Elucidating the Underlying Mechanisms of Benfotiamine-induced Cardioprotection

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Abstract

Context: Cardiovascular diseases are the leading cause of death globally. Myocardial infarction is responsible for the highest number of deaths due to cardiovascular disease.

Objective: We have previously shown that acute benfotiamine administration at the onset of reperfusion is associated with decreased infarct size and preserved contractile function in response to ischemia-reperfusion. We aimed to evaluate the involvement of the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pro-survival signaling pathways in mediating these cardioprotective effects.

Materials and Methods: *Part One* - Hearts were rapidly excised from Wistar rats and mounted on a Langendorff perfusion apparatus. After stabilization, hearts were subjected to 30 minutes of regional ischemia and 120 minutes of reperfusion. The control group received no treatment. Experimental groups were treated with 100 μM benfotiamine ± 0.1 μM Tyrphostin AG490 or Wortmannin (inhibitors of JAK2 and PI3K, respectively), dissolved in dimethyl sulfoxide. The vehicle control group received an equivalent dose of dimethyl sulfoxide. All treatments were administered for 20 minutes at the onset of reperfusion. Functional parameters were measured throughout, to test the effects of benfotiamine ± pro-survival pathway inhibitors on functional recovery. In addition, hearts were stained with Evans blue and triphenyltetrazolium chloride to assess the effects of benfotiamine ± pro-survival pathway inhibitors on infarct size.

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Part Two - Hearts that were perfused ± 30 minutes of global ischemia and ± 20 minutes of benfotiamine administration, were used to assess PI3K/Akt and JAK/STAT signaling in response to ischemia-reperfusion and benfotiamine treatment. As with previous experiments, benfotiamine was administered at a concentration of 100 μM, at the onset of reperfusion. Tissues were assessed by Western blot analysis.

Results: 20 minutes of acute benfotiamine administration at the onset of reperfusion led to a decrease in infarct size (35.6 ± 2.4% vs. 55.7 ± 5.0% [$p<0.05$]). Inhibition of PI3K/Akt signaling by addition of Wortmannin abrogated this infarct-limiting effect (51.5 ± 1.3% vs. 35.6 ± 2.4% [$p<0.05$]). However, inhibition of JAK/STAT signaling had no effect. There were no significant differences in left ventricular developed pressure, coronary flow rate or heart rate during the experiments.

In addition, 20 minutes of acute benfotiamine administration at the onset of reperfusion lead to an increase in phospho-FOXO/FOXO in the cytosolic fraction, but no significant change in phospho-STAT3/STAT3 in the nucleus.

Conclusions: Our results suggest that acute benfotiamine administration at the onset of reperfusion may act to reduce infarct size via activation of PI3K/Akt pro-survival signaling.

Opsomming

Konteks: Kardiovaskulêre siekte is die hoofoorsaak van sterftes wêreldwyd. Miokardiale infarksie is verantwoordelik vir die grootste aantal sterftes weens kardiovaskulêre siekte.

Doel: Ons het voorheen getoon dat akute benfotiamientoediening met die aanvang van reperfusie geassosieer is met 'n verkleining in die infarkgrootte, en dit het verder ook die kontraktiele funksie in reaksie op ischemie-reperfusie behou. Ons doel was om die betrokkenheid van die fosfatidielinositol 3-kinase/Akt (PI3K/Akt) en Janus kinase/seintransduseerde en aktiveerder van transkripsie (JAK/STAT) pro-oorlewings seinweg in die mediasie van hierdie kardiobeskermende effekte te evalueer.

Materiale en Metodes: *Deel een* - Harte is vinnig vanuit Wistarrotte verwyder en op die Langendorff-perfusieapparaat gemonteer. Na stabilisering is die harte blootsgestel aan 30 minute regionale ischemie en 120 minute reperfusie. Die kontrole groep het geen behandeling ontvang nie. Eksperimentele groepe is met 100 µM benfotiamien ± 0.1 µM Tirfostien AG490 of Wortmannin (inhibeerders van JAK2 en PI3K, onderskeidelik) behandel, opgelos in dimetielsulfoksied. Die draer-kontrole groep het 'n ekwivalente dosis van dimetielsulfoksied ontvang. Alle behandelings is toegedien vir 20 minute aan die begin van die reperfusie. Funksionele parameters is deurgaans gemeet om te toets vir die effekte van benfotiamien ± pro-oorlewingsweg inhibeerders op funksionele herstel. Verder is die harte met Evans-blou en trifenieltetrazoliumchloried gekleur

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om die effek van benfotiamien ± pro-oorlewingsweg inhibeerders op die infarkgrootte te bepaal.

Deel twee - Harte is vir ± 30 minute perfuseer met globale ischemie en ± 20 minute met benfotiamientoediening. Dit was gebruik om PI3K/Akt en JAK/STAT seine as gevolg van ischemie-reperfusie en benfotiamienbehandeling te ondersoek. Soos met die vorige eksperimente, is benfotiamien toegedien by 'n konsentrasie van 100 μM met die aanvang van reperfusie. Weefsel is ondersoek deur middel van Western blot analise.

Resultate: 20 minute van akute benfotiamientoediening, met die aanvang van reperfusie, het tot 'n verkleining in die infarkgrootte (35.6 ± 2.4% vs. 55.7 ± 5.0% [$p<0.05$]) gelei. Inhibering van die PI3K/Akt seinweg deur toediening van Wortmannin het die infark-beperkende effek opgehef (51.5 ± 1.3% vs. 35.6 ± 2.4% [$p<0.05$]). Inhibering van JAK/STAT seine het egter geen effek getoon nie. Daar was geen beduidende verskille in linkerventrikulêr-ontwikkelde druk, koronêre-vloeitempo of harttempo tydens die eksperimente nie.

Verder, 20 minute van akute benfotiamientoediening met die aanvang van reperfusie het 'n toename in fosfo-FOXO/FOXO in die sitosoliese-fraksie veroorsaak, maar geen beduidende verandering in fosfo-STAT3/STAT3 is in die nukleus waargeneem nie.

Gevolgtrekkings: Ons resultate suggereer dat akute benfotiamientoediening met die aanvang van reperfusie moontlik die infarkgrootte via aktivering van die PI3K/Akt pro-oorlewingsein kan verklein.

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List of Abbreviations

AGE:	advanced glycation endproducts
ANOVA:	analysis of variance
ATP:	adenosine triphosphate
Bad:	Bcl-2-associated death promoter
Bak:	Bcl-2 agonist/killer
Bax:	Bcl-2-associated X protein
Bcl-2:	B-cell leukemia/lymphoma-2
Bcl-x_L:	B-cell lymphoma-extra large
BFT:	benfotiamine
BSA:	bovine serum albumin
caspase:	cysteine-aspartic protease
cyt c:	cytochrome c
dH_2O:	distilled water
DMSO:	dimethyl sulfoxide
ECL:	enhanced chemiluminescent
EDTA:	ethylenediaminetetraacetic acid
eNOS:	endothelial nitric oxide synthase
ERK:	extracellular signal-regulated kinase
ETC:	electron transport chain
$FADH_2$:	reduced flavin adenine dinucleotide
FOXO:	forkhead box, sub-family O
G6PD:	glucose 6-phosphate dehydrogenase
GAPDH:	glyceraldehyde 3-phosphate dehydrogenase

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gp130:	glycoprotein 130
GSH:	reduced glutathione
GSK-3β:	glycogen synthase kinase-3β
GSSH:	oxidized glutathione
HBP:	hexosamine biosynthetic pathway
HCl:	hydrochloric acid
HEPES:	hydroxyethyl piperazineethanesulfonic acid
HRP:	horseradish peroxidase
IL-6:	interleukin-6
IL-6R:	interleukin-6 receptor
JAK:	Janus kinase
KCl:	potassium chloride
LVDP:	left ventricular developed pressure
MAPK:	mitogen-activated protein kinase
Mcl-1:	myeloid cell leukemia sequence-1
MEK:	mitogen-activated protein kinase/ERK kinase
$MgCl_2$:	magnesium chloride
min:	minutes
mPTP:	mitochondrial permeability transition pore
n:	sample size
NADH:	reduced nicotinamide adenine dinucleotide
NADPH:	reduced nicotinamide adenine dinucleotide phosphate
Na_3VO_4:	sodium orthovanadate
NaCl:	sodium chloride
NaOH:	sodium hydroxide
NO:	nitric oxide
PARP:	poly(ADP-ribose) polymerase

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PDK:	3-phosphoinositide-dependent protein kinase
PI3K:	phosphatidylinositol 3-kinase
Pim-1:	proviral integration site for Moloney murine leukemia virus-1
PIP2:	phosphatidylinositol 4,5-bisphosphate
PIP3:	phosphatidylinositol 3,4,5-trisphosphate
PKC:	protein kinase C
PMSF:	phenylmethyl sulfonyl fluoride
PPP:	pentose phosphate pathway
RAGE:	advanced glycation endproducts receptor
RIPA:	radioimmuno-precipitation
RISK:	reperfusion injury salvage kinase
ROS:	reactive oxygen species
rpm:	revolutions per minute
RPP:	rat-pressure product
SAFE:	survivor activating factor enhancement
SDS:	sodium dodecyl sulfate
SEM:	standard error of the mean
Ser:	serine residue
STAT:	signal transducer and activator of transcription
TBT-T:	tris-buffered saline-tween
TNF-α:	tumor necrosis factor-α
Tyr:	tyrosine residue
VEGF:	vascular endothelial growth factor
VEGFR2:	vascular endothelial growth factor receptor 2

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CHAPTER ONE: Introduction

Cardiovascular diseases are the leading cause of death globally[1]. Myocardial infarction, commonly known as a heart attack, accounts for the highest number of deaths due to cardiovascular diseases[2]. Despite improvements in patient care and the use of current treatments, millions of deaths continue to occur annually as result of myocardial infarction[3]. The treatment of cardiovascular disease and its consequences imposes a significant financial burden upon individuals and national economies, hindering development in low- and middle-income countries[4]. This burden is predicted to increase, as globalization and urbanization contribute to an increased prevalence of cardiovascular disease risk factors[5]. Therefore, an urgent need exists for effective, low-cost treatments for myocardial infarction.

The death of cardiac cells is a major consequence of myocardial infarction that can result in significantly impaired contractile function. Ischemic postconditioning (hereinafter referred to as 'postconditioning') is a strategy employed to limit cardiac cell death that was first described in 2003[6]. Although it has been applied successfully in small-scale clinical trials[7], it is not without its limitations. For example, its benefit is compromised by age and the presence of comorbidities such as diabetes and high blood pressure, both of which are common in patients being treated for myocardial infarction[8]. As a result of its limited applicability, researchers began investigating the underlying mechanisms of postconditioning. These studies focused on the pro-survival pathways activated in the heart by

postconditioning. Their aim was to find alternative means of conferring cardioprotection by targeting these pathways pharmacologically. The investigation into this new strategy of 'pharmacological postconditioning' sparked further interest in the field of cardiac cell signaling.

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway and the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway are two pro-survival signaling pathways that are activated during myocardial infarction[9,10]. Both of these pathways participate in mediating the cardioprotective effects of postconditioning[11].

Recent work by ourselves and others found that benfotiamine, a vitamin B1 derivative, may have therapeutic potential in treating myocardial infarction[12,13]. Moreover, we found that these cardioprotective effects could be induced by acute benfotiamine administration at the onset of reperfusion. However, our initial study left unanswered questions regarding the underlying mechanisms of these effects. As a result, we decided to investigate the involvement of the PI3K/Akt and JAK/STAT signaling pathways in mediating benfotiamine's effects.

In order to provide a theoretical framework for the study, the following chapter will draw attention to relevant aspects of the literature concerning cell signaling in the heart. In addition, it will describe the discovery of benfotiamine and discuss its therapeutic potential in the heart.

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CHAPTER TWO: Literature Review

The first part of this chapter focuses on the PI3K/Akt and JAK/STAT signaling pathways: a) their role in mediating the cell's response to threat, b) their involvement in postconditioning and c) the pursuit of pharmacological means to activate them. The second part of the chapter will take a closer look at benfotiamine - a therapeutic agent that may activate these pathways. In this section: a) its discovery will be described, b) its therapeutic potential in protecting the heart from hyperglycemia and myocardial infarction will be discussed, and c) its potential as a pharmacological postconditioning agent will be explored.

1. Cell signaling

The functioning of a cell relies on its ability to perceive changes in its microenvironment and respond accordingly. In addition, it must be able to send and receive messages from neighboring or distant cells. This is a crucial feature of cells comprising multicellular organisms – the capacity to cooperate[14]. These cells must communicate during growth and development. In addition, they must work together to maintain homeostasis and respond to tissue damage or disease.

When exposed to a threat, cells can employ any one of a range of survival mechanisms. For example, they can respond by rapidly synthesizing and secreting soluble proteins called cytokines. These cytokines inform

surrounding cells of the threat and recruit assistance in managing it. Cytokines are 'perceived' by neighboring cells via specific cytokine receptors embedded in their cell membranes. This message is then transmitted to the nucleus via intracellular signaling pathways such as the JAK/STAT pathway[15].

The nucleus orchestrates a cellular response to this message by altering the expression of specific genes. The level of expression of specific genes can be increased or decreased in order to facilitate cell survival and adaptation to current conditions. For example, JAK/STAT signaling is associated with upregulation of genes that promote cell survival, and downregulation of genes that promote cell death[16]. In addition to enabling survival and adaptation, gene expression can be altered to enable cells to actively counteract the threat and attempt to restore homeostasis. For example, JAK/STAT signaling is associated with increased expression of genes that promote angiogenesis, the formation of new blood vessels[17]. Thus, a cell can initiate a survival mechanism in neighboring cells, which involves JAK/STAT signaling and altered gene expression.

However, altered gene expression is not the only weapon in the cell's arsenal. A second survival mechanism that cells can employ involves the mitochondrion, which plays a crucial role in regulating cell death. When exposed to a threat, cells can activate this survival mechanism in surrounding cells by releasing substances such as adenosine[18]. When adenosine binds to the membrane-embedded receptors of surrounding cells, it leads to a variety of effects including the initiation of signaling via the PI3K/Akt signaling pathway[19].

The activation of PI3K/Akt signaling allows for rapid, transient adaptations such as the post-translational modification of existing proteins,

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thereby altering their function. For example, by phosphorylating specific proteins associated with the mitochondrion, PI3K/Akt signaling can prevent cell death[20]. Thus, a cell can initiate a survival mechanism in neighboring cells, which involves PI3K/Akt signaling and the post-translational modification of mitochondrial proteins.

Both of these survival mechanisms (altered gene expression and post-translational modification of mitochondrial proteins) are essential, since they act at the nucleus and mitochondria, key coordinators of cellular function and cell death. The response of cardiac cells during myocardial infarction is a pertinent example of the how initiating signaling via the JAK/STAT and PI3K/Akt signaling pathways allows cells to respond to a threat. The following section will briefly describe the pathology of myocardial infarction and discuss the role of these pathways in responding to it.

2. The activation of endogenous survival mechanisms during myocardial infarction

Myocardial infarction occurs when the blood supply to a particular region of the heart is interrupted. This can be caused by a blood clot that impairs blood flow in a coronary artery. The loss of blood supply, referred to as ischemia, impairs delivery of oxygen and nutrients to the region of the heart supplied by the affected artery. In addition, the removal of waste products is impaired. If this is sustained for long enough, cell death can result and a region of dead tissue, or infarct, can form[21]. Cardiomyocytes, i.e. the muscle cells of the heart, comprise more than 90% of the heart's mass and their death impairs contractile function. The size of the infarct,

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which is related to the extent of cardiomyocyte death, influences the severity of contractile dysfunction.

Multiple modes of cell death occur in the context of myocardial infarction. For a long time, necrosis (the uncontrolled death of cells, which can occur in response to trauma) was considered to be solely responsible. However, studies later identified a role for apoptosis in cell death during myocardial infarction[20]. Unlike necrosis, apoptosis (or programmed cell death) is a tightly-regulated, energy-dependent process. Intracellular signaling is crucial in regulating the initiation of apoptosis[22].

2.1 Hypoxia initiates JAK/STAT signaling

The impaired supply of oxygen to the ischemic region during myocardial infarction results in hypoxia, i.e. an oxygen deficit. This leads to impaired energy production - a serious threat to cardiomyocytes, which have a high metabolic demand. When cardiomyocytes are exposed to hypoxia, a stress-response is elicited, a number of cytokines are produced including interleukin-6 (IL-6)[23]. When IL-6 binds to its receptor (IL-6R) on neighboring cardiomyocytes, glycoprotein 130 (gp130) molecules can be recruited (see Figure 1). The association of two gp130 molecules is the final step in the formation of the cytokine receptor complex. Each gp130 molecule is constitutively associated with a JAK protein such that recruitment of two gp130 molecules brings two JAK proteins into close proximity. JAK proteins are intracellular kinases, which phosphorylate tyrosine residues in proteins. Four mammalian JAK proteins have been identified, namely JAK1, 2, 3 and Tyk2. Upon formation of the complete cytokine receptor complex, JAK proteins phosphorylate and activate

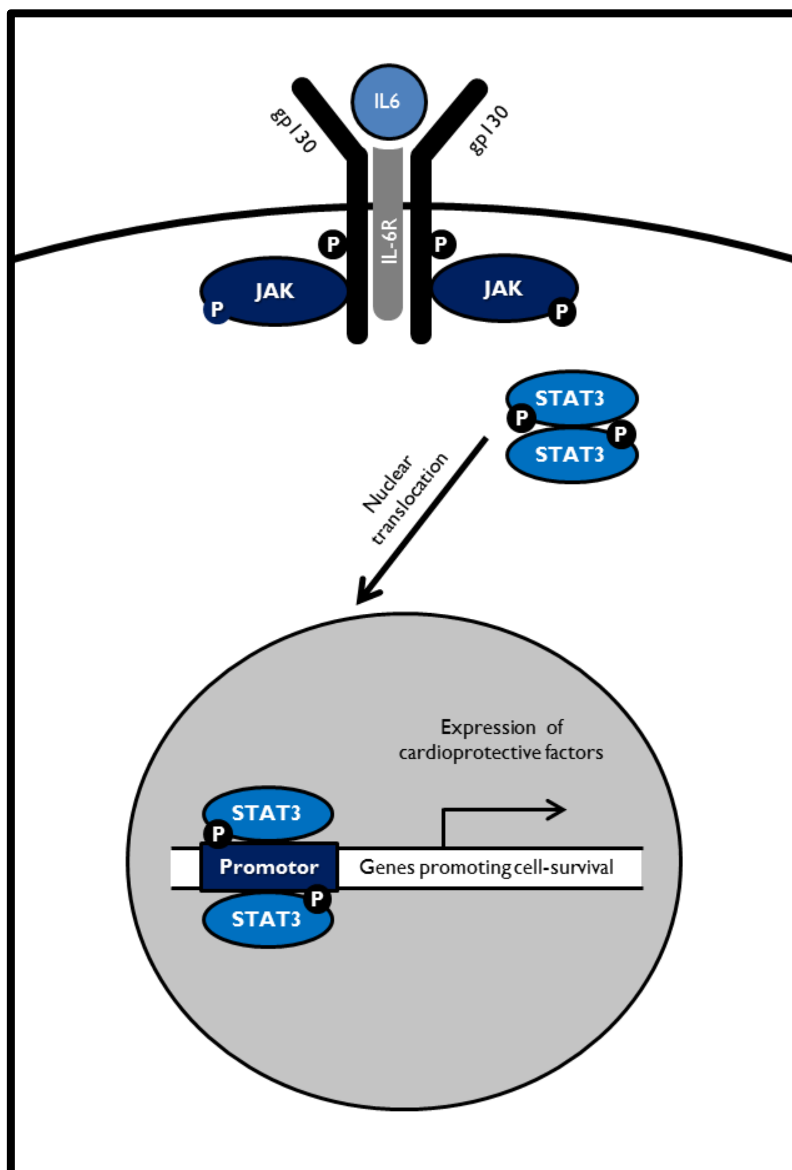

Figure 1: The initiation of JAK/STAT signaling in response to hypoxia. *IL-6 binding to its receptor recruits two gp130 molecules with their associated JAK proteins. JAK proteins phosphorylate themselves and the gp130 molecules, creating docking sites for STAT proteins to bind. Once they are bound, STAT proteins become phosphorylated before translocating into the nucleus and regulating expression of cardioprotective factors.* ***Abbreviations*** *gp130: glycoprotein 130, IL-6: interleukin-6, IL-6R: interleukin-6 receptor, JAK: Janus kinase, STAT3: signal transducer and activator of transcription.*

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themselves as well as intracellular tyrosine residues on gp130, creating docking sites for STAT proteins[15]. STAT proteins are cytosolic proteins, which are known for their role as transcription factors, which regulate transcription of a specific set of genes. Seven STAT proteins have been identified in mammals, namely STAT1, 2, 3, 4, 5a, 5b and 6. In the case of JAK/STAT signaling induced by IL-6, it is STAT3 that binds to the cytokine receptor complex. Once bound, STAT3 proteins are phosphorylated by activated JAK proteins and homodimerize. They then dissociate from the cell membrane and translocate to the nucleus in order to regulate the expression of genes that code for cardioprotective factors. For example, STAT3 activation can lead to increased production of vascular endothelial growth factor (VEGF), a cytokine that initiates angiogenesis[17]. Angiogenesis can oppose hypoxia by providing collateral blood (and oxygen) supply to the ischemic region. Thus, cardiomyocytes can counteract hypoxia and act to restore homeostasis by Il-6 release, JAK/STAT signaling and initiating angiogenesis.

In addition to opposing the threat of hypoxia by promoting angiogenesis, STAT3 is also associated with upregulation of anti-apoptotic genes such as *Bcl-2* and *Pim-1*, as well as downregulation of pro-apoptotic genes such as *Bax*[24]. In this way, JAK/STAT signaling functions to promote survival. In contrast, STAT1 has been linked to pro-apoptotic effects. This review specifically focuses on the role of JAK/STAT in pro-survival signaling, which is mediated by activation of STAT3.

The translocation of STAT3 to the nucleus and its role as a transcription factor are well-established. It was assumed that the sole function of STAT3 was regulating gene expression, however, an additional role was recently discovered. The identification of STAT3 in cardiomyocyte

mitochondria in 2009 led to the suggestion of its involvement in regulating respiration[25]. STAT3 was subsequently shown to translocate to the mitochondrion where it is thought to regulate the activity of complexes I and II of the electron transport chain[26]. In addition, it may inhibit opening of the mitochondrial permeability transition pore (mPTP), a non-selective channel of the inner mitochondrial membrane[27], and is proposed to act as an electron scavenger that reduces levels of reactive oxygen species (ROS)[28]. The consequences of these effects will be elaborated upon at a later stage since they act in concert with the effects of signaling via PI3K/Akt, another endogenous survival mechanism that exists in the heart.

2.2 Ischemia initiates PI3K/Akt signaling

In addition to inducing hypoxia and the associated IL-6 secretion, ischemia also leads to the release of adenosine by heart cells[18]. Binding of adenosine to its receptor on surrounding cells is associated with activation of the PI3K/Akt signaling pathway[19]. PI3K is a kinase that phosphorylates membrane-embedded phosphatidylinositol 4,5-bisphosphate (PIP2) to form phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 is responsible for recruiting Akt and 3-phosphoinositide-dependent protein kinase (PDK) to the membrane, bringing them into close proximity (see Figure 2). PDK, a constitutively active kinase, phosphorylates and activates Akt. Once Akt is phosphorylated, it leaves the cell membrane and prevents apoptosis by phosphorylating a wide range of proteins. Like STAT3, Akt can promote cell survival by effects that act at the mitochondrion as well as effects that act at the nucleus[29]. However, this review focuses primarily on its pro-survival effects at the mitochondrion.

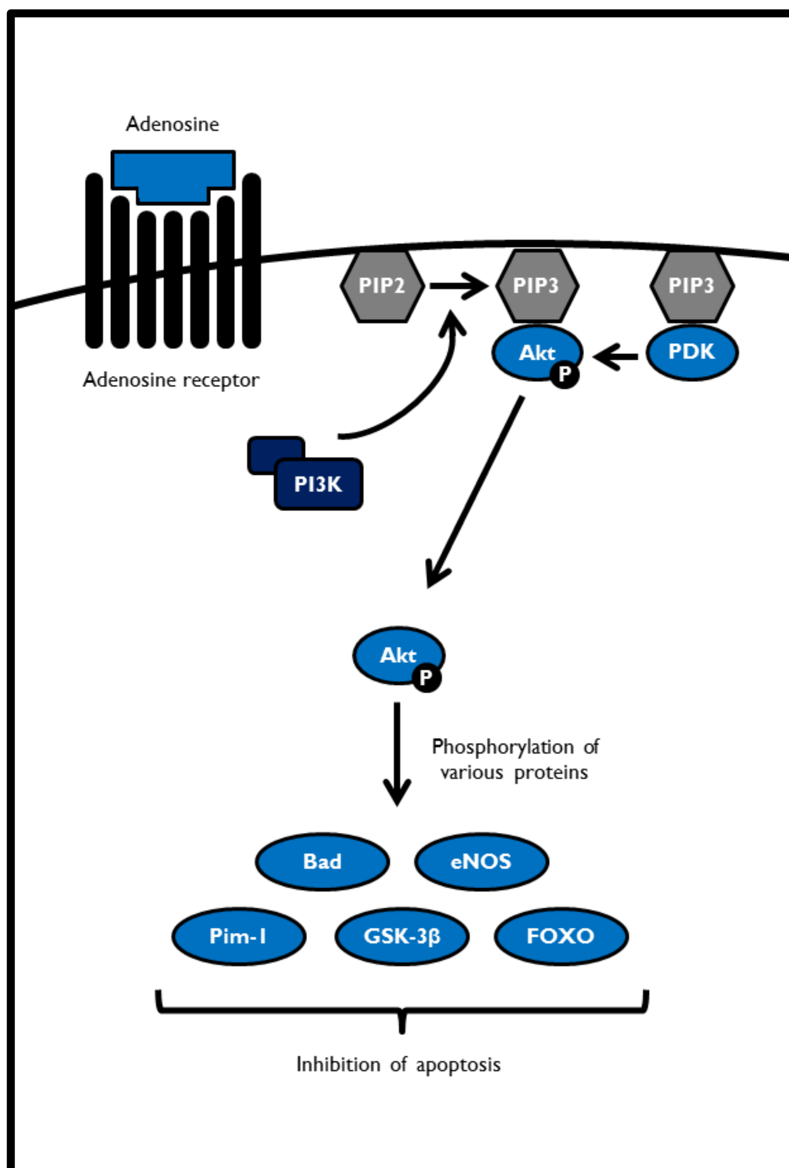

Figure 2: The initiation of PI3K/Akt signaling in response to ischemia. *Binding of adenosine to its receptor activates PI3K/Akt signaling. PI3K phosphorylates PIP2 to form PIP3, which recruits Akt and PDK to the membrane, bringing them into close proximity. PDK phosphorylates and activates Akt, which prevents apoptosis by phosphorylating proteins such as Bad, eNOS, Pim-1, GSK-3 and FOXO.* ***Abbreviations*** *eNOS: endothelial nitric oxide synthase, FOXO: forkhead box O, GSK-3ß: glycogen synthase kinase-3ß, PDK: 3-phosphoinositide-dependent protein kinase, PI3K: phosphatidylinositol 3-kinase, PIP2: phosphatidylinositol 4,5-bisphosphate, PIP3: phosphatidylinositol 3,4,5-trisphosphate.*

Activated Akt can prevent apoptosis by phosphorylating proteins such as Bad, a pro-apoptotic protein that is inactivated by phosphorylation[30]. Pim-1 can also be activated by Akt, enabling it to carry out anti-apoptotic actions of its own. These include phosphorylating and inactivating pro-apoptotic proteins and indirectly causing increases in anti-apoptotic proteins[31]. Akt can also influence gene expression through phosphorylation of transcription factors such as the forkhead box O (FOXO) proteins[32]. Phosphorylation of FOXO leads to its export from the nucleus, where it induces the expression of pro-apoptotic genes[33].

Thus, both the PI3K/Akt and JAK/STAT pathways can act to inhibit apoptosis via actions at the nucleus as well as actions at the mitochondria. Interestingly, the JAK/STAT and PI3K/Akt signaling pathways can act in concert.

2.3 JAK/STAT and PI3K/Akt cooperate to prevent apoptosis

Although both JAK/STAT and PI3K/Akt signaling can influence transcription, the following section will focus on their role in the mitochondria. Here the complementary nature of their effects are eloquently displayed.

A substantial body of evidence exists for cross-regulation between the PI3K/Akt and JAK/STAT signaling pathways. This is demonstrated in settings where PI3K is able to phosphorylate STAT3[34] and JAK2 is able to phosphorylate Akt after binding to PI3K[35]. Numerous examples exist where STAT3 activation is reported to be dependent on PI3K/Akt signaling or *vice versa,* and a number of cardioprotective stimuli activate both PI3K/Akt and

JAK/STAT signaling[36]. In addition, cases have been reported where inhibition of PI3K/Akt signaling reduced STAT3 phosphorylation in pulmonary artery endothelial cells[37] and inhibiting JAK/STAT signaling reduced Akt phosphorylation in cardiomyocytes[38]. Thus, a variety of facts support the notion that PI3K/Akt and JAK/STAT signaling are inter-regulated[39].

In addition to their inter-regulation, the notion of cooperation between these pathways is supported by their proposed convergence at the mitochondrion[40,41]. The mitochondrion is a double-membraned organelle, which is responsible for producing adenosine triphosphate (ATP) from NADH and $FADH_2$. In addition to this crucial role, mitochondria also have an important function in apoptosis. The intrinsic pathway of apoptosis is mediated by the mitochondria and the Bcl-2 family of apoptosis regulators[42]. This family of proteins can be divided into two groups: those that are anti-apoptotic (such as Bcl-x_L, Bcl-2 and Mcl-1) and those that are pro-apoptotic (such as Bad, Bax and Bak). These two groups of proteins are thought to antagonize one another until this dynamic balance is altered in favor of apoptosis. When this occurs, Bax and Bak oligomerize, forming pores that induce mitochondrial membrane permeabilization[43]. This leads to loss of membrane integrity, cytochrome c release, apoptosome formation and the initiation of apoptosis[42]. Another proposed means by which membrane integrity can be lost (causing release of pro-death factors and apoptosis initiation) is mPTP opening[44].

The initiation of apoptosis can be prevented by PI3K/Akt signaling in several ways (see Figure 3). Phosphorylation and inhibition of the pro-apoptotic protein Bad by activated Akt causes it to dissociate from the anti-apoptotic protein Bcl-x_L[30]. This increases the availability of Bcl-x_L proteins, which are freed to bind and inhibit other pro-apoptotic proteins.

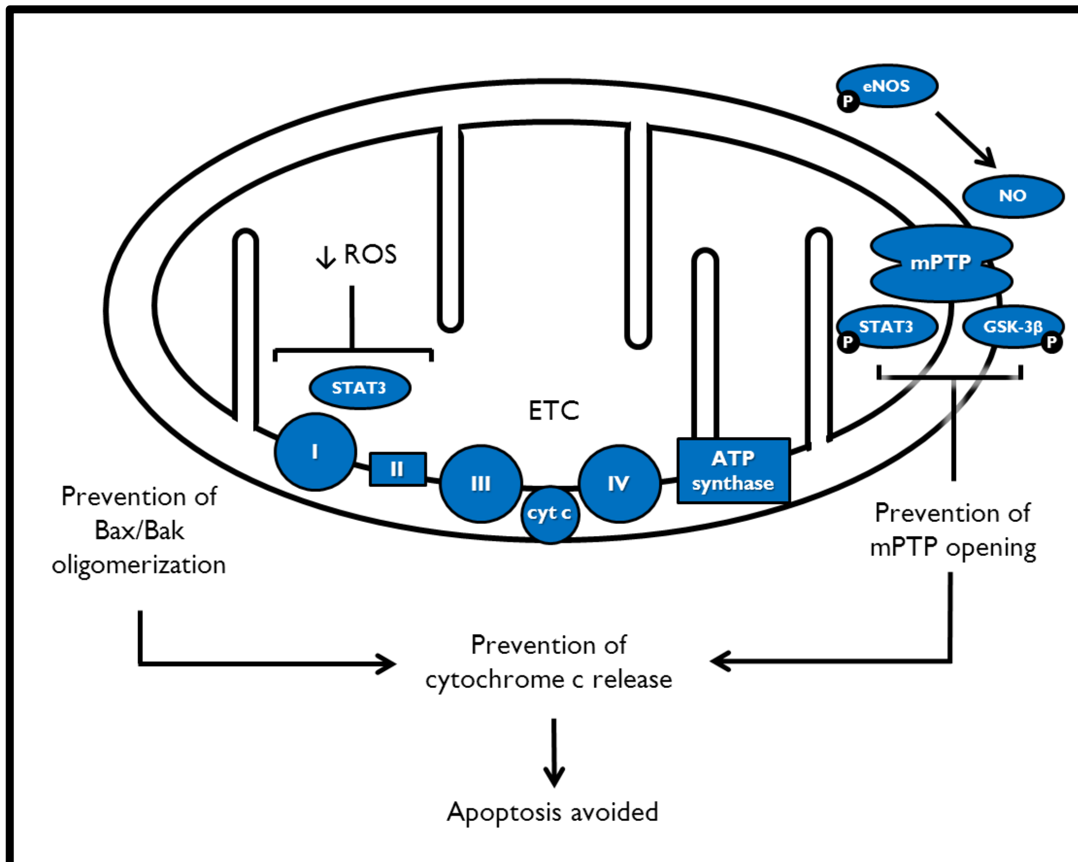

***Figure 3**: **The role of the PI3K/Akt and JAK/STAT signaling pathways in preventing apoptosis.** PI3K/Akt signaling leads to the prevention of Bax/Bak oligomerization. It also prevents opening of the mitochondrial permeability transition pore by inhibiting GSK-3β. JAK/STAT signaling and translocation of STAT3 to the mitochondrion is proposed to reduce ROS production by regulating the activity of complexes I and II of the electron transport chain. Attenuated ROS production and STAT3's proposed direct effect on the mPTP lead to the prevention of mPTP opening. Thus, PI3K/Akt and JAK/STAT signaling cooperate to prevent cytochrome c release and apoptosis initiation. **Abbreviations** cyt c: cytochrome c, eNOS: endothelial nitric oxide synthase, ETC: electron transport chain, GSK-3β: glycogen synthase kinase-3β, mPTP: mitochondrial permeability transition pore, NO: nitric oxide, PI3K: phosphotidylinositol 3-kinase, ROS: reactive oxygen species, STAT: signal transducer and activator of transcription.*

Phosphorylation of the pro-apoptotic protein Bax by activated Akt causes a change in its conformation, preventing its translocation to the mitochondrion. Bax phosphorylation may also act to promote its association with anti-apoptotic proteins. Activated Akt also causes phosphorylation and inhibition of glycogen synthase kinase-3β (GSK-3β), which leads to inhibition of mPTP opening and maintenance of the anti-apoptotic protein Mcl-1[45,46].

Activated Akt is also associated with phosphorylation and activation of endothelial nitric oxide synthase (eNOS), thereby restoring nitric oxide production, which is thought to prevent mPTP opening[47]. Therefore, PI3K/Akt signaling leads to several effects that oppose apoptosis and promote survival.

Similarly, JAK/STAT signaling also acts to prevent apoptosis. The translocation of STAT3 to the mitochondrion and its proposed regulation of complexes I and II are thought to be important in limiting electron leak from the electron transport chain and reducing superoxide production[40]. This, along with its suggested role as a ROS scavenger and its proposed direct effects on the mPTP[27] are thought to prevent mPTP opening. Therefore, signaling via PI3K/Akt and JAK/STAT can cooperate to prevent apoptosis by mutual regulation and by their converging effects at the mitochondria.

3. The penalty of reperfusion

Although PI3K/Akt and JAK/STAT signaling may assist cells in avoiding apoptosis, their capacity is limited. Restoration of blood flow (referred to as reperfusion) must eventually be initiated in order for cells

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to survive[48]. Reperfusion therapy is the primary means of treating myocardial infarction and it can be achieved in two ways. The first method is thrombolysis, which involves administering drugs to dissolve the offending blood clot(s). Another method is percutaneous coronary intervention, whereby a balloon-tipped catheter is inserted into the blocked artery and inflated to restore blood flow mechanically. The sooner reperfusion is initiated, the larger the region of myocardium that can be salvaged. However, paradoxically, the sudden restoration of blood flow can itself cause cell death, known as lethal reperfusion injury[49]. The following section will give a brief overview of the underlying pathology of lethal reperfusion injury.

The sudden restoration of blood flow at the onset of reperfusion induces a number of rapid changes (see Figure 4). These changes cause a series of perturbations at the level of the mitochondrion, which act in concert to cause cell death[48]. Firstly, the sudden restoration of oxygen and nutrient supply leads to a dramatic increase in ROS production by the electron transport chain. Excessive ROS damage the sarcolemma and impair function of the sarcoplasmic reticulum, causing calcium overload. Secondly, the removal of lactic acid, which accumulated during ischemia, and the activation of the sodium-hydrogen exchanger lead to the rapid restoration of normal pH. These changes act in concert to mediate opening of the mPTP[50].

During myocardial ischemia, the mPTP remains closed, but the sudden changes that occur at the onset of reperfusion lead to its opening. As aforementioned, opening of the mPTP leads to the initiation of apoptosis by cytochrome c release and apoptosome formation[49]. mPTP opening is thought to be a major determinant of lethal reperfusion injury[41].

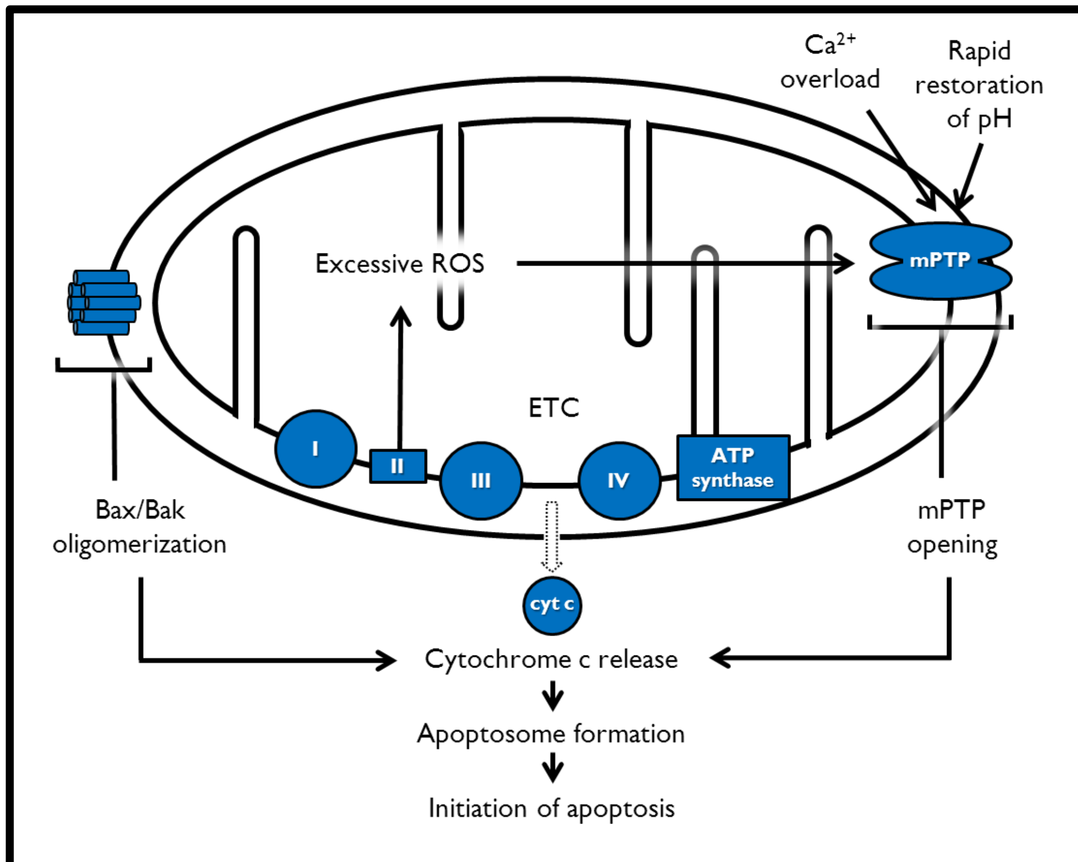

Figure 4: The underlying pathology of lethal reperfusion injury. *Restoration of oxygen and nutrient supply increases ROS production by the electron transport chain. Simultaneously, calcium levels increase and pH is rapidly restored. These changes cause opening of the mPTP, which is linked to cytochrome c release. Oligomerization of Bak or Bax is another stimulus thought to favor cytochrome c release. Cytochrome c release causes apoptosome formation and the initiation of apoptosis.* ***Abbreviations*** *cyt c: cytochrome c, ETC: electron transport chain, mPTP: mitochondrial permeability transition pore, ROS: reactive oxygen species.*

4. The promise of postconditioning

Postconditioning is a therapy that aims to reduce lethal reperfusion injury. It involves the induction of brief, alternating periods of ischemia and reflow at the onset of reperfusion. In this way, the restoration of blood flow is interrupted by intermittent periods of ischemia. In a clinical setting, this procedure would require the insertion of a balloon-tipped catheter as with percutaneous coronary intervention, and a series of alternating inflations and deflations of the balloon.

Since it was first applied in dogs in 2003, studies have demonstrated its effectiveness using various animal models[51]. The lengths of the alternating periods of ischemia and reperfusion typically vary between 1 and 3 minutes, depending on the species. The use of longer periods and the application of treatment later than the first few minutes of reperfusion proved ineffective. A reduction in infarct size was commonly selected as the primary end point and a clear role for postconditioning in reducing infarct size was soon established. Small-scale clinical trials also found postconditioning to be an effective treatment for myocardial infarction[52,53].

However, this experimental procedure is limited in terms of practicality and applicability. This procedure must be carried out within the first few minutes of reperfusion to be effective, which is challenging since most myocardial infarction patients do not have quick access to a facility for performing percutaneous coronary intervention. In addition, its benefit is impaired by the presence of comorbidities such as hyperglycemia, hypercholesterolemia and hypertension, all of which are common in patients undergoing treatment for myocardial infarction.

These limitations led researchers to investigate the underlying molecular mechanisms of postconditioning-induced cardioprotection, with the aim of pharmacologically activating them. Thus, 'pharmacological postconditioning' seeks to induce cardioprotection using a pharmacological agent that mimics the effects of traditional, mechanical postconditioning. This is a more accessible alternative with the potential to produce comparable results.

Postconditioning acts by initiating endogenous survival mechanisms in the heart. These mechanisms are referred to as the 'reperfusion injury salvage kinase (RISK) pathway' and the 'survivor activating factor enhancement pathway (SAFE). The RISK pathway encompasses signaling via the mitogen-activated protein kinase/ERK kinase (MEK)/extracellular signal-regulated kinase (ERK) 1/2 signaling pathway, i.e. the MAPK pathway, as well as the PI3K/Akt pathway[54]. The SAFE pathway involves the cytokine tumor necrosis factor-α (TNF-α) and STAT3[55,56]. Numerous studies have confirmed that the RISK and SAFE pathways confer cardioprotection when activated during reperfusion[22,57].

Despite a number of potential targets being investigated, no pharmacological postconditioning agent has gained clinical acceptance for the routine treatment of myocardial infarction as yet. Therefore, a need remains for a treatment able to mimic the effects of postconditioning, which can be implemented as a standardized treatment for myocardial infarction. Research within our group has identified a potential role for benfotiamine in pharmacological postconditioning. In the following section, the discovery and characteristics of benfotiamine will be described.

5. The therapeutic potential of benfotiamine

Benfotiamine is one of a number of thiamine (vitamin B1) derivatives (Figure 5) that have been investigated for therapeutic use. After allithiamine, a naturally-occurring thiamine derivative, was discovered in Japan in 1951[58], researchers synthesized a group of additional thiamine derivatives with improved bioavailability. They proceeded to assess the value of these compounds in treating various diseases[59].

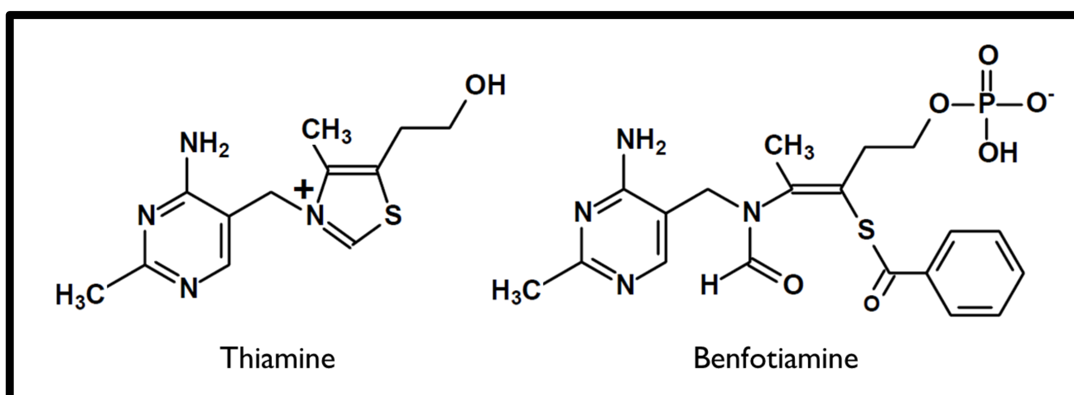

Figure 5**: **The chemical structure of thiamine and benfotiamine. *Benfotiamine is a benzoylated derivative of the phosphorylated form of thiamine.*

Benfotiamine, or S-benzoylthiamine *O*-monophosphate, has primarily been investigated as a treatment for diabetes-related nervous and cardiovascular disorders. This section focuses specifically on benfotiamine's beneficial effects on the heart. Firstly, the proposed mechanisms in diabetic cardiomyopathy are described and studies exploring benfotiamine as a treatment are discussed. Secondly, benfotiamine's potential in treating myocardial infarction is examined. Thirdly, the effects of benfotiamine on cardiac progenitor cells are described. Next, the therapeutic capacity of the

pentose phosphate pathway is explored. Finally, a potential role for benfotiamine in pharmacological postconditioning is described.

5.1 Treating diabetic cardiomyopathy

Diabetes is associated with various metabolic alterations, including hyperglycemia. Hyperglycemia, in turn, leads to a range of perturbations. In the heart, these perturbations can lead to systolic and diastolic dysfunction. When this occurs independently of atherosclerosis and hypertension, it is termed diabetic cardiomyopathy.

Recently, a unifying hypothesis was proposed describing mitochondrial superoxide as the cause of multiple hyperglycemia-induced perturbations[60–62]. Based on this hypothesis, a comprehensive explanation for the hyperglycemia-induced perturbations in diabetic cardiomyopathy was described[63]. In addition, benfotiamine was proposed as a comprehensive treatment that could counteract these perturbations[64]. These propositions will now be elaborated upon.

Hyperglycemia, or an elevated glucose concentration, is associated with increased mitochondrial superoxide (O^{2-}) production (Figure 6)[65]. This occurs as result of an overproduction of electron donors (NADH and $FADH_2$) by the citric acid cycle. Excessive mitochondrial superoxide is thought to cause DNA damage and activation of poly(ADP-ribose) polymerase (PARP), an enzyme that is responsible for DNA repair[62]. However, PARP activation can inhibit glyceraldehyde phosphate dehydrogenase (GAPDH), a key enzyme in glycolysis (the central pathway in glucose metabolism)[66]. This causes a build-up of glycolytic metabolites upstream of GAPDH, which diverge into non-oxidative glucose pathways[62].

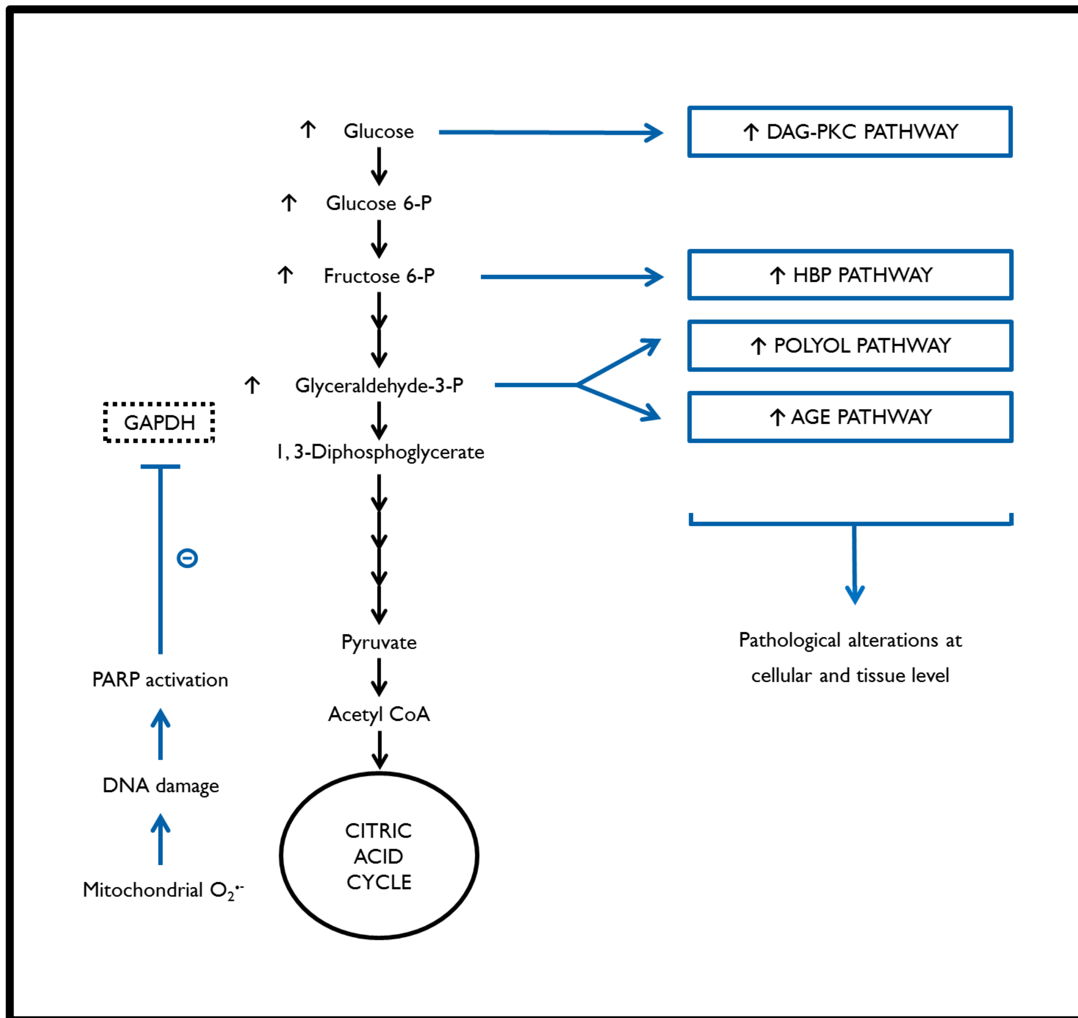

Figure 6: Hyperglycemia-induced perturbations involved in diabetic cardiomyopathy. *Under hyperglycemic conditions, mitochondrial superoxide overproduction leads to GAPDH inhibition. This leads to a build-up of upstream metabolites and excessive flux through non-oxidative glucose pathways (indicated by blue boxes). This leads to oxidative stress, systolic dysfunction and diastolic dysfunction.* ***Abbreviations*** *AGE: advanced glycation endproducts, DAG: diacylglycerol, GAPDH: glyceraldehyde phosphate dehydrogenase, HP: hexosamine biosynthetic, O_2^-: superoxide, PARP: poly(ADP-ribose) polymerase, PKC: protein kinase C.*

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More specifically, flux through the polyol pathway increases, there is an increase in advanced glycation end-product (AGE) formation, activation of protein kinase C (PKC) isoforms occurs and flux through the hexosamine biosynthetic pathway (HBP) is increased[67]. Excessive flux through these pathways in the heart, such as is thought to occur in hyperglycemia,can have detrimental effects[63].

Firstly, increased flux through the HBP causes impaired relaxation of the heart[68]. Secondly, excessive AGE formation is associated with reduced contractility, increased ventricular stiffness and impaired ventricular filling[69,70]. Thirdly, excessive flux through the polyol pathway exacerbates oxidative stress, promotes cardiomyocyte apoptosis and increases ventricular stiffness[71]. Finally, excessive activation of PKC isoforms leads to cardiac hypertrophy, impaired relaxation and increased ventricular stiffness[72]. Thus, hyperglycemia-induced perturbations are associated with oxidative stress, systolic dysfunction and diastolic dysfunction of the heart[63], which are characteristic of the diabetic cardiomyopathy[60].

In contrast, benfotiamine administration increases flux through the pentose phosphate pathway (Figure 7)[73,74]. This is thought to shunt glycolytic metabolites away from these non-oxidative glucose pathways, attenuating downstream negative effects (Figure 6). Flux though the pentose phosphate pathway increases production of ribose 5-phosphate, which is required for DNA repair, and NADPH, which regulates redox balance. The therapeutic potential of the pentose phosphate pathway will be elaborated upon at a later stage.

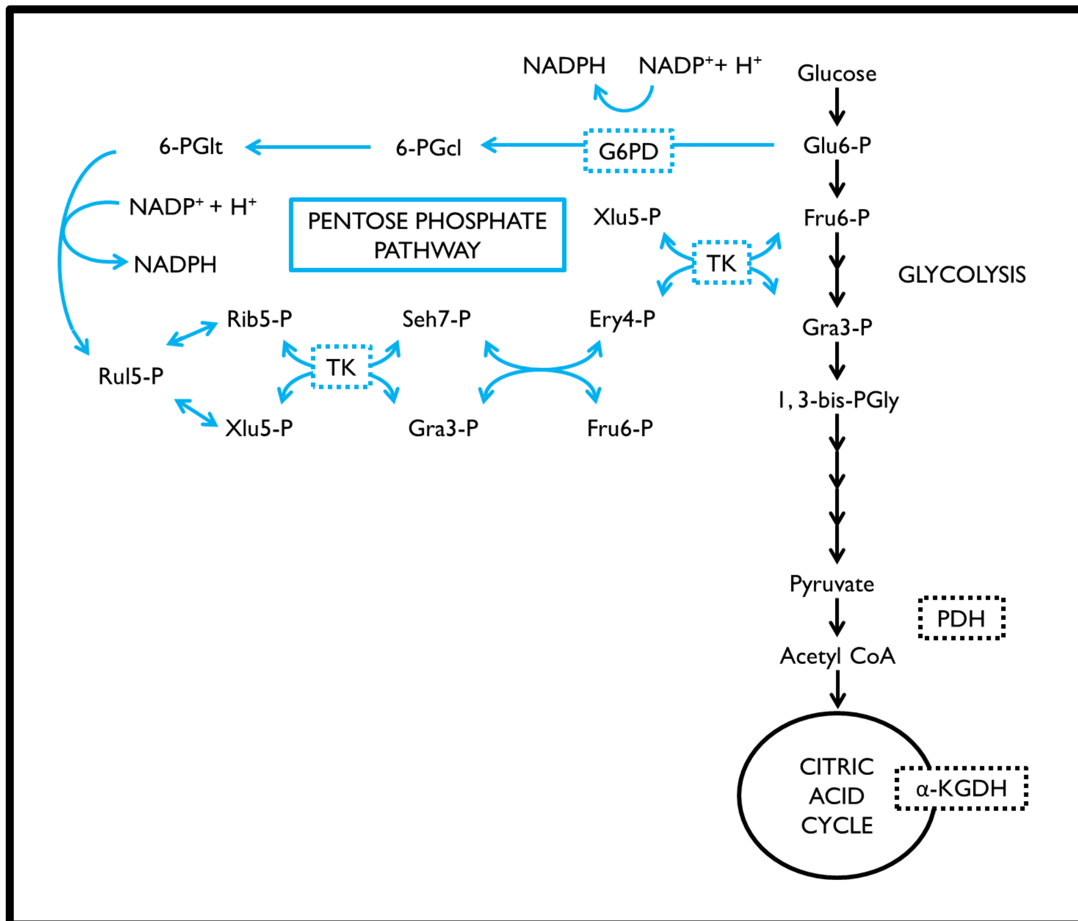

Figure 7: The role of benfotiamine in glucose metabolism. *Thiamine is a cofactor for TK, PDH and α-KGDH. Benfotiamine administration increases TK and G6PD activity, thereby increasing flux of glycolytic metabolites into the pentose phosphate pathway. This increases the production of ribose 5-phosphate and NADPH, which regulates redox balance.* ***Abbreviations*** *1,3-bis-PGly: 1,3-bisphosphoglycerate, 3-PGra: glyceraldehyde 3-P, 6-PGcl: 6-P gluconolactone, 6-PGlt: 6-P gluconate, α-KGDH: α-ketoglutarate dehydrogenase. Ery4-P: erythrose 4-P, Fru6-P: fructose 6-P, G6PD: glucose 6-phosphate dehydrogenase, Glu6-P: glucose 6-P, Gra3-P: glyceraldehyde 3-P, PDH: pyruvate dehydrogenase, Rib5-P: ribose 5-P, Rul5-P: ribulose 5-P, Seh7-P: sedoheptulose 7-P, TK: transketolase, Xlu5-P: xylulose 5-P.*

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Benfotiamine's ability to treat diabetic cardiomyopathy was tested in three studies. The first study was carried out to investigate the roles of AGEs, the AGE receptor (RAGE), and methylglyoxal (the key intermediate in AGE formation) in diabetic cardiomyopathy. Six weeks of benfotiamine treatment in mice attenuated diabetes-induced increases in collagen, methylglyoxal, RAGE and methylglyoxal-derived AGE formation in the heart[75].

The second study evaluated the effects of benfotiamine treatment on diabetes-induced contractile dysfunction. Control and diabetic mice received benfotiamine treatment for two weeks prior to cardiomyocyte isolation. Benfotiamine treatment counteracted diabetes-induced contractile dysfunction and reduced oxidative stress, as reflected by the reduced glutathione-to-glutathione disulfide ratio (GSH:GSSG)[76].

A third study was designed to investigate the efficacy of long-term benfotiamine supplementation (8 or 16 weeks) in preventing diabetic cardiomyopathy in mice. Hearts of untreated diabetic mice showed reduced activity of transketolase (TK), glucose 6-phosphate dehydrogenase (G6PD) and GAPDH. Furthermore, ROS production and cardiomyocyte apoptosis were elevated. The progression of diabetic cardiomyopathy was associated with reduced phosphorylation of STAT3, Akt, eNOS, FOXO and Bad, in addition to reduced levels of Pim-1 and Bcl-2. Benfotiamine prevented all of these alterations. Inhibition of Akt ablated benfotiamine's anti-apoptotic effects, but STAT3 inhibition had no effect. This suggests that benfotiamine's pro-survival effects are mediated by Akt. The investigators concluded that benfotiamine warrants consideration for clinical application[77].

These studies indicate that benfotiamine supplementation may have therapeutic potential as a treatment for diabetic cardiomyopathy. In addition, a potential role for benfotiamine in preventing apoptosis was identified. This pro-survival role has also been investigated in the context of myocardial infarction.

5.2 Treating myocardial infarction

Hyperglycemia is common during myocardial infarction. Chronic hyperglycemia is characteristic of diabetes mellitus, a disease with a high global prevalence, especially amongst myocardial infarction patients. In addition, acute hyperglycemia occurs during myocardial infarction in non-diabetic patients, as result of sympathetic nervous system activation in response to stress. The presence of hyperglycemia during myocardial infarction is associated with increased mortality[78]. Two studies have investigated the role of benfotiamine in treating myocardial infarction, with and without hyperglycemia.

The first study addressed the effects of chronic benfotiamine supplementation on the consequences of ischemia ± chronic hyperglycemia. In addition, the activation of pro-survival signaling was assessed[13]. In non-diabetic mice, ischemia activated an endogenous survival mechanism involving activation of the pentose phosphate pathway enzymes TK and G6PD. This was associated with vascular endothelial growth factor receptor 2 (VEGFR2)/Akt signaling and increased levels of pAkt, Pim-1, pBad and Bcl-2. This survival mechanism was impaired in diabetic mice but could be restored by benfotiamine supplementation, resulting in improved survival, reduced oxidative stress, enhanced functional recovery, improved

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angiogenesis, attenuated mitochondrial dysfunction and decreased apoptosis[13]. These findings were complemented by experiments in isolated cardiomyocytes, where benfotiamine treatment activated and preserved VEGFR2/Akt/Pim-1 signaling under hypoxic and hyperglycemic conditions.

Therefore, ischemia or long-term benfotiamine supplementation can activate a survival mechanism in the heart involving activation of enzymes of the pentose phosphate pathway and VEGFR2/Akt/Pim-1 signaling.

The second study, performed by our group, addressed the effects of acute benfotiamine administration on the consequences of ischemia-reperfusion ± acute hyperglycemia. In addition, the effects of flux through the non-oxidative glucose pathways was assessed[12]. Isolated rats hearts that underwent retrograde perfusion under hyperglycemic conditions displayed increased flux through the polyol and AGE pathways, which was associated with exacerbated oxidative stress and increased cell death. Acute benfotiamine administration at the onset of reperfusion counteracted these alterations. Hearts were exposed to ischemia and reperfusion under hyperglycemic conditions, resulting in reduced TK activity, greater PARP activation, reduced GAPDH, elevated oxidative stress and increased apoptosis. Acute benfotiamine administration once again counteracted these alterations. Interestingly, benfotiamine administration also improved functional recovery and reduced infarct size after ischemia-reperfusion, under both hypoglycemic and normoglycemic conditions.

To summarize, acute benfotiamine administration was beneficial in restoring normal glucose metabolism and attenuating hyperglycemia-induced perturbations, thereby preventing oxidative stress. In addition, benfotiamine administration reduced infarct size and improved functional recovery after

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ischemia-reperfusion, under both normoglycemic conditions and hyperglycemic conditions.

Thus, treatment with benfotiamine, chronically or acutely, can prevent the worsening of ischemia-induced dysfunction by the presence of hyperglycemia. In addition, benfotiamine treatment is associated with the activation of an endogenous survival mechanism, reduced infarct size and improved functional recovery in response to ischemia, independent of hyperglycemia-related effects. These effects warrant further investigation.

5.3 Restoring cardiogenesis

As previously discussed, cell death resulting from myocardial infarction impairs contractile function. The remaining cells are faced with a significantly increased workload, which threatens their survival. A limited number of cardiac progenitor (or stem) cells reside in the heart, which are able to differentiate into cardiomyocytes, smooth muscle cells, and vascular endothelial cells. A clinical trial recently demonstrated the ability of infused cardiac progenitor cells to increase viable heart tissue and improve restoration of function after myocardial infarction[79]. However, this crucial cardiac repair mechanism is compromised in diabetes.

A recent study found reduced abundance and proliferation of cardiac progenitor cells in diabetic mice[80]. Cardiac progenitor cells isolated from these mice displayed impaired TK and G6PD activity, higher levels of superoxide and AGEs, and inhibited Akt/Pim-1/Bcl-2 signaling. Human and mouse cardiac progenitor cells cultured in high glucose showed similar perturbations, which were associated with increased apoptosis. Benfotiamine

administration restored pentose phosphate pathway enzyme activity and cardiac progenitor cell availability and function *in vivo* and *in vitro.*

5.4 Activating the pentose phosphate pathway

The pentose phosphate pathway is most well-known for its role in producing NADPH and ribose 5-phosphate, which are essential in maintaining cellular redox balance and DNA repair. However, it has an important additional role linked to G6PD (refer to Figure 7). G6PD, the first and rate-limiting enzyme of the pentose phosphate pathway, has been associated with an endogenous survival mechanism, which prevents apoptosis and promotes angiogenesis. This will be described after a brief overview of the role of the pentose phosphate pathway in regulating cellular redox balance.

The pentose phosphate pathway is the principal intracellular source of NADPH, which plays a vital role in maintaining cellular redox balance. It has a dual function in building oxidative capacity and producing oxidative species by NADPH-dependent enzymes such as NADPH-oxidase. The antioxidant system, composed primarily of the glutathione system, catalase and superoxide dismutase, requires NADPH to function. Thus, activation of the pentose phosphate pathway may attenuate oxidative stress by increasing antioxidant capacity. However, the opposite may also be true, that is, increased pentose phosphate pathway flux may enhance ROS production by NAPDH oxidase. Of note, activation of the pentose phosphate pathway (via G6PD activation), has been linked to additional effects that are independent of NADPH production.

Increased oxidative stress can stimulate G6PD activity in rat cardiomyocytes, leading to G6PD translocation to the plasma membrane[81]. G6PD is necessary for phosphorylation and activation of VEGFR2, Akt and eNOS. VEGFR2/Akt/eNOS signaling resulted in VEGF-stimulated angiogenesis[82]. In addition, VEGFR2/Akt/Pim-1 signaling induced by ischemia (via activation of TK and G6PD) was associated with reduced apoptosis.

Therefore, when exposed to oxidative stress (associated with hyperglycemia or ischemia), cardiac cells may activate an endogenous survival mechanism involving G6PD activation and translocation to the cell membrane, leading to VEGFR2/Akt signaling. This would act to maintain redox status (as described earlier), promote cell-survival and initiate restorative angiogenesis. In support of this, G6PD overexpression is associated with increased NADPH levels and improved resistance to oxidative stress[83], while partially G6PD-deficient mice exhibit increased cardiac dysfunction following ischemia-reperfusion[81].

This mechanism appears to be compromised in diabetes; however, benfotiamine shows promise in activating and restoring it. Thus, benfotiamine may have therapeutic potential as an activator of the pentose phosphate pathway and G6PD/VEGFR2/Akt pro-survival/pro-angiogenic signaling. In this way, benfotiamine would attenuate oxidative stress (associated with hyperglycemia or ischemia-reperfusion) and reduce cell death.

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5.5 Pharmacological postconditioning

Previous work by our group showed that benfotiamine administration at the onset of reperfusion could preserve contractile function and reduce infarct size in response to ischemia-reperfusion. Benfotiamine may have therapeutic potential as a pharmaceutical postconditioning agent that activates endogenous survival mechanisms in the heart. Therefore, we set out to investigate this by assessing the cardioprotective effects of acute benfotiamine administration and the involvement of the PI3K/Akt and JAK/STAT signaling pathways.

6. Summary

Myocardial infarction is a major cause of mortality that requires effective treatment. It leads to cardiomyocyte death, which is associated with contractile dysfunction. However, the heart possesses a number of endogenous survival mechanisms that it can activate in response to threats such as ischemia and hypoxia (see Figure 8). These include signaling via the JAK/STAT and PI3K/Akt pathways (initiating cell survival responses coordinated by the nucleus and mitochondria) and activation of the pentose phosphate pathway (associated with maintenance of redox balance and activation of pro-survival/pro-angiogenic signaling). Activation of these pathways is impaired in diabetes.

Cell death due to myocardial infarction can be reduced by timeous reperfusion. However, the initiation of reperfusion can itself cause cell

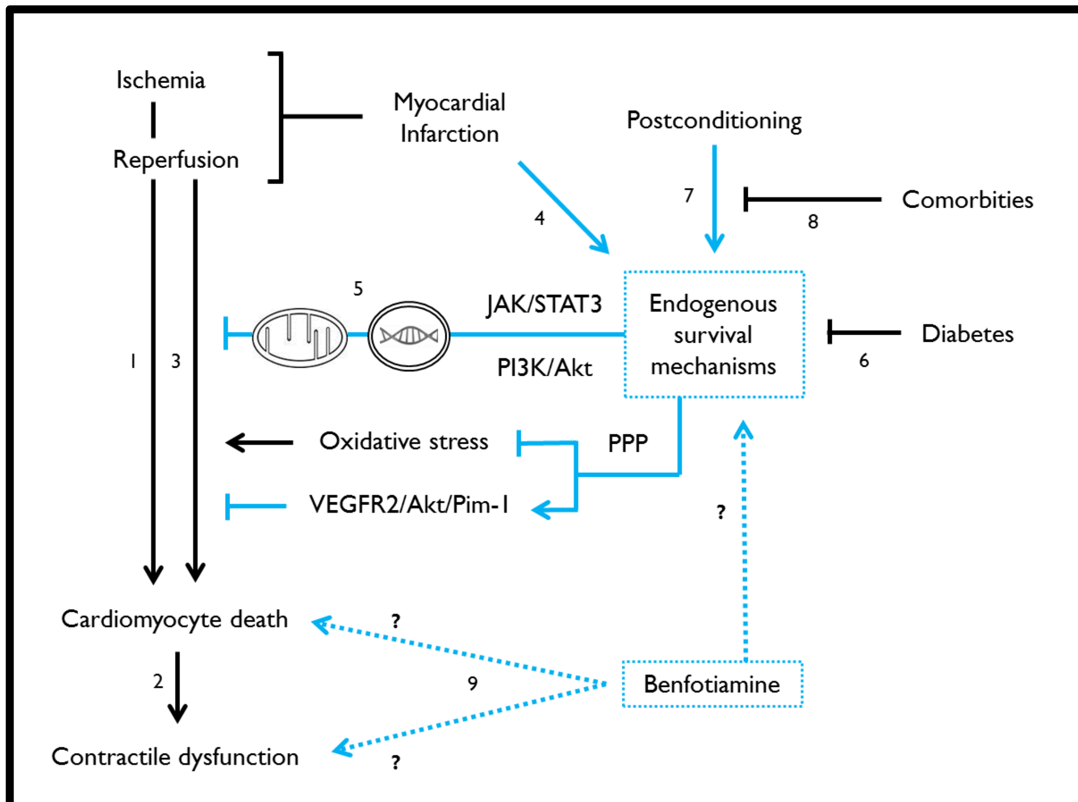

Figure 8: SUMMARY - Endogenous survival mechanisms in myocardial infarction. *1) Ischemia can result in cardiomyocyte death, which 2) impairs contractile function. The sooner blood flow is re-established, the larger the proportion of the myocardium that can be salvaged. However, 3) the initiation of reperfusion can itself cause cardiomyocyte death. When confronted with a threat, cells 4) initiate endogenous survival mechanisms, such as 5) JAK/STAT3 and PI3K/Akt signaling, which limit cell death via actions at the nucleus and mitochondrion. These survival mechanisms can be 6) impaired by diabetes or 7) activated by postconditioning. However, 8) postconditioning is impaired by comorbidities. 9) Benfotiamine may act as a pharmacological postconditioning agent that reduces cardiomyocyte death and preserves contractile function.* ***Abbreviations*** *JAK: Janus kinase, PI3K: phosphatidylinositol 3-kinase, PPP: pentose phosphate pathway, STAT: signal transducer and activator of transcription, VEGFR2: vascular endothelial growth factor receptor 2.*

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death. Postconditioning is able to attenuate lethal reperfusion injury via activation of JAK/STAT and PI3K/Akt signaling. However, it is challenged by limited accessibility and the impairment of its effects in the presence of comorbidities. A pharmacological agent that is able to mimic the effects of postconditioning may contribute significantly to the treatment of myocardial infarction. We propose benfotiamine as a potential candidate.

7. Hypothesis

Benfotiamine protects contractile function and reduces infarct size when administered during reperfusion via activation of the PI3K/Akt and JAK/STAT signaling pathways.

8. Aims

I. To determine whether benfotiamine administration at the onset of reperfusion is associated with 1) improved functional recovery and 2) reduced infarct size in response to ischemia-reperfusion

II. To test whether the effects of benfotiamine are mediated by activation of signaling via the 1) PI3K/Akt and/or 2) JAK/STAT pathways

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CHAPTER THREE: Materials and Methods

We proceeded to test our hypothesis using two approaches. In part one of the study, Langendorff isolated heart perfusions with regional ischemia and Evans blue/triphenyltetrazolium chloride (TTC) staining were used to test the effect of benfotiamine on functional recovery and infarct size after ischemia-reperfusion. Inhibitors of the PI3K/Akt and JAK/STAT pathways were employed to investigate the involvement of these pathways in mediating benfotiamine's effects.

Part two of the study involved Langendorff isolated heart perfusions with global ischemia, which were performed with the aim of producing tissues that had been subjected to varying protocols. These tissues were used to evaluate the effects of ischemia-reperfusion and benfotiamine on PI3K/Akt and JAK/STAT signaling by Western blot analysis.

The use of animals and experimental procedures in the study were reviewed and approved by the Animal Research Ethics Committee of the Faculty of Natural Sciences at Stellenbosch University [10NF_ESS01A].

Values are expressed as mean ± standard error of the mean (SEM). Functional data were analyzed using a repeated measures analysis of variance (ANOVA) with a Bonferroni post-test. Infarct size was analyzed with Tukey's multiple comparison as a post-hoc test. $P < 0.05$ was considered to be statistically significant. Analyses were done with GraphPad Prism, version 5 (San Diego, California).

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1. Benfotiamine's effects on functional recovery and infarct size - the role of pro-survival signaling

The first part of the study involved Langendorff isolated heart perfusions with regional ischemia. Functional recovery was assessed and Evans blue/TTC staining was used to evaluate infarct size after ischemia-reperfusion. Benfotiamine was administered to determine its effect on functional recovery and infarct size. Wortmannin, a PI3K inhibitor, and Tyrphostin AG490, a JAK2 inhibitor, were used to investigate the involvement of the PI3K/Akt and JAK/STAT signaling pathways in mediating these effects.

1.1 Langendorff isolated heart perfusions

Male Wistar rats (180-220 g) were euthanized with sodium pentobarbital (100 mg/kg, by intraperitoneal injection). Hearts were rapidly excised and immersed in ice-cold Krebs-Henseleit buffer to establish hypothermic cardioplegia, thereby preventing ischemic damage before perfusion could be established. Retrograde perfusion was initiated via cannulation of the aorta. The heart was perfused at constant hydrostatic pressure (100 cm H_2O ≈ 75 mmHg) with Krebs-Henseleit buffer, equilibrated with 95% O_2 and 5% CO_2 (119.4 mM NaCl, 24.9 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 0.6 mM $MgSO_4 \cdot 7H_2O$, 0.6 mM Na_2SO_4, 1.2 mM $CaCl_2 \cdot 2H_2O$, 11.0 mM glucose). The temperature of the heart was maintained at 37 ± 0.5°C by means of a temperature-controlled water jacket and monitored by insertion of a K-type implantable temperature probe into the right atrium.

1.2 Recording functional parameters

A fluid-filled, latex balloon connected to a pressure transducer was inserted into the left ventricle and adjusted until the left ventricular end diastolic pressure was 5-15 mmHg. Measurements of cardiac function (diastolic pressure, systolic pressure, heart rate, dP/dt) were recorded throughout using ADInstruments™ LabChart Pro, version 7 (New South Wales, Australia). Coronary flow rate (CFR) was measured by timed collection of coronary effluent.

Left ventricular developed pressure (LVDP) was obtained by calculating the difference between left ventricular end diastolic pressure and systolic pressure. The rate-pressure product (RPP) was calculated by multiplying heart rate with systolic pressure. Hearts that failed to establish an LVDP of at least 50 mmHg were excluded from the study.

1.3 Experimental protocol

After 20 minutes of stabilization, regional ischemia was induced by passing a 3-0 silk suture around the left anterior descending coronary artery 1-2 mm below the intersection of the pulmonary conus and left atrial appendage. The ends of the suture were threaded through a small plastic tube to form a snare, and tightened. After 30 minutes of ischemia, the snare was released to initiate reperfusion. The efficacy of ischemia was confirmed by regional cyanosis and a significant decrease in coronary flow. Various treatments were administered for the first 20 minutes of reperfusion.

Hearts were randomly assigned to one of five groups (Figure 9):

1. ***Treatment control*** — no intervention
2. ***Benfotiamine*** — 100 µM benfotiamine
3. ***Vehicle control*** — $2{\cdot}10^{-5}$ % (v/v) dimethyl sulfoxide (DMSO)
4. ***Benfotiamine + Tyrphostin AG490*** — 100 µM benfotiamine and 0.1 µM Tyrphostin AG490
5. ***Benfotiamine + Wortmannin*** — 100 µM benfotiamine and 0.1 µM Wortmannin

Wortmannin and Tyrphostin AG490 were obtained from ENZO Life Sciences (Mountain View, USA). Both treatments were dissolved in DMSO as 10 mM stock solutions and stored at -80°C. Wortmannin and AG490 were administered dissolved in Krebs-Henseleit buffer with a final concentration of 0.1 µM, consistent with dosages used in related studies[34,84,85]. Group 4 experiments were carried out in semi-darkness with the treatment reservoir and tubing covered in aluminium foil due to the light-sensitive nature of Tyrphostin AG490 . Wortmannin was added to the buffer immediately before administration due to its short half-life in aqueous solution. DMSO did not exceed $2{\cdot}10^{-5}$ % (v/v).

Benfotiamine was ordered from Sigma Aldrich (St. Louis, USA) and administered by dissolving it in Krebs-Henseleit buffer. A concentration of 100 µM was selected based on dose-response curves previously conducted by ourselves[12] and others[64].

All treatments were dissolved in Krebs-Henseleit buffer to the required final concentrations on the day of the experiment.

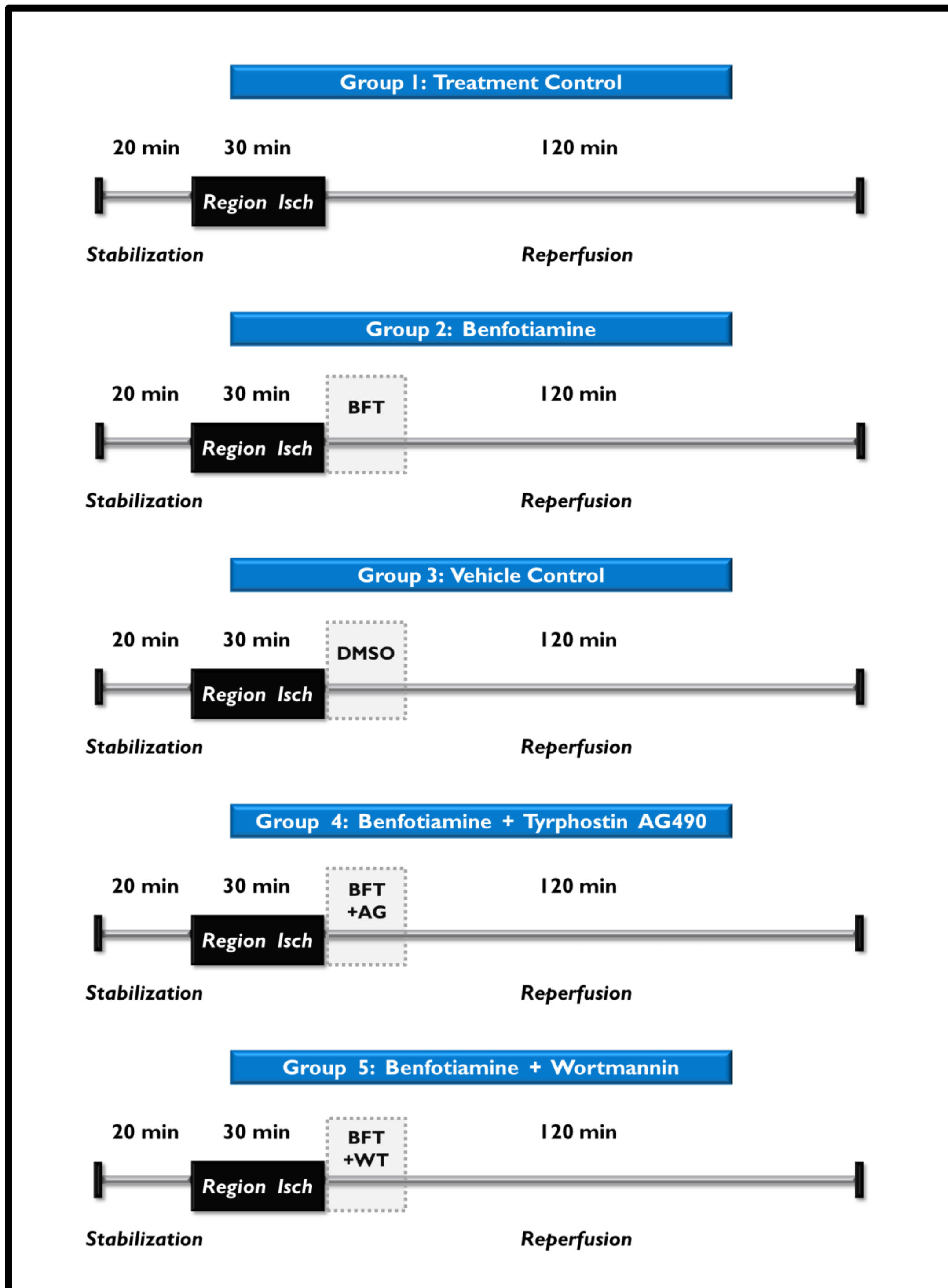

Figure 9: Experimental protocol - Part one. *Hearts underwent regional ischemia and reperfusion ± benfotiamine ± Tyrphostin AG490 or Wortmannin.* ***Abbreviations*** *Region Isch: regional ischemia, BFT: benfotiamine, DMSO: dimethyl sulfoxide, AG: Tyrphostin AG490, WT: Wortmannin.*

1.4 Determination of infarct size

Two hours of reperfusion were allowed to ensure wash-out of dehydrogenase enzymes and cofactors from non-viable tissue. At the end of reperfusion, the left anterior descending coronary artery was re-occluded by tying the suture securely. The heart was infused with 2 ml of 2% Evans Blue dye to demarcate the area at risk (i.e. the region exposed to ischemia-reperfusion). The heart was then weighed and frozen at -4°C overnight.

On the following day, hearts were defrosted slightly and cut transversely into four 2 mm slices. Slices were added to a 50 ml Falcon tube with 5 ml 1% triphenyltetrazoilum chloride (TTC) in phosphate buffer (pH 7.4) and incubated at 37°C using a waterbath. 15-20 minutes were allowed for the tetrazolium salt to react with dehydrogenase enzymes and cofactors in viable tissue to form a formazan pigment. This pigment enabled living tissue (stained deep red) to be distinguished from dead tissue (pale-colored) within the area at risk. The tubes were agitated regularly to ensure even exposure to TTC.

After TTC staining, slices were immersed in 10% formalin for 20 minutes to enhance image contrast, before being scanned. ImageJ, version 1.46r (National Institute of Health, USA) was used to measure the area at risk (as indicated by absence of Evans blue staining) and infarcted area (as indicated by a lack of Evans blue and TTC staining). Infarct size was expressed as a percentage of the area at risk.

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2. The effects of ischemia and benfotiamine on pro-survival signaling

The second part of the study involved Western blot analysis to assess activation of PI3K/Akt and JAK/STAT signaling in response to ischemia-reperfusion and benfotiamine. In order to produce tissues that had been exposed to ischemia and benfotiamine, as well as appropriate controls, Langendorff isolated heart perfusions with global ischemia were performed.

2.1 Langendorff isolated heart perfusions

Langendorff isolated heart perfusions were carried out as before (see 1.1).

2.2 Experimental protocol

After 90 minutes of stabilization, global ischemia was induced, followed by 40 minutes of reperfusion. A shorter reperfusion period was selected than in our previous protocol, since the tissues were used for biochemical analysis, but the total perfusion periods were of similar length. Benfotiamine was administered for the first 20 minutes of reperfusion.

Hearts were randomly assigned to one of four groups (Figure 10):

1. ***Non-ischemic control***	not exposed to ischemia
2. ***Non-ischemic benfotiamine***	not exposed to ischemia, 100 μM benfotiamine
3. ***Ischemic control***	30 minutes of global ischemia
4. ***Ischemic benfotiamine***	30 minutes of global ischemia, 100 μM benfotiamine

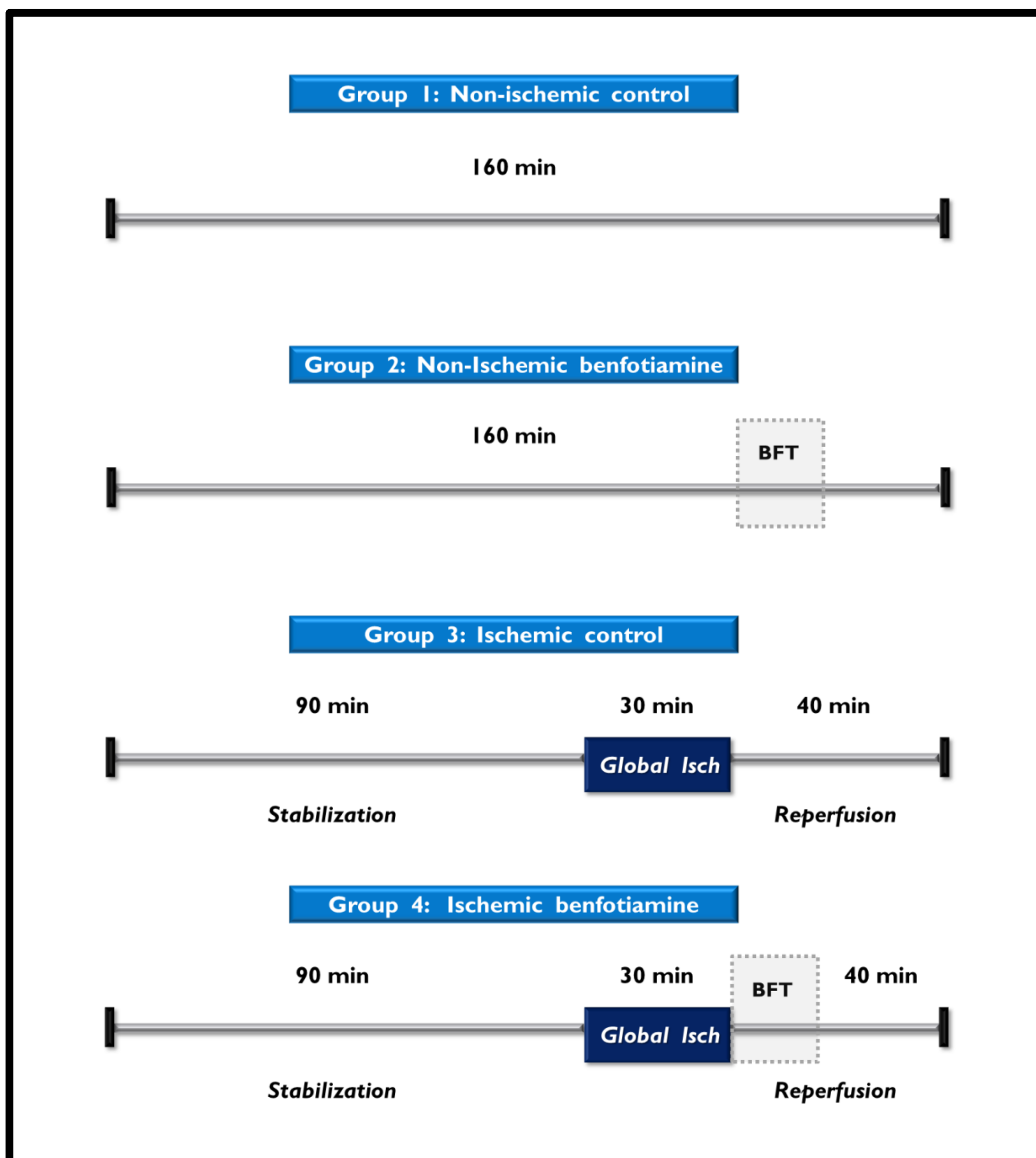

Figure 10: Experimental protocol - Part two. *Hearts were perfused ± global ischemia and ± benfotiamine.* ***Abbreviations*** *Global Isch: global ischemia, BFT: benfotiamine.*

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At the end of reperfusion, hearts were freeze-clamped with Wollenberger tongs cooled in liquid nitrogen and immersed briefly into liquid nitrogen until frozen. This was done in order to stop metabolic activity and preserve the integrity of macromolecules. Hearts were then divided into smaller sections and stored at -80°C for biochemical analysis.

2.3 Protein extraction

Frozen tissues were thawed on ice. For each heart, cytosolic proteins were isolated from some sections and nuclear proteins were extracted from other sections (as described in Appendix A). A Bradford protein assay was performed to determine the concentration of protein in each sample (Appendix B) and samples were prepared for Western blot analysis (Appendix C).

2.4 Western blot analysis

In order to assess activation of JAK/STAT signaling in response to ischemia-reperfusion and benfotiamine treatment, we used STAT3 as a marker. Secondary antibodies for STAT3 and pSTAT3 (Tyr705) (Cell Signaling Technology) were used to assess the relative quantities of these proteins in the nucleus and cytosol. We calculated the ratio of pSTAT3 to STAT3 in each compartment. Since phosphorylation of STAT3 by JAK2 causes activation and nuclear localization[16], an increased pSTAT3/STAT3 ratio in the nucleus would imply increased JAK/STAT3 signaling.

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To assess the activation of PI3K/Akt signaling in response to ischemia-reperfusion and benfotiamine treatment, FOXO1 was used as a marker. Secondary antibodies for FOXO1 and pFOXO1 (Ser256) (Cell Signaling Technology) were used to assess the relative quantities of these proteins in the nucleus and cytosol. We calculated the ratio of pFOXO1 to FOXO in both compartments. Since phosphorylation of FOXO1 by Akt causes deactivation and nuclear export[32], an increased pFOXO1/FOXO1 ratio in the cytosol would suggest increased PI3K/Akt signaling.

Western blot analysis was carried out as described in Appendix D and images were analyzed using ImageJ, version 1.46r (National Institute of Health, USA).

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CHAPTER FOUR: Results

Functional recovery

As shown in Table 1, the morphometric characteristics of rats were consistent among experimental groups.

Table 1: Morphometric characteristics of experimental rats

	n	Weight (g)	Heart weight (g)
TCon	8	210.70 ± 2.9	1.15 ± 0.07
BFT	8	204.44 ± 2.5	0.98 ± 0.03
VCon	8	206.21 ± 3.0	0.97 ± 0.04
BFT+AG	8	207.11 ± 3.4	1.03 ± 0.05
BFT+WT	8	206.33 ± 3.4	1.04 ± 0.05

TCon: Treatment control, BFT: Benfotiamine, VCon: Vehicle control, BFT+AG: Benfotiamine + Tyrphostin AG490, BFT+WT: Benfotiamine + Wortmannin. Values are mean ± SEM.

We investigated benfotiamine's effects on the recovery of cardiac function post-ischemia, by assessing a range of functional parameters. Values for rate-pressure product (RPP) and dP/dt before and after ischemia are shown in Table 2.

RPP, an indicator of cardiac workload or oxygen consumption, was increased at the onset of reperfusion in response to addition of Tyrphostin AG490 and Wortmannin. The maximum rate of increase in left ventricular pressure, dP/dt, which is a measure of contractility, did not differ between groups.

Table 2: Recovery of rate-pressure product and dP/dt

	Baseline		Reperfusion	
	RPP[1]	+dP/dt[2]	RPP[1]	+dP/dt[2]
TCon	7975 ± 601.0	2875 ± 164.7	2215 ± 321.6	1567 ± 132.8
BFT	5422 ± 1173	2423 ± 239.9	4128 ± 824.3	1554 ± 189.3
VCon	7986 ± 839.6	3057 ± 324.3	8203 ± 677.0	2000 ± 467.9
BFT+AG	10980 ± 3107	3414 ± 938.0	10780 ± 1118*	2555 ± 649.4
BFT+WT	11820 ± 977.7	2966 ± 861.2	9368 ± 2719*	2764 ± 378.9

*Baseline: values at 20-minute time point during stabilization, Reperfusion: values at 15-minute time point during reperfusion. TCon: Treatment control, BFT: Benfotiamine, VCon: Vehicle control, BFT+AG: Benfotiamine + Tyrphostin AG490, BFT+WT: Benfotiamine + Wortmannin. Values are mean ± SEM. [1]RPP: rate-pressure product (mmHg·10^3/min), [2](mmHg·10^3/sec). Values are mean ± SEM. *$p<0.05$ vs. treatment control at the same time point. n=8.*

Left ventricular developed pressure, heart rate and coronary flow rate are shown in Figure 11. No significant changes were seen in any of these parameters.

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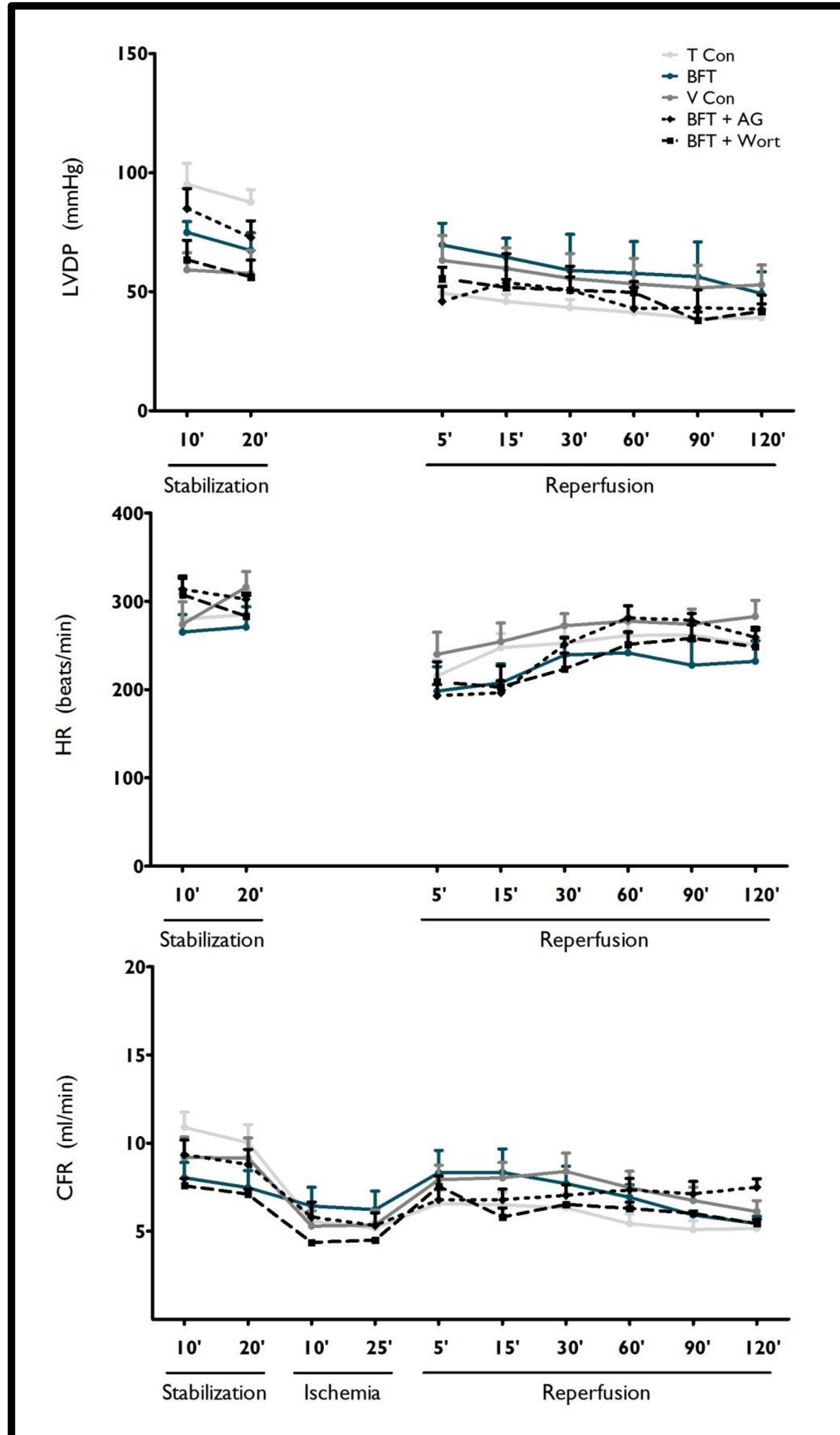

Figure 11: Functional recovery. *LVDP: left ventricular developed pressure, HR: heart rate, CFR: coronary flow rate, VCon: Vehicle control, BFT+AG: Benfotiamine + Tyrphostin AG490, BFT+WT: Benfotiamine + Wortmannin.*

Infarct size

Cell death was assessed by means of Evans blue/TTC staining. As shown in Figure 12, the area at risk did not differ between groups, indicating that the changes observed in infarct size between groups were not due to differences in the size of the region exposed to ischemia.

Benfotiamine administration led to a decrease in infarct size from 55.7 ± 5.0% to 35.6 ± 2.4% ($p<0.05$). Inhibition of PI3K/Akt signaling by addition of Wortmannin abrogated this infarct-limiting effect (35.6 ± 2.4% vs. 51.5 ± 1.3%, $p<0.05$). However, inhibition of JAK/STAT signaling had no significant effect.

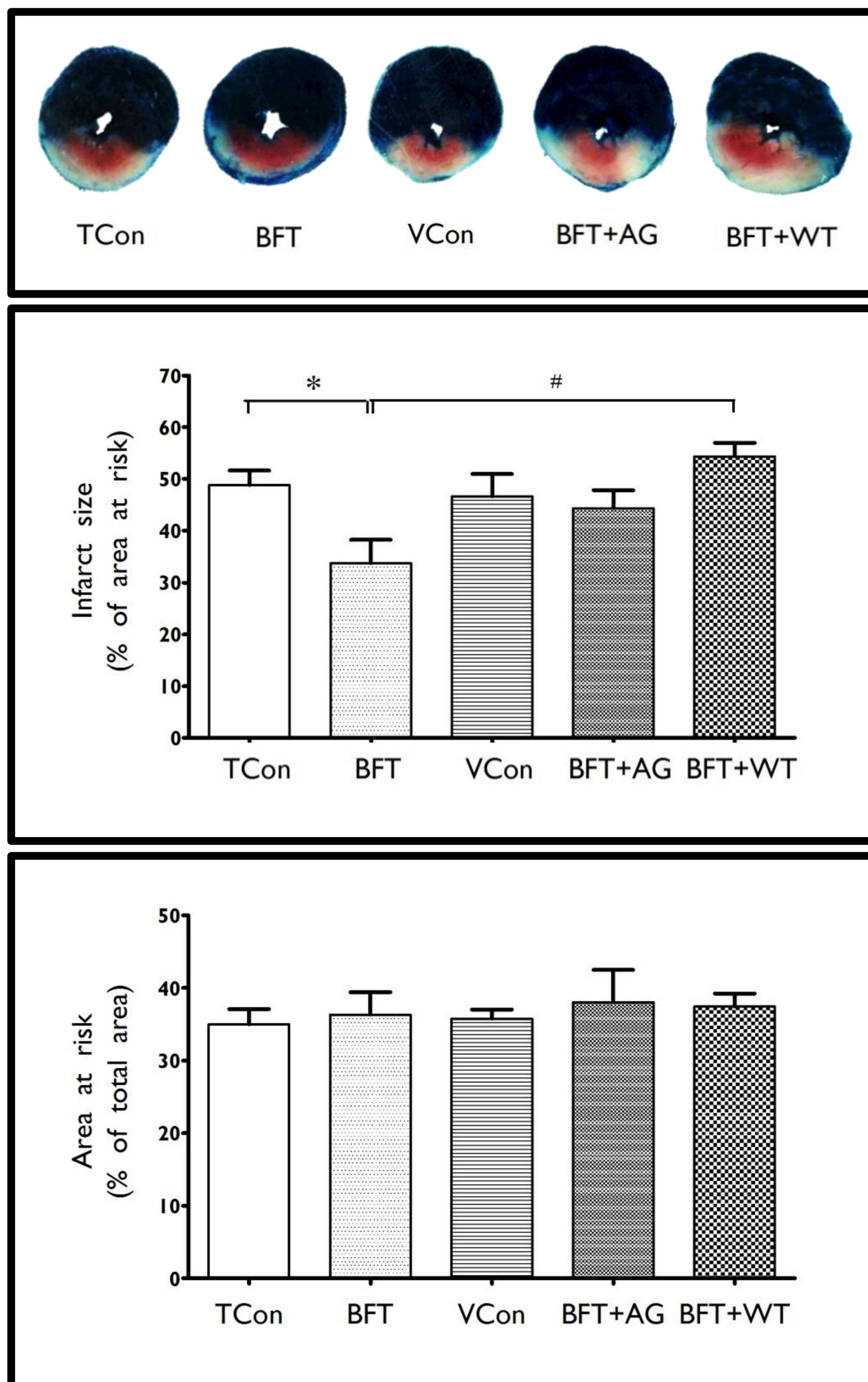

Figure 12: Infarct size. *TCon: Treatment control, BFT: Benfotiamine, VCon: Vehicle control, BFT+AG: Benfotiamine + Tyrphostin AG490, BFT+WT: benfotiamine + Wortmannin. *P<0.05 vs. TCon, #P<0.05 vs. BFT. N=8.*

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Pro-survival signaling

Tissues that were perfused ± ischemia-reperfusion and ± benfotiamine administration were assessed by Western blot analysis. pFOXO/FOXO was assessed in the cytosol as an indication of PI3K signaling. pSTAT3/STAT3 was assessed in the nucleus as an indication of JAK/STAT3 signaling. A sample size of four was used.

PI3K/Akt signaling as assessed by pFOXO/FOXO

As seen in Figure 13, the ratio of pFOXO/FOXO increased in ischemic hearts treated with benfotiamine. Ischemia or benfotiamine exposure had no significant effect on their own.

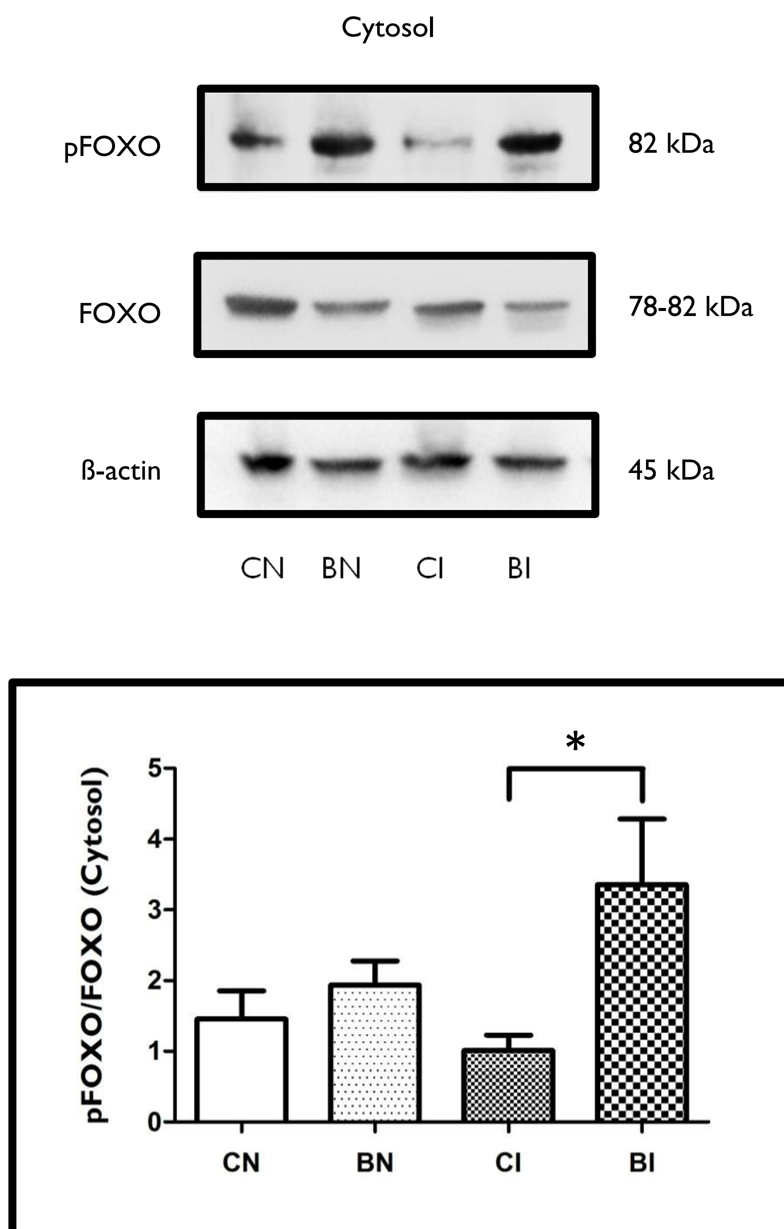

Figure 13: pFOXO/FOXO in the cytosol. *CN Non-ischemic control, BN: Non-ischemic benfotiamine, CI: Ischemic control, BI: Ischemic benfotiamine.*

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JAK/STAT signaling as assessed by pSTAT3/STAT3

The effects of ischemia and benfotiamine treatment on pSTAT3/STAT3 in the nucleus are depicted in Figure 14. There were no significant changes.

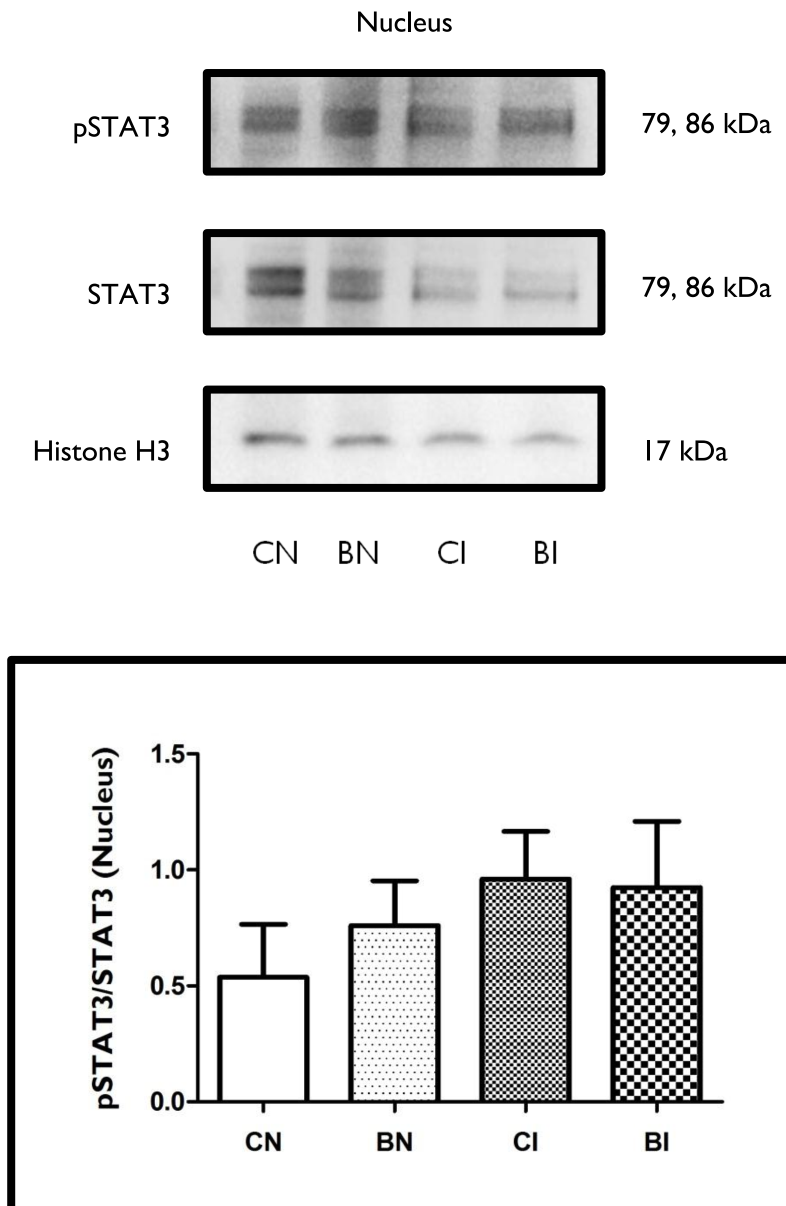

Figure 14: pSTAT3/STAT3 in the nucleus. *CN Non-ischemic control, BN: Non-ischemic benfotiamine, CI: Ischemic control, BI: Ischemic benfotiamine.*

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CHAPTER FIVE: Discussion

Benfotiamine's effect on functional recovery and infarct size - the role of pro-survival signaling

Contractile function

We assessed the effects of acute benfotiamine administration (at the onset of reperfusion) on functional recovery of the rat heart. Inhibitors of PI3K/Akt or JAK/STAT signaling were administered in addition to benfotiamine, to investigate a role for these pathways in mediating benfotiamine's effects. With the exception of an increased rate-pressure product in response to Tyrphostin AG490 or Wortmannin administration, no significant differences were found in any of the functional parameters assessed. This is in contrast to a number of related studies.

The effects of benfotiamine on cardiac contractile function were first investigated in a study by Ceylan Isik *et al.*[76] Two weeks of benfotiamine administration reduced contractile dysfunction in cardiomyocytes isolated from streptozotocin-induced diabetic mice. A second study, by Katare *et al*[77], found that 8 or 16 weeks of benfotiamine supplementation improved diastolic and systolic function in streptozotocin-induced type 1 or leptin-receptor mutant type 2 diabetic mice. In a third study, performed by the same laboratory[13], 6 weeks of benfotiamine supplementation significantly attenuated functional deterioration of hearts after myocardial infarction in both non-diabetic and diabetic mice. In each of these studies, benfotiamine

was administered by chronic supplementation, unlike in the present study, which involved acute benfotiamine administration.

A recent study, performed by our own laboratory[12], also made use of acute benfotiamine administration at the onset of reperfusion. An increase in the recovery of LVDP after global ischemia and reperfusion were induced by 20 minutes of benfotiamine administration. In comparison, the lack of significant changes seen in the present study, may be related to the specific protocols employed (regional ischemia, as opposed to global ischemia as in our previous study).

To summarize, in the current study acute benfotiamine administration at the onset of reperfusion after regional ischemia did not lead to significant changes in functional recovery between groups. This may suggest that the time of benfotiamine exposure and the extent of ischemia were not severe enough to allow for marked differences between groups in the functional parameters assessed.

Cell death

The effect of benfotiamine on cardiac cell death was initially examined in the context of diabetic cardiomyopathy. In Katare's study[77] with streptozotocin-induced type I or leptin receptor-mutant type 2 diabetic mice, benfotiamine supplementation was also linked to prevention of cardiomyocyte death. This pro-survival effect was concomitant with the restoration of Akt/Pim-1 signaling and levels of pBad and Bcl-2, as well as the restoration of STAT3-induced Pim-1 expression and the modification of

Pim-1 downstream effectors. This suggests that benfotiamine's anti-apoptotic effects may be mediated by signaling via Akt, STAT3 and Pim-1.

In vitro experiments demonstrated that benfotiamine administration could also counteract hyperglycemia-induced reductions in the levels of pSTAT3, pAkt, peNOS, and pFOXO, as well as Akt activity and nuclear localization. Of note, inhibition of PI3K and Akt abrogated Benfotiamine's anti-apoptotic effect, but STAT3 inhibition had no effect on apoptosis. Authors suggest that the induction of pAkt by benfotiamine may have compensated for STAT3 inhibition.

Additional studies found that benfotiamine supplementation reduced cardiomyocyte[13] and cardiac progenitor cell apoptosis[80]. In both cases, G6PD and VEGFR2 were involved, in addition to Akt, Pim-1, Bad and Bcl-2, in agreement with the previous study[73].

As previously discussed, these studies made use of chronic benfotiamine supplementation. However, earlier findings from our laboratory using acute benfotiamine administration were in accordance with these studies[12]. A reduction in infarct size was observed under hyperglycemic and normoglycemic conditions. This compelled us to investigate the underlying mechanisms of this effect.

In the current study, benfotiamine was administered with and without inhibitors of the PI3K/Akt and JAK/STAT pathways. Acute benfotiamine administration at the onset of reperfusion decreased infarct size from 55.7 ± 5.0% to 35.6 ± 2.4%. This effect was counteracted by inhibition of PI3K/Akt but not inhibition of JAK/STAT signaling. Thus the infarct-sparing effects of benfotiamine may not depend on JAK/STAT signaling, but appears to be mediated by PI3K/Akt.

A reduction in infarct size is expected to be accompanied by an improvement in functional recovery. However this is not always the case since the cells that were salvaged may not return to normal functioning immediately. Furthermore, a study by Lochner *et al.*[86] found that infarct size is a more reliable endpoint than functional recovery. Therefore the contrasting results from our functional recovery and infarct size measurements are not necessarily contradictory.

However, the increase in infarct size caused by administering Wortmannin in addition to benfotiamine, does not necessarily indicate attenuation of benfotiamine-induced cardioprotection. Increased cell death as result of PI3K inhibition may also be related to attenuation of endogenous survival PI3K/Akt signaling (independent of activation by benfotiamine). Therefore, we used an additional approach to test this finding.

The effects of ischemia and benfotiamine on pro-survival signaling

PI3K/Akt signaling

In the current study, an increase in pFOXO/FOXO was found in the cytosol in response to benfotiamine treatment following ischemia. An increase in PI3K/Akt signaling is associated with FOXO phosphorylation and nuclear export. Therfore, this suggests that in ischemic hearts, benfotiamine treatment activated PI3K/Akt signaling. Benfotiamine had no effect on non-ischemic tissue, while ischemia had no significant effect on untreated tissue i.e. ischemia or benfotiamine treatment in isolation was insufficient to activate PI3K/Akt signaling. However, in ischemic, treated hearts, these

stimuli may have acted synergistically to initiate PI3K/Akt signaling, an endogenous survival mechanism in the heart.

Other studies have found increased Akt phosphorylation in response to ischemia[9] or chronic benfotiamine administration[13,77,80]. These findings are corroborated by two studies in non-cardiac tissues. Gadau *et al.*[87] reported that 4 weeks of benfotiamine administration leads to Akt-mediated pro-survival and pro-angiogenic effects in ischemic diabetic mouse limbs. Furthermore, in a study by Marchetti and colleagues[88], benfotiamine administration restored endothelial progenitor cell proliferation via activation of Akt/FOXO1 signaling.

Therefore, 20 minutes of benfotiamine administration at the onset of reperfusion may reduce infarct size via activation of PI3K/Akt signaling.

JAK/STAT signaling

We did not find activation of JAK/STAT3 signaling in response to ischemia in our study. Increased JAK/STAT3 signaling has been found by others in response to ischemia and reperfusion in isolated rat hearts[89]. However, heart tissue was obtained at the end of a two-hour reperfusion period, in contrast to our study where tissues were obtained at the 40-minute time point during reperfusion.

Furthermore, we found that benfotiamine administration did not increase JAK/STAT3 signaling at the 40-minute time point during reperfusion. Benfotiamine may not activate JAK/STAT3 under these conditions or its activation may be transient. In addition, the fact that Tyrphostin AG490 administration had no significant effect on infarct size,

may be because STAT3 activation has effects which are not fully realized in the time duration or context of our study.

Using tissues obtained at a number of other time points during reperfusion (such as 10, 15, 20 and 60 minutes) in addition to those obtained at the 40-minute time point, may add valuable insight.

Katare *et al.* found STAT3 activation in the response to chronic benfotiamine supplementation in the context of diabetic cardiomyopathy[77]. Benfotiamine also reduced hyperglycemia-induced apoptosis, but this effect was not abrogated by STAT3 inhibition. Thus, benfotiamine activation in the heart in response to benfotiamine administration may be time- and/or context-dependent, leading to effects that may not directly reduce apoptosis.

Alternatively, since the increase in STAT3 activation in diabetic, treated mice during their study did not exceed levels in the non-diabetic mice, benfotiamine may have simply restored normal STAT3 activation by reducing hyperglycemia-related perturbations. In comparison, Akt and FOXO activation levels in the diabetic, treated mice did exceed levels in the non-diabetic group. The inclusion of a control group with non-diabetic, benfotiamine-treated mice would have allowed for comparison of STAT3 activation with non-diabetic, untreated mice.

Conclusion

Currently available evidence suggests that chronic benfotiamine supplementation reduces apoptosis in the heart, by activating PI3K/Akt signaling, an endogenous survival mechanism. This study found that this effect may also be induced by acute benfotiamine administration at the

onset of reperfusion following ischemia. These effects deserve further investigation since acute benfotiamine administration may have clinical applicability as a pharmacological postconditioning agent.

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Appendix A: Protein Extraction from Tissue

MATERIALS AND EQUIPMENT

Tweezers

Aluminium foil

Liquid nitrogen

Mortar and pestle

Modified radioimmuno-precipitation (RIPA) buffer

Tissue homogenizer

Homogenizing tubes

Micro-centrifuge

Vortex

Buffer A

Buffer B

PREPARING REAGENTS

100 ml modified RIPA buffer

50 mM	**Tris-HCl**	buffering agent, prevents protein denaturation
1%	**Nonidet-P40**	non-ionic detergent to extract proteins, 10% stock solution in H_2O
0.25%	**Sodium deoxycholate**	ionic detergent to extract proteins, 10% stock solution in H_2O, protect from light

1 mM	**Phenylmethyl-sulfonyl fluoride (PMSF)**	cholinesterase inhibitor/serine and cysteine protease inhibitor, 200 mM stock solution in isopropanol, store at room temperature
1 mM	**EDTA**	calcium chelator, 100 mM stock solution in H_2O, pH 7.4
1 µg/ml	**Leupeptin**	store frozen in aliquots, 1 mg/ml in H_2O
1 mM	**Activated Na_3VO_4**	tyrosine protein phosphatase inhibitor, 200 mM stock solution in H_2O
1 mM	**Sodium flouride**	serine/threonine protein kinase inhibitor, 200 mM stock solution (room temperature)
4 µg/ml	**Soyabean trypsin inhibitor**	1 mg/ml in 0.01 M phosphate buffer, pH 6.5 with 0.15 M NaCl (freeze aliquots at -20°C)
1 mM	**Benzamidine**	200 mM stock solution dissolved in dH_2O – store at -20^{0}C

I. Add 790 mg Tris base to 75 ml dH_2O. Add 900 mg NaCl and stir until solids are dissolved. Using HCl, adjust the pH to 7.4

II. Add 10 ml of 10% Nonidet-P40 to the solution

III. Add 2.5 ml of 10% Na-deoxycholate and stir until solution is clear

IV. Add 1 ml of 100 mM EDTA to the solution. Adjust the volume of the solution to 100 ml using a graduated cylinder. Aliquot and store at 2-8°C

V. On the day, add the remaining protease, phosphatase and trypsin inhibitors (100 µl of leupeptin, 500 µl of PMSF, Na_3VO_4, benzamidine, sodium flouride and soyabean trysin inhibitor). PMSF should be added immediately before use due its short half-life in aqueous solution (approximately 30 minutes)

100 x HEPES stock solution (100mM)

Dissolve 0.100 g HEPES in 100 ml dH_2O. pH to 7.9 on ice and store at 4°C

100 x Buffer A (hypotonic buffer) stock solution

- 150 mM $MgCl_2$
- 1 M KCL
- 50 mM dithiothreitol
- 20 mM PMSF

Make up in 1 L with dH_2O and store at 4°C

100 x Buffer B (high salt buffer) stock solution

- 25% glycerol
- 42 M NaCl
- 150 mM $MgCl_2$
- 20 mM EDTA
- 50 mM dithiothreitol
- 20 mM PMSF

Working solutions of Buffers A and B

Buffer A: add 10 ml Buffer A stock and 1 ml HEPES stock solution, make up to 50 ml with dH_2O in a Falcon tube and store at 4°C

Buffer B: add 10 ml Buffer B stock and 2 ml HEPES stock solution, make up to 50 ml with dH_2O Falcon tube and store at 4°C

METHOD

I. Use tweezers to wrap each piece of tissue sample in a square of aluminium foil (with foil folded 4 times) and place in liquid nitrogen

II. Use a mortar and pestle to pulverise the frozen sample under liquid nitrogen, producing a powder that is amenable to homogenization

III. Carefully unwrap the foil and, keeping cold, transfer the powdered tissue into tubes containing RIPA buffer – aim to make homogenates that are one third tissue and two thirds lysis buffer

IV. Homogenize tissues using a tissue homogenizer

V. Transfer the homogenates to fresh, pre-chilled Eppendorf tubes and keep on ice

FOR CYTOSOLIC FRACTION

VI. Centrifuge homogenates at 12000 rpm for 15 minutes at 4°C

VII. Transfer the supernatants (protein lysates) to fresh, pre-chilled Eppendorf tubes and store at -80°C

FOR NUCLEAR EXTRACT

VI. Bring homogenates to the cold room and centrifuge for 10 seconds on the highest setting

VII. Decant supernatant and resuspend pellet in 400 μL cold Buffer A

VIII. Leave on ice for 10 minutes to swell

IX. Vortex for 10 seconds and centrifuge for 10 seconds

X. Discard supernatant and resuspend pellet in 100 μL cold Buffer B

XI. Leave for 2 minutes on ice and centrifuge for 2 minutes

XII. Transfer the supernatant to fresh, pre-chilled Eppendorf tubes and store at -80°C

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Appendix B: Bradford Protein Determination

MATERIALS AND EQUIPMENT

Bovine serum albumin (BSA)

Bradford reagent

Spectrophotometer

PREPARING REAGENTS

5 x Bradford reagent stock solution

- Dilute 500 mg Coomassie brilliant blue G250 in 250 ml 95% ethanol
- Add 500 ml phosphoric acid, mix thoroughly
- Adjust the volume to 1 L with dH_2O
- Filter solution and store at 4°C protected from light

Bradford reagent

- Dilute stock solution with dH_2O
- Filter with two sheets of filter paper (solution is red/brown and turns blue when binding to protein)

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METHOD

- Switch on the spectrophotometer in advance to allow time for it to calibrate
- Thaw BSA stock solution (200 µg/ml, stored at -20 °C) and the protein samples on ice (keep on ice throughout)
- Mark 7 tubes for the protein standards and 2 for each sample
- Prepare a dilution series as follows:

BSA	dH_2O	Conc.
0	100 µl	Blank
10 µl	90 µl	2 µg
20 µl	80 µl	4 µg
40 µl	60 µl	8 µg
60 µl	40 µl	12 µg
80 µl	20 µl	16 µg
100 µl	0 µl	20 µg

- For each sample, place 95 µl dH2O and 5 µl of protein sample in duplicate Eppendorf tubes
- Vortex tubes briefly
- Add 900 µl Bradford working solution to each tube and vortex again
- Incubate tubes at room temperature for 5 minutes
- Set the spectrophotometer to 595 nm and read absorbances of standards and samples

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- If absorbance of samples falls outside the range of the highest standard, dilute with RIPA buffer and read again
- In Microsoft Excel, plot the absorbance values against the protein concentrations in µg to generate a standard curve
- Calculate the protein concentration of each sample and how much should be loaded into each well to have 50 µg of protein per well

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Appendix C: Sample Preparation

MATERIALS AND EQUIPMENT

Heating block

Laemmeli sample buffer

Sharp instrument

Vortex

Centrifuge

PREPARING REAGENTS

Laemmeli sample buffer stock solution

3.8 ml dH_2O

1.0 ml Tris-HCl, pH 6.8

0.8 ml glycerol

1.6 ml 10% SDS

0.4 ml 0.05% bromophenol blue

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METHOD

I. Set the heating block to 95°C to allow time for it to heat up

II. Make up a working solution of sample buffer by combining 850 µL Laemmeli stock solution with 150 µL ß-mercaptoethanol and vortexing (work in the fume hood)

III. Label tubes for each sample

IV. Add a quantity of sample buffer equal to one third of the total sample volume (i.e. half the volume of protein which you will add) to each tube as calculated after Bradford protein determination

V. Add the correct quantity of protein to each tube

VI. Punch holes in the lid of each tube using the sharp instrument and place on the heating block for 5 minutes

VII. Vortex each sample and centrifuge for 20 seconds

VIII. Load protein marker and samples onto gel

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Appendix D: Western Blot Analysis with Precast Gels

MATERIALS AND EQUIPMENT

Mini-Protean tetra cell tank

Electrophoresis module

Powerpac power supply

Mini-Protean pre-cast gels

Electrophoresis buffer

Protein marker

Biorad Transfer Blot

Transfer packs

Ponceau S staining solution

Tris-buffered saline-tween (TBS-T)

Non-fat milk powder or bovine serum albumin (BSA)

Primary antibody

Rotator (at 4°C)

Horseradish peroxidase-linked secondary antibody

Rotator (at room temperature)

Enhanced chemiluminescent (ECL) reagent

Biorad Chemidoc

NaOH

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DAY ONE

PREPARING REAGENTS

10 x TBS stock solution

- 48.4 g Tris
- 160 g NaCl

Dissolve in 800 ml dH_2O, adjust pH to 7.6 with concentrated HCl and make up to 2 L. Store at 4°C until needed

TBS-T working solution

Add 200 ml of 10 x TBS stock solution to a 2 L Schott bottle and fill with dH_2O. Add 2 ml of tween

10 x Electrophoresis buffer stock solution

- 60.6 g Tris base
- 288 g Glycine
- 20 g SDS (10%)

Dissolve in 2 L dH_2O. Store at 4°C until needed.

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GEL ELECTROPHORESIS

I. Remove comb by positioning both thumbs on the ridges of the comb and pushing upwards in one smooth continuous motion

II. Gently remove the green tape at bottom of gel cassette

III. Rinse wells with electrophoresis buffer using an insulin syringe, syringe wash bottle or disposable transfer pipette

IV. Set the electrode assembly to the open position on a clean flat surface

V. Place the gel cassettes, short plate facing inwards, onto the gel supports. Note that the gel will now rest at a 30° angle, tilting away from the center of the electrode assembly. If an odd number of gels are to undergo electrophoresis, use the buffer dam to complete the assembly

VI. While gently keeping the gel cassettes against the green gaskets, slide the green arms of the clamping frame over the gels, locking them into place

VII. Fill the buffer dam with electrophoresis buffer

VIII. Carefully load the samples and an appropriate protein marker

IX. Place the plate assembly into the gel tank and fill with electrophoresis buffer (700 ml for 2 gels or 1000 ml for 4 gels)

X. Electrophorese gel at 99 V until the dye front reaches the line on the bottom of the gel cassette (± 1 hour)

XI. Disconnect the cell and remove the cassette by aligning the arrow on the opening key with the arrows marked on the casette and applying downward pressure. Remove gel gently

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PROTEIN TRANSFER

I. Open the transfer pack and place the blotting paper marked "Bottom" into the Biorad Transfer Blot cassette. Place the gel on top followed by the blotting paper marked "Top". Ensure that the membrane faces the gel. Smooth out bubbles with the roller

II. Place lid on top and lock in place

III Place the cassette in the Biorad Transfer Blot and set to transfer for 7-12 minutes, depending on the size of the protein of interest

IV. Check that protein has transferred and then rinse once with TBS-T

V. Pour Ponceau S staining solution on top and swirl until red bands appear strongly

VI Pour off the stain and rinse with dH_2O until membrane is clear

VII Place membrane between two transparencies, and scan in the membrane on computer to use Ponceau S as a loading marker

BLOCKING AND INCUBATING WITH PRIMARY ANTIBODY

I. Rinse membrane 3 x 5 minutes with TBS-T

II. Block for 1 hour in 5% non-fat milk powder or 1% BSA, made up in TBS-T

III. Wash 3 x 5 minutes in TBS-T

IV. Place membrane in a 50 ml Falcon tube containing 5 µL of primary antibody in 5 ml of TBS-T (i.e. a 1 in 1000 concentration)

V. Place on rotator in 4°C walk-in fridge overnight

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DAY TWO

INCUBATING WITH SECONDARY ANTIBODY

I. Remove membrane from primary antibody

II. Wash 3 x 5 minutes in TBS-T

III. Place membrane in a 50 ml Falcon tube containing 1 µL of secondary antibody in 5 ml of TBS-T (i.e. a 1 in 5000 concentration)

IV. Place on rotator at room temperature for 1 hour

V. Wash membrane 3 x 5 minutes in TBS-T

PROTEIN VISUALIZATION AND IMAGE ANALYSIS

I. Prepare ECL by combining reagents A and B in a 1:1 ratio in a foil-covered tube. Add 1 ml to membrane, covering surface completely by tilting gently

II. After 2 minutes, transfer the membrane to the tray of the Biorad Chemidoc

III. Select the “Image Lab” program from the computer’s desktop

IV. The start page will pop up – choose an existing protocol or create a new one. Modify protocol settings as required by selecting the relevant item under “Protocol Set Up”

V. Select the “Gel Imaging” option and make a selection in the “Application” section using the “Select” button.

VI. Select “Protein Gels” from the drop-down menu and choose the applicable option, for example “Ponceau”. The appropriate required filter and illumination settings will appear

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VII. Set the "Imaging area" to 'Biorad Criterion Gel". The imaging area will be displayed on the right hand side

VIII. Specify the optimum exposure, for example, "Intense Bands". Default color is auto-selected

IX. Select "Lane and Band Detection" to do molecular weight analysis

X. Select the required level of sensitivity

XI. Select "Analyze MW" then select molecular standard used, the lane it is in and the regression method "Linear (semi-log)"

XII. Select "Generate a report" – specific output and name it

XIII. Get a protocol summary and save it

XIV. Load the gel into the imager and click on the "Position Gel" button. A live image of the gel appears, allowing you to adjust its position and the zoom setting as required

XV. Click on "Run Protocol". When the images and report appear, save to an appropriately labeled folder

STRIPPING AND REPROBING

I. Wash membrane 2 x 5 minutes with dH_2O

II. Strip membrane by washing with 0.2 M NaOH for 5 minutes

III. Wash membrane 2 x 5 minutes with dH_2O

IV. Block for 1 hour in 5% non-fat milk powder or 1% BSA made up in TBS-T

V Wash 3 x 5 minutes in TBS-T

VI. Proceed with normal Western blot procedure (incubating with primary antibody overnight)