The role of Phosphodiesterase 3 (PDE3) in heart protection

By

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: March 2016

ABSTRACT

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Introduction: During a heart attack the blood supply to the heart is reduced significantly, known as ischemia (Reimer & Jennings, 1991). Energy generating pathways that require oxygen become inhibited in the ischemic tissue. The energy decline leads to an increase in ions (calcium, hydrogen, sodium) inside the ischemic heart cells, leading to tissue damage, which is enhanced by reperfusion (Nakamura et al 1999, Piper et al 2004). Hypercontracture, mitochondrial membrane potential and cell death are the hallmarks of ischemia/reperfusion injury. β-adrenergic receptor (β-AR) activation by sympathetic stimuli increases the intracellular cyclic AMP levels, which activates PKA and enhance cell damage during ischemia. Phosphodieterases (PDEs) have been found to play a role in the sensitivity of cardiomyocytes to anoxia induced cell death (Geisbuhler et al., 2002), and PDE3 is known to play a role in β-AR mediated signalling (Christ et al., 2009). Therefore the involvement of PDE3 was evaluated as a regulator of cell survival in the presence and absence of β-AR stimulation (β1-, β2- and β3-AR, separately and together), and insulin administration.

Aims: The main aim of this study is to investigate the effective role of the PDE isoform 3, in the cardioprotection induced by insulin versus damage induced by the β-AR and to identify the possible mechanisms involved.

Methods: Cardiomyocytes were isolated from Male Wistar rat hearts and cultured overnight in 96-well plates for simulated ischemia/reperfusion experiments the next day. Twelve experimental groups were tested in all objectives including (1) Control; (2) Control + PDE 3 inhibitor; (3) Insulin; (4) Insulin + PDE 3 inhibitor; (5) Dobutamine; (6) Dobutamine + PDE 3 inhibitor; (7) Formoterol; (8) Formoterol + PDE 3 inhibitor; (9) Isoproterenol; (10) Isoproterenol + PDE 3 inhibitor; (11) BRL-37344; and (12) BRL-37344 + PDE 3 inhibitor. These treatments were applied during 20 minutes simulated ischemia induced by 3mM SDT and 10mM 2DG, followed by 60 minutes reperfusion. Cell viability was determined by staining cells with JC-1 and images of cells were captured using fluorescence microscopy. The cells were analysed according morphology and fluorescence intensity.

Results: In cardiomyocytes that were subjected to 20 minutes simulated ischemia and 60 minutes reperfusion, 3mIU insulin mediated cardioprotection through decreasing cell hypercontracture and increasing cell viability. β2-AR stimulation through the agonist 10uM formoterol also led to protection regarding the cell parameters.

Cardiomyocyte protection during ischemia was also elicited when the PDE3 enzyme were inhibited by 10uM milrinone. β1-AR stimulation with 10uM dobutamine appears to have no significant effect regarding damage or protection, while β3-AR stimulation with 10uM BRL37344 resulted in protection.

Conclusion: Cardioprotection was elicited with the inhibition of the PDE3 enzyme, but did not have any significant effect on insulin-mediated cell protection. One of the most remarkable observations made, was the fact that β2-AR stimulation with formoterol and β3-AR stimulation with BRL-37344 the most effective triggers of cardioprotection.

OPSOMMING

Die rol van fosfodiesterase 3 (PDE3) in hart beskerming.

Inleiding: Tydens 'n hartaanval word die bloedtoevoer na die hart aansienlik verminder, wat bekend staan as isgemie (Reimer & Jennings, 1991). Energie genererende weë wat suurstof benodig word geïnhibeer in die isgemiese weefsel. Die afname in energie lei tot 'n toename in ione (kalsium, waterstof, natrium) in die isgemiese selle, wat lei tot weefsel skade, wat vererger word deur herperfusie (Nakamura et al 1999, Piper et al 2004). Hiperkontraksie, mitochondriale membraan potensiaal en die verlies van selle deur seldood is kenmerke van isgemie/herperfusie beskadiging. β-adrenerge (β-AR) aktivering deur sintetiese stimuli verhoog die intrasellulêre sikliese AMP vlakke, wat PKA aktiveer en selskade verder verhoog tydens isgemie. Daar is bevind dat fosfodiesterases (PDEs) 'n rol speel in die sensitiwiteit van kardiomiosiete aan anoksie geïnduseerde seldood (Geisbuhler et al., 2002), en dit is bekend dat PDE3 ‘n rol speel in β-AR seintransduksiepaaie (Christ et al., 2009). Gevolglik is die betrokkenheid van PDE3 geëvalueer as 'n reguleerder van seloorlewing in die teenwoordigheid en afwesigheid van β-AR sein stimuli (β1-, β2- en β3-AR, afsonderlik en tesame), en insulien toediening.

Doelwitte: Die hoofdoel van hierdie studie is om die effektiewe rol van die PDE isoform 3 te ondersoek, in die miokardiale beskerming gebied deur insulien teenoor skade veroorsaak deur die β-AR, en om die moontlike meganismes wat betrokke is te indentifiseer.

Metodes: Kardiomiosiete is geïsoleer vanaf Wistar rotharte en oornag in 96 putjie-plate gekultiveer vir gesimuleerde isgemie/herperfusie eksperimente die volgende dag. Twaalf eksperimentïele groepe is getoets in alle doelwitte, insluitend (1) Kontrole; (2) Kontrole + PDE 3 inhibitor; (3) Insulien; (4) Insulien + PDE 3 inhibitor; (5) Dobutamien; (6) Dobutamien + PDE 3 inhibitor; (7) Formoterol; (8) Formoterol + PDE 3 inhibitor; (9) Isoproterenol; (10) Isoproterenol + PDE 3 inhibitor; (11) BRL-37344; en (12) BRL-37344 + PDE 3 inhibitor. Hierdie behandelings is aangewend tydens 20 minute gesimuleerde isgemie, wat bewerkstellig is deur 3mM STD en 10mM 2DG, gevolg deur 60 minute herperfusie. Seloorlewing is bepaal deur die selle met JC-1 te kleur en afbeeldings van selle is gemaak deur middel van fluoresensie mikroskopie. Die selle is ontleed volgens morfologiese en fluoresserende intensiteit.

Resultate: In kardiomiosiete wat blootgestel was aan 20 minute gesimuleerde isgemie en 60 minute herperfusie, het 3mIU insulien miokardiale beskerming bewerkstellig deur sel hiperkontraksie te verminder en seloorlewing te verhoog. β2-AR stimulering deur die agonis 10uM formoterol het ook gelei tot beskerming ten opsigte van die sel parameters.

Kardiomiosiet beskerming tydens isgemie was ook teweeg gebring met die inhibering van die PDE3 ensiem deur 10uM milrinone. β1-AR stimulering met 10uM dobutamien het geen beduidende effek rakende skade of beskerming getoon nie, terwyl β3-AR stimulering met 10uM BRL37344 tot beskerming gelei het.

Gevolgtrekking: Miokardiale beskerming is bewerkstellig deur die inhibering van die ensiem PDE3, maar dit het nie enige noemenswaardige uitwerking op insulien-bemiddelde sel beskerming gehad nie. Een van die mees merkwaardige waarnemings was die feit dat β2-AR stimulering met formoterol en β3-AR stimulering met BRL-37344 die mees doeltreffende bewerker van miokardiale beskerming was.

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LIST OF ABBREVIATIONS

Units of measurement:

% - percentage

Ml - microlitre

Mg - microgram

ml - milliliter

g - gram

M - molar

min - minute

H - hour

mM - millimole

µM - micromole

Other abbreviations:

ARCMs: adult rat cardiomyocytes

ATP: adenosine triphosphate

β-ARs: beta-adrenergic receptors

BBS: Blebbistatin

CVD: cardiovascular disease

ECM: extracellular matrix

HA: hyaluronan HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IHD: ischemic heart disease

JC-1: 5, 5¢, 6, 6¢-tetrachloro-1, 1', 3', 3'-tetraethylbenzimidazolocarbocyanine iodide

µm: micrometer

PBS: phosphate buffered saline

PI: propidium iodide

R/G: red/green

Ins: insulin

For: Formoterol

Dob: Dobutamine

Iso: Isoproterenol

Mil: Milrinone

Rep: Reperfusion

Isc: Ischemia

WHO: World Health Organisations

NOS: nitric oxide synthase

iNOS: inducible nitric oxide synthase

eNOS: endothelial nitric oxide synthase

NO: nitric oxide

ROS: Reactive Oxygen Species

RISK: Reperfusion Induced Salvage Kinases

PKG: protein kinase G

PKC: protein kinase C

PI3-K: phosphoinositide 3-kinase

PKB/Akt: protein kinase B

cAMP: cyclic adenosine monophosphate

cGMP: cyclic guanosine monophosphate

PKA: protein kinase A

Cco: cytochrome-c oxidase

NCDs – non-communicable diseases

AMI - Acute myocardial infarction

SR - sarcoplasmic reticulum

PLB - phospholamban

MyBP-C - myosin binding protein-C

VSMC - vascular smooth muscle

ICER - inducible cAMP early repressor

IC - intermittent claudication

L/E - laminin entactin

ARF - arformoterol

CHAPTER 1

1.1 Introduction

During a heart attack, the blood supply to the heart is reduced significantly. This event is known as ischemia (Reimer & Jennings, 1991). As a result of poor oxygen and energy substrate delivery together with poor metabolic waste removal, mitochondrial ATP production becomes impaired in the ischemic tissue (Depre et al., 1999, Verma et al., 2005). The energy levels of the heart reduces rapidly and leads to an increase in ions (calcium, hydrogen, sodium) inside the ischemic heart cells, leading to tissue damage (Nakamura et al., 1999, Piper et al., 2004).

Reperfusion, known as a therapeutic intervention, is applied to salvage heart tissue and increase oxygen supply (Braunwald & Kloner, 1985). Conversely, in addition to the beneficial effects (increase in oxygen) of reperfusion, there is the sinister side which inflicts further damage and cardiomyocyte death (Yellon & Hausenloy, 2007).

Energy depletion and tissue damage is worsened by elevated levels of adrenaline and nor-adrenaline, via β-adrenergic receptor (β-AR) activation by sympathetic stimuli, which increases the intracellular cyclic AMP levels, and consequently activates PKA and enhances cell damage during ischemia (Schömig, 1990, Makuala et al., 2005). However we found that insulin, known as a cardioprotective agent, increases cAMP levels in the rat heart during ischemia, and this thereby confers protection (Lopes et al., 2008). This cardio-protection depends on phosphodiesterase (PDE) activity.

PDEs are enzymes that are known to degrade cAMP and thereby act as gates that traffic cAMP to specific intracellular compartments (Fischmeister et al., 2006, Lissandron and Zaccolo, 2006). Not enough emphasis has been given to the role of PDEs in ischemia/reperfusion of the heart, although the inhibition of PDE3 prior to ischemia has been shown to be cardioprotective under experimental conditions (Sanada et al., 2001, Rao and Xi, 2009).

1.2 Aims and Objectives

The main aim of this study will be to investigate the effective role of the PDE isoform 3, in the cardioprotection induced by insulin versus damage induced by the β-AR stimulation and to identify the various mechanisms involved.

Objectives:

- To identify the lowest concentration of the following agents whereby they exert their optimal effect (cardioprotection/damage) :
    - Insulin (0.3IU, 3IU, 30IU)
    - Isoproterenol (100nM)
    - Formoterol (10nM, 10µM, 100µM)
    - Dobutamine (10nM, 10µM, 100µM)

- Does the inhibition of PDE 3 improve or reduce: (a) insulin-mediated cell protection and/or (b) β-AR induced cell damage in isolated rat heart cells during simulated ischemia/reperfusion?
- What are the changes in total cellular ATP and intracellular Ca^{2+} during simulated ischemia/reperfusion in isolated rat cardiomyocytes, when: (a) protected by insulin signalling, (b) damaged by β-AR, (c) insulin-induced protection is reduced or enhanced by the inhibition of PDE 3 identified in Objective 1.
- How does the intracellular cAMP levels and compartments in the cytosol of cardiomyocytes change during simulated ischemia/reperfusion in the presence of insulin versus β-AR signalling, and how does the inhibition of the PDE 3 influence those cytosolic cAMP compartments?

CHAPTER 2 – Literature Review

2.1 Cardiovascular diseases and Epidemiology

Cardiovascular disease (CVD), which is defined as any disease of the heart and blood vessels, is a major cause of disability and premature death throughout the world. The World Health Organisation (WHO), estimated that 17.3 million people died of cardiovascular disease in 2008, accounting for 30% of all deaths worldwide (Alwan, 2011). Of these deaths; approximately 7.3 million were caused by heart attacks while 6.2 million deaths were caused by stroke (Mendis et al., 2011). In 2010, non-communicable diseases (NCDs) were estimated to account for 29% of all deaths in South Africa (figure 2.1), with CVD contributing the highest percentage (WHO, 2011). It is estimated that more than 70% CVD associated deaths occur in the low and middle income countries (Alwan, 2011). A concerning reality is that by the year 2030, the expected number of deaths due to CVDs, will increase from 17.3 million to 23.3 million (Mathers & Loncar, 2006; Alwan, 2011; Smith, 2012).

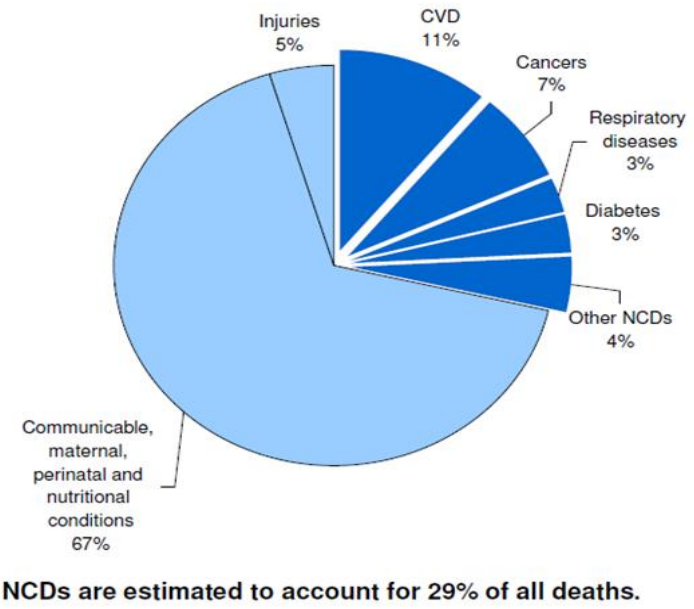

Figure 2.1: Proportional mortality (% of total deaths, all ages) in South Africa during 2010 (Source: World Health Organization - NCD Country Profiles, 2011).

Ischemic heart disease (IHD) remains the leading cause of human mortality, especially in the developed world, resulting in about 7.6 million deaths worldwide (Mathers and Loncar, 2006). According to the projections made by Murray & Lopez (1997), heart disease is set to remain a major contributor to mortality in years to come. IHD (6.6%) as of 2010 was the second leading cause of deaths in South Africa, the first being HIV/AIDS (Norman et al., 2006).

Bradshaw and colleagues conducted a study indicating that IHD causes the highest mortality rate in the Western Cape, accounting for 12% of deaths per year (Bradshaw, 2004).

Ischemic heart disease is usually not found in isolation, but is often a single facet of the so-called metabolic syndrome (Bonora et al., 2003; Caglayan et al., 2005). Although there are tremendous advances in the knowledge of the causes of IHD, ischemia/reperfusion (I/R) remains the leading contributing factor to cardiac dysfunction and infarct size (I/R will be described in section 2.3). The prevalence of I/R injury can be reduced by cardiac self-protective mechanisms called ischemic pre-, per- and post-conditioning. This will be discussed in greater detail later in the text.

2.1.1 Major risk factors for Ischemic heart diseases

IHD has a number of well determined risk factors and increased research has focused on identifying and understanding these risk factors, especially with the high incidence rate globally as well as in South Africa. These major risk factors can be classified as: behavioural (poor diet, lack of exercise, excessive alcohol use and smoking) and metabolic disorders (hypertension, hypercholesterolemia, diabetes mellitus, high blood pressure etc.) (Opie, 2004b; Mendis et al., 2011). Other risk factors include stress, age, gender and heredity (Mendis et al., 2011).

2.2 Consequences of Ischemic heart diseases: Myocardial infarction

2.2.1 Acute myocardial infarction

Acute myocardial infarction (AMI) is the event mainly responsible for the harmful impact of IHD on survival and actually influencing the global health burden (Murray CJ &Lopez AD, 1997). AMI or heart attack ischemia can be caused by the obstruction of blood flow (ischemia) to coronary arteries of the myocardium and can be a life threating condition if not treated (Opie, 2004a). Atherosclerosis is one of the main contributing factors of AMI and is caused by accumulation of fibrous plaques within the walls of the coronary arteries, appearing as ‘’fatty streaks’’ (Opie, 2004a, Epstein & Ross, 1999). The resulting effect is associated with high blood cholesterol and lipid levels (Epstein & Ross, 1999).

2.3 Ischemia and Reperfusion: an introduction

2.3.1 Ischemia

Ischemia, whether it is due to a pathological state or not, is a condition described by the inadequate blood flow to an area/organ leading to a deficiency of oxygen and nutrients and a build-up of toxic waste products, which cause damage and dysfunction to the affected tissue (Baju, 2005). Therefore an imbalance between oxygen supply and demand exists (Opie, 2004b).

Deprivation of oxygen can cause several metabolic changes within cardiomyocytes. These changes (figure 2.2) include a decreased adenosine triphosphate (ATP) level, which is a result of a shift from aerobic- to anaerobic metabolism (Jennings & Yellon, 1992). The energy deficiency within the heart serves as a critical trademark of this ischemic event. Another alteration is the simultaneous accumulation of protons which results in decrease intracellular pH (acidosis) (Jennings & Yellon, 1992). The decrease in blood supply during ischemia also prohibits the removal of possible toxic metabolites.

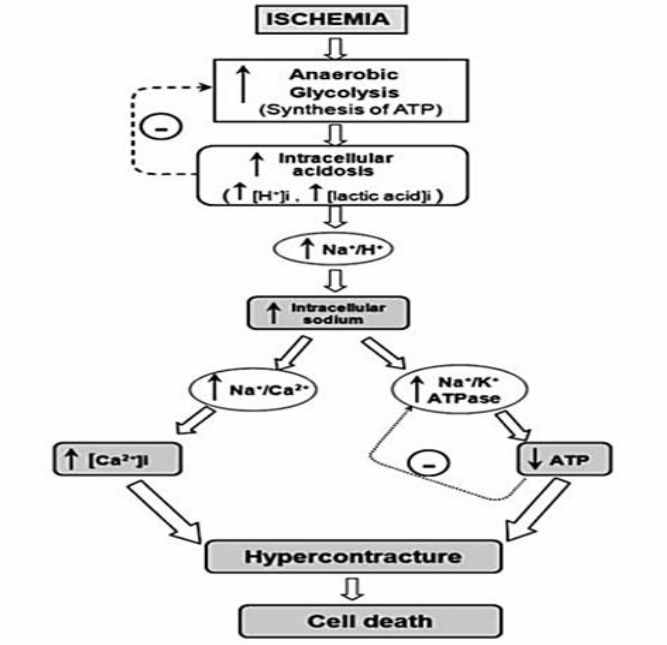

Figure 2.2: Schematic representation of the main events contributing to rapid lethal cardiomyocyte injury during ischemia and hypercontracture-cell death (Source: Luna-Ortiz et al., 2011).

With the ATP levels reaching a detrimental low concentration, the ATP-dependent membrane pumps are unable to transport ions across the membrane, resulting in ion imbalances that can cause membrane damage and cell death (Nakamura et al, 1999).

An example of this damage occurs when the sodium/hydrogen ion exchanger exports protons in exchange for sodium entering the cardiomyocyte. The sodium influx can be maintained via the sodium/potassium ATPase, only with the present of sufficient ATP levels through glycolysis, but the occurrence of prolonged ischemia, inhibits this effect (Avkiran & Marber, 2002).

A decrease in ATP levels can also be contributed through the stimulation of beta-adrenergic receptors (β-ARs) in response to catecholamine's (Schömig, 1990). The latter is responsible for the stimulation of G protein-coupled receptor (GPCR), generating cAMP by means of adenylyl cyclase (AC) (Zaccolo & Movsesian, 2007).

cAMP then activates protein kinase A (PKA), the main downstream effector of cAMP, leading to increased intracellular calcium that result in muscle contracture (Janse, 2004). The combination of these detrimental intracellular changes, eventually leads to cell death.

Cell contracture is defined as sustained shortening and stiffening of the myocardium (Piper et al., 2003). On the other hand, ischemic contracture can be the result of either insufficient production of ATP (<100 μmol/L), which cannot dissociate the actin myosin cross bridges, or insufficient energy, which cannot restore resting cytosolic calcium levels (Grossman & Barry, 1980).

2.3.2 Reperfusion

The high incidence rate of death associated with IHDs has led to increased research to identify effective cardioprotective interventions. It is generally agreed that the best strategy to reduce myocardial infarct size is to re-establish perfusion of the affected tissue as soon as possible. This is achieved by means of reperfusion, a protective intervention, which involves the transport of nutrients and oxygen to the affected tissue and/or metabolites away from it. Two important factors play an important role in the efficacy of early reperfusion, i.e. the degree of tissue injury and duration of ischemic insult (Jennings & Reimer, 1983).

However, in addition to the beneficial effects of reperfusion ("two-edged sword"), there is the sinister side which inflicts further damage, collectively known as reperfusion injuries, including myocardial stunning, reperfusion arrhythmias and myocyte hypercontracture (Piper et al., 2004; Opie 2004b). Hypercontracture occurs during the early phase of reperfusion when contractile activation is excessively amplified via the restoration of ATP synthesis in the presence of elevated cytosolic Ca^{2+} concentration (Piper et al., 1998; Siegmund et al., 1992).

Without the intervention of reperfusion all the ischemic tissue will eventually be lost. However, it is also well documented that reperfusion causes intensive contraction (opening of calcium channels) and poor relaxation within the myocardium (Hausenloy & Yellon, 2013). In the case were contracture affects the entire heart, this normally happens after global ischemia, it has been labelled as the ‘’stone heart’’ phenomenon (Piper et al, 2003).

Even though reperfusion is a paradoxical treatment for ischemia associated damage, this remains the only way to salvage reversibly damaged tissue. Thus ischemia and reperfusion go hand in hand and therefore a ‘’link’’ between ischemia and reperfusion is established with literature often referring to ischemia/reperfusion (I/R). It is therefore necessary to investigate possible methods whereby I/R injury can be reduced.

2.4 Natural infarct-sparing mechanisms

Countless direct cardioprotective therapies have been claimed to reduce infarct size in animals, however, few have been reproducible and none has been translated effectively into clinical practice. The treatment of reperfusion injury requires measures beyond timely reperfusion. In the laboratory, three major discoveries that can significantly lessen infarct-related injury have changed our understanding of cell survival:

1. Ischemic Preconditioning (1986)
2. Per-Treatment
3. Postconditioning (2003)

A serendipitous discovery by Murry et al. (1986), show how the exposure of a heart to 4 episodes of 5 minutes ischemia with intermittent reperfusion before a long period of sustained ischemia of 40 minutes, reduced the infarct size by 75%. This phenomenon was termed ischemic preconditioning (IPC) and is regarded as the strongest form of in vivo protection against ischemia. The efficacy of preconditioning (figure 3) in terms of cardioprotection can be seen in infarct size reduction (Murry et al., 1986), functional recovery improvement (Cave & Hearse, 1992) and in certain cases, decline in reperfusion arrhythmias (Shiki & Hearse, 1987). It should be noted that IPC has been presented in all species tested, even man (Yellon et al., 1993).

The mechanism behind IPC can be defined as a “trigger (adenosine)” - “mediator (protein kinases)” – “end-effector (mitochondria)” pathways (Yellon & Downey, 2003). The discovery of ischemic preconditioning is regarded as the single most important development in the field of ischemic biology in the last 20 years. The enormous interest in elucidation of the mechanism of protection could lead to development of pharmacological mimetics to be used in the clinical setting.

While pre-conditioning are deemed as the most effective and powerful mechanism to reduce myocardial infarct size, it is not possible in the clinical setting to pre-treat individuals prior to AMI. The second mechanism for protection against ischemia is termed remote ischemic conditioning/perconditioning. Remote ischemic conditioning (RIC) is a cardioprotective mechanism whereby one or cycles of non-lethal ischemia reperfusion is applied to an organ or tissue remote from the heart (Lim & Hausenloy, 2012). Przyklenk was the first to describe this phenomenon and demonstrated that the application of this mechanism can reduce the size of the myocardial infarction significantly (Przyklenk et al., 1993). In this study drug treatment will be administered during the sustained period of ischemia (per-treatment) to stimulate cardioprotection.

In 2003, Zhao and coworkers identified another mechanism of protection against ischemia called postconditioning (Zhao et al., 2003). They described this event as a series of brief ischemia and reperfusion cycles applied from the onset of reperfusion, which also reduces infarct size (figure 2.3) (Zhao et al., 2003). It has been well documented that postconditioning is just as effective in reducing infarct size as IPC (Zhao et al., 2003). In previous years, the damage of reperfusion injury was limited by using other reperfusion intervention such as initial hypoxic reperfusion (Serviddio et al., 2005) and pressure-controlled initial reperfusion (Selimoglu et al., 2007).

Cardioprotection elicit by preconditioning and postconditioning share certain events which include the following: activation of survival kinases ERK and PI3K/Akt (RISK pathway), adenosine triphosphate-sensitive potassium channel and closure of the mitochondrial permeability transition pore (Zhao et al., 2003).

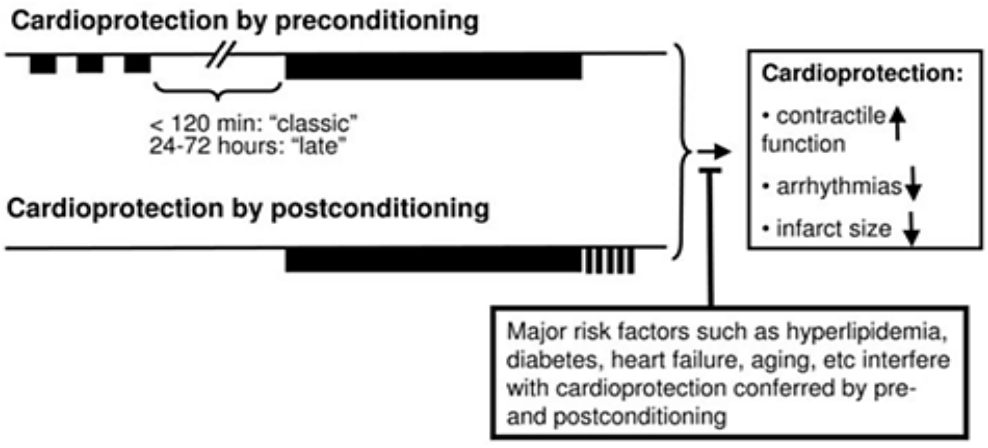

Figure 2.3: Cardioprotection against ischemia and reperfusion injury via mechanisms termed as ischemic preconditioning and postconditioning (Source: Ferdinandy et al., 2007).

2.5 Cell death: necrosis and apoptosis in ischemia/reperfusion

Cell death within the heart can be attributed to the following factors:

1) Apoptosis
2) Necrosis
3) Autophagy

This study will focus on two distinct forms of cell death, apoptosis (programmed cell death), as well as necrosis, which have been associated with ischemia/reperfusion injury. Early studies initially revealed that necrosis was the only form of cell death due to ischemia/reperfusion. It was reported that after a period of 90 minutes global ischemia, up to 92% of cell death was due to necrosis (Freude et al., 2000).

Necrosis refers to a process whereby the membrane fails due to the loss of ATP. The result is the swelling of the cell followed by the emission of the cellular content such as lactate dehydrogenase (LDH), activating inflammation (Hunot & Flavell, 2001; van Vuuren, 2008). LDH is thus a known marker to measure necrotic death.

However, the cell death in the injured myocardium can also be ascribed to apoptosis. In contrast to the above, Anversa et al. (1998) observed that up to 86% of cell death in a large infarct, following coronary occlusion, could be attributed to apoptosis. The phenomenon apoptosis, which is an energy dependent process, causes the cell to die without compromising membrane integrity.

This form of cell death requires caspase activation, resulting in endonucleases digesting chromosomal DNA into internucleosomal fragments (Hunot & Flavell, 2001).

When programmed cell death is initiated, pro-apoptotic proteins known as Bax and Bak are transported into the cytosol. Here they bind to BCL-XL found on the surface of the mitochondria, and activate pathways that lead to mitochondrial rupture, depolarization and release of cytochrome C (Youle & Strasser, 2008).

Finally, Autophagy, or cellular self-digestion, is a cellular pathway involved in protein and organelle degradation. It refers to the dynamic rearrangement of intracellular membranes to allow an organized breakdown and recycling of cytoplasmic portions (Mizushima et al., 2008). Death of myocardial cells is a catastrophic event as dead myocytes cannot be replaced by division of surviving myocytes.

2.6 The second messengers cAMP and cGMP

The second messengers, cyclic adenosine 3, 5 -monophosphate (cAMP) and cyclic guanosine 3, 5 -monophosphate (cGMP), act as important regulators of various cellular functions and morphological processes within the myocardium. Acute changes in the cytosolic levels of cyclic nucleotides can be the result of heart failure and cardiomyopathy that impair normal function (Lohse et al., 2004; Lohmann et al., 1991). Upon stimulation of the β-AR, the downstream effector adenylyl cyclase causes the production of cAMP (Zaccolo et al., 2007).

cAMP-dependent protein kinase (PKA) is recognized as the main downstream effector of cAMP, activating PKA catalytic subunits (Schaub et al., 2006). In the heart, cAMP plays a pivotal role in regulating the strength and frequency of cardiac contraction (heart rate) and relaxation through activating PKA. This, in turn, phosphorylates the L-type calcium channels, ryanodine receptors, phospholamban, and troponin I (Zaccolo et al., 2007).

According to literature, ischemia causes high levels of cAMP to accumulate within cardiac muscle cells which can be detrimental to the heart (Lubbe et al., 1992). Inside the cardiomyocytes, adenylyl cyclase increases cAMP production in response to ischemia and β-AR activation (Podzuweit et al., 1996). Furthermore, PDEs are repressed by a reduction in pH that results from the rapid ATP hydrolysis and this inhibits cAMP degradation (Podzuweit et al 1996). The harmful effects of excessive cAMP during ischemia are accompanied by increased Ca^{2+} entry in the sarcolemma causing intracellular Ca^{2+} overload (Tsien, 1983). Thus, hypercontracture is induced in Ca^{2+} overloaded cardiomyocytes, ultimately leading to cell death through necrosis.

Conversely, cGMP is produced through nitric oxide (NO) activation of guanylyl cyclase's (GC) and natriuretic peptide receptors (NPRs). cGMP is known to reduce inotropy and alter numerous metabolic responses through the activation of its downstream effector, protein kinase G (PKG) (Zaccolo et al., 2007). Both contractility and hypertrophy are known to be negatively modulated by cGMP, and the latter also mediates apoptosis in cardiomyocytes (Tsai & Kass, 2009).

As a result of opposite effects of PKA- and PKG-mediated phosphorylation on target proteins, the two second messengers exert contrasting influences on cardiac function (Shah et al., 2000). During ischemia a number of changes occurs such as energy loss (-ATP) and an increase in ions inside the ischemic cardiomyocte. Elevated catcholamine levels and subsequent β-AR activation can aggravate these detrimental effects through increased levels of intracellular cAMP. Increased cAMP levels can ultimately amplify contractility and the opening of the mPTP, which leads to apoptosis and eventually cell death. However, during ischemia, increased levels of cGMP can oppose the detrimental effects of cAMP through increasing muscle contraction and decreasing contracture (Zaccolo et al., 2007).

2.7 β-adrenergic receptors (β-AR)

The Beta-adrenergic receptors (β-ARs) are a class of G-protein-coupled receptors (GPCRs) which shares the common feature of 7-transmembrane spanning domains. Sympathetic stimulation by catecholamines (norepinephrine and epinephrine) causes these receptors to regulate a wide range of biological processes. This can include myocardial contractility and relaxation, cell growth, cell survival and cell death. The scientist, Ahlquist (1948), was the first to differentiate adrenergic receptors into alpha- and beta-adrenergic receptors (Ahlquist, 1948).

The uses of pharmacological and cloning methods have revealed three human genes that express three subtypes, β1, β2, and β3. These subtypes are thought to mediate death and survival signals, respectively (Emorine et al., 1989; Byland et al., 1995).

The classical signalling pathway involves agonists such as catecholamine's to bind to β-AR which in turn causes conformational changes and the activation of the classical Gs-adenylyl cyclase (AC)-cAMP-PKA signalling pathway. It is known that all three β-AR subtypes are distributed in a variety of tissues, within the heart of different species. (Skeberdis et al., 1997, Bylund et al., 1998). In the mammalian rat heart the β1-subtype tends to dominate distribution (85%) and is found in the ventricle rather than the atrium (Buxton et a., 1987).

β1- and β2-subtypes are both responsible for elevated levels of the intracellular messenger, cAMP, through the stimulation of the Gs protein that activates AC (Molenaar & Parsonage, 2005; Brodde et al., 2006) (figure 2.4). However, the β2-subtype can also stimulate the Gi protein, which in turn causes an inhibitory effect on the production of cAMP.

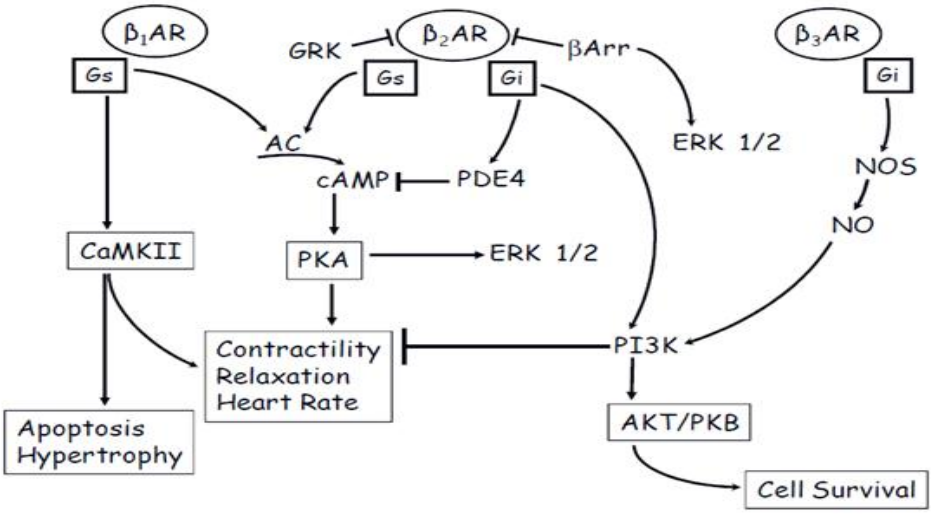

Figure 2.4: Subtype-specific signalling pathways of cardiac β-ARs (Source: Lohse, Engelhardt and Eschenhagen, 2003; Zheng et al., 2005).

When cardiac β1-AR becomes stimulated, it causes an increase in automaticity (chronotropy), conduction velocity (dromotropy) and contraction force (inotropy) (Kaumann, 1989; Bristow et al., 1990). These effects are mediated through the activity of PKA.

Furthermore, Gauthier et al. (1996) compiled evidence that the expression of β3 adrenergic receptors in human ventricles is evident (Gauthier et aI., 1996). The β3-AR differs from β1-AR and β2-AR with regard to their molecular structure as well as their pharmacological profile (Bylund et al., 1994). A common feature shared by both the β2- and β3-AR is the fact that they both stimulate the Gi protein associated with the PI3-kinase (figure 4.1). This signalling pathway activates Akt/PKB resulting in cell survival.

2.7.1 The β-adrenergic receptor signalling in the heart

The β-AR isoforms found in the heart have different affinities for different ligands (Hoffmann et al., 2004). These receptors play an integral role in normal cardiac function and in heart failure, and are dynamically regulated to maintain homeostasis.

PKA is known to phosphorylate a host of Ca^{2+} handling proteins. These can include the sarcolemma L-type Ca^{2+} channels (Zhao et al., 1994; Gerhardstein et al., 1999), ryanodine receptors (Marx et al., 2000) and sarcoplasmic reticulum (SR) membrane proteins, phospholamban (PLB) (Simmerman and Jones, 1998), troponin I and C protein (Sulakhe and Vo, 1995) and myosin binding protein-C (MyBP-C) (Kunst et al., 2000).

As a result of this phosphorylation the cardiomyocyte contractile behaviour becomes affected via increased Ca^{2+} influx into the sarcoplasmic reticulum (SERCA) and modulating myofilament Ca^{2+} sensitivity (Troponin) (Figure 4.1).

Under ischemic conditions; the second messenger (cAMP) is known to accumulate in the cardiomyocyte, which can be attributed to the increased cAMP production via AC in response to β-AR activation. However, the decrease in pH that results from the rapid ATP hydrolysis causes a hold in cAMP degradation due to inhibition of the PDEs (Podzuweit et al., 1996).

The pharmacological activation of the β-adrenergic signal transduction pathway via ligands/agonist such as formoterol and dobutamine are thought to elicit either protection or damage (Bhushan et al., 2012, Pantos et al., 2003, Wang et al., 2013). In view of the above, the next section will focus on this possible agonists and the functional role they play in cardiology.

2.7.2 Formoterol and Dobutamine acting as agonist in β-AR signalling

During ischemia a number of changes occurs such as energy loss (-ATP) and an increase in ions inside the ischemic cardiomyocyte. Energy depletion and tissue damage is worsened by elevated levels of adrenaline and nor-adrenaline, via β-AR activation by sympathetic stimuli, which increases the intracellular cyclic AMP levels. All these events can be harmful and ultimately leads to cell death.

There is continued interest in developing more selective β-AR agonists and pharmacological agents that can be applied during ischemia to elicit cardioprotection.

One such agent is known as formoterol. The long acting β2-AR agonist, formoterol, can exert a survival signal through GPCR activation within the cardiomyocytes (Wills et al., 2012). This β2-adrenergic receptor binding results in the production of the second messenger, cAMP, through adenylyl cyclase, which contributes less to cardiac contractility than β1-AR.

According to the literature, only a few research papers has been published regarding dobutamine mediated activation of β-AR during ischemia and its effect on cardiomyocytes. This thus gives as the opportunity to investigate this event in our study and also inspect other agonist and their role in such a setting.

Dobutamine is a sympathomimetic drug popular for treating heart failure in clinic settings (Tuttle & Mills, 1975). This drug's primary mechanism is the direct activation of the β1-receptor which is linked to an adenine nucleotide regulatory cascade via heterotrimeric Gs proteins. The resulting activation causes an increase of adenylyl cyclase activity which is responsible for the conversion of ATP to cAMP (Shulz et al., 1992).

Isoproterenol is a non-selective beta-adrenergic agonist. It has the ability to bind to both β1/β2-AR and can be used for the treatment of bradycardia. By activating β1-AR it induces positive chronotropic and inotropic effects (Shen & Howard, 2008). On the other hand, by binding to β1-AR, the agonist can instigate cardiomyocyte apoptosis which leads to heart failure (Zhou et al., 2013).

2.8 Insulin: a protecting agent during ischemia/reperfusion

Reperfusion of ischemic myocardium is a necessary mechanism to restore cardiac function, but with detrimental effects such as lethal myocardial cell damage (Piper et al., 2004; Garcia-Dorado, 2004). The phenomenon ‘’reperfusion injury’’ of cardiomyocytes occurs normally within the first few minutes of reperfusion. Taking this in consideration, any protection against this form of injury must be applied before or immediately upon the onset of reperfusion. With the use of interventions to alter cardiac mechanical function and energy metabolism, myocardial tolerance to ischemia/reperfusion can possibly be improved.

Researchers have investigated whether insulin will elicit a potential protecting effect against myocardial reperfusion injury when administered at the time of reperfusion. Insulin, which is a pleiotropic hormone, exerts various effects on the central nervous system, immune system, glucose metabolism, the central nervous system, immune and cardiovascular system (Wooi et al., 2012).

2.8.1 Protection against reoxygenation-induced hypercontracture

A clinical trial conducted by van der Horst and colleagues found that patients treated with a Glucose–Insulin–Potassium scheme (GIK) at the onset of reperfusion had a reduced in-hospital mortality risk (van der Horst et al., 2003). A number of studies have been conducted, with accumulating evidence stating that insulin provides myocardial protection by mechanisms acting at the very beginning of reperfusion (Jonassen et al., 2001). One such experimental study, done by Abdallah and colleagues, identified the possible protection mechanism of insulin against reoxygenation-induced hypercontracture within cardiomyocytes (Abdallah et al., 2006).

At the start of reoxygenation, clearance of calcium from the cytosol can effectively reduce the development of hypercontracture (Abdallah, 2005). If this event does not happened, myocardial cells end up with excessive cytosolic calcium, leading to high cytosolic calcium peaks causing an uncontrolled contractile activation of the myofibrils, and hence hypercontracture (Siegmund B, Schluter KD, Piper HM, 1993; Siegmund et al., 1997).

The mechanism whereby insulin-mediates protection against reoxygenation-induced hypercontracture is defined as follows. The activation of NO synthase through insulin, causes the downstream activation of PKG. This leads to the increased phosphorylation of phospholamban and consecutively activates SERCA.

With the activation of SERCA, large amounts of cytosolic Ca^{2+} can be removed by the sarcoplasmic reticulum. An increased Ca^{2+} removal into the SR causes an accelerated recovery of cytosolic Ca^{2+} control and prevents excessive myofibrilar activation, e.g., hypercontracture (Abdallah et al., 2006).

2.8.2 RISK pathway

The Reperfusion Injury Salvage Kinase (RISK) pathway, also known as the pro-survival kinases, plays an important role during the early stages of reperfusion, which recently, has promoted a renewed interest in cardioprotective reperfusion approaches (Hausenby DJ & Yellon DM, 2004).

After ischemia-reperfusion insults, cardiomyocytes have an integral program for survival via the recruitment of innate pro-survival kinase cascades (figure 4). The RISK pathway consists of PI3-kinases and p42/p44 extra-cellular signal-regulated kinases (ERK1/2) (Yang et al., 2005, Tsang et al., 2004). The latter are both activated by ischemia-reperfusion injury or by pharmacological PostC (pharmacological PostC through the use of insulin). Further downstream, Akt (protein kinase B) and MEK kinases (MEK kinase = MAPK/ERK kinase; mitogen-activated protein kinase), are both important components of the cell survival pathway and have shown to elicit anti-apoptotic effects (Luna-Ortiz et al., 2011).

Davidson and colleagues reported that when the PI3K-Akt kinases are phosphorylated via insulin, the cardiomyocytes can be protected against oxidative stress by inhibition of mPTP (Davidson et al., 2006). Thus, the pharmacological activation of the RISK pathway by insulin reduces the possibility that the mPTP will open, protecting the myocyte against ischemia-reperfusion injury.

The potential mechanisms (figure 2.5) through which the RISK pathway mediates the inhibition of mPTP opening are as follow: 1) the downstream target glycogen synthase kinase-3b (GSK-β) is phosphorylated and inhibited by Akt (Juhaszova et al., 2004) 2) phosphorylation of endothelial NO synthase (eNOS), that results in the production of nitric oxide. The latter is known to inhibit mPTP opening (Kim et al., 2004) 3) finally, the phosphorylation of Bcl-2-associated death promoter (BAD) by means of p70S6 kinase, thereby negating its pro-apoptotic effect. From this we can confirm that the insulin-mediated RISK pathway delays mPTP opening and protects the cells from injury and eventually cell death.

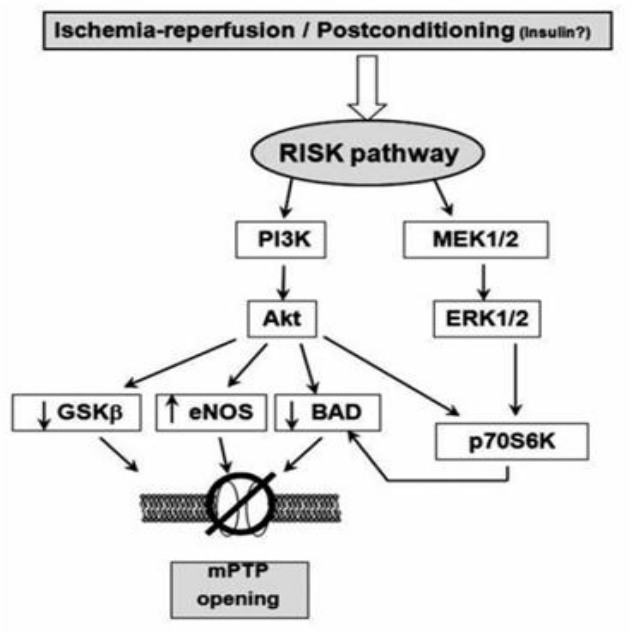

Figure 2.5: Scheme showing the proposed mechanism of protection via insulin-mediated RISK pathway (Source: Luna-Ortiz et al., 2011).

2.8.3 Glucose homeostasis

Although the primary cardioprotective action of insulin is considered to be through the RISK pathway, increased glucose uptake during ischemia is also recognised as an important event in cardiomyocte protection. Insulin signalling has numerous effects on glucose metabolism and occurs by means of the PI3K pathway and the mitogen-activated protein kinase pathway (Wooi NG et al., 2012). The insulin receptor substrate is phosphorylated (activated) when insulin binds to the membrane receptor.

The activated substrate becomes associated with PI3K, which in turn activates a number of downstream effectors. These effectors include: Akt, PKC, and the mammalian target of rapamycin (mTOR) (Wooi NG et al., 2012).

When both Akt and PKC are activated, the intracellular glucose transporter 4 (GLUT4) proteins are translocated to the plasma membrane where they enhance the peripheral uptake of glucose molecules (Wooi NG et al., 2012). Ultimately, an increased intracellular glucose concentration will result in amplified production of ATP that is needed for ion-pump activity, which is responsible for normal ion homeostasis. In addition to glucose uptake, activation of Akt also promotes glycogenesis by inhibiting glycogen synthase kinase-3, the known enzyme responsible for inactivating glycogen synthase (Wooi NG et al., 2012). Severe ischemia can also inhibit glycolysis mainly by decreasing delivery of glucose to ischemic cells, also by glycogen depletion and glycolytic enzyme inhibition.

From the above, we can state that insulin exerts cardioprotective effects via glucose-dependent and -independent mechanisms. Contradictory to this, certain complications regarding the use of insulin as protective agent exists. Numerous studies have confirmed that a high pharmacological-insulin dose is required to exert optimal effect. (Wooi NG et al., 2012). Optimal protection also requires a precise concentration, which in terms of various species can differ from one to another. Therefore, taking these complications in consideration, the search for another potentially cardioprotective agent, to be administered during ischemia/reperfusion, is one of the main objectives of this study.

2.9 Phosphodiesterase Enzymes

The cyclic nucleotide phosphodiesterases (PDEs) are described as a family of enzymes that selectively catalyse the hydrolytic degradation of 3' cyclic phosphate bonds of adenosine cAMP and/or guanosine 3' ,5' cyclic monophosphate cGMP (Bender et al., 2006; Beavo et al., 2006). The hydrolysis of these cyclic nucleotides specifically controls the levels and localization of both cAMP and cGMP, thereby mediating their return to the basal state (Lugnier, 2006).

The PDE superfamily, often referred to as class I cyclic nucleotide PDEs, consist of 11 members (PDE1-11) designated according to their structure, kinetics, substrate specificity, and regulatory mechanisms (Francis et al., 2001; Bender & Beavo, 2006). A conserved catalytic domain (C-domain) is shared among the PDEs, but the amino acid sequence outside this region is known to differ immensely (Francis et al., 2011).

The structure of PDEs consists of highly divergent N-terminal containing structural elements which allows selective responses from the various PDEs to specific regulatory signals (Manganiello, 2003). Regulation of PDEs can be conducted on both a genetic level and diverse biochemical mechanisms such as the following: phosphorylation/dephosphorylation, allosteric binding of cGMP or cAMP, binding of Ca^{+2}/calmodulin, and various protein-protein interactions (Bender & Beavo, 2006).

2.9.1 Phosphodiesterases in Functional Compartments

Each of the 11 members can be classified according to its substrate specificity (Francis et al., 2001). This means each PDE have a preference for either hydrolysing cAMP (PDE 4, 7 and 8), or cGMP (PDE 5, 6 and 9), or (PDE 1-3, 10 and 11) possess dual specificity to degrade both cAMP and cGMP (figure 2.6) (Bender & Beavo, 2006). The PDEs 1-4 are found to be expressed in various tissues, whereas the other members of the family are more restricted to a specific tissue such as the kidney and skeletal muscle.

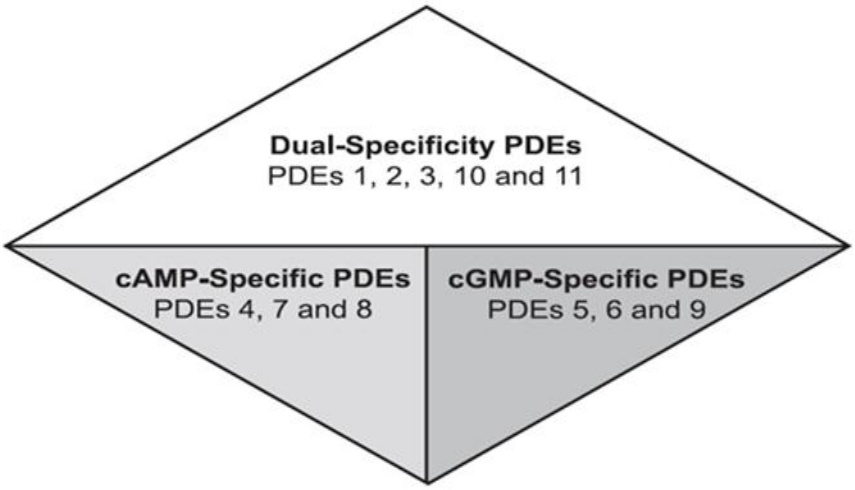

Figure 2.6: Depiction of mammalian families of phosphodiesterases (PDEs) according to preferences for either cAMP or cGMP. Dual-specificity PDEs hydrolyze both cyclic nucleotides (Source: Francis et al., 2011).

Cardiac mRNA expression has been reported for the following 7 members of the PDE superfamily, PDE1–5, PDE8a, and PDE9a (Miller & Yan, 2010; Zang & Kass, 2011). The PDE activity also plays an integral role in the regulation of cross-talk between cAMP and cGMP signalling pathways (Zaccolo & Movsesian, 2007).

Zaccolo and cowokers also stated that within the cell, a specific distribution of cyclase's (adenylyl or guanylyl) in close vicinity with PDEs exist, causing gradients of cAMP or cGMP to form, with the highest cyclic nucleotide concentration close to cyclase enzymes and the lowest in PDE residing areas (Lomas & Zaccolo, 2013).

The subcellular distribution of PDE3 and PDE4 isoforms was shown by Mongillo et al. (2004), who also provided evidence of functional compartmentalization of PDE isoforms through their differential effects on β-AR signalling (Mongillo et al., 2004).

2.9.2 Characterization and properties of phosphodiesterase 3 (PDE3)

2.9.2.1 Overview

PDE3 which is recognized as the cGMP inhibited PDE, has been extensively studied over the last few years and consist of two isoforms, PDE3A and PDE3B (Shakur et al., 2001). With regards to genetic splicing, it has been found that the PDE3A isoform has three variants (PDE3A1, 2, 3) (Choi et al., 2001; Wechsler et al., 2002). It has been identified that both PDE3A2 and PDE3A3 variants acts as alternate start site truncations of PDE3A1. Various sized PDE3B proteins have also been described, but no PDE3B splice or alternative start variants have been identified (Bender & Beavo, 2006).

The two subfamily genes contains a catalytic region with a high degree of amino acid identity (<80%) with very similar kinetic properties shared amongst them. Their physiological functions make them useful for cardiotonic drug targets (Bender & Beavo, 2006). The PDE3 family possesses a unique biochemical property which enables it to hydrolyse both cAMP and cGMP, but in such a manner that the hydrolysis of cAMP is inhibited by cGMP under in vivo and in vitro conditions (Bender & Beavo, 2006).

This is because PDE3 exhibits a Vmax (maximum enzyme velocity) for cAMP that is tenfold higher than its Vmax for cGMP. With this taken in consideration, PDE3 acts as a functionally cAMP-hydrolyzing enzyme and cGMP is a competitive inhibitor (Bender & Beavo, 2006; Degerman et al., 1997). Therefore PDE 3 isoforms have been labelled as “the cGMP-inhibited cAMP PDE”. They are further distinguished through regulation of their activity by phosphorylation pathways including the PKA and PI3K/PKB pathways.

Also present in the structure of both PDE isoforms (3A & 3B) is an insert in the catalytic domain which is not present in other PDEs (Bender & Beavo, 2006). According to He and coworkers, this 44-aa insert may be involved in cGMP-PDE3 sensitivities (He et al., 1998). Another special feature is that the N-termini of each isoform comprises of both a large and a small hydrophobic domain (figure 2.7) (Wechsler et al., 2002). These domains are thought to play an integral role in protein localization.

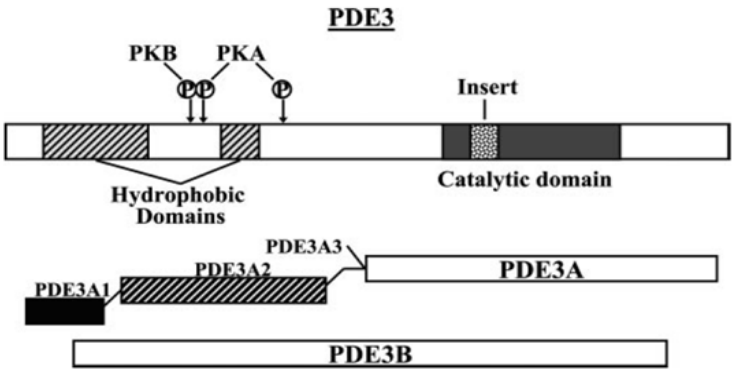

Figure 2.7: The cGMP-inhibited PDE. PDE3 activity can be regulated by both PKA and PKB phosphorylation. The hydrophobic domains are thought to be involved in protein localization (Source: Bender & Beavo, 2006).

2.9.2.2 Localization

The PDE3 can be bound to either the cytosol or membranes of different organelles. It has been documented to be associated to the sarcoplasmic reticulum (Lugnier et al., 1993), Golgi apparatus (Geoffroy et al., 2001), plasma membrane (Okruhlicova et al., 1996, 1997) as well as the nucleus envelope (Lugnier et al., 1999b).

Both the isoforms are expressed in a variety of tissues within the human body and have distinct but overlapping localizations. For instance, the PDE3A is found in abundance in cells of the cardiovascular system, including cardiac myocytes, vascular smooth muscle (VSMC) and platelets (Shakur et al., 2001).

In contrast, PDE3B is the major PDE in adipocytes, hepatocytes, as well as in several cardiovascular tissues (Liu & Maurice, 1998; Shakur et al., 2001). Studies conducted by Liu and coworkers, have described that both PDE3A and PDE3B isoforms have been expressed in blood vessels of the cardiovascular system within the rat heart (Liu & Maurice, 1998).

The three known isoforms, PDE3A1, PDE3A2 and PDE3A3, rise from the PDE3A gene, and varies only in the sequence near the N-terminus (Roa et al., 2009). It has been documented that the PDE3A1 is not well expressed within the cardiomyocyte, but the other two isoforms are highly expressed in both vascular smooth muscle and cardiomyocytes (Wechsler et al., 2002).

Shakur and colleagues found that PKA activates both PDE3A and PDE3B within the heart, which results in a negative feedback on the levels of cAMP found in the cell (Shakur et al., 2001). Bender and colleagues have also suggested that the PDE3 enzyme may play a pivotal role in mediating tachyphylaxis (inadequate response to drug administration) in response to long term β-agonist stimulation.

There have been several PDE3B proteins found, which differ greatly in size, but their origin is still to be established, yet PDE3A appears to represent the dominant isoform (Bender & Beavo, 2006). Nevertheless, PDE3B1 proteins have recently been discovered within mice heart tissue and it has been stated to account for about 30 present of the PDE3 activity (Patrucco et al., 2004).

2.9.2.3 Regulation of myocardial contractility by PDE3

When investigating the cardiovascular system, two important facts are well-known. To begin with, increased levels of cAMP induce positive inotropic effect in the heart, whereas cGMP decreases cardiac contraction and induces vasorelaxation when its levels become increased. As a result of their cyclic nucleotide inactivating role, PDEs play a major role in the fine regulation of these functions.

As stated before, PKA is activated through cAMP elevation, which in turn is responsible for the phosphorylation and activation of multiple substrates. The latter plays a role in the contraction and relaxation within cardiomyocytes through the regulation of Ca^{2+} transients and contractile protein phosphorylation.

This includes the cardiac RyR2, the Ca^{2+}-ATPase SERCA2 in the SR and LTTCs in the sarcolemma (Knight & Yan, 2012; Yan et al., 2007; Matthew, 2003). These proteins play an integral role in Ca^{2+} re-uptake and when PKA phosphorylates LTCC, which leads to increased Ca^{2+} influx into cells.

Regarding the cardiovascular system, several studies have indicated the involvement of PDEs regulating cardiac contractility through modulation of cAMP or cGMP signalling, and PDE 3's involvement will be discussed below.

When investigating the regulation of cardiac contractility, PDE3 is the most studied due to the key inotropic effect PDE3 inhibitors have in laboratory animals and humans. When PDE3 becomes inhibited, PKA phosphorylates and activates PLB and RyR2 which increases the SR Ca^{2+} uptake, but it seems that LTCC channel activity is not regulated (Yan et al., 2007; Kerfant et al., 2007).

The modification of the SR therefore appears to be the primary PDE3-mediated regulation of contractility and not through plasma membrane-associated, Ca^{2+} signalling. Packer and coworkers (Packer et al., 1991) showed when PDE3 is inhibited pharmacologically. It caused an increase in both heart rate and contractile force within humans, and these effects in tandem lead to increased cardiac function and coronary blood flow (Packer et al., 1991). This ultimately can be beneficial to patients with congestive heart failure, but only in the early phase.

Though PDE3 had been characterised through its inotropic and chronotropic effects in the heart, the specific isoform involved has not been well documented until the development of PDE3A and PDE3B deficient mice. This associates the PDE3A isoform as dominant in regulating contractility. In an experiment conducted by Sun and colleagues, they found that within PDE3A knock-out (KO) mice, the heart rate increased significantly in contrast to PDE3B (Sun et al., 2007).

Although it seems that the PDE3B isoform has a lesser impact in cardiac PDE3 activity, in the mouse heart it has been revealed that this isoform associates with the αPI3Kγ complex, which is essential for the PDE3B activity in the signalling complex (Patrucco et al., 2004). PDE3B-mediated cAMP degradation is controlled by PI3Kγ, in conjunction with a kinase independent mechanism, which is known to control cardiac contractility and protects against pressure overload-induced cardiac damage (Patrucco et al., 2004).

On the contrary, Packer and coworkers found that αPI3Kγ is not necessarily required in PDE3 activity in a subcellular compartment containing the SR Ca^{2+}-ATPase. However, its presence in PDE4, suggest that the αPI3Kγ complex is associated with different PDE isoforms found in distinct subcellular domains (Kerfant et al., 2007).

2.9.2.4 PDE3 and Cardiac myocyte apoptosis

Apart from the second messenger's cAMP and cGMP being involved in modulating myocyte contraction, they also play a pivotal role in regulating myocyte gene expression, which can result in pathological cardiac remodelling and subsequent cardiac dysfunction (Knight & Yan, 2012). In addition, myocyte hypertrophy, apoptosis, fibroblast activation and proliferation are associated with pathological cardiac remodelling and numerous PDEs, including PDE3, seems to be implicated in these processes providing promise for pharmacological intervention.

It appears that PDE3A plays a vital role in regulating cardiomyocyte survival. In experimental animals, chronic inhibition of PDE3A or PDE3A knockdown has been shown to potentiate cardiomyocyte apoptosis via activation of the transcriptional repressor protein ICER (inducible cAMP early repressor) (Ding et al., 2005).

With the latter being activated, CREB-mediated transcription becomes down regulated as well as anti-apoptotic proteins such as Bcl-2, causing apoptosis (Ding et al., 2005). It has been well documented that down regulation of PDE3A expression and up regulation of ICER have been found to be present in animals suffering from heart disease, and humans hearts with ischemic and dilated cardiomyopathies (Ding et al., 2005; Francis et al, 2011; Yan et al., 2007; Abi-Gerges et al., 2009).

In the experiment conducted by Ding and coworkers, Sprague-Dawley rats were used and infused with angiotensin II (pro-apoptotic stimuli) (0.7 mg/kg per day) for 7 days via osmotic minipumps to induce apoptosis (Kim et al., 2001).

2.10 PDE3 Inhibitors: Introduction and Milrinone

When PDE3 activity becomes inhibited, cAMP levels becomes elevated within the cardiac muscle and this leads to increased rate of both developed force as well as the rate of muscle relaxation (Knight & Yan, 2012). Parallel to this, inhibition can further lead to reduced total peripheral resistance, enhanced coronary blood flow, reduced pulmonary vascular resistance, reduced right atrial pressure, and venodilation (Knight & Yan, 2012). Therefore, milrinone, amrinone, and enoximone, which are the most common known PDE3 inhibitors, have become potent drugs for the acute treatment of heart failure, due to their inotropic actions (Baim et al., 1983; Jaski et al., 1985). Nevertheless, there are some studies that propose significant differences among these PDE3 inhibitors with respect to the relative magnitude of these effects.

Zausig and colleagues conducted a study where they compared the dose dependent effects of milrinone, amrinone, and enoximone in isolated guinea pig hearts (Zausig et al., 2006). They discovered that milrinone increased contractility at lower concentrations (10^{-6}M) when compared to the other inhibitors. At a higher concentration (10^{-4}M), milrinone also increased coronary flow and thus oxygen supply which in itself can play a major role in reperfusion injury (Zausig et al., 2006).

Milrinone is the most well studied PDE3 inhibitor and most extensively used in clinical practise. It has the unique ability to inhibit both PDE4 and PDE5 at a concentration of 1mM (Lugnier, 2006). The vasodilatory and contractile effects of some PDE inhibitors, in addition to possible cardioprotection, make research on these agents an especially promising area of investigation.

Table 2.1 represents general characteristics of the various PDEs and their affinity for the second messengers, tissue distribution and selective chemical inhibitors. This review will focus on PDE3 and its selective inhibitors.

Table 2.1: The basic characteristics of PDE and their selective chemical inhibitors (Source: Roa & Xi, 2009).

PDE isoform	cGMP K_m (μmol/L)	cAMP K_m (μmol/L)	Tissue distribution	Inhibitors	IC_{50}	Ref
PDE3A	0.02–0.15	0.18	Heart; vascular smooth muscle; platelets; oocyte; kidney	Milrinone	150 nmol/L	[3,18,254–256]
PDE3B	0.28	0.38	Vascular smooth muscle; adipocytes; hepatocytes; kidney; β cells; developing sperm; T lymphocytes; macrophages	Amrinone	16.7 μmol/L	
				Enoximone	1.8 μmol/L	
				Pimobendan	2.4 μmol/L	
				Cilastazol	200 nmol/L	
PDE4A	–	2.9–10	Ubiquitous	Rolipram	1 μmol/L	[3,18]
PDE4B	–	1.5–4.7	Ubiquitous	Ro 20-1724	2 μmol/L	
PDE4C	–	1.7	Lung; testis; neuronal cells	Cilomilast	120 nmol/L	
PDE4D	–	1.2–5.9	Ubiquitous	Roflumilast	0.8 nmol/L	
PDE5A	2.9–6.2	290	Platelets; vascular smooth muscle; brain; lung; heart; kidney; skeletal muscle	Sildenafil	4 nmol/L	[3,18]
				Vardenafil	0.1 nmol/L	
				Tadalafil	2 nmol/L	
				Udenafil	6 nmol/L	

2.10.1 Effects of PDE3 inhibitors on myocardial ischemia/reperfusion injury

PDE inhibitors are known to elicit pre-and postconditioning-like effects, and it is our interest to determine this mechanism of action and the significant role it can play in clinical cardiology.

From previous research that has been done in our laboratory regarding this subject, it has been found that PDE activity and cyclic nucleotide levels undergoe alteration during ischemia and ischemic preconditioning in isolated perfused rat hearts (Lochner et al., 1998).

Early evidence presented by Lowe and colleagues, suggested that milrinone exerts a cardioprotective effect. They observed improvement of contractile recovery in an isolated perfused rabbit heart following hypoxia/reperfusion injury with the administration of 10μM milrinone (Lowe et al., 1992). In contrast, milrinone treated post-myocardial infarction (MI) had no substantial effect on the survival rate of rats (Levijoki et al., 2001).

2.10.1.1 Differential effects of Amrinone and Milrinone in ischemia/reperfusion

Other PDE3 inhibitors, such as amrinone, have also caused improved contractile recovery after ischemia/reperfusion in the isolated rat heart (Zucchi et al., 1990). Decreased levels of creatine kinase and lactate dehydrogenase in the coronary effluent also indicated that amrinone lessened I/R injury in isolated rat hearts. The authors attributed this protective effect to the possibility of altered Ca^{2+} handling by amrinone in the myocardium (Rechtman et al., 2000).

The differential cardioprotective effect of the PDE3 inhibitors such as amrinone and milrinone can be recognised in the diverse way they modulate inflammatory signalling. For instance, when the different amrinone concentrations (50, 100, 250 μmol/L) were tested in two-day-old Wistar rat cardiomyocytes, NFκB activation by endotoxin (LPS) was inhibited (Chanani et al., 2002). On the other hand, milrinone to some extent plays a role in potentiating TNF driven NFκB activation without affecting LPS induced NFκB activation (Chanani et al., 2002).

In view of the above, the significant effect that numerous PDE inhibitors have on inflammatory signal transduction pathways and mediator production within the clinical setting is of our utmost interest. Largely, it seems that amrinone has the potential to affect diverse pro-inflammatory stimuli, whereas milrinone does not. Taking this into thought, it might be of our interest to investigate this PDE inhibitor in more depth regarding cardioprotection within isolated cardiomyocytes.

2.10.1.2 Protection by Esmolol and Milrinone at late-ischemia and early reperfusion

With the continuous struggle to translate ischemic preconditioning to clinical application for patients with acute myocardial infarction (AMI), recent research in ischemic postconditioning unlocks a new prospect to overcome this problem (Huang et al., 2011). Both the RISK pathway and the activation of Akt have been identified as possible mechanisms in postconditioning to reduce infarct size and mediate heart protection (Hausenloy et al., 2004; Ovitz et al., 2010). Sanada and colleagues found that direct myocardial PKA activation in combination with the PD3 inhibitor milrinone at reperfusion level can lead to cardioprotection, since milrinone is known to lead to activation of PKA (Haikala et al, 1997; Sanada et al., 2001).

To determine whether the myocardial infarct size (IS) of the left ventricle (LV) can be reduced, a study was performed by Huang and co-workers (Huang et al., 2011). In their experiment they made use of the β1-adrenergic receptor blocker, esmolol, and phosphodiesterase 3 inhibitor, milrinone, during late ischemia and early reperfusion (Huang et al., 2011). The cardiomyocytes were cultured for 24 hours, whereafter hypoxia was induced. The latter was done by layering mineral oil over media covering the cells for 2 hours, followed by 60 minutes re-oxygenation.

A previous study has indicated that a very high dose of β1-AR blocker esmolol given at the onset of reperfusion reduces LV-IS (Geissler, 2002). In the study done by Huang and co-workers, they used a rat model with the heart being exposed to 30 minutes of ischemia followed by 4 hours of reperfusion. The drugs were introduced individually (esmolol; milrinone) and in conjunction (esmolol + milrinone) to the heart and infused over 10 minutes (from the last 5min of ischemia to the first 5min of reperfusion).

To investigate the in vivo intra-ischemic myocyte protection of esmolol + milrinone, they studied the effect of an adult rat mixed cardiomyocyte culture preparation (mixed = intrinsic cardiac adrenergic (ICA) cells) saline (control), esmolol (1 nmol/L), milrinone (4.7 μmol/L) or esmolol + milrinone were individually applied to the myocyte culture.

Their findings were as follow: The treated rat hearts showed IS-reduction when they received reperfusion treatment of esmolol, milrinone or esmolol + milrinone, respectively. When compared to the saline control, the respective administration of esmolol and milrinone resulted in 35% and 59% more IS reduction compared to when the drugs were given individually. Examining the myocyte cultures, they found that treatment with esmolol, milrinone and esmolol + milrinone led to reduced cell death by 5.5%, 13.3% and 16.8%, respectively (figure 2.8), compared with saline (control).

In contrast, when the culture was treated with esmolol + milrinone in the presence of Akt 1/2 kinase inhibitor, it was noted that the myocyte death were higher when compared to that of the saline control. Thus, the inhibition of Akt through the Akt 1/2 kinase inhibitor causes insufficient postconditioning mediated heart protection through this signalling pathway (Hausenloy and Yellon, 2004; Ovitz et al., 2010).

Another encouraging finding is that when the LV myocardium was treated with esmolol, milrinone or esmolol + milrinone during early reperfusion, a reduction in overall cardiomyocyte apoptosis by 23%, 37% and 60%, respectively, were observed when compared to the saline control. In contrast, when both Rp-cAMPS (PKA inhibitor) and Akt-inhibitor were administered in conjunction with esmolol/milrinone, an increase IS was noticed compared to the saline control. According to the data a basal tonic PKA-Akt signalling exists during ischemia/reperfusion and disruption of this specific pathway can lead to reperfusion injury.

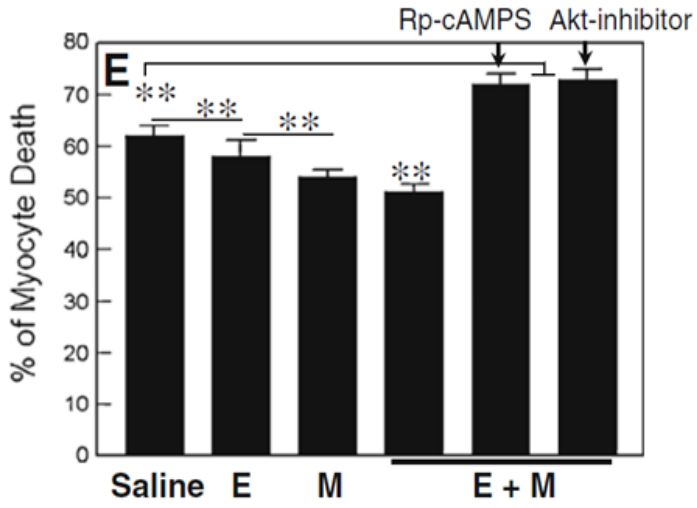

Figure 2.8: The effects of intra-ischemic application of esmolol, milrinone or E + M in the absence and presence of Rpc AMPS or AKT 1/2 kinase inhibitor on myocyte death rates during hypoxia/re-O_2 in myocyte culture preparation (Source: Huang et al., 2011)

In conclusion, Huang and co-workers demonstrated that brief infusion period of esmolol+milrinone at late-ischemia and early-reperfusion can result in LV-IS reduction. This cardioprotective effect is associated with the activation of PKA/Akt-dependent anti-apoptotic signalling.

These findings suggest that many PDE3 inhibitors are capable of causing postconditioning-like effects either by themselves or in conjunction with other approaches such as β-AR modulation. Although the mechanisms still remain to be elucidated through additional studies, the cardioprotective effects that include improvements in viability are indeed very attractive.

2.10.1.3 Comparison of the effects of Cilostazol and Milrinone on cAMP-PDE activity, intracellular cAMP and calcium in the heart

In the following study, Shakur and colleagues investigated the basic different cardiotonic effects the PDE3 inhibitors cilostazol and milrinone would have in the rabbit heart (Shakur et al., 2002). They especially looked at changes in intracellular cAMP and calcium with the administration of these inhibitors. Previous studies have indicated that cilostazol is commonly used for effective treatment of symptoms of intermittent claudication (IC), which is known as a manifestation of lower-extremity peripheral arterial occlusive disease (Yasuda et al., 1985; Elam et al., 1998). It has also been reported that cilostazol's adverse effects include headache, diarrhea and palpitations (Shakur et al., 2002).

A recent study, done by Cone and coworkers, compared the effect of cilostazol (>15 μM) and milrinone (>10 μM) on the rabbit heart and interestingly found that milrinone elicited a stronger positive inotropic response than cilostazol (Cone et al., 1999).

As PDE3 is recognised as the main cAMP hydrolysing enzyme in the heart, at least six other PDE isoforms have been discovered within this organ (Weishaar et al, 1986; Hetman et al, 2000). The L-type Ca^{2+} channels found within isolated ventricular myocytes are regulated by PDEs 1–4 (Verde et al., 1999). The numerous PDE families' involvement in compartmentation of cAMP signalling responses has also been well documented in cardiomyocytes (Jurevicius et al., 1996; Elks & Manganiello, 1985).

In the experiment, rabbit hearts were used to obtain isolated ventricular myocytes by means of a conventional enzymatic dissociation method (Liu et al., 1996). Thereafter, the potential differences in the effects of both drugs on cAMP and calcium signalling pathways were examined by measuring intracellular changes in cAMP and calcium. The effects of cilostazol and milrinone on cAMP-PDE activity were also performed on failing human hearts to determine whether these drugs can be implicated in therapeutic use (Shakur et al., 2002).

Investigating the results, it was found that within microsomal versus cytosolic fractions from rabbit heart, the cilostazol exhibited greater selectivity than milrinone for the cAMP-PDE activity (Shakur et al., 2002). The difference can be attributed to the fact that less cytosolic cAMP-PDE activity was inhibited by cilostazol compared to milrinone (Shakur et al., 2002). Examining the comparison with the failing human hearts, there was no significant difference in the inhibition of total cytosolic cAMP-PDE activity by cilostazol and milrinone at any concentration.

This difference can be ascribed to other mechanism by which cilostazol and milrinone exerts their effects in human. When the cAMP and calcium levels were compared in the isolated cardiomyocytes, milrinone (>10 μM) caused greater elevations in intracellular cAMP and calcium than cilostazol (Shakur et al., 2002). This indicates that milrinone acts as an effective PDE3 inhibitor, causing PDE3 activity to be inhibited, and resulting in elevated cAMP levels.

In summary, the most updated clinical and laboratory evidence demonstrating the significant effect of PDE3 inhibitors (milrinone, amrinone and cilostazol) regarding cardioprotection has been reviewed. The putative molecular and cellular mechanisms underlying the modulatory effects of PDE3 inhibitors were discussed in view of contractile function and myocyte viability in the normal and ischemic hearts. Various protein kinase and the second messenger, cAMP and cGMP, are some of the key role players forming the signal transduction cascades that result in the positive inotropic and pro-survival phenotype counteracting myocardial I/R injury.

As mentioned in the introduction, ischemic heart disease remains the number one cause of mortality throughout the world and has increased in many developing countries as a result in changes in economic, nutrition and environment. For that reason, the emphasis must be placed in the vital investigations in this dynamic area which will lead to well-designed and controlled clinical trials, ultimately implicating the use of PDE3 inhibitors as a primary drug treatment in promoting cell survival under critical situations, such as ischemic heart attack.

2.11 Clinical applications of PDE3 inhibitors in heart failure

When looking at the clinical application of PDE3 inhibitors, it is evident that the focus has been on treating patients with heart failure through positive inotropy. Alongside this, the hemodynamic and vasodilatory effects of these inhibitors are also to be taken into consideration. Yet, with the above mentioned purposes, the clinical trials of PDE3 inhibitors have been found inadequate.

In 1989, a study published by DiBianco and co-workers, found increased incidence of ventricular arrhythmias in patients with heart failure when they were treated with milrinone and/or digoxin (DiBianco et al., 1989). A double-blind study, known as the Prospective Randomized Milrinone Survival Evaluation (PROMISE), involved the treatment of 1,088 patients associated with New York Heart Association (NYHA) class III with milrinone therapy (40 mg oral daily) (Packer et al., 1991). They discovered that the milrinone therapy was responsible for a 28% increase in mortality when compared to the placebo. More interestingly, the milrinone treated patients had a higher incidence of hypotension and syncope.

Even though PDE3 inhibitors are known to be regularly used in the treatment of heart failure, in light of the above contradictory findings, a determination of whether the risks of treatment outweigh the benefits remains to be established.

CHAPTER 3 - Materials and Methods

3.1 Animals

This study made use of male Wistar rats weighing between 210 - 350g. The animals were held in the Health and Biomedical Sciences Animal Facility at the University of Stellenbosch, Tygerberg Campus until the time of experimentation. The animals had free access to food, standard lab chow, and water. On the day of experimentation, the rats were anaesthetized by means of an intra-peritoneal injection of pentobarbital.

3.2 Ethical approval

For the use of all animals, an approval by the Ethics committee of the University of Stellenbosch (Faculty of Health Sciences) was given, protocol number SU–ACUM13-00018. The project conformed to the conditions described in the “Revised South African National Standard for the care and use of Animals for Scientific purposes” (South African Bureau of Standards, SANS 10386, 2008).

3.3 Chemicals and drugs

The chemicals and drugs were purchased from the following establishments and are indicated in the table below. All necessary chemicals are stored at -4 ˚C and -20˚C.

Table 3.1: All agents and suppliers

Sigma Aldrich, St Louis, MO, USA	**HEPES**
	Sodium pyruvate
	Sodium chloride (NaCl)
	2, 3-Butanedione Monoxime (BDM)
	Sodium hydrosulphite
	Laminin entactin
	Creatine
	Taurine
	Carnitine
	M199 with Hank's salts
	Blebbistatin
	Protease IV
	2–deoxy-glucose
	JC-1-stain
	LDH Assay Kit
Roche	Bovine serum albumin (BSA) fraction V
	BSA fatty acid free (FAF)
Worthington	Collagenase Type II
Eli Lilly	Insulin
BD Biosciences	Laminin/entactin (L/E)
	Penicillin/Streptomycin (pen/strep)
Merck	Sodium pentobarbital
	D-glucose
	Calcium chloride ($CaCl_2$)
	Potassium chloride (KCl)
	Disodium hydrogen phosphate (Na_2HPO_4)
	Sodium dihydrogen phosphate (NaH_2PO_4)
	Magnesium sulphate ($MgSO_4$)
	Soduim chloride (NaCl)
Donated by Dr. R Salie (Tocris)	Formoterol

3.4 Pre-treatment of 96-well plates with cell culture adhesive

In all experiments black, clear bottom 96-well tissue culture plates (SIGMA) were used. The cell culture adhesive, laminin entactin (L/E), was prepared to a final concentration of 100μg/ml in phosphate buffered saline (PBS) with 1.2mM Ca^{2+}. The plates were then coated by adding 5μl or 7μl in the centre of the each well. Thereafter, the 96-well plates were placed in an incubator (5% CO_2 and 37˚C) overnight. On the day of the experiment, each well was washed with 100μl/well of PBS containing 1.2mM $CaCl_2$ and allowed to dry in the laminar flow hood. Plating of the cardiomyocytes could then commence.

3.5 Isolation of Adult Rat Ventricular Cardiomyocytes

The Langendorff perfusion system was assembled in a laminar Flow Cabinet and thoroughly rinsed with sterile ethanol and left to dry. The cardiomyocyte isolation technique was based on a protocol published by Fischer et al. (1991). All the necessary isolation buffers (appendix I) were prepared and sterilised in advance and were continuously gassed with 5% CO_2 and 95% O_2 before and/or during usage. The rats were anesthetized with 0.3ml/mg of sodium pentobarbital. This was followed by sterilization of the rat in 70% ethanol and dissection took place inside a laminar flow hood. The heart was excised and arrested in an ice cold PBS buffer (Buffer A) containing 0.5mM Ca^{2+}. Buffer A contained in mM: KCl 6; Na_2HPO_4 1; NaH_2PO_4 0.2; $MgSO_4$ 1.4; NaCl 128; HEPES 10; D-glucose 11, sodium pyruvate and insulin 100mIU.

The aorta of the heart was then mounted on the cannula of a Langendorff apparatus with buffer A gassed with 5% CO_2 and 95% O_2 at 37°C. Thereafter retrograde perfusion took place with a constant flow (~9ml/min) of calcium free buffer A through the coronary arteries to get rid of any excess blood. After 5 minutes, heart digestion commenced by switching the perfusion from buffer A to buffer B. The latter contained 0.5% BSA fraction V, BSA (FAF), 440U/ml collagenase Type II, 0.2mg/ml protease IV and 18.0mM BDM added to buffer A.

The first 10ml of the digestion buffer was discarded and the remaining buffer was then recirculated through the heart. 40μl $CaCl_2$ of a 100mM stock (final $[Ca^{2+}]$ = ~ 0.1mM) was added 10 minutes and 20 minutes after recirculation (final $[Ca^{2+}]$ = ~ 0.2mM). The perfusion continued until the heart appeared soft, swollen and soapy (25-35 minutes).

After the digestion procedure was completed, the ventricles were cut off and placed into a petri dish which contained 15ml of buffer D (10ml buffer C [buffer A, 0.5% BSA (FFA), 0.5% BSA fraction V, 9.0mM BDM] + 5ml buffer B). The cardiomyocytes were then separated from the ventricular tissue by gentle agitation in buffer D with a tweezer until complete cell dissociation.

The suspension containing the cells was filtered through a nylon filter (200-200µm) into a 50ml Falcon conical tube. The cardiomyoctes were left to sediment for approximately 10 minutes and were thereafter centrifuged at 50xg for 1 minute.

The supernatant, containing dead cells was removed from the tube until there was just a visible pellet of cells left at the bottom. The next step was to raise the calcium gradually to a final concentration of 1.2mM, while separating live rod cells from dead round cells. The cell pellet was resuspended in 6ml buffer E1 (0.6mM $CaCl_2$), transferred to a new 15ml conical tube and left for 10 minutes to sediment by gravity. The supernatant was then removed, resuspended in 6ml buffer E2 (0.9mM $CaCl_2$), transferred to a new tube and sedimented for 7 minutes. Lastly, the supernatant was yet again removed and the pellet was placed in 6ml buffer E3 (1.2mM $CaCl_2$) in a fresh tube and left to sediment for 5 minutes.

After the calcium raising was completed, the final pellet was transferred to 6ml buffer F (buffer E3 containing 10µM blebbistatin (BBS) and 1.2mM $CaCl_2$). Finally, the cardiomyocytes were ready to be assessed for viability. The materials for the different buffers and stains/indicators are listed in the Appendix I.

3.6 Assessment of cardiomyocyte viability

The viability of the cells was assessed by removing 150µl from the 6ml cell suspension (buffer F) and delivered by pipette into the two chambers of the improved Nubauer haemocytometer (SIGMA). Thereafter, the haemocytometer was placed under the light microscope (Nikon) to count the cells. The cardiomyocytes were divided into two categories, where rod shaped cells were considered viable and round shaped cells considered non-viable. The percentage of each category was then determined and only cultures with >70% viability was used. A density of 2500-3000 total cells/well at 100µl/well was calculated and resuspended in 12ml culture buffer per 96-well plate. The cells were then seeded in the clear bottom 96-well plates that were pre-coated with L/E and cultured overnight at 5%CO_2 and 37˚C.

3.7. Experimental conditions tested

Twelve experimental groups were tested in all objectives during simulated ischemia/reperfusion, including (1) Ischemic control, (2) PDE 3 inhibitor, (3) Insulin, (4) Insulin + PDE 3 inhibitor, (5) Dobutamine, (6) Dobutamine + PDE 3 inhibitor, (7) Formoterol, (8) Formoterol + PDE 3 inhibitor, (9) Isoproterenol, (10) Isoproterenol + PDE 3 inhibitor (11) BRL and (12) BRL + PDE 3 inhibitor.

Milrinone is a known PDE3 inhibitor that was used in this project, while dobutamine was used to activate β1-AR and formoterol to activate β2-AR. Isoproterenol is a non-selective beta-adrenergic agonist (β1/β2). Within each experiment, each of the various conditions and time points were done in duplicate, while all experiments were repeated three times on three different experimental days to generate an n = 4.

3.7.1 Simulated ischemia/reperfusion (SIR)

3.7.1.1 Experimental procedure

The necessary buffers for the experimental day were prepared which include: normoxic buffer and ischemic buffer. Overnight cultured cardiomyocytes were overlayed with 100µl normoxic buffer and incubated for 10 minutes. Thereafter, the normoxic buffer was replaced with fresh normoxic buffer and placed back into the incubator for another 10 minutes. This ‘’washing’’ step was done twice to ensure that all unnecessary debris/dead cells were removed from the cell population in the wells (30min wash period, figure 3.1). Each solution was pipetted at 100µl per well unless specified otherwise.

This was followed by 10 minutes pre-ischemic incubation. During this incubation period, cells were pre-treated with milrinone for 5 and 10 minutes respectively then subjected to 20 minutes simulated ischemia (figure 3.2). Ischemia was induced with 10mM Sodium dithiosulfite (SDT), which inhibits oxidative phosphorylation, and 3mM 2 De-oxy D-glucose (2-DG) that inhibits glycolysis. Formoterol was administered during both stimulated ischemia (per-treatment) and 15 minutes reperfusion (post-treatment) (figure 3.1).

After both the ischemic and staining period, cardiomyocytes underwent a double normoxic wash. JC-1 stain was used to stain the cells and this commenced after 15 minutes reperfusion (figure 3.1/3.2). Once the staining of cells were completed (10-15 minutes), the plate was placed back in the incubator for colour development (30 minutes). In this study there were numerous drugs and PDE inhibitors tested at various time points in the experimental protocol.

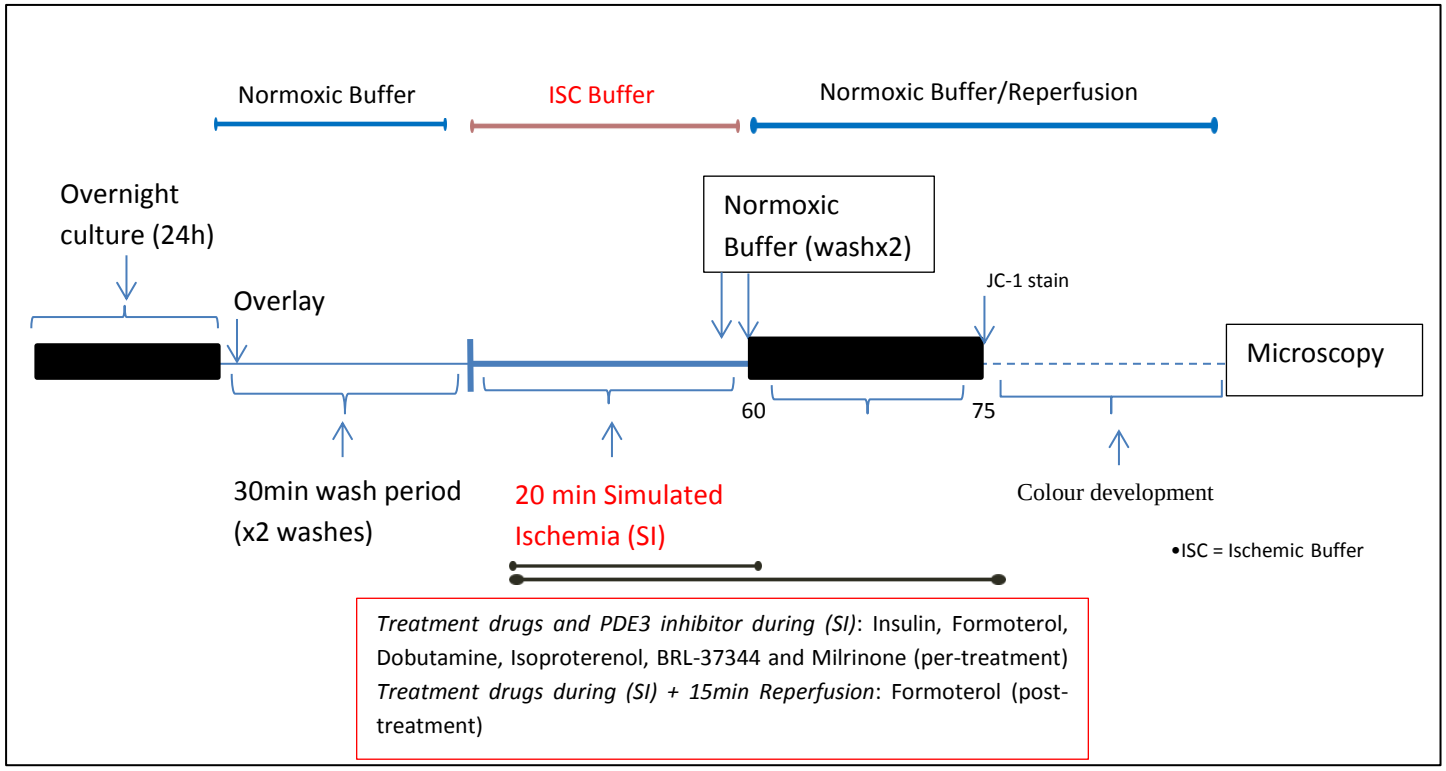

Figure 3.1: Per-and Post-treatment experimental procedure (simulated ischemia/reperfusion)

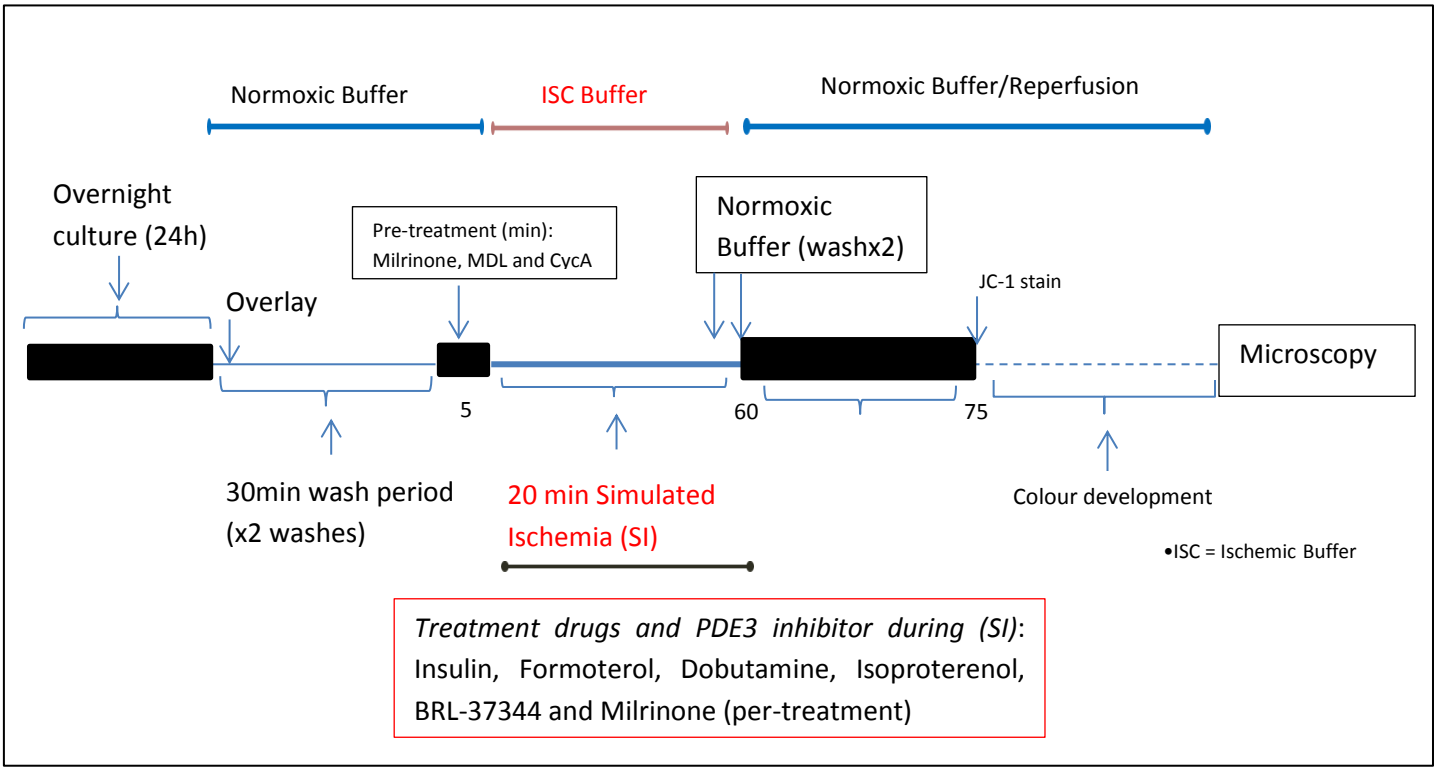

Figure 3.2: Pre-treatment experimental procedure (simulated ischemia/reperfusion)

3.7.1.2 Staining of cardiomyocytes

During the experimental procedure (section 3.7.1.1), JC-1 (apoptosis indicator) staining was done 15 minutes after reperfusion. The staining of cardiomyocytes with JC-1 was done for a period of 10 minutes, followed with double medium wash and stabilized for 30 minutes in the incubator. The fluorescent marker, JC-1, is a mitochondrial membrane probe that expresses a red/orange fluorescence in a healthy mitochondria and green fluorescence in the cytosol when excited. However, during ischemia, the cell becomes apoptotic, causing the mitochondria to lose its membrane potential difference, resulting in a green fluorescence in the cytosol and no red fluorescence (Perry et al., 2011).

3.8 Assay Kits

3.8.1 Cell viability: LDH-Cytotoxicity Activity Assay Kit

Lactate dehydrogenase (LDH) activity was measured and used as a marker for cell death. A LDH-Cytotoxicity Assay Kit II was used to determine the LDH-Cytotoxicity in the cardiomyocytes at the end of ischemia. Cell death or the cytotoxicity was evaluated by the quantification of plasma membrane damage. Lactate dehydrogenase (LDH), which is a stable enzyme, was released into the cell culture medium when the plasma membranes underwent damage

The absorbance of all controls and samples were measured using the FLOUstar Ω plate reader. The plate reader was set for different reaction times and the absorbance was measured at 450nm and 650nm. The reaction time could be decreased or increased depending on the colour development (abcam, LDH-Cytotoxicity Assay Kit II manual, 2012).

3.9 Data collection and fluorescence analysis

The analysis of the cell experiments was done by using the fluorescence microscope and capturing individual images of cells that represent one field view per replicate (well) at 10× objectives. The program NIS-Elements Basic Research was used to capture the photos. Three cell parameters were investigated: cell length, cell morphology (rod or round shaped) and the red/green fluorescence intensity (mean). The Fiji analysis software (*Image J*) was used to determine these parameters for each image.

For cell length, each rod and round shaped cell was individually measured using the straight, segmented line function within the software. The cell morphology was determined by using the cell counter and counting the total number of rod shaped (live) and round shaped (dead) cells. The fluorescence ratio of each well was calculated as the mean red over the mean green, expressed as R/G ratio.

3.10 Statistical analysis

All the data obtained in the experiments are expressed as the mean ± standard error of the mean (SEM) n = (3-5)*3. Graphpad Prism (version 5.0) was used for graph analysis. The one-way or two-way analysis of variance (ANOVA) was used to determine variance between different groups followed by multiple comparisons with Bronferonni post hoc test. Probability values less than <0.05 ($p < 0.05$) was considered statistically significant.

CHAPTER 4 – Results

The results in this study were obtained by investigating the following three parameters at the end of simulated ischemia/reperfusion, in 24h cultured primary cardiomyocyte populations:

- Cell hypercontracture (*cell length*)
- Cell mitochondrial membrane potential (*Early apoptosis, red/green fluorescence intensity ratio (R/G))*
- Cell viability *(% viable cells*)

Ischemia/Reperfusion experimental data with isolated cardiomyocytes:

4.1 Effect of insulin on survival of cardiomyocytes.

In order to investigate the possible role of PDE3 in insulin-mediated protection, a cardioprotective concentration of insulin first had to be determined. Three concentrations (0.3mIU, 3mIU and 30mIU) of insulin were tested and the following images (figure 4.1) are representative of those conditions.

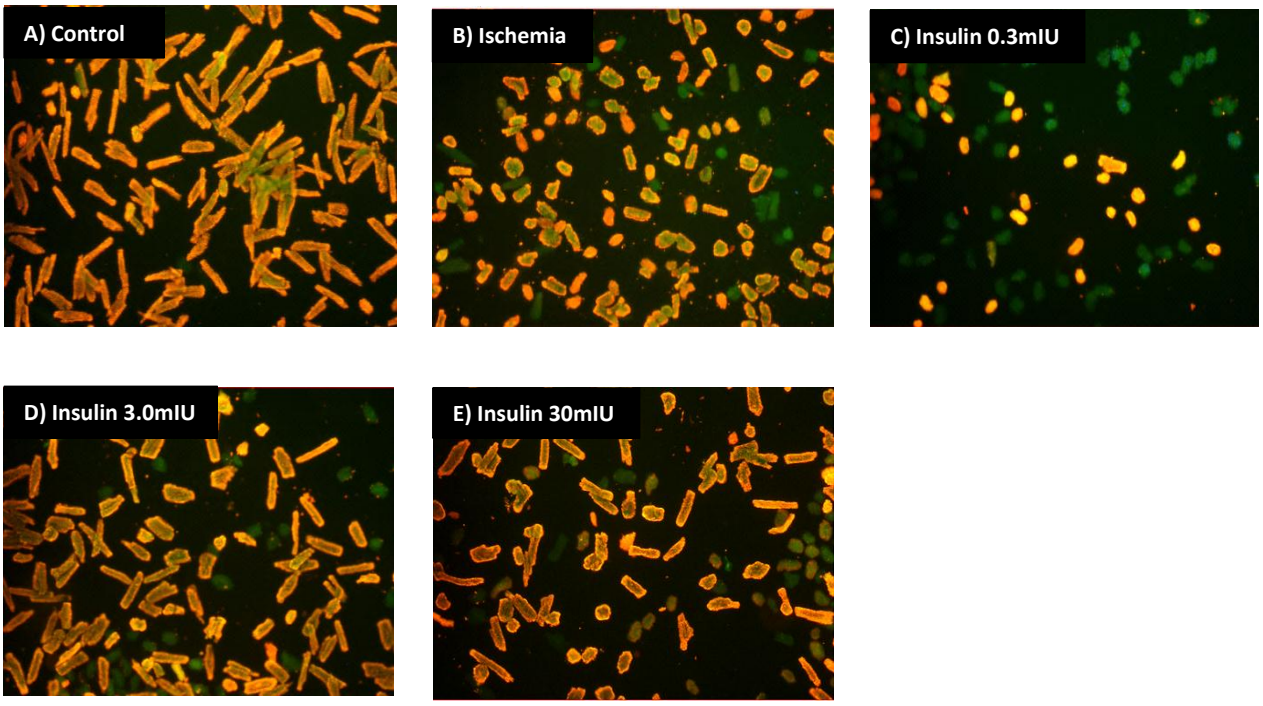

Figure 4.1: Fluorescence images of ventricular cardiomyocytes subjected to 30 minutes simulated ischemia with and without insulin, followed by 60 minutes reperfusion and JC-1 staining. ***A)*** Normoxic control cells with no drug treatment, ***B)*** 30 minutes chemical ischemia ***C)*** Ischemia with 0.3mIU insulin ***D)*** Ischemia with 3mIU insulin and ***E)*** Ischemia with 30mIU insulin treatment.

The microscope images (Figure 4.1A-E, n = 3) were analysed to determine the degree of cardiomyocyte hypercontracture (cell length, figure 4.2) and percentage viable cardiomyocytes (number of rod shaped cells over total cells x 100, figure 4.3) in response to insulin administration during 30 minutes simulated ischemia. Figure 4.1A, the normoxic control group is an illustration of a healthy cell population, predominantly rod shaped with a high red fluorescence intensity and low green fluorescence intensity. This treatment group yielded 92.2 ± 1.7% rod shaped cells on average (figure 4.3) with an average cell length of 94.72 ± 3.64um (figure 4.2).

30 Minutes chemical ischemia followed by 60 min reperfusion (see section 3.7.1 for experimental procedure) led to a great insult on the cells with morphology changes from rod to round shaped cells (hypercontracture), indicating cell death (figure 4.1B). At the end of ischemia/reperfusion, the length of cardiomyocytes was 45.67 ± 3.01um, indicating hypercontracture, which was associated with a loss in mitochondrial membrane potential as can be seen by the decrease in red fluorescence intensity (figure 4.1B).

During the ischemic phase, the cardiomyocytes were treated with three different insulin concentrations. Cell hypercontracture and viability did not increase significantly with the administration of 0.3mIU insulin (figure 4.1C), when compared to the ischemic control (cell length: 55.40 ± 4.51um vs 45.67 ± 3.01um, figure 4.2; cell viability: 33.37 ± 10.0% vs 13.05 ± 3.09%, figure 4.3). With an increase in insulin concentration to 3mIU (figure 1D), there was a significant increase ($p < 0.01$) in the percentage rod shaped cell population (42.35 ± 11.07% vs 13.05 ± 3.09%) and cell length (69.02 ± 6.26um vs 45.67 ± 3.01um, $p< 0.01$) compared to the ischemia control. The highest insulin concentration, 30mIU, also seems to increase the rod shaped cells significantly (40.57 ± 6.73% vs 13.05 ± 3.09%, $p < 0.05$, figure 4.3) with no effect on hypercontracture. Thus exerting a protective effect, but not to the same extend as the 3mIU insulin concentration (figure 4.1E).

Based on these results, insulin treatment at a concentration of 3mIU, improves both cell contracture and cell viability during 30 minutes chemical ischemia. No insulin effects were noticed under normoxic conditions. Thus, 3mIU insulin was selected to be used in experiments done for the remainder of the study.

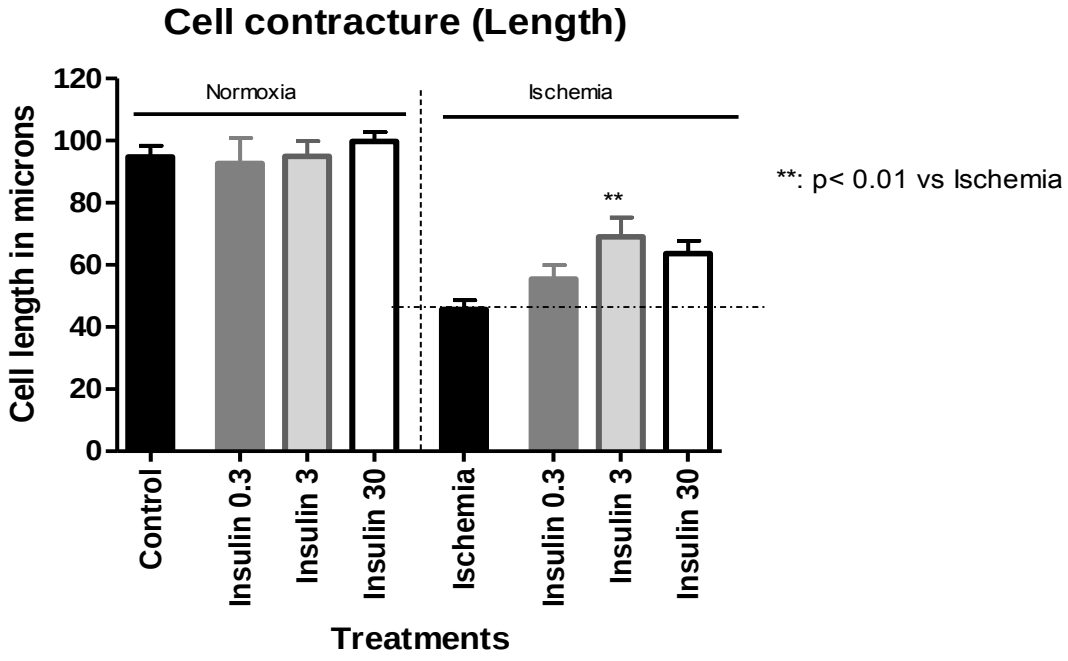

Figure 4.2: Effect of insulin (0.3mIU, 3mIU, and 30mIU) on cell length (hypercontracture) in micrometre during normoxic and after ischemia/reperfusion. Chemical ischemia was induced for 30 minutes, followed by 60 minutes reperfusion. Sample size n = 3. **P < 0.01 versus ischemia, all other symbols in this section represents comparisons between groups excluding ischemia control, example #.

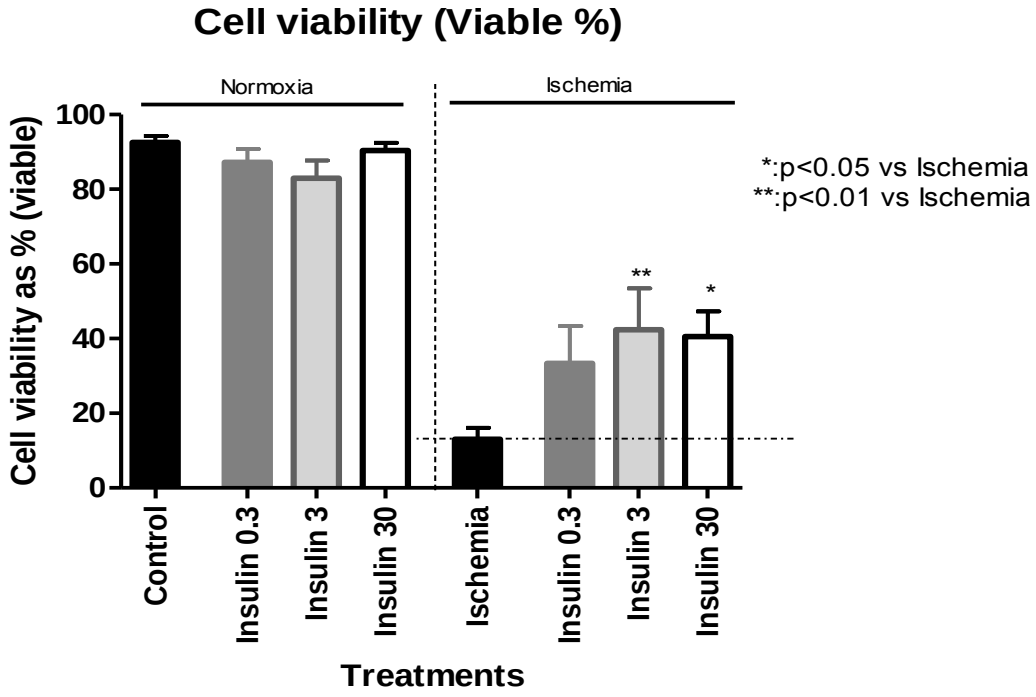

***Figure 4.3*:** Effect of insulin (0.3mIU, 3mIU, and 30mIU) on the cell viability during normoxic and ischemic conditions. Chemical ischemia induced for 30 minutes, followed by 60 minutes reperfusion. Sample size n = 3. *P < 0.05 and **P < 0.01 versus ischemia.

4.2 Effect of milrinone (PDE3 inhibitor) on insulin-mediated cardiomyocyte protection.

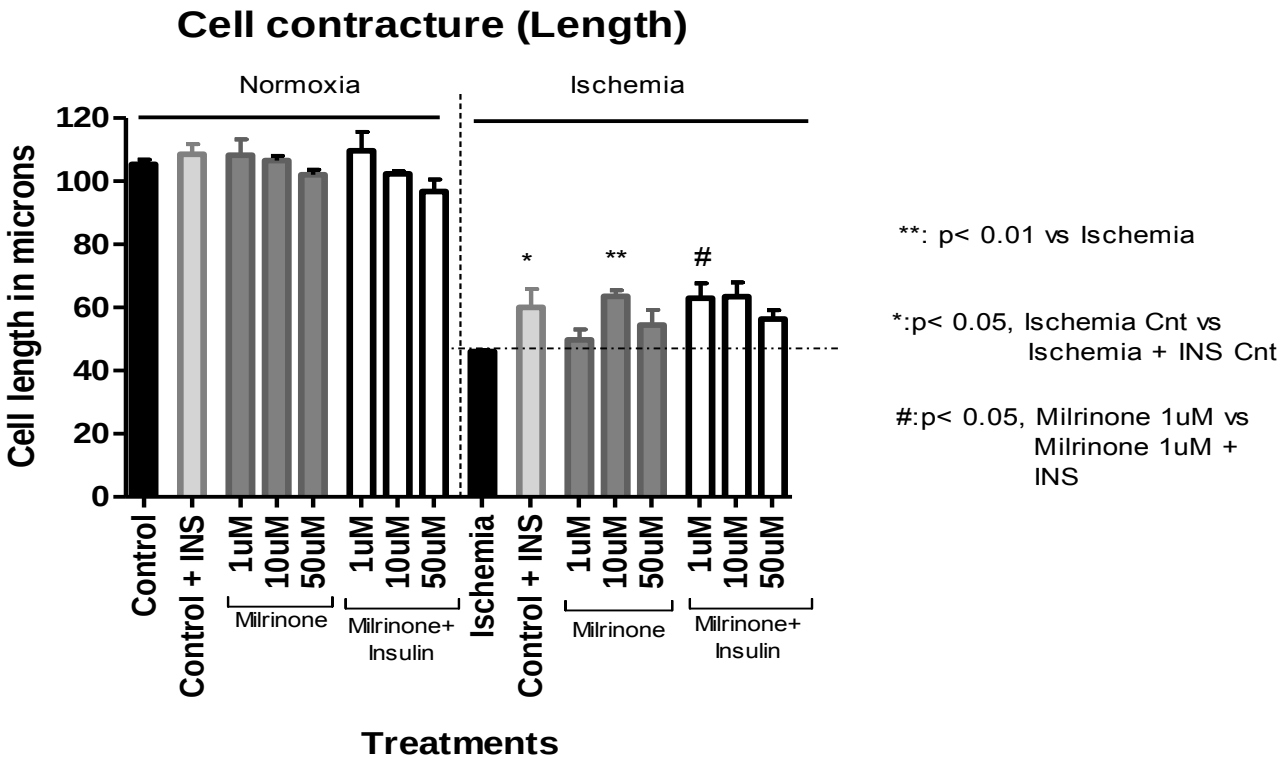

***Figure 4.4*:** Milrinone was tested at 1μM, 10μM and 50μM concentrations during ischemia in the presence and absence of insulin (3mIU) administration to determine cell length (hypercontracture) in micrometre. Milrinone and insulin were tested under both normoxic and ischemic conditions. Chemical ischemia was simulated for 20 minutes followed by 60 min reperfusion and cell contractures were investigated. Sample size n = 3. *P < 0.05 and **P < 0.01 versus ischemia.

From figure 4.4, it is evident that no significant differences were found under normoxic conditions between the milrinone concentrations and milrinone concentrations + insulin, when compared to the normoxic control. Conversely, both 10μM milrinone (63.55 ± 1.87um) and the 3mIU insulin control (60.05 ± 5.75um) administered during ischemia show significantly ($p \leq 0.05$) less cell hypercontracture, when compared to ischemia (46.09 ± 0.45um). There is also a significant difference ($p \leq 0.05$) between groups treated during ischemia with 1μM milrinone (49.71 ± 3.35um) compared to 1μM milrinone + 3mIU Insulin (62.94 ± 4.72um). Yet protection was probably provided by the 3mIU insulin when combined with 1uM milrinone, given that 3mIU insulin could exert protection on its own.

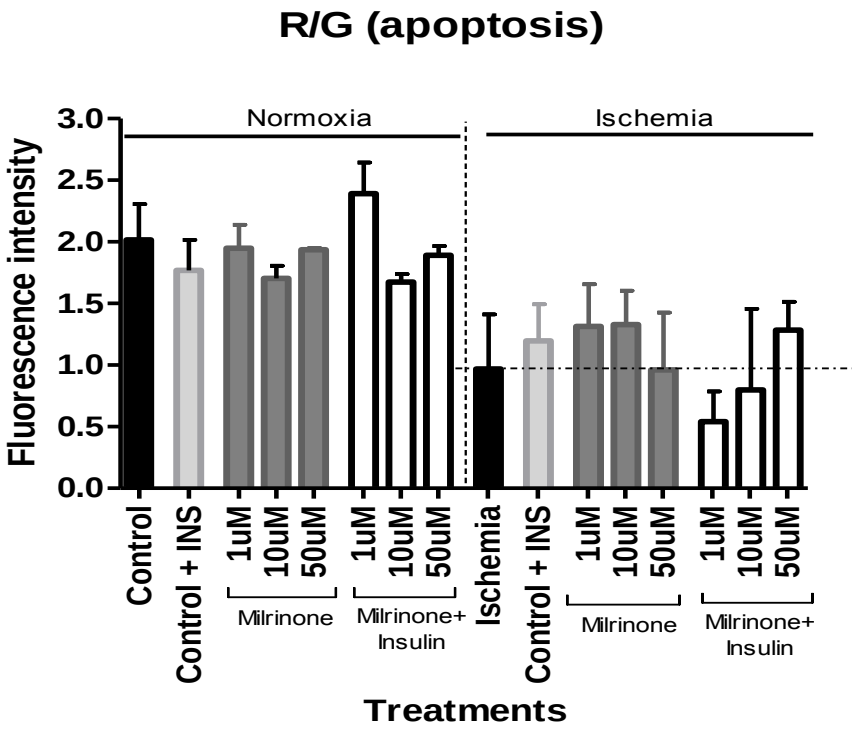

Figure 4.5: Milrinone was administered at 1µM, 10µM and 50µM concentrations during ischemia in the presence and absence of 3mIU insulin, and mitochondrial membrane potential (R/G) investigated at the end of reperfusion. Chemical ischemia was simulated for 20 minutes, followed by 60 minutes reperfusion. Sample size n =3.

There were no differences in R/G ratio found between the control groups during normoxia (figure 4.5). Drugs were administered during 20 minutes ischemia, but no significant differences were seen amongst the PDE3 inhibitor (milrinone) concentrations. It is evident that errors bars are quite large which might be the reason why no significant differences are seen amongst the groups.

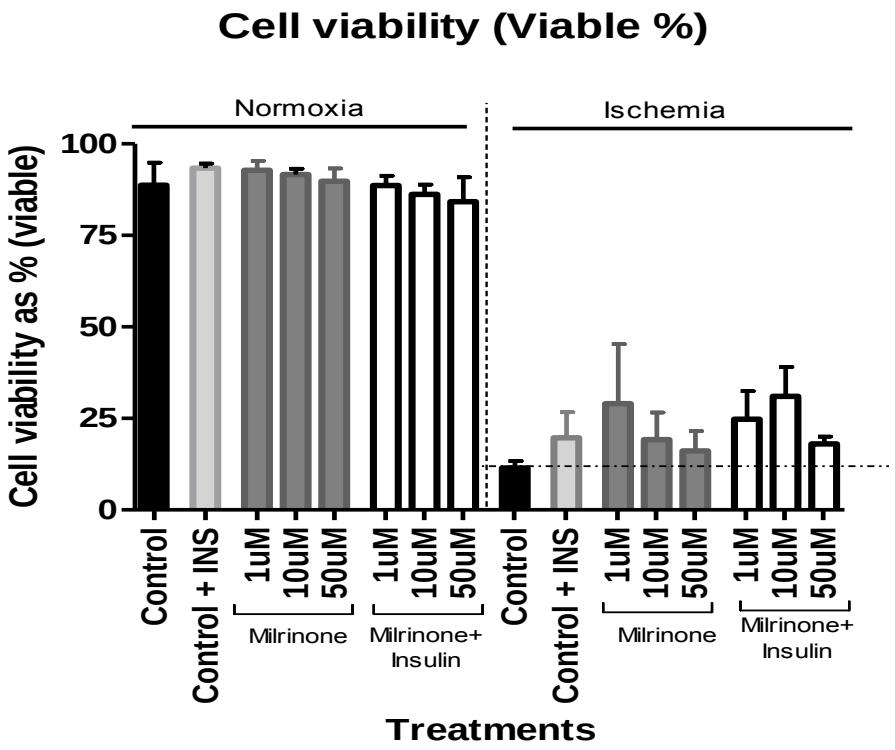

Figure 4.6: Milrinone was administered at 1μM, 10μM and 50μM concentrations during ischemia in the presence and absence of insulin (3mIU) to determine cell viability. The same experimental protocol and time points were followed as seen from the previous two parameters (Chemical ischemia was simulated for 20 minutes, followed by 60 minutes reperfusion). Sample size n = 3. P < 0.0001: normoxia control versus ischemia control.

Investigating each treatment group during normoxia, the data shows no significant differences in cell viability percentage (figure 4.6). After sustained ischemia, the cell viability decreased significantly ($p \leq 0.0001$) from 88.74 ± 6.11 (control normoxia) to 11.33 ± 1.94 (control ischemia), but there were no differences amongst any of the ischemia groups.

4.3 The effect of the PDE3 Inhibitor Milrinone and the non-selective β-AR agonist (Isoproterenol) on Insulin mediated protection.

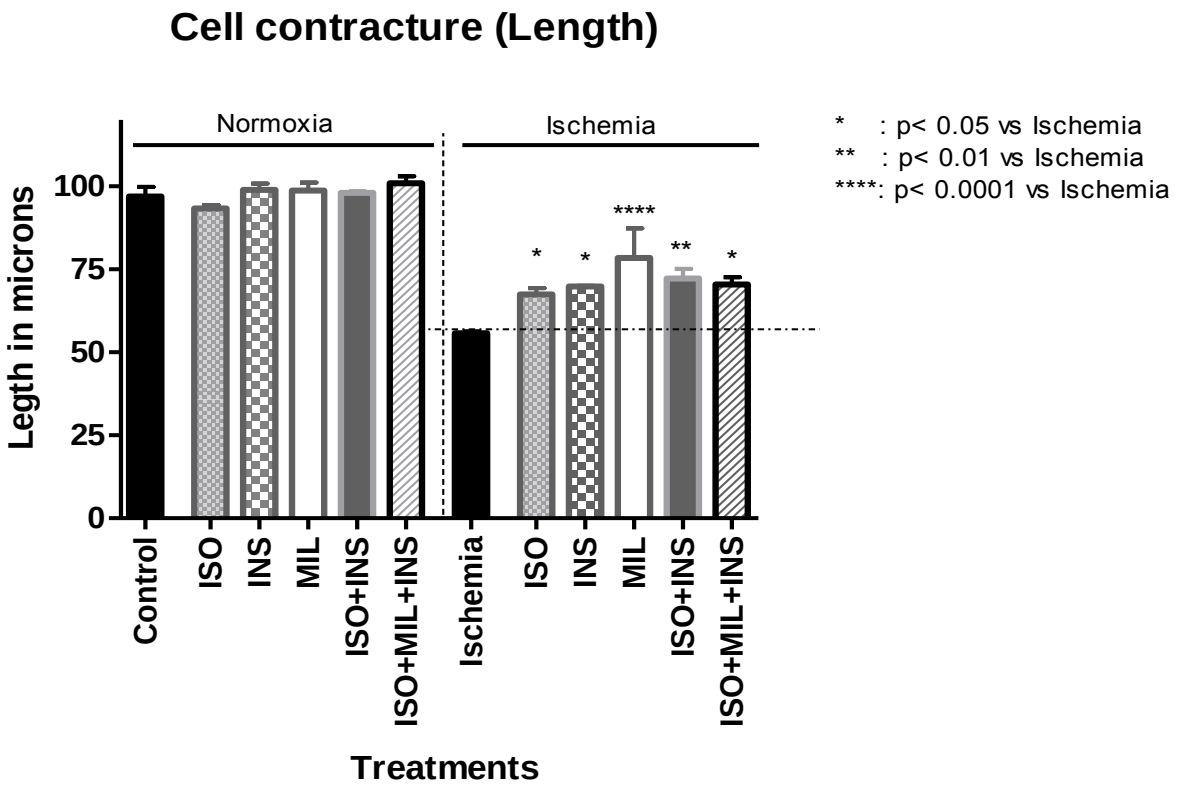

Figure 4.7: Cell length (hypercontracture) data of cardiomyocytes when treated with isoproterenol (100nM), insulin (3mIU) and milrinone (10μM) during ischemia. Chemical ischemia was simulated for 20 minutes, followed by 60 minutes reperfusion. Agents were given independently and in combination. Sample size n = 4. *P < 0.05; **P < 0.01; ****P< 0.0001 versus ischemia.

There were no differences in cell length between the treatment groups when compared to the control group during normoxia (figure 4.7). Cell length increased significantly when cardiomyocytes were treated during ischemia with 100nM isoproterenol (67.40 ± 1.90um, $p < 0.05$), 3mIU insulin (69.84 ± 0.21um, $p < 0.05$), and 10μM milrinone (78.40 ± 8.98um $p < 0.0001$), compared to the ischemic control (55.7 ± 0.54um). When isoproterenol and insulin were given in combination a significant difference ($p < 0.01$) was also noticed when compared to the ischemic control (72.22 ± 2.88 vs 55.7 ± 0.54um). This was also evident for the treatment group, isoproterenol + insulin + milrinone (70.40 ± 2.22 vs 55.7 ± 0.54um).

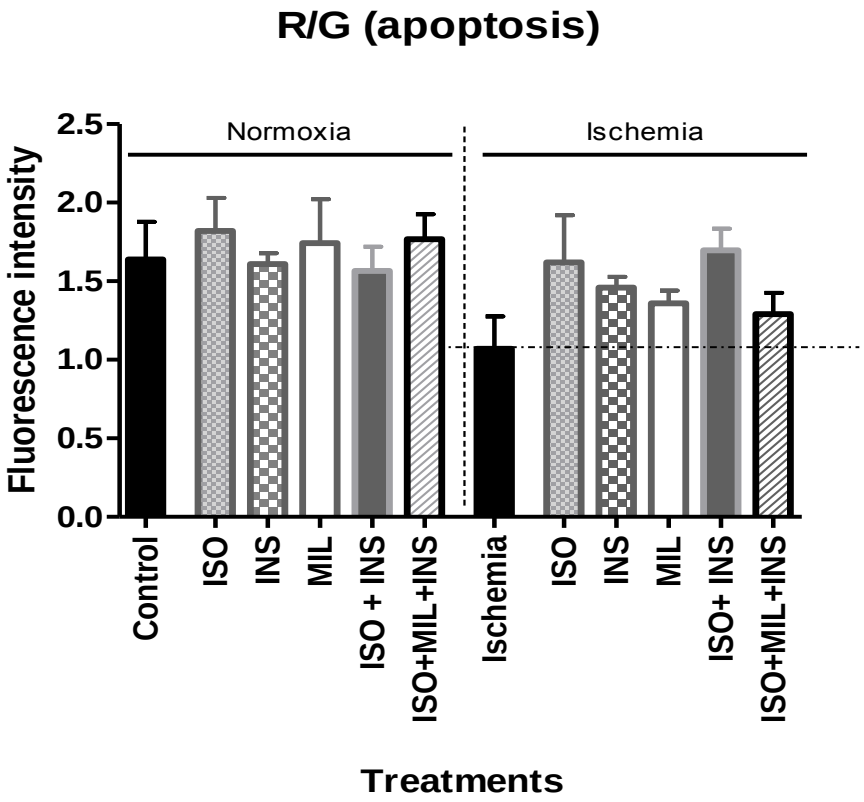

Figure 4.8: Cell viability as a measure of JC-1 red over green fluorescence (R/G) ratio. Isoproterenol (100nM), insulin (3mIU) and milrinone (10μM) were administered during normoxic and ischemic conditions respectively. Chemical ischemia was simulated for 20 minutes. Sample size n = 4.

There were no difference between the control group and the various treatment groups during normoxia or ischemia (figure 4.8). Thus, not ischemia or any of the drugs had an effect on R/G.

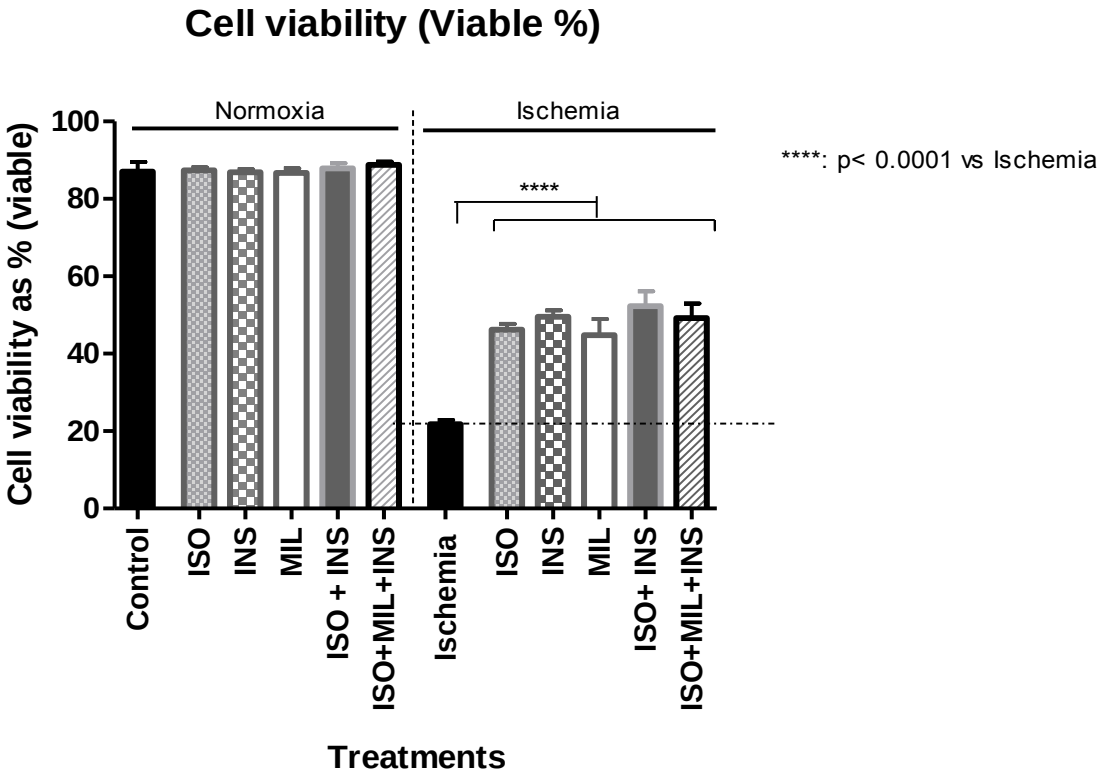

Figure 4.9: Percentage viable cardiomyocytes after ischemia/reperfusion, when treated with isoproterenol (100nM), insulin (3IU) and milrinone (10µM) during ischemia. As from the two previous parameters, identical experimental procedure was followed. Chemical ischemia was simulated for 20 minutes, followed by 60 minutes reperfusion. Sample size n =4. ****P< 0.0001 versus ischemia.

No significant differences were observed between the control group and the treatment groups under normoxic conditions (figure 4.9). Cell viability was however significantly (p< 0.0001) improved during ischemic treatment with isoproterenol (46.17 ± 1.57%), insulin (49.55 ± 1.68%) and milrinone (44.79 ± 4.19%), compared to the ischemia control (21.8 ± 1.06%) group. This was also the case with the combination treatments, isoproterenol + insulin (52.27 ± 3.83%) and isoproterenol + insulin + milrinone (49.22 ± 3.74%) when compared to the ischemia control group (21.8 ± 1.06%).

4.4 The effect of β2-AR agonist, Formoterol, as ischemic Per-treatment and Post-treatment.

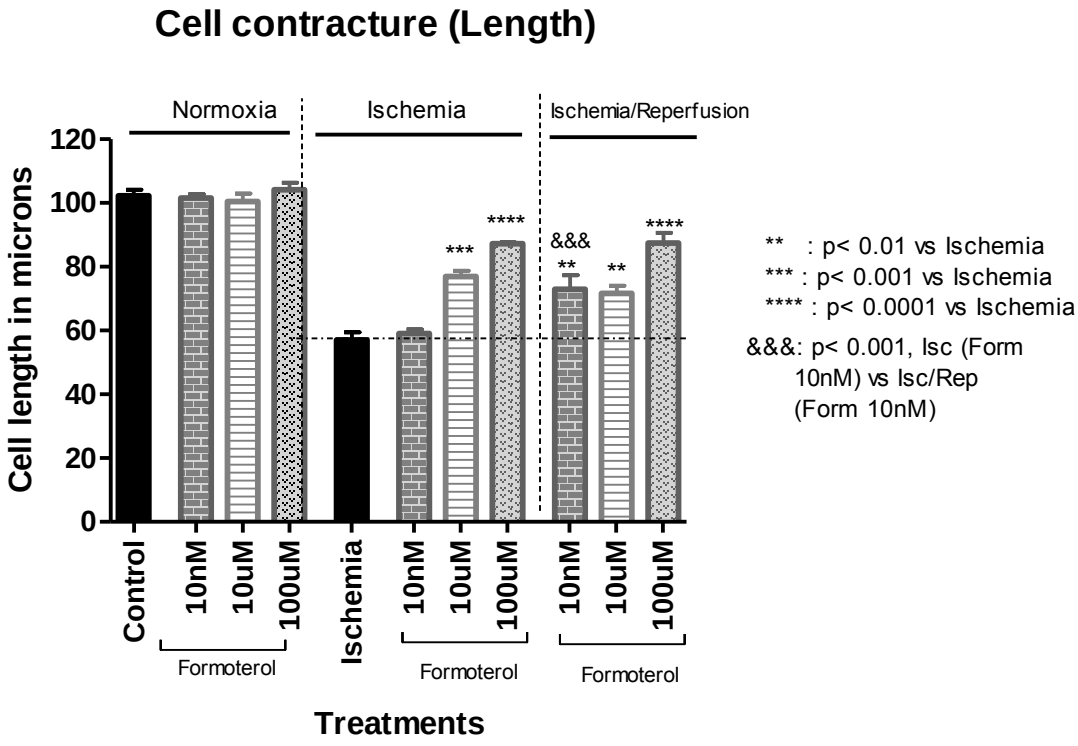

Figure 4.10: Cell length (hypercontracture), comparing formoterol treatment during 20 minutes ischemia with treatment during 20 minutes ischemia and the first 15 minutes of reperfusion. Three formoterol concentrations (10nM, 10μM and 100μM) were tested. Sample size n = 4. **P < 0.01; ***P< 0.001; ****P< 0.0001 versus ischemia.

In figure 4.10, no differences were noted between the treatment groups compared to the control group during normoxia. When the agonist is applied during ischemia, a significant decrease occurs in cell hypercontracture, indicated by an increase in cell length, with 10μM formoterol (76.98 ± 1.77um, p< 0.001) and 100μM formoterol (87.20 ± 0.47um, p< 0.0001) versus control ischemia (52.87 ± 1.13um). A significant increase in cell length was induced with all three formoterol concentrations (10nM: 72.97 ± 4.34um and 10μM: 71.68 ± 2.34um; p< 0.01, 100μM: 87.41 ± 3.19um; p< 0.001), when administered during ischemia/reperfusion, compared to the ischemia control group (61.19 ± 3.89um).

The 10nM formoterol also significantly decreased the cell contracture when it was administered during ischemia/reperfusion compared to administration only during ischemia (&&&: 72.97 ± 4.34um vs 59.06 ± 1.33um, p< 0.001). The overall effect of formoterol seems to be protective in both per-treatment and per- + post-treatment regarding cell length during ischemia.

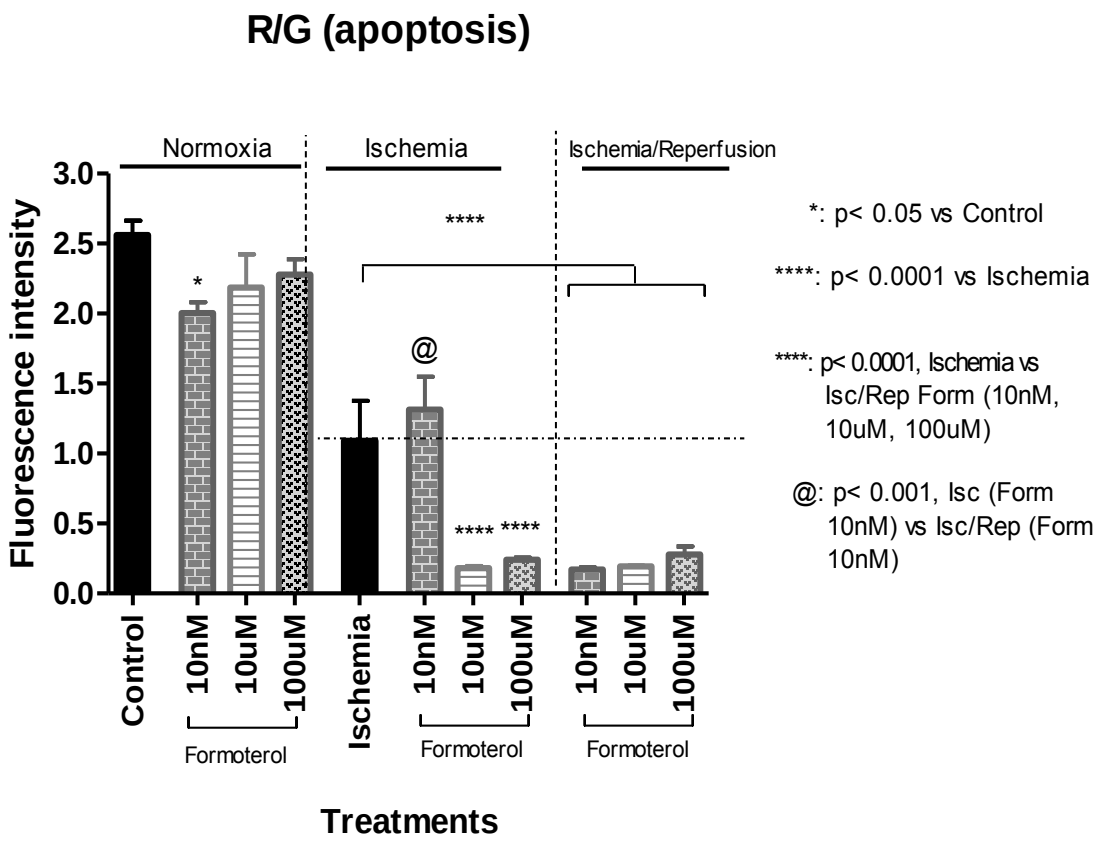

Figure 4.11: JC-1 fluorescence intensity (early apoptosis) measured as ratio of red over green (R/G). Formoterol (10nM, 10µM and 100µM) treatment during 20 minutes ischemia compared to continues treatment during 20 minutes stimulated ischemia and the first 15 minutes of reperfusion. Sample size n = 4. *P < 0.05 versus control;***P< 0.001; ****P< 0.0001 versus ischemia.

The fluorescence intensity has decreased in the formoterol 10nM vs control group during normoxia (2.00 ± 0.10 vs 2.56 ± 0.07). Figure 4.11 shows a marked decrease (p< 0.0001) in the R/G with 10µM (0.18 ± 0.01) and 100µM (0.27 ± 0.02) formoterol treatment during 20 minutes ischemia groups compared to the ischemia control (1.75 ± 0.25). A similar reduction (p< 0.0001) in R/G was also found with all three formoterol concentrations (10nM, 10µM and 100µM) given during ischemia/reperfusion compared to the ischemia control group. The results also indicate a significant (p< 0.001) decrease in intensity when 10nM formoterol was administered during ischemia/reperfusion (0.17 ± 0.01) compared to only during ischemia (@: 1.31 ± 0.23).

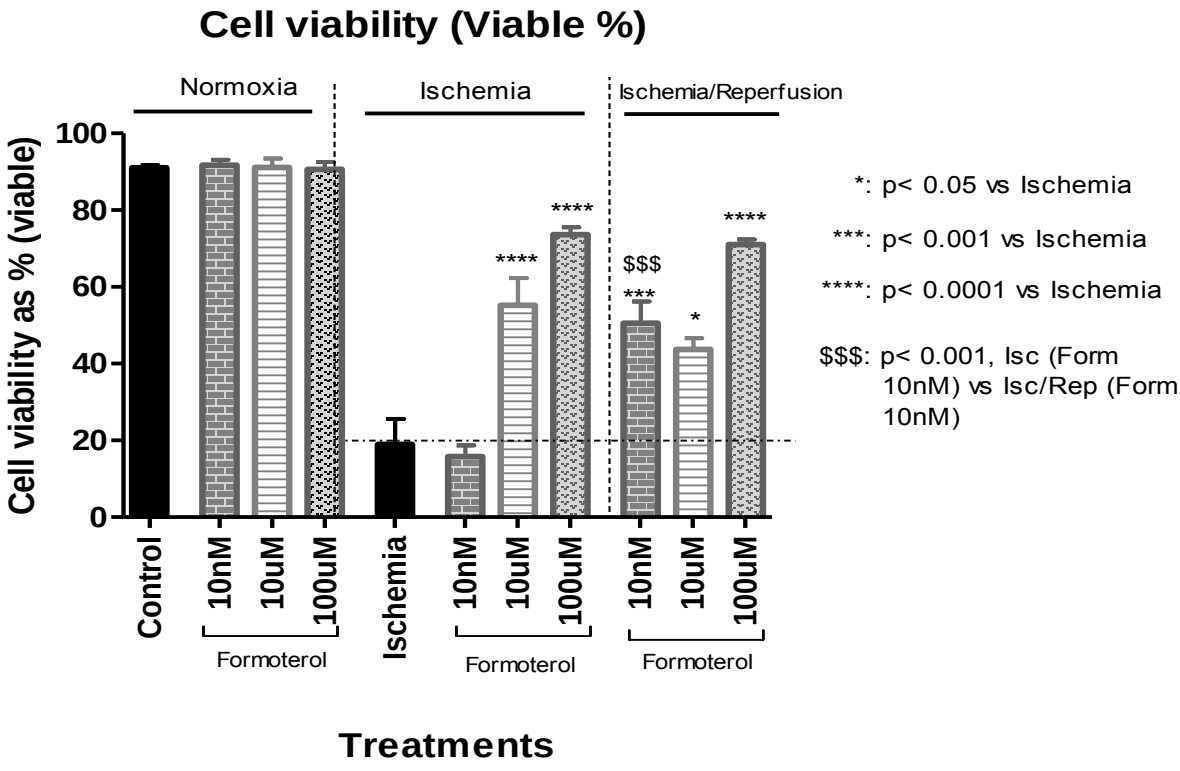

Figure 4.12: Cell viability after formoterol treatment during 20 minutes simulated ischemia and the first 15 minutes of reperfusion, compared to ischemia treatment only. Three formoterol concentrations (10nM, 10uM and 100uM) were tested to determine optimal β-AR activation dosage. Sample size n =4. *P < 0.05; ***P< 0.001; ****P< 0.0001 versus ischemia.

No significant decrease in cell viability was associated with any of the formoterol (10nM, 10μM and 100μM) concentrations tested during normoxia (figure 4.12). The percentage cardiomyocyte viability was significantly (p< 0.0001) increased when 10μM (55.17 ± 7.12%) and 100μM formoterol (73.6 ± 1.90%) were administered during 20 minutes chemical ischemia (9.5 ± 0.83%). All three formoterol treatment concentrations applied during ischemia/reperfusion significantly improved cell viability when compared to the ischemia control group (10nM: 50.57 ± 5.61%; 10μM: 43.72 ± 2.93% and 100μM: 71.0 ± 1.38% vs ischemia: 28.42 ± 12%). Interestingly, when formoterol 10nM was given during ischemia and reperfusion ($$$: 50.57 ± 5.61%), the viability percentage was notably higher (p< 0.001) compared to treatment just in ischemia (15.75 ± 2.98%). A dose of 10uM was chosen to elicit protection via β2-AR for further experiments.

4.5 The influence of PDE3 Inhibitor (Milrinone) on the effect of β1-AR agonist (Dobutamine) and β2-AR agonist (Formoterol) during ischemia.

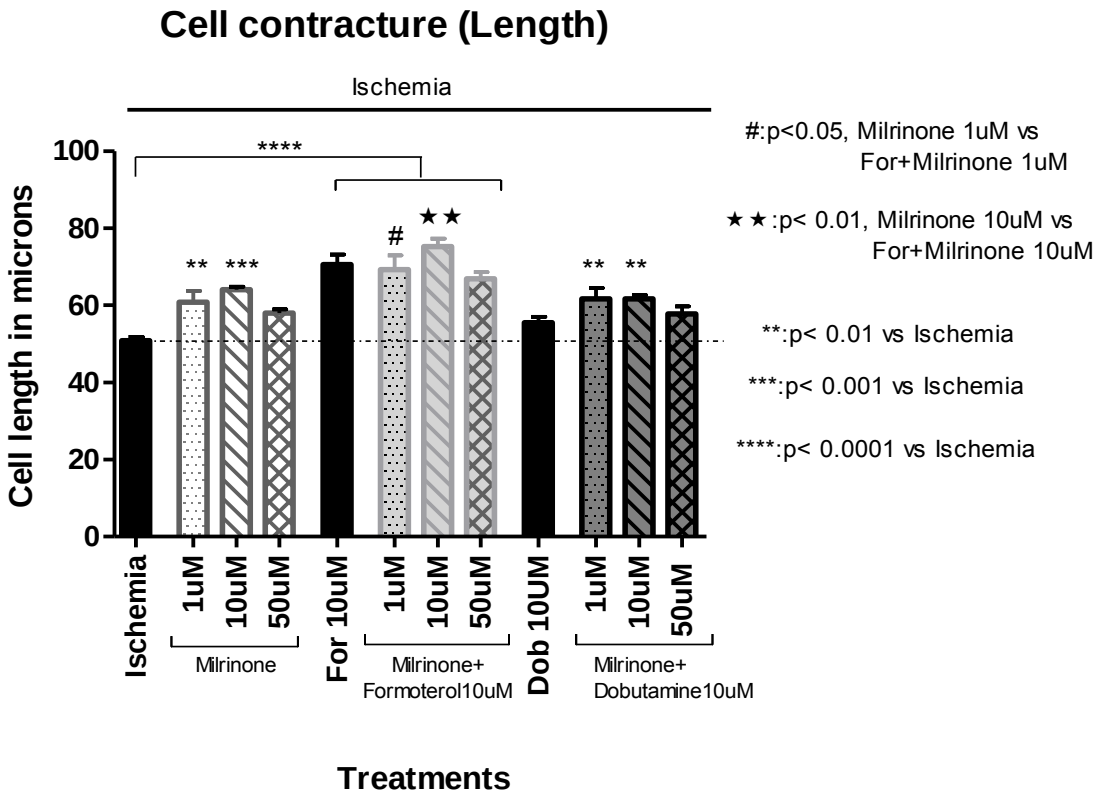

Figure 4.13: The effect of Milrinone (1μM, 10μM and 50μM), dobutamine (10μM) and formoterol (10μM) administered during 20 minutes simulated ischemia and 60 minutes reperfusion on cell length (hypercontracture). Milrinone was given with and without β2 agonist (formoterol) and β1 agonist (dobutamine) as a treatment in the respective groups. Sample size n = 4. *P < 0.05; **P < 0.01; ***P< 0.001; ****P< 0.0001 versus ischemia.

A dobutamine concentration of 10μM was used in this study, which was decided based on the literature (Wang et al. 2013) and dose response tests done in our lab. In figure 4.13 hypercontracture was significantly (p< 0.0001) decreased in cardiomyocytes treated during ischemia with milrinone at concentrations of 1μM (60.83 ± 2.92um) and 10μM (64.06 ± 0.83um), compared to the ischemia control group (50.76 ± 1.02um). With the administration of 10μM formoterol in conjunction with all three milrinone concentrations, the cell hypercontracture was also decreased significantly compared to ischemia (1μM: 69.28 ± 2.59um; 10μM: 75.32 ± 2.03um and 50μM: 66.94 ± 1.64um vs ischemia: 50.76 ± 1.02um). However, the formoterol + milrinone treated groups did not promote more protection than the formoterol group (70.61 ± 2.21) alone. Regarding the β1 agonist, treatment groups dobutamine + milrinone 1μM (61.68 ± 2.87um) and dobutamine + milrinone 10μM (61.69 ± 0.89um) showed an increase in cell length compared to ischemia group (50.76 ± 1.02um).

Dobutamine 10μM alone does not elicit protection, while milrinone + dobutamine treatment groups provided no more protection when compared to milrinone concentrations alone. A remarkable finding from the data in figure 4.13 is that as soon as β2-specific agonist formoterol is given as a treatment with milrinone, the cell hypercontracture seems to decrease even more (increase in length) in comparison with milrinone treatment on its own. This is seen with formoterol + milrinone 1μM (#: 69.28 ± 3.70um) versus milrinone 1μM (60.83 ± 2.92um, p< 0.05) and formoterol + milrinone 10μM (★★: 75.32 ± 2.03um) versus milrinone 10μM (64.06 ± 0.83um, p< 0.01) treatment groups.

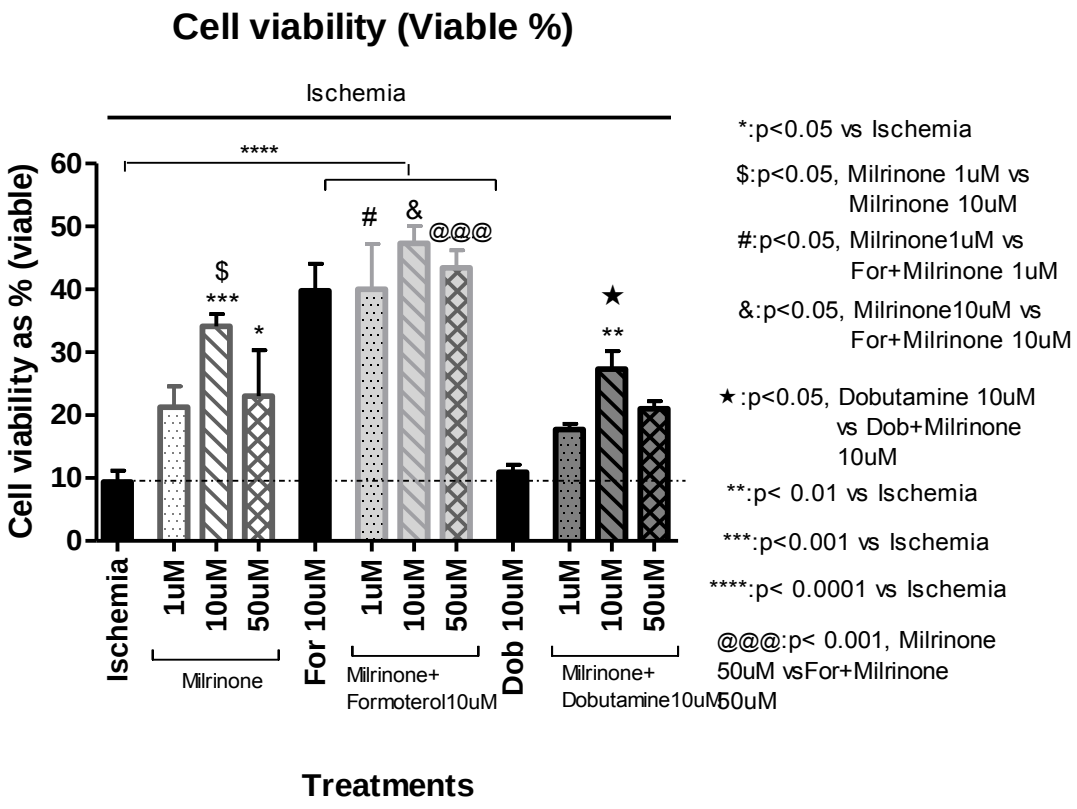

Figure 4.14: Effect of milrinone (1μM, 10μM and 50μM), dobutamine (10μM) and formoterol (10μM) on % cell viability when administered during 20 minutes simulated ischemia. Milrinone was given with and without formoterol and dobutamine as a treatment. Values are expressed as the mean ± SEM. Sample size n = 4. *P < 0.05; **P < 0.01; ***P< 0.001; ****P< 0.0001 versus ischemia.

Complementary to the cell hypercontracture data (figure 4.13), milrinone significantly increased the cell viability (figure 4.14) at concentrations of 10μM (34.12 ± 1.94%, p< 0.001) and 50μM (23.0 ± 7.33%, p< 0.05) compared to the ischemia control group (9.37 ± 1.79%). With the administration of 10μM formoterol in conjunction with the different milrinone concentrations, the cell viability increased much more (p< 0.0001) than the milrinone only treated groups when compared to ischemia (formoterol 10μM 39.77 ± 4.30%; formoterol + 1μM milrinone 40.02 ± 7.21%; formoterol + 10μM milrinone 47.32 ± 2.74% and formoterol + 50μM milrinone 43.4 ± 2.82% vs ischemia: 9.37 ± 1.79%).

With regards to dobutamine, only the 10μM dobutamine + 10μM milrinone treatment group showed any significance increase compared to the ischemia control group (27.32 ± 2.84 vs 9.37 ± 1.79%, p< 0.01).

The cell viability also increased significantly with a higher dosage of the milrinone as seen when comparing differences to ischemia (9.37 ± 1.79%) between 1μM milrinone (21.27 ± 3.30%), and 10μM milrinone ($: 34.12 ± 1.94%, p< 0.01). From this we can state that an increase in the milrinone dosage caused an increase in cell viability during ischemia.

As established from figure 4.14, when formoterol is given as a treatment with milrinone, the cell viability in this instance seems to increase even more in comparison with milrinone treatment on its own. This is seen with formoterol + milrinone 1μM (#: 40.02 ± 7.21%) versus 1μM milrinone: 21.27 ± 3.30%, p< 0.05) and formoterol + milrinone 10μM (&: 47.32 ± 2.74%) versus 10μM milrinone: 34.12 ± 1.94%, p< 0.05) treatment groups. The overall result of inhibiting the PDE3 enzyme can thus be thought to have a protective effect during ischemia considering the increase in cell contracture and viability.

4.6 Pre- and Per-treatment of β1-AR agonist, Dobutamine and β2-AR agonist, Formoterol in combination with PDE 3 Inhibitor, Milrinone.

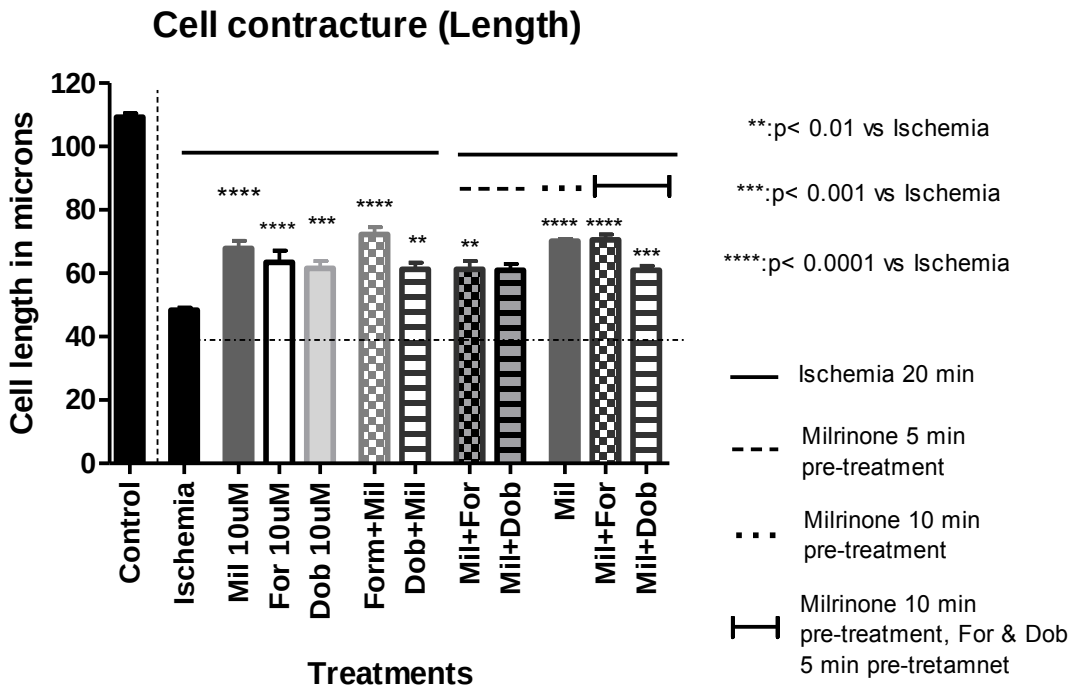

Figure 4.15: Cell length (hypercontracture) data of Pre- and Per-treatment with agents during chemical ischemia. 10μM Milrinone was given 5 and 10 minutes before chemical ischemia (pre-treatment) respectively and during 20 minutes sustained chemical ischemia (per treatment). 10μM Dobutamine and 10μM formoterol were both given 5 minutes before ischemia (pre-treatment) and thereafter during 20 minutes sustained chemical ischemia (per treatment). Sample size n = 4. *P < 0.05; **P < 0.01; ***P< 0.001; ****P< 0.0001 versus ischemia.

In figure 4.15 the cell hypercontracture significantly decreased (increased cell length) compared to control ischemia (48.38 ± 0.78um) when 10μM milrinone (67.82 ± 2.42um, p< 0.0001), 10μM formoterol (63.44 ± 3.74um, p< 0.0001) and 10μM dobutamine (61.57 ± 2.27um, p< 0.001) were individually administered during chemical ischemia. A similar significant increase in cell length was seen when milrinone was applied in combination with formoterol (72.26 ± 2.25um, p< 0.0001) and dobutamine (61.20 ± 2.19um, p< 0.01) compared to the ischemic control group (48.38 ± 0.78um). When milrinone was given 5 minutes before sustained ischemia, only the milrinone + formoterol treatment showed any significant difference (61.29 ± 2.54um vs ischemia: 48.38 ± 0.78um, p< 0.01).

10 Minutes pre-treatment with milrinone (70.16 ± 0.68um) did not decrease cell hypercontracture any more than milrinone during ischemia only (67.82 ± 2.42um, p< 0.0001), when compared to ischemia control group (48.38 ± 0.78).

Finally, when the cardiomyocytes were pre-treated with milrinone for 10 minutes plus 5 minutes pre-treatment of each agonist, a significant increase in cell length was also observed (milrinone + formoterol: 70.52 ± 1.70um; p< 0.0001 and milrinone + dobutamine: 60.92 ± 1.44um; p< 0.001) versus ischemia (48.38 ± 0.78um), but no further protection was seen when compared to the formoterol and dobutamine treatment groups on their own.

From the above, it can be noticed that none of the two pre-treatment time points had any effect in increasing the cell length any further compared to per-treatment during sustained ischemia.

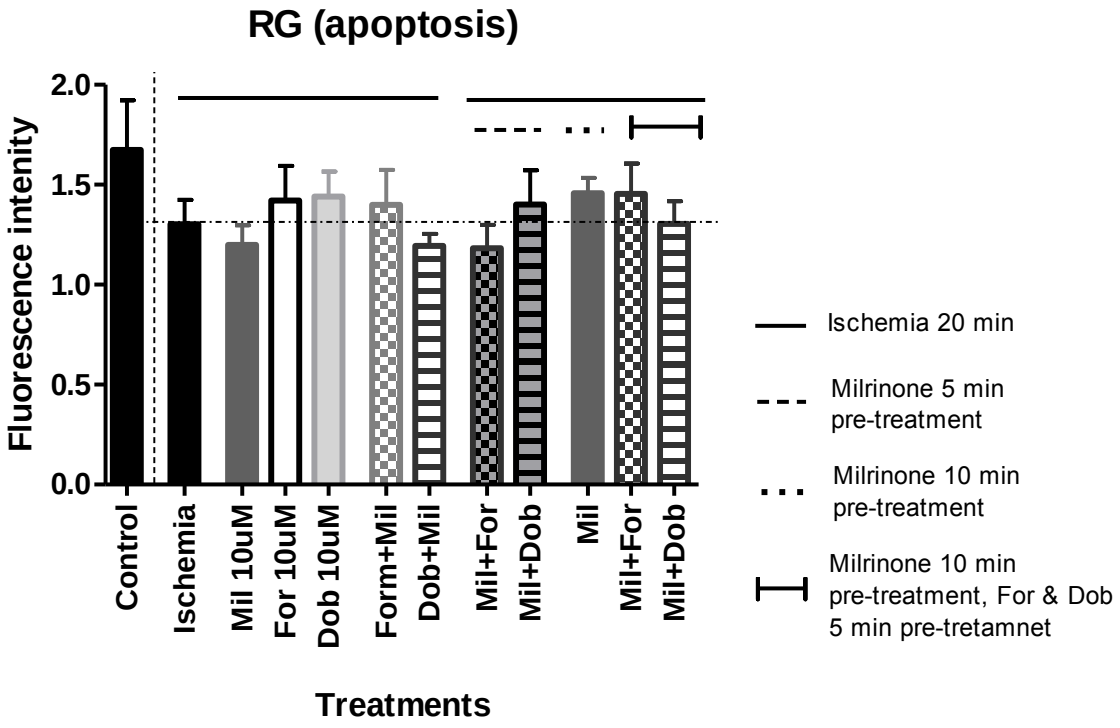

Figure 4.16: Mitochondrial membrane potential (R/G) data of Pre- and Per-treatment of agents during chemical ischemia. 10uM Milrinone was given 5 and 10 minutes before chemical ischemia (pre-treatment) respectively and during 20 minutes sustained chemical ischemia (per treatment). 10μM Dobutamine and 10μM formoterol were both given 5 minutes before ischemia (pre-treatment) and thereafter during 20 minutes sustained chemical ischemia (per treatment). Sample size n = 4.

The data shows that there were no significant differences during ischemia amongst the different treatment groups regarding mitochondrial membrane potential. Thus neither ischemia nor any agent had an effect on mitochondrial function.

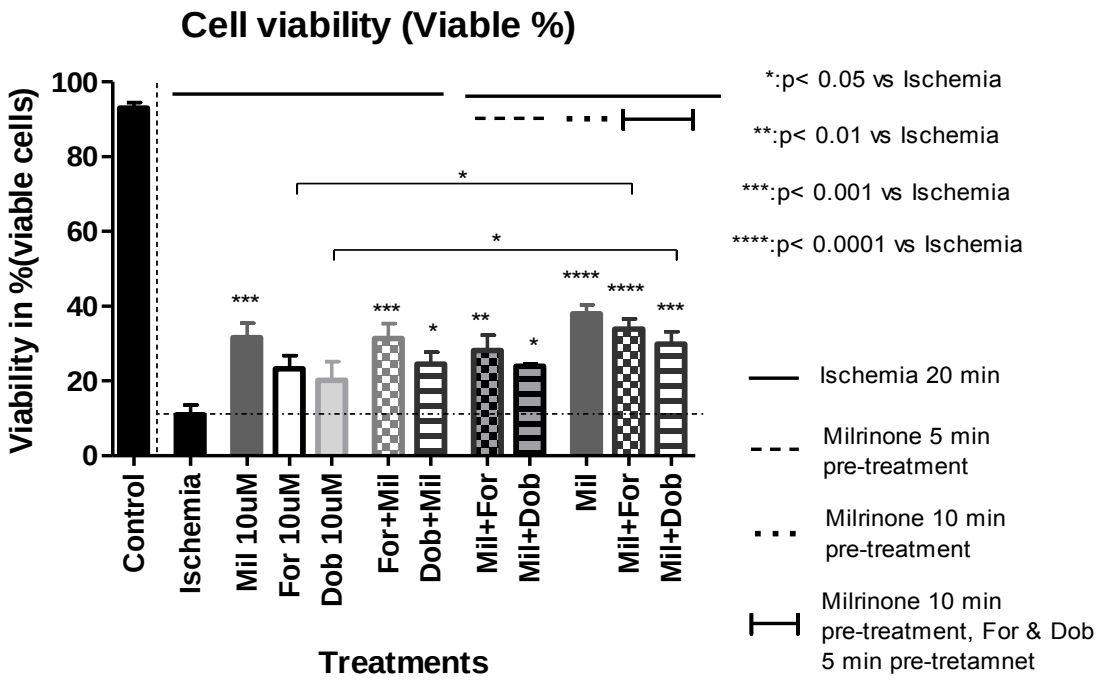

Figure 4.17: Cell viability (viable %) data of Pre- and Per-treatment of agents during chemical ischemia. 10uM Milrinone was given 5 and 10 minutes before chemical ischemia (pre-treatment) respectively and during 20 minutes sustained chemical ischemia (per treatment). 10μM Dobutamine and 10μM formoterol were both given 5 minutes before ischemia (pre-treatment) and thereafter during 20 minutes sustained chemical ischemia (per treatment). Sample size n = 4. *P < 0.05; **P < 0.01; ***P< 0.001; ****P< 0.0001 versus ischemia.

Cell viability increased significantly with 10μM milrinone treatment during ischemia (31.5 ± 3.99% vs ischemia: 10.95 ± 2.55%, p< 0.001). Similar to the protection seen on hypercontracture (figure 4.15), both 10μM formoterol + 10μM milrinone (31.37 ± 3.55%) and 10μM dobutamine + 10μM milrinone (24.45 ± 5.00%) significantly improved viability compared to the ischemia control group (10.95 ± 2.55). This protection was however similar to that of milrinone alone during ischemia.

Five minutes pre-treatment before ischemia and per-treatment with milrinone in combination with β1 (28.15 ± 4.10%, p< 0.05) and β-2 agonist (23.87 ± 0.74%, p< 0.01), also increased the cell viability significantly compared to ischemia (10.95 ± 2.55%).

Between all the treatment groups, pre-treatment of milrinone for 10 minutes was seen to increase the cell viability the most compared to the ischemia control group (37.97 ± 2.29% vs ischemia: 10.95 ± 2.55%, p< 0.0001). With the last two treatment groups, pre-treatment with PDE inhibitor for 10 minutes plus 5 minutes pre-treatment of each agonist, a significant increase in cell contracture was also observed similar to that of cell hypercontracture.

4.7 The effect of per-treatment with PDE3 Inhibitor (Milrinone) in combination with, β1-agonist, β2-agonist and non-selective β-AR agonist.

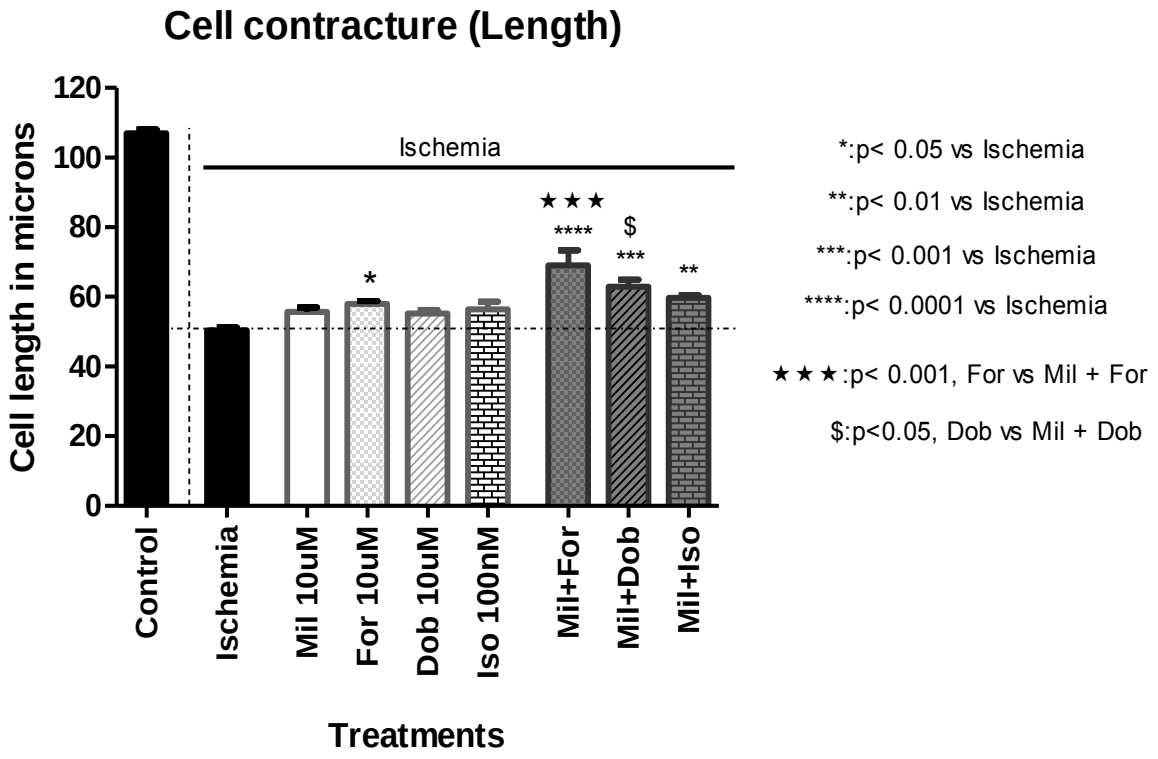

Figure 4.18: Cell length (hypercontracture) data of per-treatment with 10μM milrinone in combination with 10μM dobutamine, 10μM formoterol and 100nM isoproterenol. The different treatments were administered during 20 minutes stimulated chemical ischemia. Sample size n = 4. *P < 0.05; **P < 0.01; ***P< 0.001; ****P< 0.0001 versus ischemia.

From figure 4.18, the 10μM formoterol (57.90 ± 0.89um; p< 0.05) caused a significant decrease in cell contracture (increased length) during simulated ischemia (50.33 ± 0.95um). None of the two other agonists had any significant effect on the cell hypercontracture. Regarding combination treatment, it is evident that as soon as milrinone is given with each agonist respectively, cell contracture decreases in all three treatment groups compared to the ischemia control (50.33 ± 0.95um): milrinone + formoterol (69.01 ± 4.35um; p< 0.0001), milrinone + dobutamine (62.96 ± 1.94um; p< 0.001) and milrinone + isoproterenol (59.76 ± 0.68um; p< 0.01).

There was a significantly better cell hypercontracture recovery also seen with milrinone + formoterol (★★★: 69.01 ± 4.35; p< 0.001) versus formoterol group (57.90 ± 0.89). Decreased cell hypercontracture was also detected when milrinone + dobutamine ($: 62.96 ± 1.94; p< 0.05) was compared to dobutamine group (55.25 ± 0.98) during ischemia.

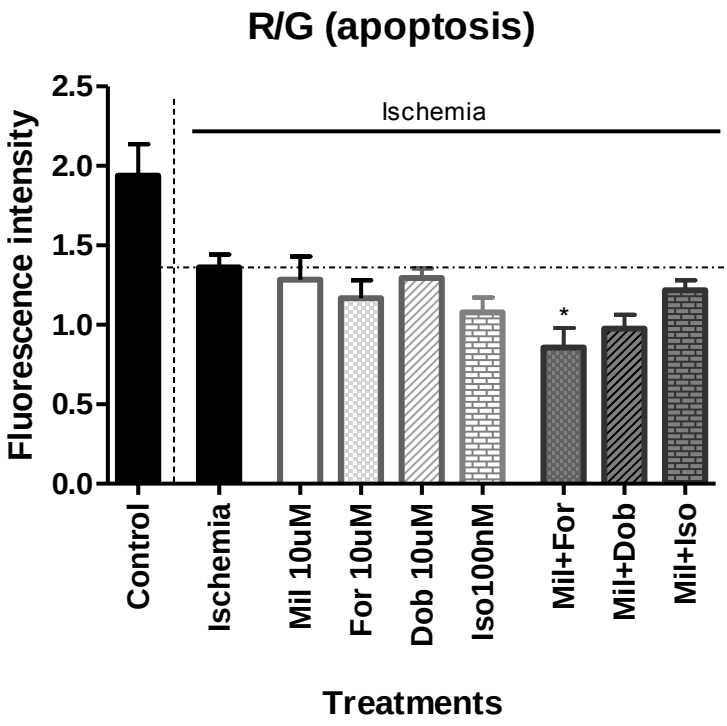

Figure 4.19: Mitochondrial membrane potential (R/G) of per-treatment with 10µM milrinone in combination with, 10µM dobutamine, 10µM formoterol and 100nM isoproterenol. The different treatments were administered during 20 minutes stimulated chemical ischemia. Sample size n = 4. *P < 0.05 versus ischemia.

The only significant difference (figure 4.19) regarding fluorescence intensity is seen with the treatment group of milrinone + formoterol during ischemia (0.85 ± 0.12 vs 1.36 ± 0.08; p< 0.05). This indicates that formoterol might exert a harmful effect on the cardiomyocytes, which is contradictory to the findings in the cell contracture parameter.

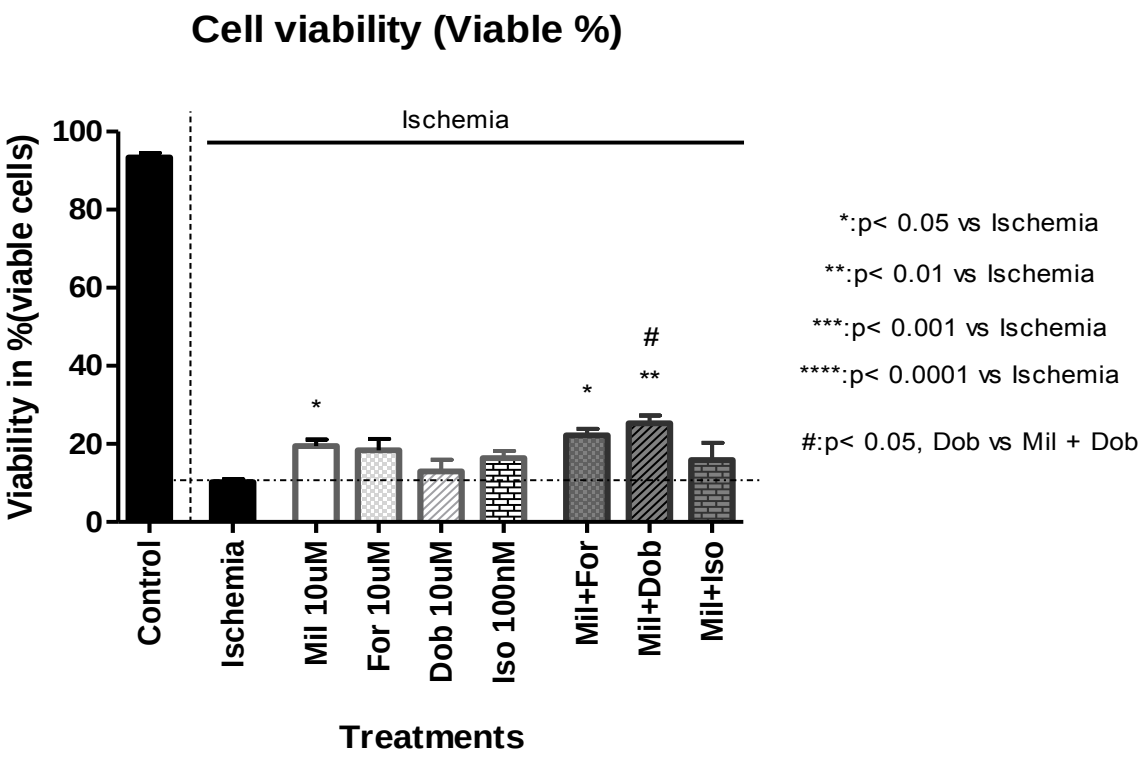

Figure 4.20: Percentage viable cardiomyocytes after per-treatment with 10μM milrinone compared to, or in combination with, 10μM dobutamine, 10μM formoterol and 100nM isoproterenol. The same experimental protocol was followed as seen from the cell contracture parameter. Sample size n = 4. *P < 0.05; **P < 0.01 versus ischemia.

The treatment of 10μM milrinone (19.47 ± 1.68%; p< 0.05) significantly increases cell viability compared to the ischemia control group (10.20 ± 0.79%). As seen in the case of the cell hypercontracture parameter (figure 4.18), cell viability increases significantly when 10μM milrinone is given in combination with 10μM formoterol (22.2 ± 1.69%; p< 0.05) and 10μM dobutamine (25.27 ± 2.04%; p< 0.01). Then again, when these agonists are administered individually, no viability recovery is noted during chemical ischemia. The overall effect of these agents seems to lean towards the protection of the cardiomyocytes during the intervention of 20 minutes simulated ischemia.

4.8 The effect of pre- and per-treatment with PDE3 Inhibitor (Milrinone) in combination with the non-selective β1/2-AR agonist and β3 agonist.

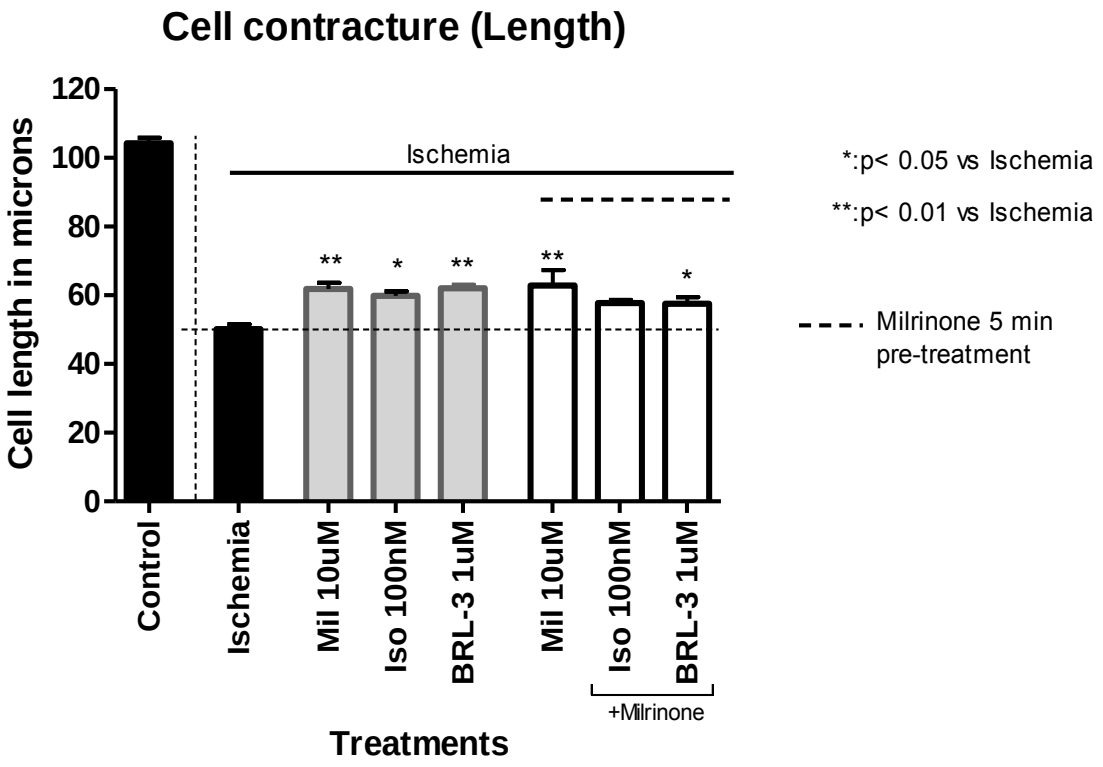

Figure 4.21: Cell length (hypercontracture) data of pre- and per-treatment with milrinone in combination with non-selective B1/2-AR agonist, isoproterenol and β3 agonist, BRL-37344. Milrinone was given 5 minutes prior to (pre-treatment) and during 20 minutes chemical ischemia (per-treatment), whereas agonists were only given during ischemia. Sample size n = 4. *P < 0.05; **P < 0.01 versus ischemia.

From figure 4.21 it is evident that cell hypercontracture significantly decreased (increased length) in both the 10μM milrinone (61.85 ± 1.75um; p< 0.01) and 100nM isoproterenol (59.82 ± 1.29um; p< 0.05) groups compared to the ischemia control group (50.17 ± 1.35um). The same effect was also seen with the 1μM BRL-37344 (62.01 ± 0.98um; p< 0.01) versus the ischemia control group (50.17 ± 1.35um). When the cardiomyocytes were pre-treated with the 10μM milrinone 5 minutes before sustain ischemia, a significant decrease in cell hypercontracture was seen (62.01 ± 4.48um vs 50.17 ± 1.35um; p< 0.01).

The latter however did not enhance the cell length any further than just per-treatment of milrinone during 20 minutes sustained ischemia (62.01 ± 4.48um vs 61.85 ± 1.75um). Lastly, a significant decrease in hypercontracture was also observed with the combination treatment group, 10μM milrinone + 1μM BRL (57.54 ± 1.92um; p< 0.05), compared to ischemia control.

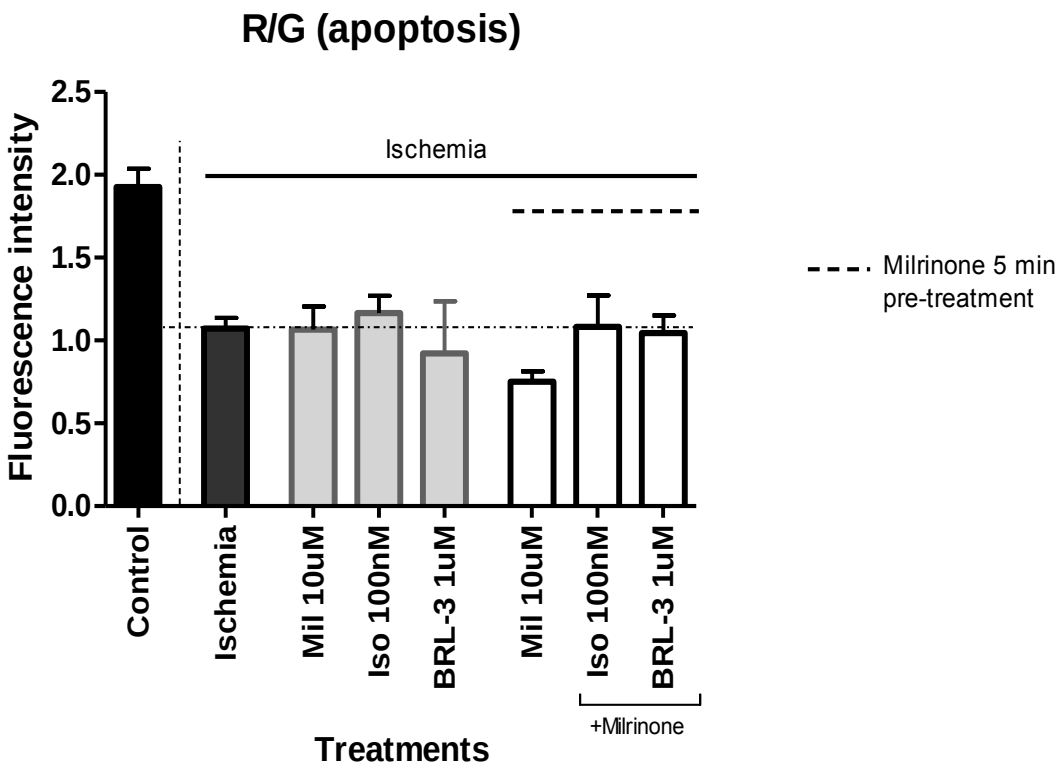

Figure 4.22: Mitochondrial membrane potential of pre- and per-treatment with 10µM milrinone in combination with, 100nM isoproterenol and 1µM BRL-37344. Milrinone was given 5 minutes prior to (pre-treatment) and during 20 minutes chemical ischemia (per-treatment), whereas agonists were only given during ischemia. Sample size n = 4.

The data in figure 4.22 shows that there were no significant differences during ischemia amongst the treatments groups regarding mitochondrial membrane potential.

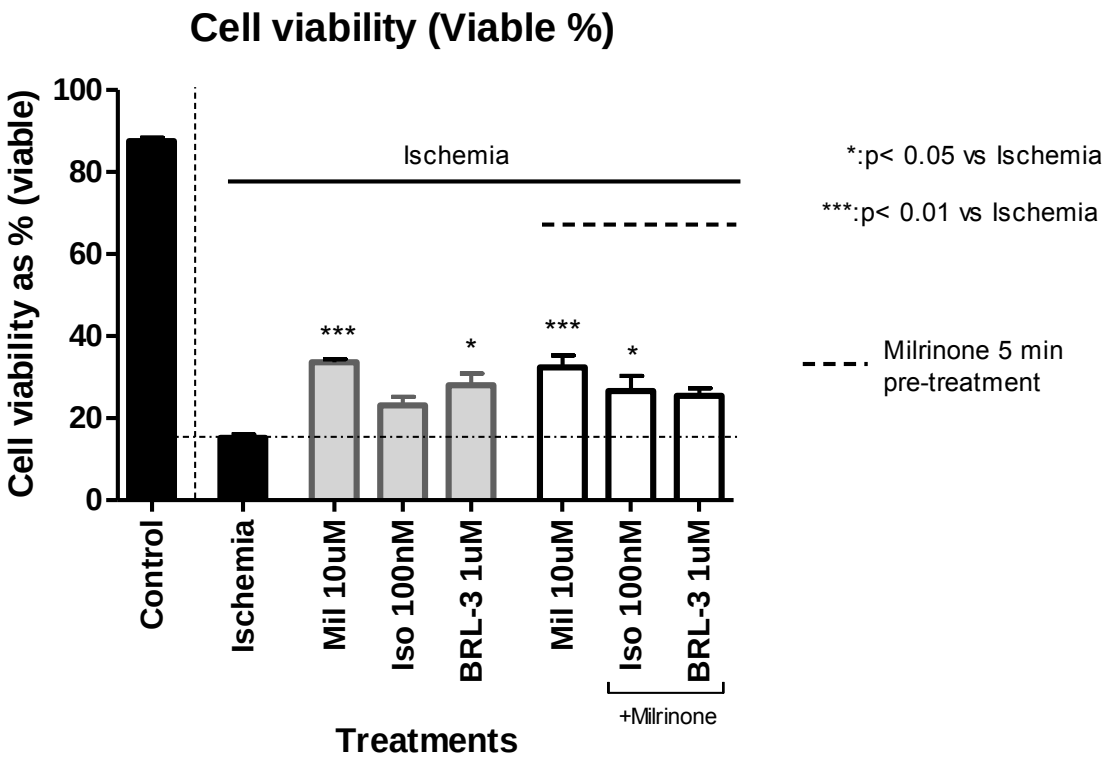

Figure 4.23: Percentage viable cardiomyocytes data of pre- and per-treatment with 10μM milrinone in combination with, 100nM isoproterenol and 1μM BRL-34377. Milrinone was given 5 minutes prior to (pre-treatment) and during 20 minutes chemical ischemia (per-treatment), whereas agonists were only given during ischemia. Sample size n = 4.*P < 0.05; ***P< 0.001 versus ischemia.

Cell viability percentage in figure 4.23 recovered significantly with 10μM milrinone (33.64 ± 0.81%; p< 0.001) and 1μM BRL-37344 (28.05 ± 2.85%; p< 0.05) administration respectively during 20 minutes ischemia (15.22 ± 0.81%). There was a significant improvement seen in cell viability with 5 minutes pre-treatment of 10 μM milrinone followed with combination treatment of 10μM milrinone + 100nM isoproterenol vs ischemia control (26.62 ± 3.68% vs 15.22 ± 0.81%; p< 0.05).

A further increase was noted with pre- and per-treatment of 10μM milrinone (32.40 ± 2.91%; p< 0.001) compared to the ischemia control group (15.22 ± 0.81%). It thus appears that inhibition of the PDE3 enzyme causes a protective effect, but pre-treatment of this agent does not further enhance any protection seen with per-treatment during sustained ischemia.

CHAPTER 5 – Discussion

The most significant observations made in this thesis are the following: (i) 3mIU insulin can mediate cardioprotection in cardiomyocytes during simulated ischemia/reperfusion; (ii) β2-AR signalling activation during ischemia with 10μM formoterol leads to protection; (iii) protection can be elicited when the PDE3 enzyme is inhibited during ischemia by 10μM milrinone; (iv) β1-AR stimulation with 10μM dobutamine appears to have no significant effect on cell survival, while (v) β3-AR stimulation with 10μM BRL37344 during ischemia exerts protection.

5.1 Effect of insulin on survival of cardiomyocytes

In order to evaluate the role of PDE3 in insulin-mediated protection in the present study, three insulin concentrations (0.3mIU, 3mIU and 30mIU) were administered during ischemia to first identify an optimal dose for protection against ischemia/reperfusion injury. 3mIU Insulin was found to be cardioprotective, by reducing hypercontracture (figure 4.2) and increasing cell viability of cardiomyocytes (figure 4.3). This is in alignment with known insulin-induced cardioprotective effects described in the literature, such as reduced infarct size, reduced hypercontracture and improved cell survival (Jonassen et al., 2001, Yu et al., 2008).

The precise mechanism through which insulin protection is elicited is still unclear, but according to the literature there are several possibilities. Insulin has been shown to be cardioprotective in animal models, activating PI3K and reducing post-ischemic myocardial apoptotic death as well as protecting against acute myocardial infarction in humans (Jonassen et al., 2001; Baines et al., 1999; Malmberg et al., 2002; Diaz et al., 1998). Early administration of insulin at reperfusion reduced myocardial infarction through an Akt and p70s6 kinase dependent signalling pathway, figure 5.1 (Jonassen et al., 2001). An insulin concentration of 1mIU and 5mIU was used in their study to elicit myocardial protection, which falls within the same dose range as the 3mIU insulin used in this thesis to exert protection.

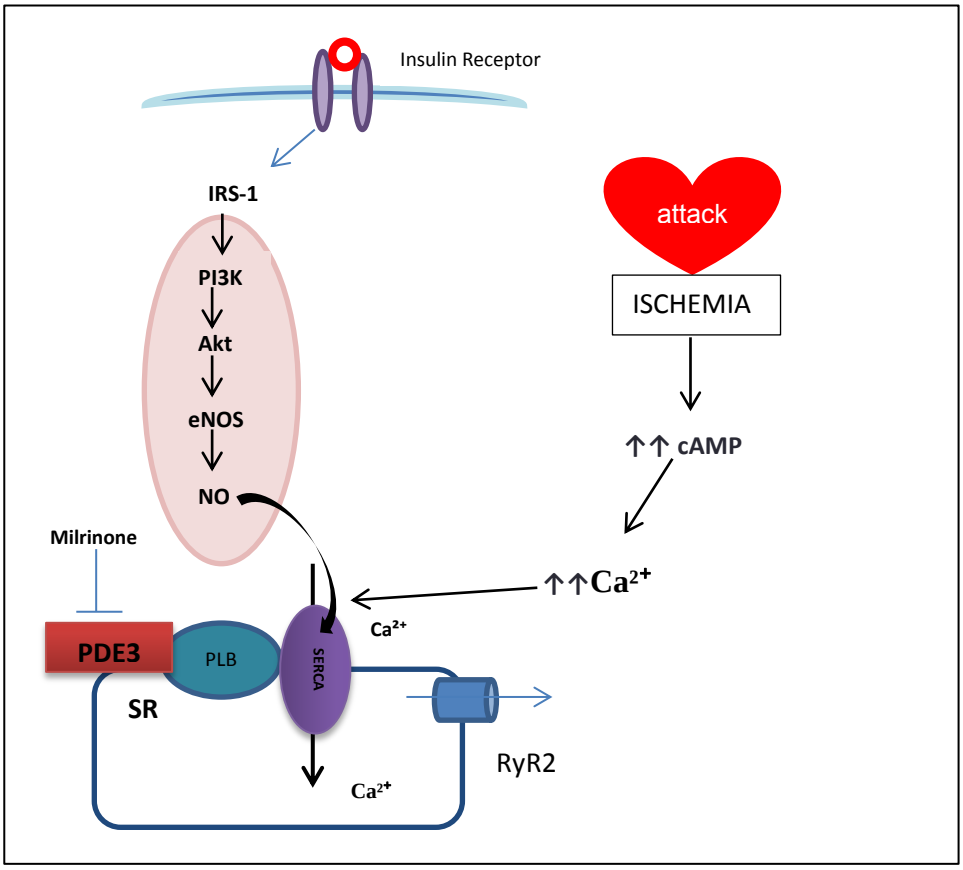

***Figure 5.1**:* Insulin-mediated protection through PI3K–Akt–eNOS–NO survival signalling, which reduces intracellular calcium accumulation by increased SR calcium uptake through SERCA during ischemia/reperfusion that reduces hypercontracture. Ischemia in the absence of insulin cause intracellular calcium overload in response to elevated cAMP, leading to hypercontracture.

5.1.1 Effect of milrinone (PDE3 inhibitor) on insulin-mediated protection

The PDE3 inhibitor milrinone was tested at 1μM, 10μM and 50μM concentrations during ischemia and the results showed that 1μM and 10μM milrinone decreased cell hypercontracture, which is an indication of improved cell relaxation (figure 4.4). Thus, ischemia in the absence of PDE3 inhibition induced hypercontracture and reduced cell viability, which was only improved when PDE3 was inhibited during ischemia. Notably, this indicates that active PDE3 in ischemic cardiomyocytes is damaging, which is similar to the findings by Huang et al (2011).

Huang and co-workers (2011) found that the administration of 4.7μM milrinone during late ischemia and early reperfusion reduced infarct size in vivo, and reduced myocyte death in cardiomyocyte cultures during hypoxia, they found that treatment with milrinone reduced cell death by 13.3% (Huang et al., 2011). The latter evidence confirms that milrinone at 10μM should be adequate to inhibit the PDE3 enzyme effectively, and thereby contribute to cardiac protection.

Interestingly, with simultaneous administration of milrinone concentrations (1μM, 10μM and 50μM) in the presence of insulin (3mIU), a similar protective effect was seen when compared to milrinone treatment only, but with no further decline in hypercontracture. Thus, it is evident from the results that milrinone 10μM and insulin 3mIU exerts the same degree of protection and might possibly mediate their protective effects through similar mechanisms and/or with their end effectors possibly overlapping.

Hypercontracture is an ischemia/reperfusion pathology that develops in response to ATP loss, intracellular calcium overload and mPTP opening, which are all essentially linked through intracellular calcium handling (Bonz et al., 1998, Ladilov et al., 1999, Siegmund et al., 1990). A mechanism that might possibly be shared between insulin and PDE3 inhibition is improved calcium handling, which is a known mechanism whereby insulin protects adult cardiomyocytes against reoxygenation-induced hypercontracture (Abdallah et al., 2006). These authors showed that insulin induce the clearance of calcium from the cytosol through enhanced calcium sequestration into the sarcoplasmic reticulum (SR), mediated through SERCA activation.

This protective mechanism is dependent on the activation of survival pathways that involve PI3-kinase, eNOS, and PKG (Abdallah et al., 2006) as seen in figure 5.1. Yu et al also demonstrated an insulin-mediated protective mechanism through improved calcium handling (Yu et al., 2008). In their experimental study 100nM insulin improved recovery of contractile function through enhancement of SERCA2a activity, in cardiomyocytes subjected to simulated ischemia/reperfusion.

The localization of PDE3 at the SR (Fischmeister et al., 2006) puts this enzyme at an ideal place to regulate cAMP and cGMP concentrations at the SR, and therefore also their respective targets PKA and PKG. It is thus conceivable that during ischemia when PDE3 is active, cAMP and/or cGMP levels might be kept low at the SR, which would prevent PKA and/or PKG activation mediated phospolamban phosphorylation. SERCA activity would be reduced and intracellular calcium increased (figure 5.1). However, if PDE3 is inhibited during ischemia, then cAMP and/or cGMP levels might increase at the SR and allow phospholamban phosphorylation through PKA and/or PKG, allowing SERCA to reduce intracellular calcium and hypercontracture.

In order to test this theory, intracellular levels of Ca^{2+} must be measured in the presence and absence of insulin and PDE3 while SERCA is inhibited, but this was not possible in this study. Attempts to measure intracellular calcium was unsuccessful because sodium dithionite, which is used to induce simulated ischemia, bleached the fluo-4 intracellular calcium dye as is elaborated. An alternative would be to measure intracellular calcium in a hypoxia model.

5.1.2 The effect of PDE3 Inhibitor (Milrinone) on the non-selective β-AR agonist (Isoproterenol) and Insulin

A decrease in cell hypercontracture (increase in length) was seen when cardiomyocytes were treated with 100nM isoproterenol, 3mIU insulin and 10μM milrinone respectively and together, during 20 minutes simulated chemical ischemia (figure 4.6). This was also seen with the cell viability, where the % rod shaped cells increased with each of these treatments during ischemia (figure 4.7). It is important to note that the cardioprotective effect elicited by isoproterenol was unexpected, given that isoproterenol is generally administered during ischemia/reperfusion to mimic the detrimental effects of β-AR signalling (Zhu et al., 2000, Yu et al., 2008).

The combination treatment of isoproterenol (100nM) + insulin (3mIU) also decreased cell contracture and increased cell viability when compared to the ischemia control, but combination treatment did not alter these effects further when measured against individual treatments of drugs.

Insulin is able to diminish the contractile response to β-AR stimulation and block isoproterenol-elicited cardiac dysfunction as well as cell injury in myocardial ischemia/reperfusion (Yu et al., 2008). This mechanism of insulin induced cardioprotection involves the PKA-mediated Ca^{2+} transient to be inhibited and promotion of improved post-ischemic Ca^{2+} handling (Yu et al., 2007). The opposite was however found in this study where both 3mIU insulin and 100nM isoproterenol administration led to protective effects during ischemia/reperfusion.

5.2 Effect of β2 agonist, Formoterol, as ischemic Per-treatment and Post-treatment

Three concentrations (10nM, 10μM, 100μM) of the β2 agonist, formoterol were tested during 20 minutes ischemia and 60 minutes reperfusion (figure 3.1). Both 10μM and 100μM concentrations of formoterol administered during ischemia resulted in a decline in cell hypercontracture after ischemia/reperfusion (figure 4.10). Yet, all concentrations (10nM, 10μM and 100μM) of formoterol administered during ischemia/reperfusion decreased cardiomyocyte hypercontracture and increased cell viability, and thus ultimately led to cardiomyocyte protection as is found by Bhushan et al. (2012). The use of arformoterol (ARF), to stimulate acute β2-adrenoreceptor led to the activation of cAMP and downstream effectors such as PKA, Akt, eNOS and enhancement of NO bioavailability, which reduced myocardial cell death and preserved cardiac function after ischemia/reperfusion (Bhushan et al., 2012).

It is thus evident that formoterol in this thesis activated β2-AR, causing the activation of downstream effectors that led to cardioprotection during simulated ischemia/reperfusion. The cardioprotection of β2-AR stimulation might here possibly be dependent on the activation of PI3K-PKB/Akt and ERK p44/p42 MAPK during the administration of formoterol (figure 5.2), given that these pathways have been shown to promote protection by the β2-AR (Chesley et al., 2000).

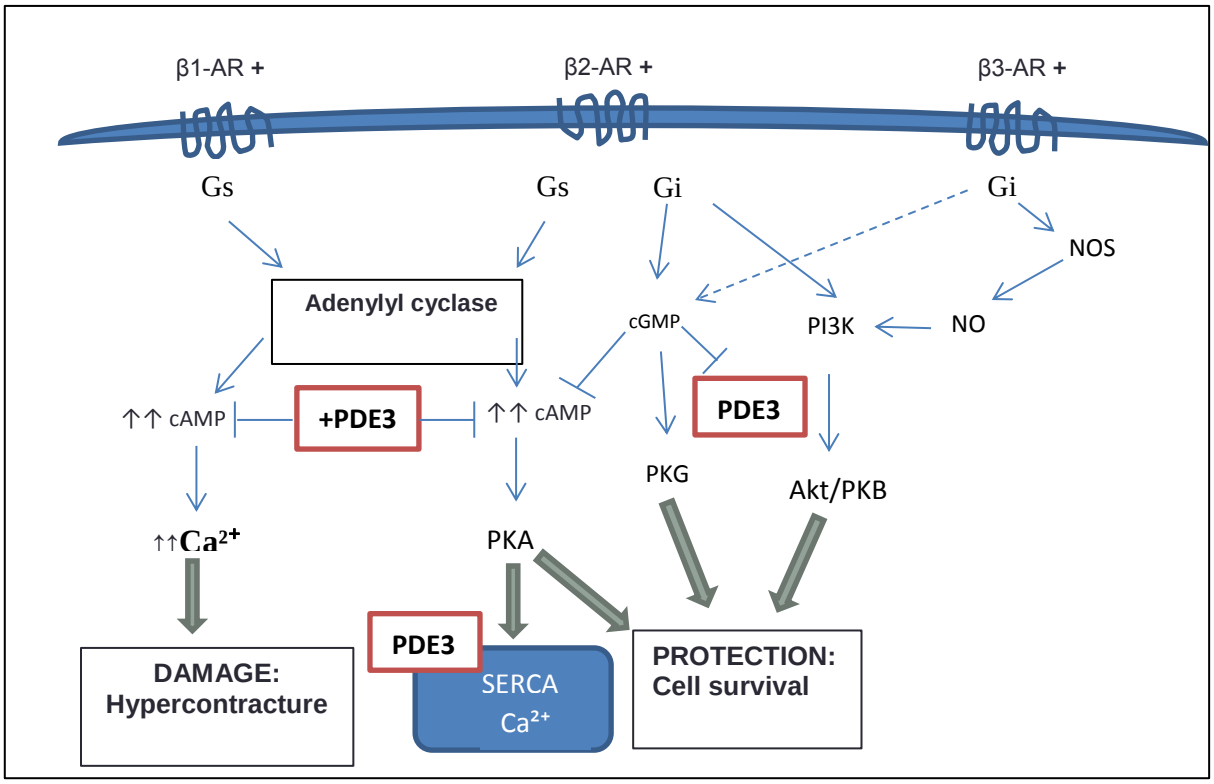

Figure 5.2: Subtype-specific signalling pathways of cardiac β-ARs.

5.2.1 Effect of β1 agonist, dobutamine, as ischemic Per-treatment

Given that β1-AR is associated with ischemia/reperfusion damage, different concentrations (1μM, 10μM and 100μM) of the β1-AR agonist dobutamine, was administered during ischemia to find a concentration that would enhance hypercontracture (data not shown, experiments done by Smith A (an MSc student)). None of the doses could exacerbate hypercontracture, and 10μM was chosen to continue experiments with, given it is higher than the receptor binding affinity (K (D) = 2.5 muM in rat heart) and Wang et al. (2013) used a 1μM dobutamine concentration in their study.

The results obtained in the present study demonstrated how the activation of the β1 AR via 10μM dobutamine had contrasting effects to the literature, regarding cell survival parameters. Contrary to expectations, in some cases the activation of this signalling pathway led to decreased cell hypercontracture and increased cell viability (figure 4.15) and some instances had no affect (figure 4.13).

This inconsistency might be attributed to the fact that during ischemia the full damaging effect on the cardiomyocytes was already achieved, and therefore no further damage could be induced via dobutamine-mediated stimulation of β1-AR signalling.

5.2.2 The influence of PDE 3 Inhibitor (Milrinone) on the effect of β1 agonist (Dobutamine) and β2 agonist (Formoterol) during ischemia.

Of the three milrinone concentrations tested (1μM, 10μM, 50μM), 10μM milrinone was the only concentration that consistently decreased cell hypercontracture and improved the cell viability during simulated ischemia (figure 4.4 & 4.13). The effect of milrinone was investigated in combination treatment with the β1 agonist (Dobutamine) and β2 agonist (Formoterol) during ischemia, to determine whether the inhibition of PDE3 affects these β-AR signalling effects.

As previously discussed, the stimulation of β1-AR signalling through binding of dobutamine did not have any significant effect on either contracture or viability of cardiomyocytes. Combination treatment of milrinone 10μM + dobutamine 10μM however caused a decrease in contracture (increased length) and significantly increased cell viability (figure 4.13). This protective effect of milrinone (milrinone + dobutamine) did not affect those parameters any further than treatment of milrinone on its own during ischemia, and therefore milrinone ultimately did not influence dobutamine.

When milrinone was given in combination treatment with formoterol (milrinone 10μM + formoterol 10μM), cell hypercontracture seemed to decrease significantly and an increase in cell viability was observed, but these effects were not more substantial than formoterol 10μM treatment alone, as the latter already exerted the maximum protection (figure 4.13). Treatment of milrinone thus had also no effect on formoterol treatment. By measuring cAMP and cGMP, information can be gained regarding possible associated signalling, but due to time constraints this was unfortunately not done.

Formoterol, just like insulin, might also mediate its cardioprotective effects through PDE3 inhibition or through an overlapping end effector, given that similar degree of protection was elicited with formoterol and milrinone, administered together and separate.

β2-AR stimulation with arformoterol reduced infarct size in mice heart in vivo through the activation of cAMP-PKA-Akt-eNOS-NO (Bhushan et al 2012). Referring to figure 5.2, β2-AR stimulation through formoterol can activate PKA, which will increase SR sequestration of Ca^{2+} or activate PKG, which can, all together, lead to cardioprotection in this study during ischemia/reperfusion. Another possible mechanism of cardioprotection via formoterol-mediated β2-AR activation, is the fact that cGMP is able to inhibit PDE3, which results in increased levels of cAMP (figure 5.2).

One of the many localizations of PDE3 enzyme is near SERCA (figure 5.1 and 5.2) and inhibition thereof can possibly result in the transformation of PLB from the dephosphorylated state to a phosphorylated state, resulting in SERCA activity and increase uptake Ca^{2+}. This can thus act as a potential protecting mechanism which exert the same function as PKA phosphorylation of PLB and could be the reason why β2-AR signalling through formoterol does not show any more protection.

Regards to dobutamine and PDE3 inhibition, it might be probable that β1-AR stimulation via dobutamine and milrinone targets the same downstream effectors such as cAMP in the signalling cascade and therefore can possibly eliminate one another's underlying mechanisms.

5.2.2.1 Pre- treatment of β1 agonist, Dobutamine and β2 agonist, Formoterol in combination with PDE3 Inhibitor, Milrinone.

To investigate whether protective or damaging effects could be further enhanced through β-AR stimulation and PDE3 inhibition, cardiomyocytes were pre-treated with both β-agonists and milrinone. The combination treatments, 10μM milrinone + dobutamine 10μM/formoterol 10μM, with milrinone pre-treated 5 minutes before ischemia and during 20 minutes simulated ischemia, did not alter the effects compared to per-treatment of these agents.

Milrinone also did not decrease hypercontracture any further when administered 10 minutes before simulated ischemia (figure 4.15). Thus, the inhibition limit of the PDE3 enzyme was reached with per-treatment of milrinone.

Five minutes preconditioning with dobutamine (10μM) resulted in protection of the rat heart against ischemia/reperfusion injury in the form of postischemic contractile dysfunction (Asimakis & Conti, 1995). In contrast, our study showed that when milrinone was pre-treated for 10 minutes in combination treatment with dobutamine 10μM/formoterol, each pre-treated for 5 minutes before ischemia respectively, no enhanced effects were seen regarding cell contracture (figure 4.15) or cell viability (figure 4.17).

The reason for this is that when milrinone, formoterol and dobutamine were given as per-treatment, their optimal effect has been reached as regards to protection or damage. Therefore, pre-treatment of milrinone and the β-agonists did not have a substantial effect. No effects were seen with mitochondrial membrane potential during ischemia and this can be as a result of very large error bars.

It might also be possible that formoterol protects cardiomyocytes through mitochondrial biogenesis during simulated ischemia. It has been found that mitochondrial dysfunction is a result of ischemia/reperfusion and β2-AR agonist formoterol can induce mitochondrial biogenesis which then stimulates cell repair and regeneration (Wills et al., 2012). The formoterol mediated stimulation of β2-AR signalling pathway may regulate mitochondrial function in cardiac tissue (Wills et al., 2012).

5.3 The effect of pre- and per-treatment with PDE3 Inhibitor (Milrinone) in combination with β3 agonist (BRL-37344).

This study illustrated that the selective β3-AR agonist, BRL-37344 significantly decreased the cell hypercontracture (figure 4.21) accompanied by an increase in cell viability (figure 4.23) during simulated ischemia. Thus, this form of protection in isolated cardiomyocytes might be mediated by inhibitory G-protein coupled NOS–NO signalling (figure 5.2). The production of NO causes the generation of cGMP which can possibly inhibit PDE3, resulting in reduced cAMP induced stimulation of contractility or PDE3 inhibition can increase cAMP levels (figure 5.2). Niu et al. (2013) demonstrated in vivo cardioprotective effects through β3-AR stimulation with nNOS identified as the key downstream effector in mediating heart protection (Niu et al., 2013).

Another possible manner in which the BRL-37344 mediated β3-AR stimulation can cause a decrease in hypercontracture and increase in cell viability is through the NO-PI3K-PKB/Akt signalling pathway (figure 5.2) which promotes cell survival as seen with the cell viability parameter in this study (figure 4.23). Heusch et al. (2011) have also found that NO may play a crucial role in cardioprotection against ischemia/reperfusion and might possible activate one of these proposed signalling pathways.

When the cardiomyocytes were treated with β3-AR agonist in combination with milrinone 10μM, the cell contracture and cell viability remained unchanged. Both 1μM BRL-37344 and 10μM milrinone produced maximum protection when administered independently during ischemia. As in the case of the other two β-AR agonist, BRL-37344 seems to have no effect on the mitochondrial membrane potential.

CHAPTER 6 - Conclusion

It has been shown that acute myocardial ischemia can cause stimulation of cellular signals which result in cell damage, and if not inhibited, can lead to detrimental effects and even cell death. Consequently, cardioprotective strategies can be implicated to modify crucial signal-transduction pathways to improve functional recovery of the heart after ischemia/reperfusion. Throughout this study isolated cardiomyocytes were subjected to 20 minutes simulated ischemia followed by 60 minutes reperfusion. Survival endpoints that were measured at the end of reperfusion include cell hypercontracture, % cell viability and mitochondrial membrane potential.

Studies showed that stimulation of β-ARs can activate downstream signalling pathways and consequently elicit cardiac protection (β2-AR, β3-AR) or damage (β1-AR) during ischemia/reperfusion. Insulin is a known cardioprotective agent that can oppose detrimental signals from β-ARs and was therefore included in this study. The main aim was to evaluate the role of PDE3 in mediating damage versus protection by the aforementioned stimuli. The reason for the focus on PDE3 is due to its role in the regulation of β-ARs in contractility during normoxic conditions, and because it has previously been shown to elicit protection in rat hearts in vivo when administered before ischemia (Sanada et al., 2001), or during the end of ischemia and beginning of reperfusion (Huang et al., 2011).

Agonist doses first had to be optimized for protection to be elicited by insulin, formoterol (β2-AR agonist) and BRL-37344 (β3-AR). The same had to be done for inducing damage through dobutamine (β1-AR) and isoproterenol (β1- and β2-AR). The data indicated that 3mIU insulin can elicit cardioprotection, indicated by decreasing cell hypercontracture and increasing cell viability. β2-AR stimulation with 10μM formoterol and β3-AR stimulation with 1μM BRL-37344 also induced cardioprotection as indicated by a significant improvement in % cell viability and decreased cell hypercontracture. These results are in accord with the literature (Wills et al., 2012, Bhushan et al., 2012, Watts et al., 2013).

The involvement of 10μM dobutamine in stimulating β1-AR appeared to have no significant effect on any of the three parameters investigated. Dobutamine was expected to increase cell hypercontrature and decrease % cell viability according to most of the literature, which is contradictory to our findings (Pantos et al., 2003). This complexity is also seen with Wang et al. (2013), which elicited a protecting effect with dobutamine while Angelos et al. (2002) found damaging effects with dobutamine administration. Hence, the role of β1-AR activation via dobutamine needs to be further evaluated since this agonist did not affect cardio performance in any manner during ischemia/reperfusion. In this study, it was also demonstrated that β1/β2-AR stimulation by 100nM isoproterenol predominantly decreased cell hypercontracture and % cell viability, which is also in contrast to the opinion in literate (Yu et al., 2008).

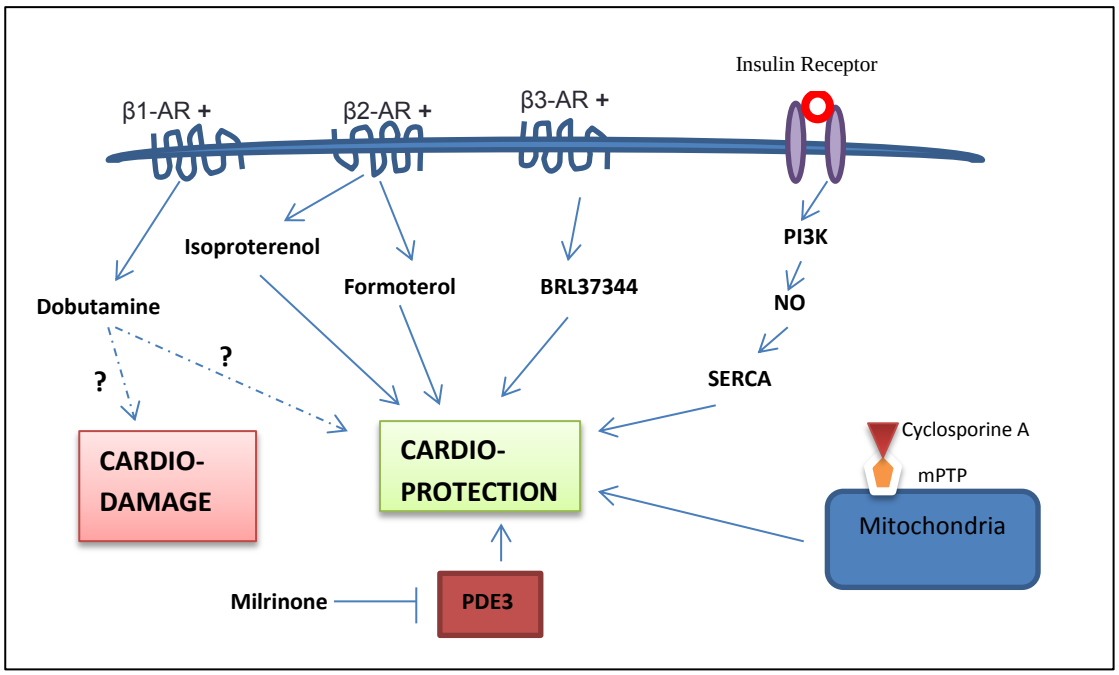

Figure 6.1: Image showing a summary of the sequence of signalling cascades and drugs that were shown to be involved in the cardioprotection or cardio-damage in cardiomyocytes during ischemia/reperfusion in this study.

10μM Milrinone elicited an equivalent decrease in cell hypercontracture with a simultaneous increase in the % cell viability during ischemia/reperfusion, as was elicited by 3mIU insulin, 10μM formoterol and 1μM BRL-37344. This indicates that active PDE3 during ischemia is detrimental to cardiomyocyte survival and must therefore be inhibited to protect cardiomyocytes from ischemia/reperfusion injury. It is possible that insulin, β2-AR and β1-AR mediate protection through PDE3 inhibition, and therefore protection could not be enhanced by combining milrinone with each cardiopotective agonist.

Alternatively, if these cardioprotective agents do not mediate protection by inhibition of PDE3, then another possible explanation is that they share common end effectors, for example mPTP opening, which is associated with intracellular calcium accumulation. The involvement of mPTP through JC-1 preloading and intracellular calcium with Fluo-4 preloading (no data shown) was evaluated, but due to the bleaching effects of SDT, the fluorescence labels were destroyed and no conclusion could be made. These parameters must be evaluated in the future using a model of hypoxia, which would preserve fluorescent labels preloaded in the cells before ischemia.

Due to the absence of mechanistic data in this thesis, it is impossible to predict which mediators and effectors are involved in mediating protection of PDE3, insulin, β2- and β3-AR agonist, but certain pathways can be identified from the literature to consider for future evaluation.

In this regard intracellular calcium handling through PI3K-PKB-NO-SERCA pathway (figure 6.1) should be considered given that this pathway is used by insulin to reduce hypercontracture (Abdallah et al., 2006), which is a pathology that insulin, formoterol, BRL-37344 and milrinone significantly reduced. mPTP opening should also be considered given its close association with elevated intracellular calcium during reperfusion (Ruiz-Meana et al., 2007).

The work from this thesis identified agonists that can induce cardioprotection in 24h cultured adult cardiomyocytes, which might mediate protection through PDE3 inhibition, but further research is required to identify the mechanisms involved.

Future directions:

Further research to be done:

(i) Investigating the changes in total cellular ATP and intracellular Ca^{2+} during ischemia/reperfusion in isolated rat cardiomyocytes, when protected by insulin signalling or damaged by β-AR *(due to time constraints we were not able to investigate this objective).*

(ii) Studies should investigate the combinational use of milrinone with other drugs, example β1/2-AR blockers, and the possible long-term effects thereof.

(iii) Focus should be placed on the hypoxia method as an alternative to simulate ischemia in experiments and the precise 2-DG concentration used in the ischemic buffer.

(iv) Using specific assay kits to determine the intracellular cAMP levels and compartments in the cytosol of cardiomyocytes change during simulated ischemia/reperfusion.

(v) Ischemia mPTP opening or closure (Cyclosporine A) as a possible downstream effector of PDE3 inhibition.

(vi) Introducing protein sequencing to determine the exact localization of PDE3 enzyme in the cardiomyocte.

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APPENDIX I

Buffer A		
Reagent	*Final Concentration*	*In 250ml (1 heart)*
PBS	1x	247.5ml
D-Glucose	11mM	495mg
Pyruvate	2mM	55mg
Insulin (100IU)	30IU	75µl
Pen/Strep	1%	2.5ml

Buffer B		
Reagent	*Final Concentration*	*In 50ml (1 heart)*
BSA (Fatty acid free)	0.5%	275mg
Collagenase II 275u/mg	440IU/ml	90mg
Protease IV	10mg	
2.3 Butanedione Monoxim (BDM)	18mM	91.2mg

Buffer C		
Reagent	*Final Concentration*	*In 50ml (1 heart)*
Buffer A	1x	50ml
BSA (Fatty acid free)	0.5%	250mg
Bovine Serum Albumin (BSA)	0.5%	250mg
BDM	9mM	45.6mg

Buffer D		
Reagent	*Final Concentration*	*In 15ml (1 heart)*
Buffer C	2/3x	10ml
Buffer B	1/3x	5ml
$CaCl_2$ (100mM)	0.3mM	45µl

Buffer E			
	Reagent	Final Concentration	6ml/Ca2+ raise (1 heart)
E1	Buffer C	1x	6ml
	$CaCl_2$ (100mM)	0.6mM	36µl
E2	Buffer C	1x	6ml
	$CaCl_2$ (100mM)	0.9mM	54µl
E3	Buffer C	1x	6ml
	$CaCl_2$ (100mM)	1.2mM	72µl

Buffer F and Buffer G for cardiomyocyte plating (without insulin and BDM)		
Reagent	*Final Concentration*	*In 6ml (1 heart)*
Buffer C	60%	3.6ml
CaCl2 (100mM)	1.2mM	36l
CMCB	40%	2.4ml
17mM Blebbistatin	10M	3.5l

Cardiomyocyte Culture Buffer (CMCB)			
MW in g/mol	*Reagent*	*1 x [C]*	*In 500ml*
Hank's solution	M199	1x	485ml
(Stock)	1M Hepes	10mM	5ml
110	Pytuvate	5mM	275mg
131.13	Creatine	5mM	328mg
197.7	Carnitine	5mM	495mg
125.15	Taurine	5mM	315mg
BSA (FAF)		1.5%	5g
Pen/Strep		1%	5ml
Warm to 37C and adjust pH to 7.4 Filter-sterilize and store at 4C			

Normoxic washing buffer	
Buffer A (no insulin)	210ml
CMCB	140ml
1.2mM Ca2+	2.52 ml

Ischemic buffer:	
M199 Hanks solution	495ml
Pen/Strep	5ml
Ischemic wash	
40% Ischemic buffer	20ml
60% PBS + Ca2+	30ml
Add	
Sodium dithionite (SDT) (174.11 mg in 1ml Milli-Q water)	10l/ml
2-deoxy-D-glucose (2-D-G) (164.16mg in 1ml Milli-Q water	3l/ml
pH ischemic wash 6.4	

JC-1 for staining	
CMCB	6ml
JC-1 stock	30l
Propidium iodide for staining	
100M PI stock	60l
CMCB	6ml
Fluo-4	
50mg aliquot dissolved in 46l Pluronic F-127:	
Dissolved Fluo-4	60l
CMCB	6ml

SNARF-5	
50mg aliquot dissolved in 86l Pluronic F-127	
Dissolved SNARF-5	60l
CMCB	6ml

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