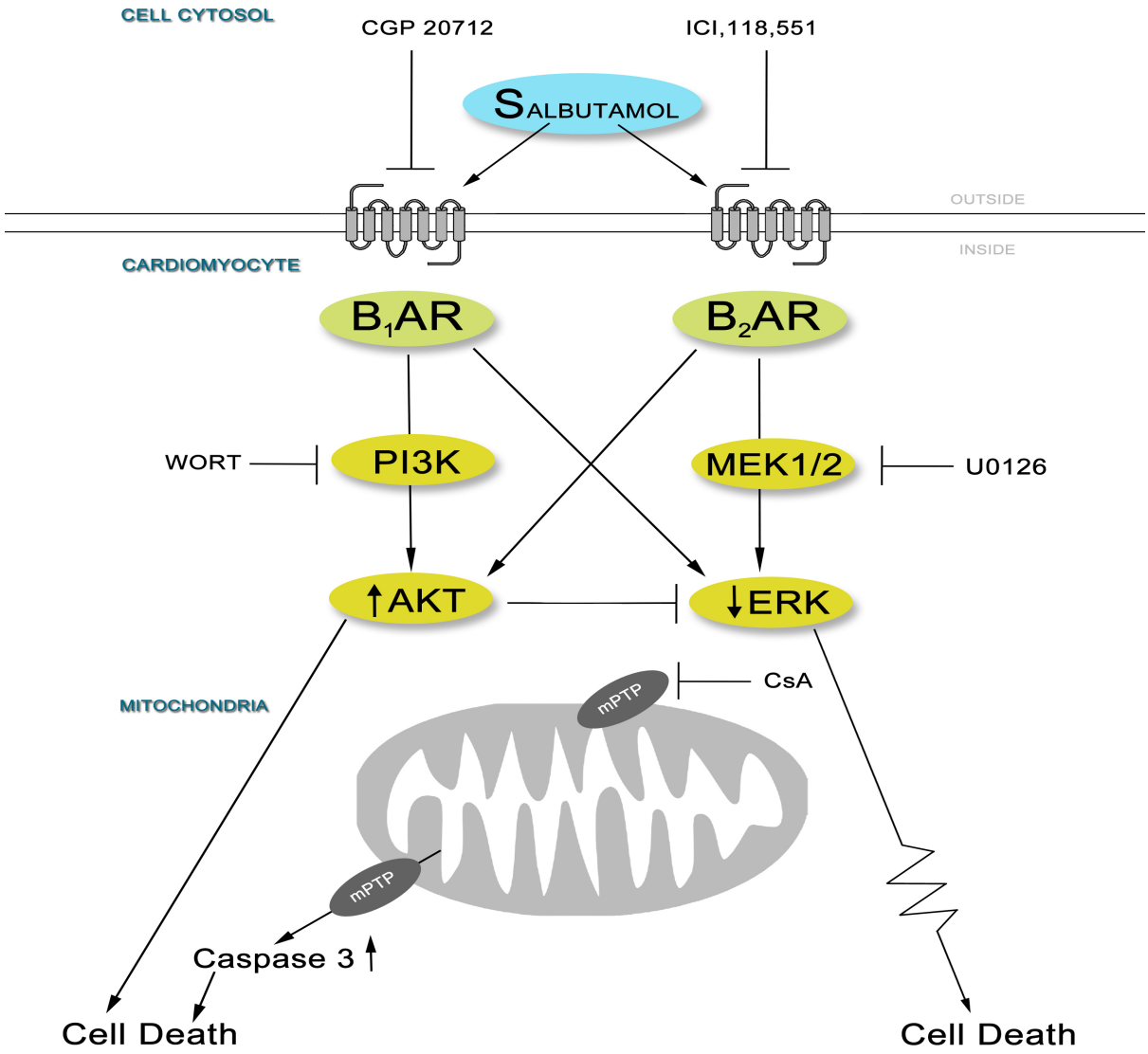


Figure 8.1 Proposed signalling pathway of Salbutamol mediated toxicity in cardiomyocytes. Activation of either β_1AR or β_2AR can increase phosphorlated Akt and decrease phoshorylated Erk. The use of U0126 inhibited MEK1/2 pathway activation in the rat heart, whilst PI3K inhibitor Wortmannin (Wort) decreased expression of p-Akt. Elevated levels of p-Akt were shown to surpress levels of p-Erk expression. Further down the signalling cascade, Cyclosporin A (CsA) was shown to inhibit opening of the mPTP, whilst Salbutamol meditated injury via elevated p-Akt signalling activated a significant increase in cleaved caspase 3 concnetration initiating cell death (Caspase 3).

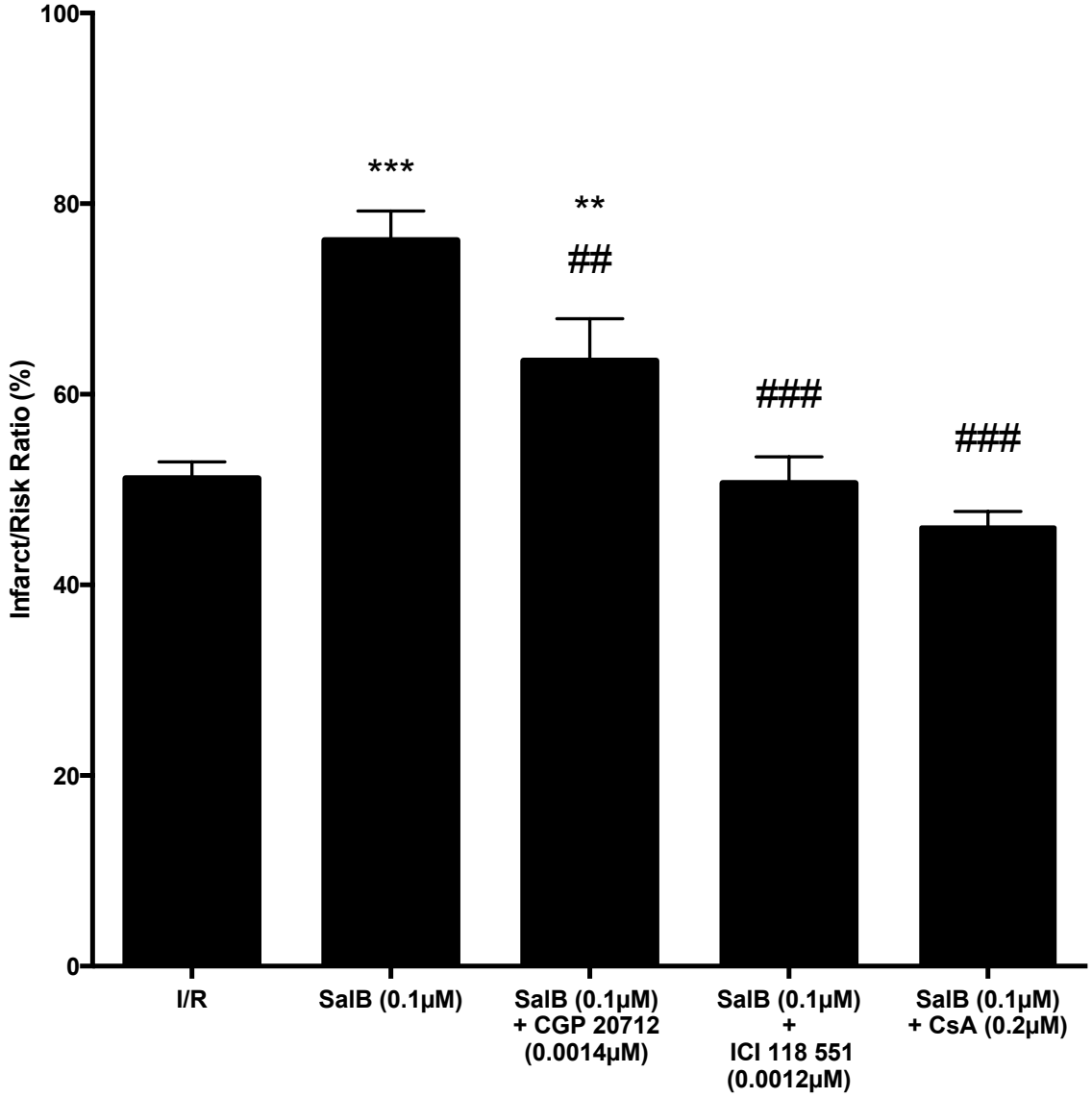


Figure 8.2 Summary graph illustrating Infarct to Risk ratio (%) of hearts treated with Salbutamol in the presence of β_1AR antagonist CGP, 20712, β_2AR antagonist ICI 118, 551 and Cyclophilin D inhibitor Cyclosporin A (CsA). Data extracted from previous chapters. $^{***}p<0.001$ vs. I/R, $^{**}p<0.01$ vs. IR, $^{###}p<0.001$ vs. SalB (0.1μM), $^{##}p<0.01$ vs. SalB (0.1μM).

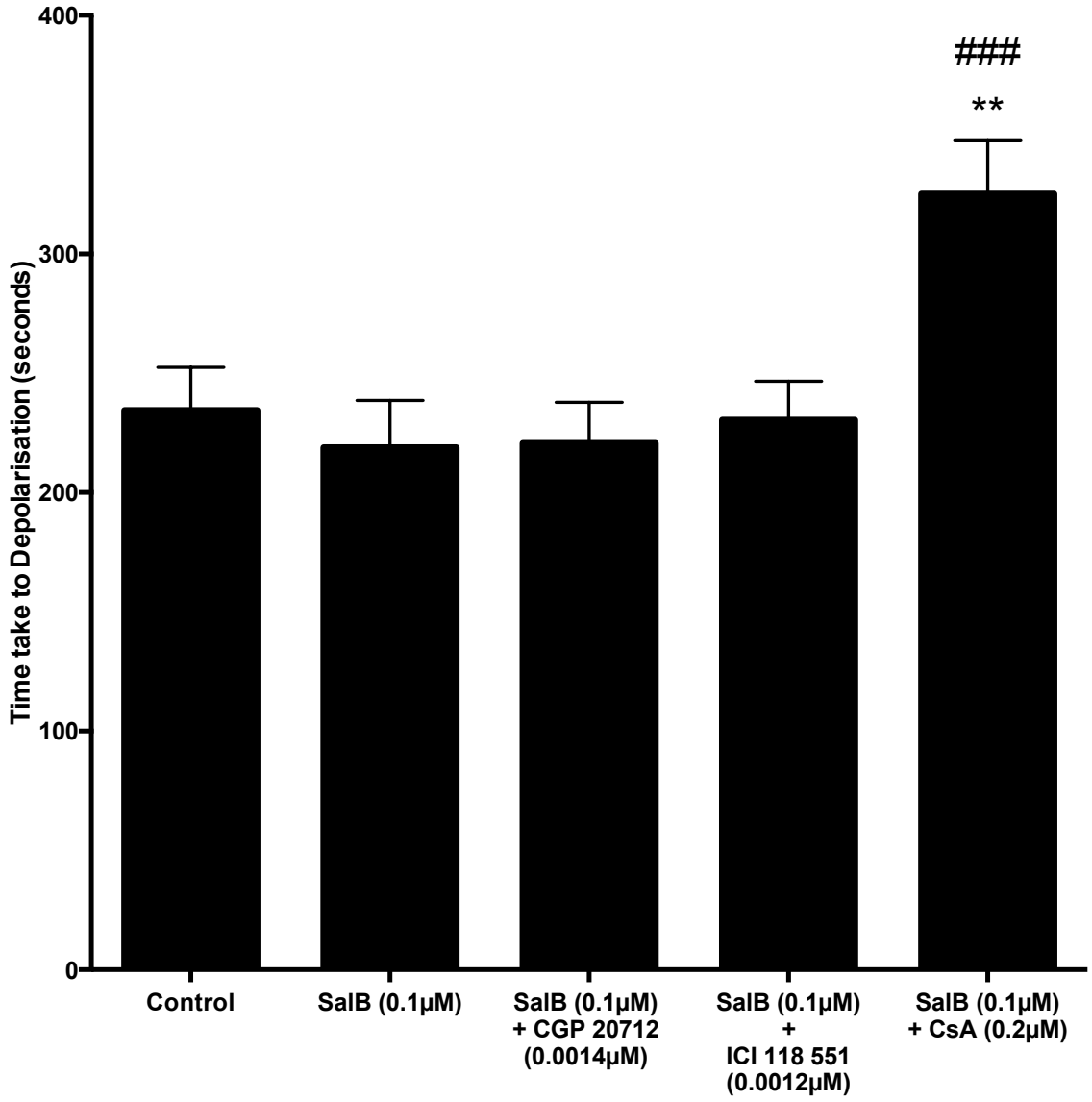


Figure 8.3 Summary graph illustrating the effects of Salbutamol on time taken to depolarisation in isolated cardiomyocytes in a model of oxidative stress in the presence of β_1AR antagonist CGP, 20712, β_2AR antagonist ICI 118, 551 and Cyclophilin D inhibitor Cyclosporin A (CsA). Data extracted from previous chapters. $^{**}p<0.01$ vs. Control, $^{###}p<0.001$ vs. SalB (0.1μM).

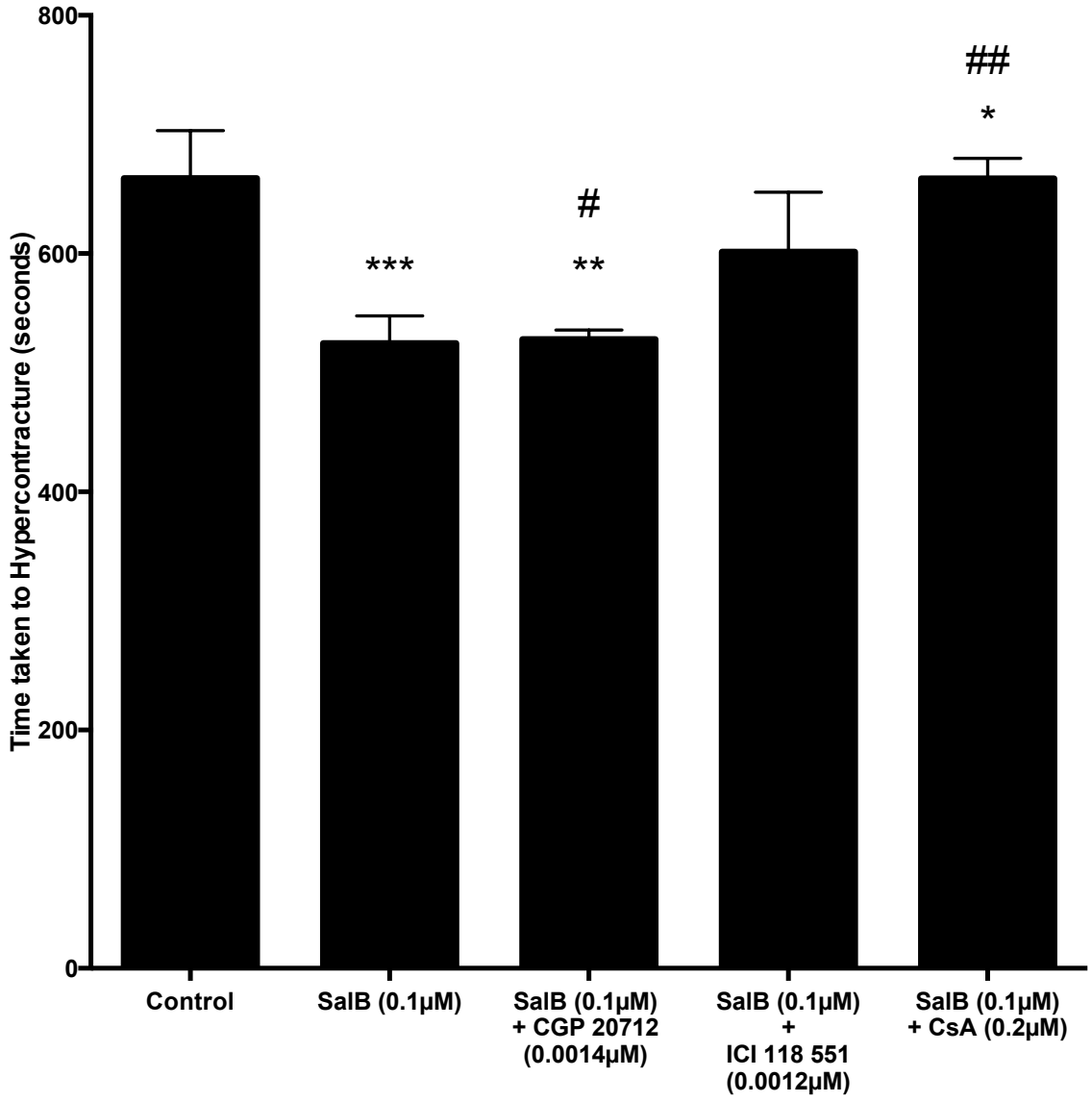


Figure 8.4 Summary graph illustrating the effects of Salbutamol on time taken to hypercontracture in isolated cardiomyocytes in a model of oxidative stress in the presence of β_1AR antagonist CGP, 20712, β_2AR antagonist ICI 118, 551 and Cyclophilin D inhibitor Cyclosporin A (CsA). Data extracted from previous chapters. $^{***}p<0.001$ vs. Control, $^{**}p<0.01$ vs. Control, $^{\#\#}p<0.01$ vs. SalB (0.1μM), $^{\#}p<0.05$ vs. SalB (0.1μM).

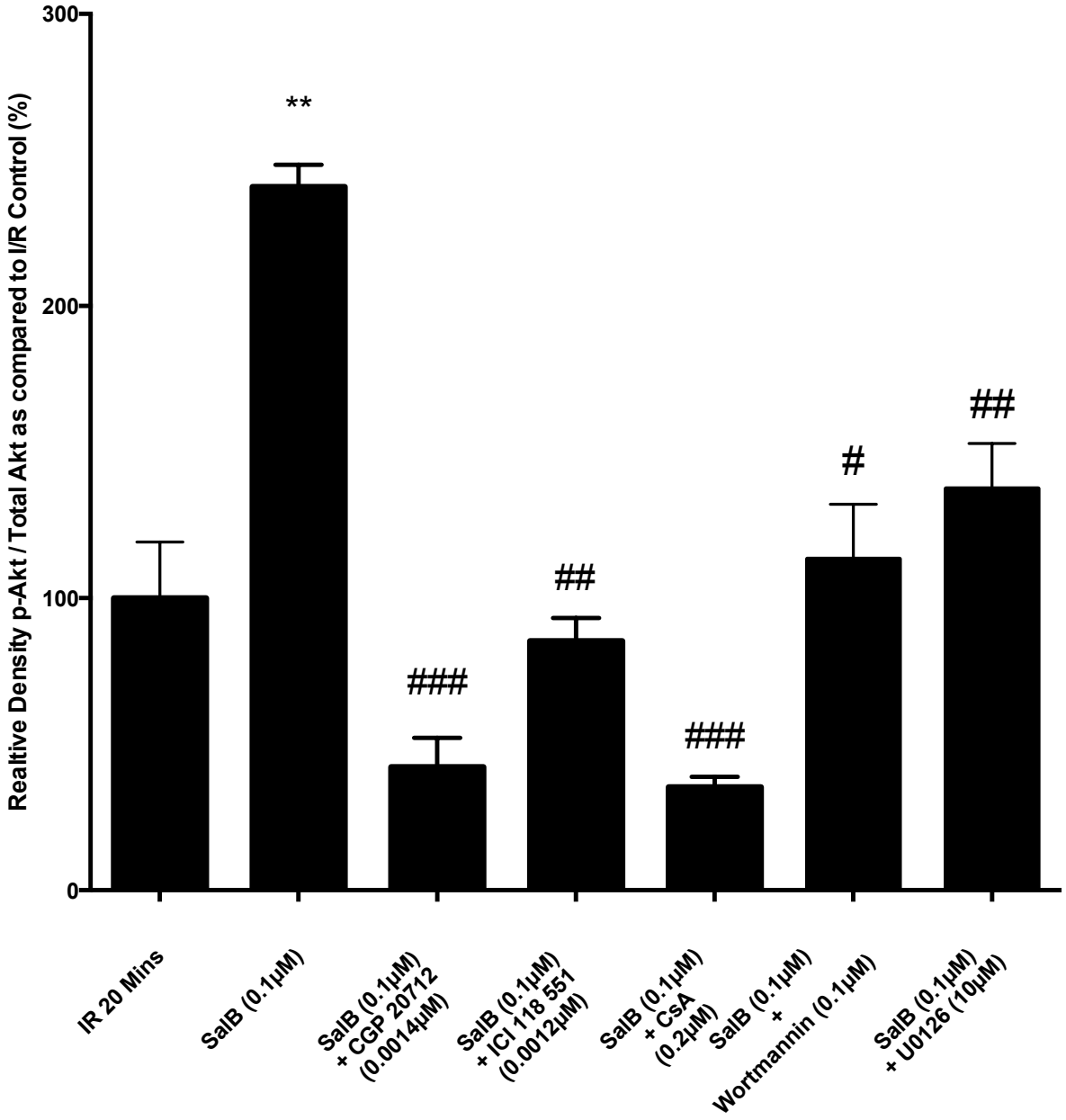


Figure 8.5 The effects of Salbutamol on the expression of phosphorylated Akt (Ser473) in the presence of β_1AR antagonist CGP, 20712 (0.0014μM), β_2AR antagonist ICI 118, 551 (0.0012μM) and Cyclophilin D inhibitor CsA (0.2μM), Wortmannin (0.1μM) and U0126 (10μM). Hearts exposed to 35 minutes ischaemia and 20 minutes of reperfusion. Data extracted from previous chapters. $^{**}p<0.01$ vs. IR 20 Mins, $^{###}p<0.001$ vs. SalB (0.1μM), $^{##}p<0.01$ vs. SalB (0.1μM), $^{#}p<0.05$ vs. SalB (0.1μM).

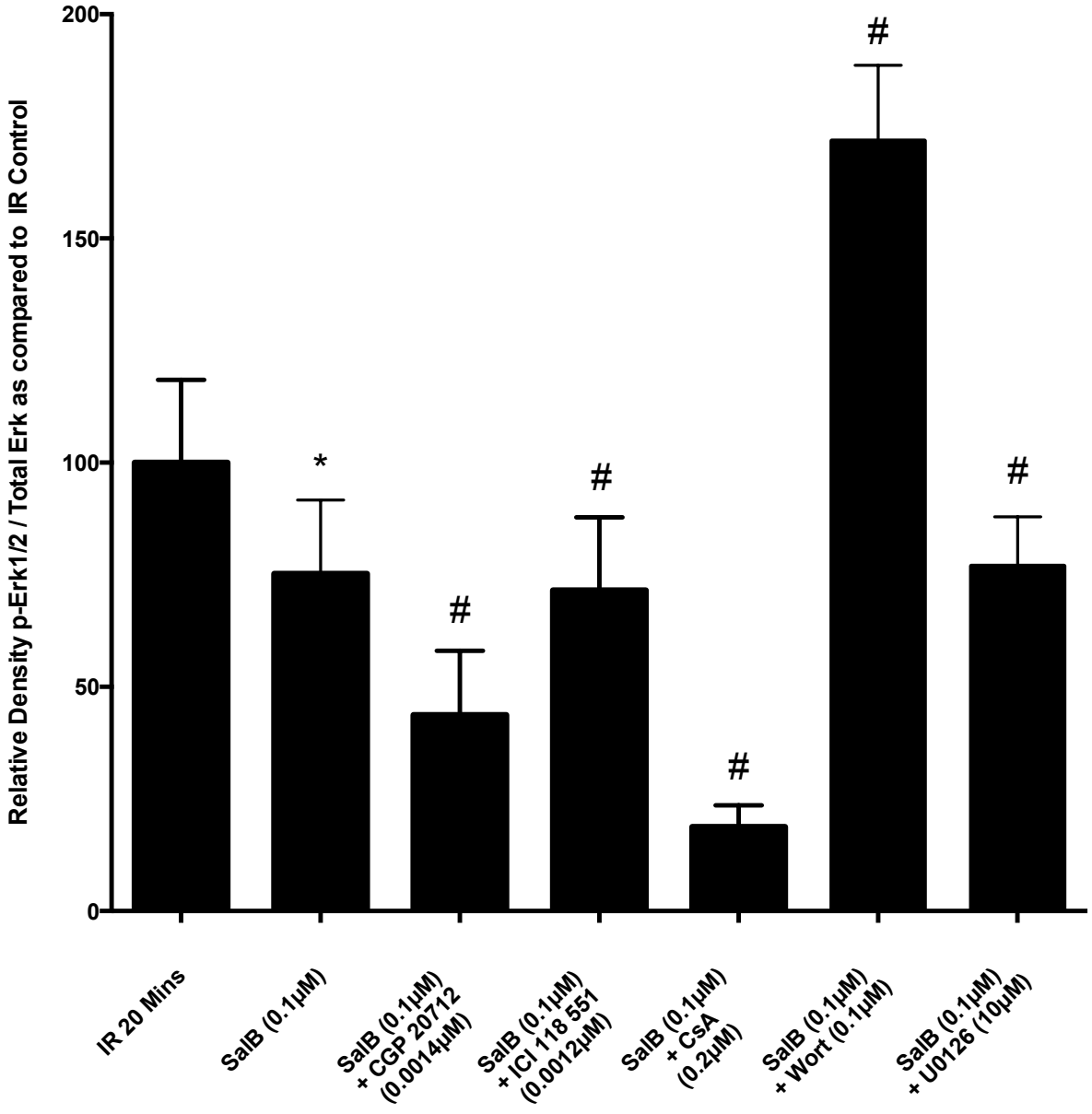


Figure 8.6 The effects of Salbutamol on the expression of phosphorylated Erk (p44/p42) in the presence of β_1AR antagonist CGP, 20712 (0.0014μM), β_2AR antagonist ICI 118, 551 (0.0012μM) and Cyclophilin D inhibitor CsA (0.2μM), Wortmannin (0.1μM) and U0126 (10μM). Hearts exposed to 35 minutes ischaemia and 20 minutes of reperfusion. Data extracted from previous chapters. $^{*}p<0.05$ vs. IR 20 Mins, $^{\#}p<0.05$ vs. SalB (0.1μM).

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