

Proteasome Inhibitors: A novel therapy that blunt hyperglycemia-induced cardiac contractile dysfunction

by

Büin Adams

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Supervisor: Professor M. Faadiel Essop

Co-supervisor: Dr. RF. Mapanga

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Declaration

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ABSTRACT

Introduction

Diabetes is considered a major threat to human health in both developed and developing nations. Cardiovascular disease which is common in diabetic patients has increased the overall disease affliction. Moreover, stress-induced hyperglycemia has led to increased mortality and morbidity in patients with an acute myocardial infarction (MI), whether the patient has diabetes or not. In addition, acute MI might stem from stress-induced hyperglycemia capability to increase inflammation and oxidative stress resulting in a worse functional cardiac outcome. Hyperglycemia-induced oxidative stress can similarly result in the formation of miss folded or damaged proteins that may be eliminated by the ubiquitin-proteasome system (UPS).

Futhermore, hyperglycemia-induced oxidative stress can also result in dysregulation of the UPS that removes these misfolded proteins. Additionally, an increasing body of evidence implicates UPS dysfunction in cardiac diseases and hyperglycemia which has been associated with increased inflammation and blunted cardiac function in response to ischemia-reperfusion. Literature however is blurred whether a reduction or a rise in the UPS is damaging with hyperglycemia and in response to ischemia-reperfusion. In light of this, we hypothesized that UPS inhibitors such as Z-Leu-Leu-Leu-al (MG-132) and lactacystin, protects the rat heart against ischemia-reperfusion under hyperglycemic perfusion conditions.

Materials and Methods

Isolated rat hearts were perfused *ex vivo* with Krebs-Henseleit buffer containing 33 mM glucose vs. controls (11 mM glucose) for 60 min, followed by 20 min global ischemia and 60 minutes reperfusion \pm PI treatment (MG-132 and lactacystin), anti-inflammatory (Ibuprofen) and anti-oxidant (NAC). Infarct size was determined using Evans Blue dye and 1% 2,3,5-triphenyl tetrazolium chloride (TTC) staining with 20 minutes regional ischemia and 2 hours reperfusion \pm PI's treatments. Tissues were collected at the end of the global ischemia experiments and analyzed for UPS activity, oxidative stress, apoptosis and inflammation.

Results

Our data expressed a reduced cardiac contractile function in response to ischemia and reperfusion under hyperglycemic conditions as well as an increase in UPS activity. PI treatment resulted in cardio-protection for *ex vivo* rat heart model exposed to ischemia and reperfusion under hyperglycemic conditions as well as ibuprofen and NAC. In parallel lactacystin treatment significantly decreased myocardial oxidative stress, apoptosis, and inflammation which provided cardio-protection in response to ischemia and reperfusion under hyperglycemic conditions.

Conclusions

This study shows that acute hyperglycemia elicits myocardial oxidative stress, apoptosis and inflammation that in time results in an increase in contractile dysfunction following ischemia and reperfusion. However, we found that PI treatment with both MG-132 and lactacystin blunted high glucose-induced damaging effects which resulted in a robust cardio-protection in response to ischemia and reperfusion under hyperglycemic conditions, by reducing oxidative stress, decreasing apoptosis and limiting inflammation. A parallel outcome was observed at baseline although the underlying mechanisms driving this process still need to be clarified. Our findings indicate that the UPS may be a unique therapeutic target to treat ischemic heart disease in diabetic patients, and non-diabetic individuals that present with stress-induced hyperglycemia. In summary, this thesis established that PIs act as a novel cardio-protective intervention to treat acute hyperglycemia with associated cardiovascular complications.

Uittreksel

Inleiding

Diabetes word beskou as 'n baie groot problem vir menslike gesondheid vir beide die ontwikkel en onontwikkelende lande. Kardiovaskulêre siekte wat normaal met diabetiese pasiente geassosieer word veroorsaak 'n toeneemende druk, wat hierdie siekte laat toeneem. Verder meer vergroot stresgeïnduseerde hiperglukemie die mortaliteit van pasiente met of sonder diabetes wat akute miokardiale infarksie onder lede het. Akute miokardiale infarksie kan ook ontstaan van stresgeïnduseerde hiperglukemie se bekwaamheid om meer inflammasie en oksidante stress te veroorsaak wat in 'n meer swakker funksionele kardiaale toestand. Hiperglukemiegeïnduseerde oksidatiewe stres ook tot wanregulering van die ubiquitien-proteosoomsisteem (UPS) wat wangevoude proteïene verwyder, aanleiding gee. Kontrasterende data bestaan van verhoogde/verlaagde UPS aktiwiteit, sowel as met hiperglukemie en/of in reaksie tot isemie-reperfussie. As gevolg hiervan, hipotetiseer ons dat Z-Leu-Leu-Leu-al (MG-132) and lactacystin as 'n nuwe kardiobeskeringsmiddel kan optree deur miokardiale oksidatiewe stress, inflammasie en UPS aktiwiteit te verlaag in reaksie op isemie-reperfussie tydens akute hiperglukemiese toestande kan verlaag.

Materiale en Metodes

Geïsoleerde rotharte is ex vivo met Krebs-Henseleit buffer, wat, 33 mM glukose vs. kontrole (11 mM glukose) bevat, vir 60 min geperfuseer, daarna is dit deur 20 min globale isemie gevolg en 60 min reperfussie ± PI behandeling (MG-132 and lactacystin), anti-inflammatoriese behandeling (Ibuprofen) en antioksidant behandeling (NAC). Infarkgrootte is bepaal deur Evans bou kleursel en 1% 2,3,5-tripheniel tetrazoliumchloried (TTC) kleuring met 20 minute regionale isemie, en 2 uur reperfussie ± PI's behandeling. Weefsels is aan die einde van die globale isemie eksperimente versamel, en vir oksidatiewe stres, apoptose en inflammasie ontleed.

Resultate

Ons data toon aan dat kardiaale kontraktiele funksie in reaksie op isgemie-reperfussie onder hiperglukemiese toestande verlaag het asook 'n toename in UPS aktiwiteit veroorsaak. PI behandeling het gelei tot kardiaale beskerming vir *ex vivo* rotharte wat aan isgemie-reperfussie onder hiperglukemiese toestande blootgestel was sowel as ibuprofen en NAC. Parallel hiermee het lactacystin oksidatiewe stres, apoptose, inflammasie, en UPS aktiwiteit na isgemie-reperfussie, verlaag in reaksie isgemie-reperfussie onder hiperglukemiese toestande.

Gevolgtrekking

Hierdie studie het bevind dat akute hiperglukemie, miokardiale oksidatiewe stres lei tot oksidante stress, apoptose, en inflammasie na kontraktiele wanfunksionering na isgemie-reperfussie lei. Ons het bevind dat beide MG-132 en lactacystin behandeling, hoë glukose-geïnduseerde skadelike effekte onderdruk, en kardiaale-beskerming in reaksie op isgemie-reperfussie onder hiperglukemiese toestande ondervind was deur oksidante stress, apoptose, en inflammasie te verlaag. 'n Soorgelyke effek is tydens die basislyn waargeneem, alhoewel die onderliggende meganisme wat hierdie proses meer ondersoek instel. Ons bevindinge dui op dat die UPS 'n nuwe behandeling teiken kan word in isgemie-geïnduseerde reperfussie onder akute en chroniese hoë glukose toestande. In opsomming, het die tesis belowend bevindinge gevind wat 'n nuwe terapeutiese intervensie vir die behandeling van akute hiperglukemie met geassosieerde kardiovaskulêre komplikasies gebruik kan word.

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DEDICATION

This thesis is dedicated to my family but most especially to my late mother Portland Adams. Thank you for teaching me to love hard and the true meaning of sacrifice you will always be with me. These last four months has been the most trying time in my life but your strength and determination that I have seen and emulated has pulled me through. You were there at the beginning of this chapter in my life but were unable to see it fulfilled so close to the end. I carry you in my heart; I carry you in my mind and everything I do. You will never be forgotten and your teaching will live within me forever

My eternal thanks and gratitude

Love your son

Buin

TABLE OF CONTENTS

Declaration	ii
Abstract (English)	iii
Uittreksel (Afrikaans)	v
Acknowledgements	vii
Dedication	viii
Table of contents	ix
List of Tables	xii
List of Figures	xiii
List of Conference Proceedings	xv
List of Abbreviations	xvi
Units of Measurements	xxii

CHAPTER 1 Literature review

1.1	Background	1
1.2	Implication of the UPS	2
1.3	The dire effects of diabetes mellitus	3
1.4	Cardiac metabolism	5
1.4.1	Substrate use of the heart under normal physiological conditions	5
1.5	Glucose uptake and metabolism	5
1.6	FA uptake and utilization	7
1.7	Interplay between glucose and FA	8
1.8	Alterations of cardiac metabolism during diabetes/ hyperglycemia	10
1.9	Detrimental effects of hyperglycemia related to the development of CVD	11
1.10	Hyperglycemia-induced effects and CVD onset	12
1.10.1	Hyperglycemia induced oxidative stress	12
1.10.2	Hyperglycemia-induced non-oxidant pathway activation	15
1.10.2.1	Polyol pathway	16
1.10.2.2	Hexosamine pathway	17
1.10.2.3	DAG-PKC pathway	17
1.10.2.4	Advanced glycation end-products and associated receptors	17
1.11	Metabolic changes during ischemia and reperfusion	18
1.12	Ionic imbalances during ischemia and reperfusion	20
1.13	Myocardial reperfusion injury	21
1.14	Various modes of cell death in CVDs	23
1.14.1	Necrosis	23

1.14.2	Apoptosis	24
1.14.2.1	Extrinsic /external/death receptor pathway	24
1.14.2.2	The intrinsic/mitochondrial pathway	25
1.14.3	Autophagy	27
1.14.4	Cross talk between the ubiquitin–proteasome system and autophagy	29
1.15	Structure and general function of the UPS	30
1.16	The cardiac 26S proteasome	32
1.17	Proteasome inhibitors	33
1.17.1	Peptide aldehydes	34
1.17.2	Epoxyketones	34
1.17.3	Boronic acids	34
1.17.4	Vinyl sulfones	34
1.17.5	β -lactones	34
1.18	Proteasomal involvement during myocardial ischemia and reperfusion	35
1.19	UPS and inflammation - hyperglycemia and I/R	38
1.20	Conclusion	42

CHAPTER 2 Methods and Materials

PI: a novel cardio-protective agent that blunts hyperglycemia-induced contractile dysfunction

2.1	Animals and ethics statement	43
2.2	Drugs and chemicals used	43
2.3	<i>Ex-vivo</i> perfusions global ischemia during simulated acute hyperglycemia	43
2.4	<i>Ex-vivo</i> regional ischemia and reperfusion during simulated acute hyperglycemia	46
2.5	Determination of infarct size	46
2.6	Western blot analysis	47
2.7	Proteasome activity measurements	49
2.8	Glutathione assay Kit	50
2.9	Statistical analysis	50

CHAPTER 3 Results

Acute PI treatment neutralizes hyperglycemia-mediated contractile dysfunction following ischemia and reperfusion

3.1	Evaluation of <i>ex vivo</i> heart function during global ischemia and reperfusion (simulated acute hyperglycemia) \pm MG-132	51
3.2	Assessment of infarct size following regional ischemia and reperfusion (simulated acute hyperglycemia \pm MG- 132)	55
3.3	The effects of MG-132 treatment on myocardial proteasomal activity in hearts exposed to ischemia and reperfusion under high glucose settings	57
3.4	Evaluation of <i>ex-vivo</i> heart function during global ischemia and reperfusion (simulated acute hyperglycemia) \pm lactacystin	61
3.5	Assessment of infarct size following regional ischemia and reperfusion	

	(simulated acute hyperglycemia) ± lactacystin	63
3.6	Lactacystin treatment diminishes proteasomal activity	65
3.7	Comparative analyses of treatment modalities	66
3.8	Effects of lactacystin treatment on myocardial ROS levels and apoptosis	69
3.9	Lactacystin blunts inflammation in hearts exposed to ischemia and reperfusion under high glucose conditions	72
3.10	Autophagy upregulated in lactacystin - treated hearts subjected to ischemia and reperfusion	73
3.11	Reduction in eNOS activation due to lactacystin under high glucose conditions	75
3.12	Lactacystin blunts GR2K under high glucose conditions	75
CHAPTER 4 Discussion		
4.1	Introduction	77
4.2	Cardio-protection using PIs after ischemia-reperfusion under simulated hyperglycemic conditions	78
4.3	PI blunts inflammation in hearts exposed to ischemia-reperfusion under high glucose conditions	81
4.4	Other mechanisms involved in proteasome inhibition-mediated cardio-protection	82
4.5	Conclusion	83
4.6	Future directions	83
4.6.1	Limitations of the stud	83
	References	84
APPENDICES		113

LIST OF TABLES

CHAPTER 1

Table 1.1: Interpretation of blood glucose levels

Table 1.2: Regulation of the cardiac UPS in myocardial ischemia

CHAPTER 2

Table 2.1: Investigational heart grouping

Table 2.2: Antibodies employed for Western blotting analysis

CHAPTER 3

Table 3.1: Coronary flow and end-diastolic pressure (EDP) under high glucose vs. baseline glucose conditions hearts

Table 3.2: Coronary flow, end-diastolic pressure, heart rate and velocity of contraction for hearts perfused under high glucose (33 mM) vs. control (11 mM) \pm NAC and

Ibuprofen

Table 3.3: Effects of lactacystin on ex vivo coronary flow and end-diastolic pressure during I/R

LIST OF FIGURES

CHAPTER 1

- Figure 1.1: Glucose uptake and oxidation under normal conditions
- Figure 1.2: Fatty acid uptake and oxidation under normal conditions
- Figure 1.3: The Randle cycle showing the interrelationship between myocardial fatty acid and glucose metabolism
- Figure 1.4: The production of superoxide ($O_2^{\bullet-}$) by mitochondrial complex I and III
- Figure 1.5: Mitochondrial overproduction of superoxide activates four major pathways of hyperglycemic
- Figure 1.6: Apoptotic pathway in cardio myocytes
- Figure 1.7: The ubiquitin-proteasome pathway
- Figure 1.8: Model of NF- κ B activation by hyperglycemia
- Figure 1.9: A schematic presentation of the activation of the transcription of NF- κ B

CHAPTER 2

- Figure 2.1: Schematic diagrams showing the perfusion protocols for evaluation of the effects of various drug treatments

CHAPTER 3

- Figure 3.1: High glucose-induced cardiac contractile dysfunction in rat hearts exposed to high glucose conditions vs. baseline glucose concentrations
- Figure 3.2: Acute MG-132 treatment increases cardiac contractile function under baseline glucose and high glucose perfusion conditions reflected by improved LVDP and RPP vs. untreated hearts
- Figure 3.3: MG-132 treatments had no effect on HR and dP/dt max
- Figure 3.4: MG-132 administration exhibited cardio-protective properties following regional I/R under high glucose perfusion conditions
- Figure 3.5: Increased proteasomal activity in hearts subjected to I/R under high glucose Conditions
- Figure 3.6: MG-132 reduces high glucose-induced chymotrypsin-like proteasomal activity following I/R

- Figure 3.7: NAC and Ibuprofen treatment elicit increased cardiac contractile function under baseline and high glucose conditions
- Figure 3.8: Lactacystin treatment during reperfusion improves contractile function at baseline and under high glucose condition following I/R
- Figure 3.9: The effects of lactacystin treatment on HR and dP/dt max
- Figure 3.10: Lactacystin administration leads to cardio-protection following regional I/R
- Figure 3.11: Lactacystin decreases high glucose-induced chymotrypsin-like proteasomal activity after I/R
- Figure 3.12: Comparative assessment of PI treatment during reperfusion improves function recovery at baseline and under high glucose conditions following I/R
- Figure 3.13: Comparison of treatment modalities with I/R under high glucose conditions
- Figure 3.14: Comparison infarct size percentage decreased for MG-132 vs. lactacystin
- Figure 3.15: Comparison of proteasomal activity between MG-132 vs. lactacystin
- Figure 3.16: Anti-oxidant effects of lactacystin in hearts subjected to I/R under baseline and high glucose conditions
- Figure 3.17: Anti-apoptotic effects of lactacystin in hearts subjected to I/R under high glucose conditions
- Figure 3.18: Anti-apoptotic effects of lactacystin in hearts subjected to I/R under control and high glucose conditions
- Figure 3.19: Anti-inflammatory effects of lactacystin in hearts subjected to I/R under control and high glucose conditions
- Figure 3.20: Lactacystin increases autophagy in hearts subjected to I/R under control and high glucose conditions
- Figure 3.21: Lactacystin blunts eNOS in hearts subjected to I/R under control and high glucose conditions
- Figure 3.22: Lactacystin blunts GR2K in hearts subjected to I/R under control and high glucose conditions

LIST OF CONFERENCE PROCEEDINGS

National

- Büin Adams, Mapanga RF and Essop MF. Novel therapeutic approaches to treat ischemic heart disease in diabetic patient. SEMSDA Annual conference University of Johannesburg (16-18 August 2013).Abstract accepted for poster presentation.

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LIST OF ABBREVIATIONS

ACC	acetyl-CoA carboxylase
AGE(s)	Advanced glycation end-product(s)
AIF	apoptosis inducing factor
AMI	acute myocardial infarction
AMP	adenosine monophosphate
AMPK	adenosine monophosphate protein kinase
Apaf-1	adaptor protein apoptotic protease activating factor-1
APO-1	apoptosis antigen 1
ARC	apoptosis repressor with caspase recruitment domain
ARE/EpRE	antioxidant/electrophile responsive gene promote element
ATP	adenosine triphosphate
<u>B</u>	
Bad	Bcl-2 associated death promoter
Bax	Bcl-2-associated X protein
Bcl-2	B cell leukemia/lymphoma-2
Bcl-xL	B cell leukemia/lymphoma-x-isoform
BH3	Bcl-2 homology domain 3
Bid	BH3-only interacting protein domain
Bim	Bcl-2 like protein 11
BNIP3	Bcl2)/adenovirus E1B interacting protein 3
β2ARs	β2-adrenergic receptors
<u>C</u>	
Ca ²⁺	calcium
CAC	citric acid cycle
CD36	cluster of differentiation 36
CHD	coronary heart disease
CHIP	Hsp70-interacting protein
CO ₂	carbon dioxide
CPT-I/II	carnitine palmitoyl transferase I/II
CuSOD	copper superoxide dismutase

CVD	cardiovascular disease
<u>D</u>	
DAG	diacylglycerol
DHAP	dihydroxy acetone phosphate
DISC	death-inducing signaling complex
DNA	deoxy-ribonucleic acid
dP/dtmax	maximal velocity of contraction
<u>E</u>	
eNOS	endothelium nitric oxide synthase
ETC	electron transport chain
<u>F</u>	
FABPpm	fatty acid binding protein (plasma membrane)
FADD	Fas-associated death domain protein
FADH ₂	reduced flavin adenine dinucleotide
FAO	fatty acid oxidation
FAs	fatty acids
FATP	fatty acid transporter
<u>G</u>	
GADPH	glyceraldehyde-3-phosphate dehydrogenase
GDH	glycerophosphate dehydrogenase
GFAT	glutamine:fructose-6-phosphate amidotransferase
GLUT-1	Glucose transporter-1
GLUT-2	Glucose transporter-4
GO	glucose oxidation
GPCR	G protein coupled-receptor
GRK2	G-protein-coupled receptor kinase 2
GSH	glutathione
GSSG	glutathione disulphide
GST	glutathione S-transferase
<u>H</u>	
H ⁺	hydrogen ion
H ₂ O	water

H ₂ O ₂	hydrogen peroxide
HF	heart failure
HR	heart rate
OH [•]	hydroxyl radical
<u>I</u>	
I/R	ischemic and reperfusion
IAP	inhibitor of apoptosis
ICAM-1	intracellular adhesion molecule
Iκβ	interleukin kappa beta
IL-6	interleukin 6
IRS	insulin receptor substrate
<u>K</u>	
K ⁺	potassium
Keap1	Kelch ECH associating protein 1
<u>L</u>	
LAMP2	lysosome-associated membrane protein 2
LC3	light chain 3
LPL	lipoprotein lipase
LVDP	left ventricular developed pressure
<u>M</u>	
MAFbx	atrogin-1/ muscle atrophy F-box
MCD	malonyl-CoA decarboxylase
MDM2	murine double minute 2
MG	methyglyoxal
MI	myocardial infarction
MnSOD	manganese superoxide dismutase
mPTP	mitochondrial permeability transition pore
mTOR	mammalian target of rapamycin
MuRF	muscle RING finger
<u>N</u>	
Na ⁺	sodium
Na ⁺ /H ⁺	sodium/hydrogen

$\text{Na}^+\text{-HCO}_3$	sodium bicarbonate
NaCl	sodium chloride
NAD	nicotinamide dinucleotide
NADH	nicotinamide adenine dinucleotide hydrogen
NADP^+	oxidized nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NBR1	Neighbor of BRCA1 gene 1 protein
NF- $\kappa\beta$	Nuclear factor-kappa beta
NO	nitric oxide
Nox	NADPH oxidase
NQO1	NAD (P) H dehydrogenase [quinone] 1
Nrf2	nuclear factor erythroid 2 p45-related factor 2
<u>Q</u>	
$\text{O}_2^{\bullet-}$	superoxide radical
ONOO^-	peroxynitrite
<u>P</u>	
p62	nucleoporin p62
p62/SQSTM1	sequestosome 1
PARP	poly(ADP)ribose polymerase
PB1	polymerase basic 1
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PFK 1/2	phosphofructokinase $\frac{1}{2}$
PI	proteasome inhibitors
PI 3-K	Phosphatidyl inositol-3-kinase
PIC	proinflammatory cytokines
PKB	protein kinase B
PKC	protein kinase C
PPAR- α	peroxisome proliferator alpha
PTP	permeability transition pore

R

RIP	receptor-interacting protein
ROS	reactive oxygen species
RPP	rate pressure product

S

	Protein
SCD	sudden cardiac death
SDS	sodium dodecyl sulfate
SEM	standard error of means
Smac/DIABLO	second mitochondria-derived activator of caspase/direct IAP-binding
SOD	superoxide dismutase
Sodium/potassium	Na ⁺ /K ⁺
Sp1	specificity protein 1

T

T2DM	type 2 diabetes mellitus
tBID	truncated BID
TG	triacylglycerides
TNF- α	Tumour necrosis factor alpha
TTC	2,3,5-triphenyl tetrazolium chloride

U

Ub	ubiquitin
UBA	ubiquitinated-associated domain
UBL	ubiquitin-like domain
ucNOS	uncoupling nitric oxide synthase
UCP	uncoupling proteins
UDP	uridine diphosphate
UDP-GlcNac	uridine diphosphate acetylglucosamine
UPS	ubiquitin proteasome system
USA	United States of America

X

XIAP	X-linked inhibitor of apoptosis protein
------	---

Z

ZnSOD

zinc superoxide dismutase

UNITS OF MEASUREMENT

%	percent/percentage
AU	arbitrary units
g	gram
Hz	Hertz
kDa	kilodalton
mg	milligram
min	minutes
mM	millimolar
mmol/l	millimoles per liters
nm	nanometre
°C	degrees Celsius
µg	microgram
µM	micromolar
µmol/L	micromoles per litre

CHAPTER 1

1.1 Background

The dramatic increase in obesity and non-insulin-dependent diabetes mellitus (Type 2) during the past few decades has led to it being considered a major threat to human health in both developed and developing nations (**Wild S et al., 2004; Bradshaw D et al., 2007**). Since cardiovascular complications and mortalities are common in diabetic patients (**Mazzone T et al., 2008; Boudina S and Abel ED., 2007; Barr EL et al., 2007**) this further increase the overall burden of the disease. It is therefore a necessity for a comprehensive understanding of the underlying biochemical and molecular mechanisms orchestrating the development of Type 2 diabetes and the onset of cardiovascular diseases (CVD). Various CVD are associated with diabetes such as coronary heart disease (CHD), myocardial infarction. Metabolic derangements play a fundamental role in the onset of diabetes and CVD. Here elevated metabolite availability, e.g. hyperglycemia, defined as an increase in glucose in the bloodstream, can cause oxidative stress that may contribute to the onset of diabetic cardiomyopathy (**Ceriello A., 2000; Smart EJ and Li X-A, 2007**). Such metabolic abnormalities together with other subcellular defects and abnormal gene profiles may lead to cardiac cell death (**Cai L et al., 2002**) and by extension, contractile dysfunction.

Diabetic patients have not benefitted as much from the progresses in the management of associated risk factors such as obesity, dyslipidemia, and hypertension that led to decreased mortality for non-diabetic individuals burdened with CHD (**Kempler P., 2005**). The above-mentioned risk factors do not fully explain the excess risk for CHD associated with diabetes. CHD is caused due to a blockage or narrowing (stenosis) of the arteries that supply blood to the heart muscle, often due to a buildup of fatty plaque inside the arteries. A severe enough blockage can cause an ischemic event and if not treated promptly part of the heart muscle dies due to lack of oxygen also known as myocardial infarction (**Boudina S and Abel ED., 2007; Barr EL et al., 2007**). Therefore in recent years, much attention has been given to the association of mortality and morbidity risk and the incidence of hyperglycemia in patients admitted to intensive care units following acute myocardial infarction (MI), irrespective whether patients were diabetic or not (**Capes SE et al., 2003**). Acute hyperglycemia has also been independently associated with impaired left ventricular function (**Ishihara M et al., 2003**) and a larger infarct size

due to an increased incidence of the no-reflow phenomenon (**Iwakura K et al., 2003**). A worse myocardial performance was also found in patients with acute MI and associated hyperglycemia (**Poulsen SH et al., 2000**).

In an attempt to understand the mechanisms linking hyperglycemia to CVD there are several proposals how exactly such damage may occur. There is convincing evidence suggesting a key role for hyperglycemia-induced oxidative stress as a pivotal mechanism leading to myocardial cell death by increased endothelial cell apoptosis (**Valgimigli M et al., 2003**). Furthermore, there is the suggestion that the damaging effects of stress hyperglycemia with an acute MI can stem from its ability to increase inflammation. Here amplified inflammatory reactions are linked to decreased cardiac function (**Marfella R et al., 2003**). Additionally, hyperglycemia-induced oxidative stress can result in the formation of excess misfolded or damaged proteins – usually eliminated by the ubiquitin-proteasome system (UPS) – with detrimental consequences for cardiac function.

1.2 Implication of the UPS

An increasing body of evidence implicates UPS dysfunction in cardiac diseases and hyperglycemia. This has been associated with enhanced inflammation and blunted cardiac function in response to ischemia-reperfusion (I/R) (**Evans JL et al., 2002; Palombella et al., 1994; Toth A et al., 2006**). The literature, however, is “blurred” whether a reduction or a rise in the UPS activity is damaging/protective with hyperglycemia and in response to I/R (**Kuhn D et al., 2006; Marfella R et al., 2009; Powell SR et al., 2006; Tsukamoto O et al., 2010**). An in-depth study on proteasome alterations in diabetic hearts has not been reported thus far, though greater proteasome functions were implicated as a damaging consequence in hearts exposed to high glucose levels (**Mapanga RF et al., 2012**). However, since increased reactive oxygen species (ROS) is associated with hyperglycemia within the context of both diabetes and myocardial ischemia, it is plausible that oxidative modification of proteasome subunits may alter proteasome function in this instance (**Powell SR., 2006**).

The role of proteasome dysfunction within the context of diabetes and associated CVD remains to be determined as it remains uncertain whether an increased or decreased UPS is harmful with hyperglycemia and/or in response to I/R. For example, **Pye et al. (2003)** found that myocardial reperfusion injury is reduced by proteasomal inhibitors, while

others determined that UPS over-activity may augment the risk of complications during myocardial ischemia in diabetic patients (**Marfella R et al., 2009**). By contrast, others established that proteasomal deficiency may add to the detrimental effects of myocardial ischemia (**Bulteau AL., 2001**). Supplementary studies are consequently essential to determine the mechanisms underlying dysfunctional UPS in the heart under these conditions. In light of this, we hypothesized that proteasomal inhibitors (PI) (Lactacystin or Z-Leu-Leu-Leu-al [MG-132]) prevents the metabolic processes that occur during acute hyperglycemia with I/R; hence it is cardio-protective. Therefore, this thesis was designed to investigate the role of proteasome inhibition as a novel therapeutic intervention to help improve cardiac function following I/R under hyperglycemic conditions. The next section will look at the various forms and effects diabetes has on human physiology.

1.3 The dire effects of diabetes mellitus

Diabetes mellitus is one of the most common chronic diseases worldwide, and continues to rise in number and significance as changing lifestyles lead to reduced physical activity and increased obesity (**World Health Organisation., 2006**). This condition is characterized as a group of metabolic diseases where individuals display elevated glucose levels (Table1) either due to insufficient insulin secretion by the pancreas, or because cells do not respond to the insulin that is produced (**Boussageon R et al., 2011**). Classical symptoms of diabetes normally experienced are polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased appetite) (**Rother KI., 2007**). Diabetes can be classified into four main categories: type 1, type 2, gestational diabetes, and secondary to other underlying disease or cause.

Table 1.1 Interpretation of blood glucose levels

Fasting Blood glucose	2 hour after 75g glucose load	Temporary diagnosis
<5.6 mmol/l	<7.8 mmol/l	Normal
5.6 – 7.0 mmol/l	7.6 – 11.0mmol/l	IGT (“ Impaired Glucose Tolerance”)
>7.0 mmol/l	>11.1 mmol/l	Diabetes

Diabetic Care 27, Supp 1.1, 2004.

Type 1 diabetes mellitus is characterized by loss of the insulin-producing beta cells of the islets of Langerhans in the pancreas, thus leading to insulin deficiency. This type can be further classified as immune-mediated or idiopathic (**Lambert P and Bingley PJ., 2007**). The majority of type 1 diabetes is of the immune-mediated nature, where beta cell loss is due to a T-cell-mediated autoimmune attack. Type 2 diabetes mellitus (T2DM) is characterized by excessive hepatic glucose production, decreased insulin secretion, and insulin resistance. A number of lifestyle factors are known to play a significant role in the development of T2DM, including: obesity, lack of physical activity, poor diet, stress, and urbanization (**Bugger H and Abel E., 2008**). Gestational diabetes is well-defined as the first onset of diabetes mellitus in women during pregnancy caused by a surplus production of glucocorticoids (**Lawrence JM et al., 2008**). The fourth type of diabetes is a condition where hyperglycemia manifests with pancreatic or hormonal disease, or secondary to drug or chemical exposure, and also with certain genetic syndromes (**Malik VS et al., 2010; National Diabetes Data Group, 1995**).

The rate of diabetes incidence has increased prominently over the last 50 years in parallel with obesity due to growing westernization of diets; jeopardizing the working class as indicated by increased T2DM prevalence (**Epidemiology of Diabetes Interventions and Complications (EDIC) study., 2003**). Recent data show that there are approximately 285 million persons diagnosed with T2DM (compared to around 30 million in 1985), and that this number is expected to rise to 439 million by the year 2030 (**Marcovecchio ML et al., 2011**). T2DM is typically a chronic disease that is associated with a shorter life expectancy (**Fasanmade OA et al., 2008**). This is partly due to a number of complications including: 2-4 times risk of CVD (already defined above), including ischemic heart disease and MI. Diabetes is also associated with long-term cardiovascular complications such as atherosclerosis (**Fasanmade OA et al., 2008**), thus further contributing to the overall burden of disease (**Hayashi T et al., 2008**). The prevalence, incidence, and mortality of CVD are unusually amplified in persons with diabetes compared with those without diabetes. In light of this, the current study focused on the role of acute hyperglycemia and its link to MI. The next section therefore discusses the substrate alterations that occur in both acute and chronic hyperglycemia.

1.4 Cardiac metabolism

Chronic hyperglycemia that occurs with diabetes is mainly due to derangements in substrate metabolism which may impair cardiac function with I/R (**Rahimi R *et al.*, 2005**) as well as long term complications. Acute hyperglycemia involving glucose levels that are extremely high is a medical emergency and can rapidly produce serious complications (such as fluid loss through osmotic diuresis). It is most often seen in persons who have uncontrolled insulin-dependent diabetes (**Ceriello A., 2000; Smart EJ and Li X-A, 2007**) and is independently associated with impaired left ventricular function (**Ishihara M *et al.*, 2003**) as mentioned before. With diabetes, the heart uses fatty acids (FAs) more prominently resulting in both diastolic and systolic cardiac dysfunction (**Anderson D and Rodrigues B., 2006**). We will thus next discuss cardiac energy metabolism of the normal adult mammalian heart for a better understanding of biochemical and molecular mechanisms involved in cardiac pathophysiology under hyperglycemic conditions.

1.4.1 Substrate use of the heart under normal physiological conditions

The heart has a high demand for provision of energy. The energy currency of the heart is adenosine triphosphate (ATP) and an entire pool of ATP is consumed 6-8 times per minute (**Ashrafian H., 2011**). To sustain this high energy demand a wide variety of energy sources are used such as carbohydrates, lactate, FAs, amino acids and ketones. Here FAs and carbohydrates constitute the major energy sources under normal physiological conditions. For a normal functioning heart, 70% of ATP assembly is generated by Fatty acid oxidation (FAO), whereas glucose and lactate accounts for the remainder (**Gertz E.W *et al.*, 1988; Neely J.R *et al.*, 1972**).

1.5 Glucose uptake and metabolism

Glucose is the major carbohydrate used by the heart and its uptake is dependent on the transmembrane glucose gradient and the content of the sarcolemmal glucose transporters GLUT1/4. GLUT1 is more prominent at the sarcolemma and represents basal cardiac uptake (**Kraegen EW *et al.*, 1993**) while GLUT4 is more dominant in the adult heart. Such transporters are usually located within an intracellular pool and translocate to the sarcolemmal membrane in a process mediated by insulin (**Barnard RJ and Youngren JF., 1992; Fischer Y *et al.*, 1997**). However, GLUT-mediated glucose uptake can also be stimulated by insulin-independent mechanisms such as adenosine

monophosphate (AMP)-activated protein kinase (AMPK) which promotes GLUT4 redistribution to the sarcolemmal membrane (**Hausenloy DJ., 2012; Li J et al., 2004**). Once inside, glucose is broken down into pyruvate through glycolysis.

Phosphofructokinase-1 (PFK-1) is the rate limiting enzyme within the glycolytic pathway which catalyzes the production of fructose 1, 6-bisphosphate from fructose 6-phosphate (see Figure 1.1). PFK-1 is activated by fructose 2, 6-bisphosphate, which is formed from fructose 6-phosphate catalysed by PFK-2. Following glycolysis, the pyruvate generated is transported into mitochondria and decarboxylated to acetyl-CoA, a step mediated by the multi-enzyme complex pyruvate dehydrogenase (PDH) (**Bowker-Kinley MM et al., 1998; Holness M and Sugden M., 2003**). PDH can be phosphorylated and inactivated by pyruvate dehydrogenase kinase (PDK). Acetyl-CoA then enters the Krebs cycle also known as the citrate acid cycle (CAC), which will be used throughout the rest of the thesis, where it is eventually broken down into water (H₂O) and carbon dioxide (CO₂) for ATP generation (**Hausenloy DJ., 2012; Huang B et al., 2003**) (Figure 1.1). As mentioned earlier, the heart also utilizes FAs; thus the next section will discuss its uptake and breakdown for energy production.

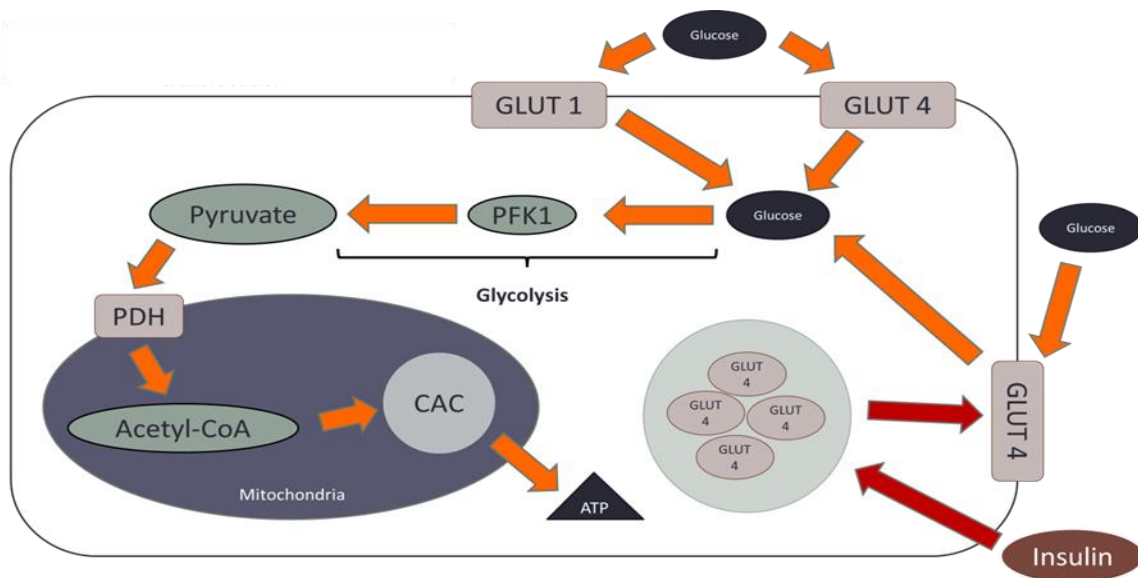


Figure 1.1 Glucose uptake and oxidation under normal conditions. Insulin-mediated GLUT 4 translocation. GLUT 1 and 4 (glucose transporter 1 and 4); ATP (adenosine triphosphate, P (phosphate residue); PFK-1 (phosphofructokinase 1); PDH (pyruvate dehydrogenase); CAC (citric acid cycle). Modified from An D and Rodrigues B., 2003

1.6 FA uptake and utilization

As the heart has limited capacity to synthesize and store FA's, it relies on a continuous exogenous supply. Circulating FAs are supplied to the heart via two sources, i.e. adipose tissue and triglyceride (TG)-rich lipoproteins (**Augustus AS *et al.*, 2006**). This occurs through hydrolysis by lipoprotein lipase (LPL). FAs are then taken up into the cardiomyocyte by three transporters: cluster of differentiation 36 (CD36), FA transport protein (FATP), and FA binding protein plasma membrane (FABP_{PM}) (**Luiken JJ *et al.*, 1999; Ibrahimi A *et al.*, 1999; Luiken JJ *et al.*, 2002**) (Figure 1.2). FAs are then converted to fatty acyl-CoA, which is transported into the mitochondrion through carnitine palmyoyltransferase-1/2 (CPT-1/CPT-2). Inside the mitochondrion, fatty acyl-CoA undergoes β -oxidation to generate acetyl-CoA, which is then further oxidized within the CAC. FA utilization can be regulated through various mechanisms such as activation of the transcriptional modulator, peroxisome proliferator-activated receptor- α (PPAR- α). When activated PPAR- α increases the expression of several enzymes involved in FA oxidation (**Dressel U *et al.*, 2003**) (Figure 1.2). Malonyl-CoA is generated through carboxylation of acetyl-CoA [a reaction catalyzed by acetyl-CoA carboxylase (ACC)], inhibiting CPT-1 and FA oxidation. AMPK inhibits ACC which relieves its inhibition on CPT-1 and promotes FA oxidation. Similarly, malonyl-CoA decarboxylase (MCD) decreases malonyl-CoA by decarboxylating it to acetyl-CoA, thereby enhancing CPT-1 and FA oxidation (**Dyck JR *et al.*, 1988; Dyck JR *et al.*, 2004**) (Figure 1.2).

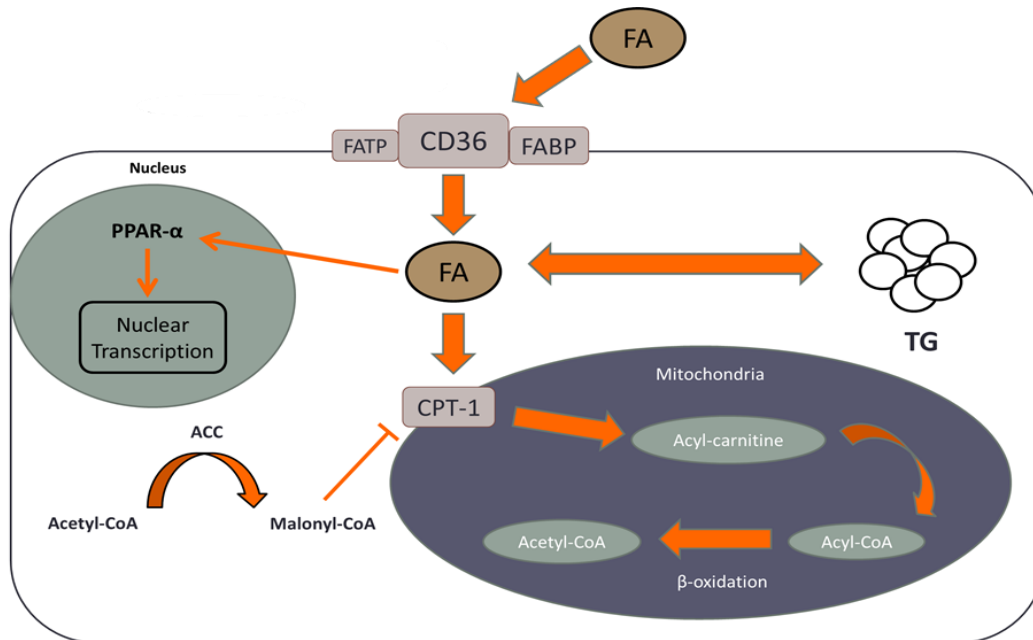


Figure 1.2 Fatty acid uptake and oxidation under normal conditions. CD36 (cluster of differentiation 36); FATP (fatty acid transport protein); FABP (plasma membrane fatty acid binding protein); FA (fatty acid); TG (triglycerides); ACC (acetyl-CoA carboxylase); PPAR- α (Peroxisome proliferator-activated receptor- α); CPT-I (carnitine-palmitoyl transferase I); CACT (carnitine/acylcarnitine transferase). Modified from An D and Rodrigues B., 2003

1.7 Interplay between glucose and FA

The regulation of glucose and FA metabolism does not occur independently but rather a unique cross-talk exists between these substrates (Randle PJ *et al.*, 1963; Saha AK *et al.*, 1997). Here FAs impair basal and insulin-stimulated glucose uptake and oxidation (an event referred to as the Randle cycle) and impacts glucose utilization at numerous levels (Randle PJ *et al.*, 1963) (Figure 1.3). Accumulation of FAs and augmented intracellular FA derivatives, such as fatty acyl CoA, diacylglycerol and ceramide impair insulin-mediated glucose uptake through inhibition of insulin receptor substrates and protein kinase B (PKB) (Grundleger ML and Thenen SW., 1982; Itani SI *et al.*, 2000 Stanley W *et al.*, 2005). These FA metabolites trigger a serine kinase cascade, involving protein kinase C- θ and I κ B kinase- β (interleukin kappa beta kinase- β) resulting in the serine phosphorylation of insulin receptor substrate (IRS) (Itani S.I *et al.*, 2000). This in turn interferes with its ability to phosphorylate and activate phosphatidylinositol 3-kinase and PKB which decreases glucose uptake (Kim JK *et al.*, 2004; Yuan M *et al.*, 2001). Increased intracellular FA levels (as observed in diabetes) can stimulate PPAR- α and thereby promote the expression of FA oxidation genes, including pyruvate dehydrogenase

kinase-4 (PDK4), which is known to inhibit PDH and pyruvate flux (**Newsholme EA and Randle PJ., 1964**). PDK4 is also inhibited by increased acetyl-CoA to free CoA, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) to nicotinamide adenine dinucleotide (NAD) or oxidized nicotinamide adenine dinucleotide phosphate (NADP⁺) ratios produced by high rates of FAO, thus further inactivating PDH. The augmented acetyl-CoA to free CoA ratio also causes the build-up of intracellular citrate and this can block PFK and glycolysis (**Kim JK *et al.*, 2004; Holness MJ and Sugden MC., 2003**). This results in activation of PDK4 and phosphorylation of PDH to its inactive form (**Bowker-Kinley MM *et al.*, 1998; Holness MJ and Sugden MC., 2003**). In addition, acetyl-CoA inhibits the rate of flux through active dephosphorylated PDH.

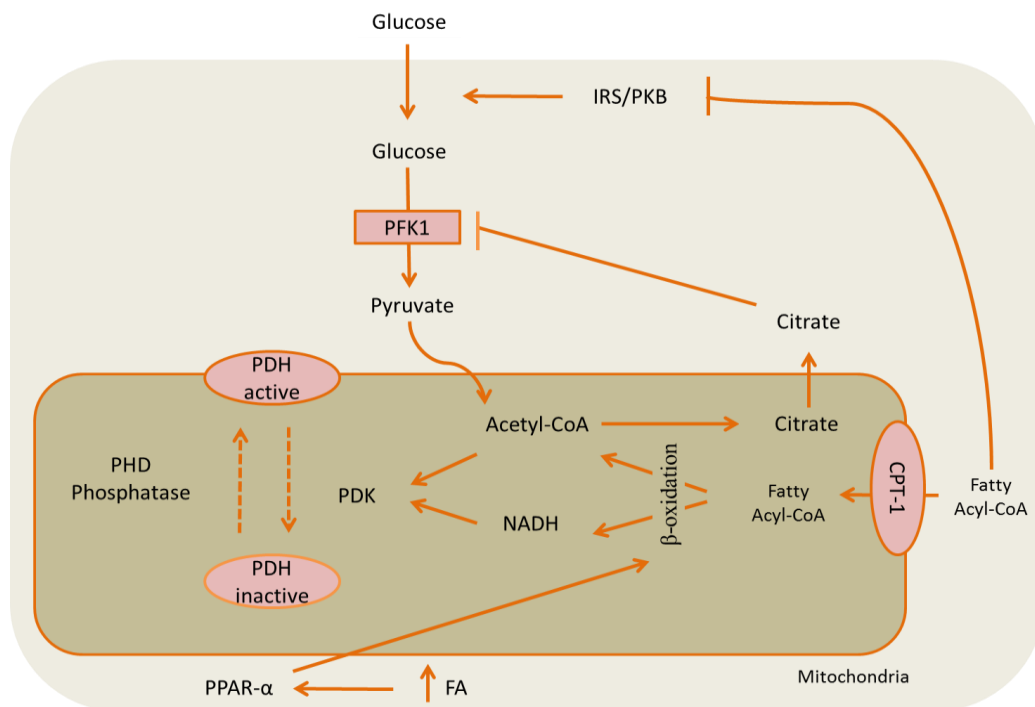


Figure 1.3 The Randle cycle showing the interrelationship between myocardial fatty acid and glucose metabolism. IRS/PKB (insulin receptor substrate/protein kinase B); PFK-1 (phosphofructokinase-1); PDH (pyruvate dehydrogenase); PDK (pyruvate dehydrogenase kinase); CPT-1 (carnitine palmitoyl transferase-1); PPAR- α (peroxisome proliferator-activated receptor- α); NADH (nicotinamide adenine dinucleotide hydrogen); FAs (fatty acids). modified from An D and Rodrigues B., 2003

Thus in some pathophysiological disease states (e.g. diabetes and myocardial infarction) high FA utilization impairs glucose utilization thereby contributing to hyperglycemia and insulin resistance (**Jagasia D *et al.*, 2001; Maki M *et al.*, 1997**). In addition, as the heart

adapts to high FA consumption for ATP generation there is a decreased cardiac efficiency since more oxygen will be consumed with less energy produced. This increases the risk of cardiac dysfunction especially during ischemia when reduced oxygen supply is available for FAO. Furthermore, under these conditions the heart also increases its dependence on glucose as fuel substrate (**Derave W et al., 2000; Jensen J et al., 1997**).

However, the heart has the ability to promptly switch substrate selection in order to accommodate diverse physiological and pathological conditions involving extracellular hormones, substrate availability and workload (energy demand) (**Ashrafian H., 2011; Hausenloy DJ., 2012**). Although switching of substrates guarantees that a continuous ATP generation is provided to retain heart function, it is also associated with detrimental effects such as decreasing cardiac efficiency. Cardiac efficiency is the amount of work produced by the heart per energy (oxygen) consumed. It is dependent not only on the efficiency of producing ATP but also on the efficiency of using energy to produce contractile work (**Ashrafian H., 2011**). FAs are therefore less efficient as more oxygen is required to produce ATP. If the FA supply to the heart rises, assuming sufficient oxygen availability (non-ischemic conditions), the rate of FAO increases and glucose oxidation (GO) decreases; followed by FAO inhibiting GO (**Ashrafian H., 2011**). Therefore in certain conditions where circulating free FAs are elevated, e.g. ischemia and T2DM, there can be a decrease in cardiac efficiency (**Hausenloy DJ., 2012**). The next section will now focus on substrate metabolism under pathophysiological conditions of interest in this thesis i.e. diabetes/hyperglycemia and MI as well as I/R.

1.8 Alterations of cardiac metabolism during diabetes/ hyperglycemia

Hyperglycemia is a common symptom for all types of diabetes and is the main risk factor predisposing to the development of micro- and macrovascular complications (**Luiken JJ et al., 2003; Sowers JR et al., 2001; Wilson PW., 2001**). There are two forms, i.e. chronic hyperglycemia (diabetes) and acute hyperglycemia that can occur in both diabetic and non-diabetic individuals (**Stanley W et al., 2005; United Kingdom Prospective Diabetes Study (UKPDS) Group., 1998**). Hyperglycemia is a consequence of decreased glucose clearance and augmented hepatic glucose production, as a result of decreased of glucose to glycolysis thereby resulting in a reduction in GO (**Carroll R et al., 2005; Young ME et al., 2002**). The impaired recruitment of GLUT4 to the cell membrane upon insulin stimulation that may occur as a result of alterations in the insulin receptor

substrates (IRS)/phosphoinositide-3 kinase (PI 3-K)/PK β also known as Akt signaling pathway is also implicated (Clerk LH *et al.*, 2002; Young ME *et al.*, 2003). Together with this (especially with chronic hyperglycemia), enhanced lipolysis in adipose tissue and higher hepatic lipoprotein synthesis can dramatically increase circulating free FAs and TG (Clerk L.H *et al.*, 2002). The outcome of hyperglycemia on the heart is described in the section below.

1.9 Detrimental effects of hyperglycemia related to the development of CVD

The chronic activation of such mechanisms (as discussed above) can lead to the onset of diabetic cardiomyopathy. Diabetic cardiomyopathy is characterized by ventricular dysfunction that occurs independently of coronary artery disease and hypertension (An D and Rodrigues B., 2003). This condition is characterized by an increased stiffening of the left ventricular wall with associated accumulation of connective tissue and insoluble collagen (Anguera I *et al.*, 1998; Rodrigues B *et al.*, 1995; Saito F *et al.*, 2003) and irregularities of numerous proteins that control ion flux, specifically intracellular calcium (Golfman LS *et al.*, 1996; Tahiliani AG and McNeill JH., 1986). The underlying causes of the diabetic cardiomyopathy remain unclear, although several studies indicate that it could also arise as a consequence of various metabolic alterations such as hyperglycemia (Lopaschuk GD., 1989; Lopaschuk GD., 2002; Lopaschuk GD *et al.*, 1994).

Hyperglycemia can contribute to the progress of vascular complications through numerous mechanisms such as activation of the 1) polyol and 2) hexosamine pathways, 3) activation of diacylglycerol (DAG) - PKC, 4) increased oxidative stress and 5) the enhanced production of advanced glycation end-products (AGEs) (Ohnishi T *et al.*, 2005; Wendt T *et al.*, 2006; Koya D *et al.*, 1997; Brownlee M., 2001). These mechanisms induce endothelial and cardiomyocyte dysfunction and will be later discussed. In support for this role, recent epidemiological studies demonstrated that chronic hyperglycemia is an independent risk factor for coronary heart disease; although the exact mechanism(s) underlying this association are not completely clear (Duckworth W *et al.*, 2009; Home P *et al.*, 2009). Abundant experimental evidence from animal studies shows that acute hyperglycemia blunts endothelium-dependent vasodilatation in isolated aortas from normal and diabetic animals (Kuusisto J *et al.*, 1995; Durante W *et*

al. 1988). Acute MI is the major contributor to cardiovascular deaths with diabetes and it often progresses into end-stage heart failure (HF) (Ishihara M *et al.*, 2003). Furthermore, stress-induced, acute hyperglycemia in non-diabetic patients with acute MI is associated with increased in-hospital deaths (Masaharu I., 2012). This implies that the affliction of diabetes still warrants much attention and requires a complete understanding of the basic molecular mechanisms as it will allow the development of effective, novel therapies which would aim to reduce the development of the diabetic related complications. To further understand the effects of hyperglycemia, the next section discusses some of the mechanisms whereby hyperglycemia can lead to CVD development.

1.10 Hyperglycemia-induced effects and CVD onset

1.10.1 Hyperglycemia induced oxidative stress

Both chronic and acute hyperglycemia may mediate damaging effects through a sequence of secondary transducers, particularly ROS (Jaswal JS *et al.*, 2011). Oxidative stress exists when the production of ROS is greater than its removal by intracellular antioxidant systems (Jaswal JS *et al.*, 2011). The resultant elevation of ROS has ample deleterious effects on the cardiovascular system e.g. cellular damage by oxidation; disruption of vascular hemostasis through interference with nitric oxide (NO), and by modulation of detrimental intracellular signaling pathways (Brownlee M., 2001). Although under physiologic states most intracellular ROS arise from mitochondria, this may be different within the context of disease onset (Boudina S, Abel ED., 2007). For example, the NADPH oxidase enzymes are also known as ROS generators. These enzymes act as catalysts for electron transfer from NADPH to molecular oxygen, resulting in the generation of free radicals (Li L and Renier G., 2006). Through interaction with an assortment of transcription factors, redox signaling impacts the expression of growth-related genes and in turn disturbs contractile function. A surge in ROS also leads to deoxyribosnucleic acid (DNA) damage and activation of poly (ADP ribose) polymerase (PARP) as a reparative enzyme (Ihnat M *et al.*, 2007). There is also evidence that greater cytosolic ROS generation fuels mitochondrial ROS overproduction, which underscores the pathogenic prominence of ROS generation from non-mitochondrial sources (Ihnat M *et al.*, 2007; Zhou L *et al.*, 2010).

As discussed earlier, mitochondrial ROS is usually the major source of oxidative stress associated with hyperglycemia. Here more glucose is oxidized in the CAC cycle, which

drives greater electron input [NADH and FADH₂ (flavin adenine dinucleotide, reduced)] into the electron transport chain (ETC). There are four protein complexes in the mitochondrial electron transport chain, called complex I, II, III, and IV (Figure 1.4). When glucose is metabolized through the CAC, it generates electron donors (**Chen Y et al., 2005; Genova M et al., 2001**). The main electron donor is reduced nicotinamide adenine dinucleotide (NADH), which provides electrons to complex I (**Carroll J., 2005**). The other electron donor generated by the CAC cycle is FADH₂, formed by succinate dehydrogenase, which donates electrons to complex II (**Cecchini G., 2003**). Electrons from both these complexes are passed to coenzyme Q, and then transferred to complex III, cytochrome-C, complex IV, and finally to molecular oxygen, to then be reduced to water (**Wallace DC., 1992**). The ETC is organized in this way so that the level of ATP can be precisely regulated. This generates what is in effect a voltage across the inner mitochondrial membrane. The energy from this voltage gradient drives the synthesis of ATP by ATP synthase (**Wallace DC., 1992; Trumpower BL., 1990**). Alternatively, uncoupling proteins (UCPs) (Figure 1.4) keeps the rate of ATP generation constant through this process.

Under hyperglycemic conditions the voltage gradient across the mitochondrial membrane increases until a critical threshold is reached due to the excess electron flux via NADH and FADH₂. At this point the electron transfer inside complex III is blocked (**Korshunov SS et al., 1997**), causing the electrons to build up in coenzyme Q, and thereby generating superoxide (Figure 1.4). The mitochondrial isoform of the enzyme superoxide dismutase (SOD) usually degrades this to hydrogen peroxide, which is then converted to water and oxygen. ROS produced can also damage the ETC producing complexes itself thereby further decreasing its activity and exacerbating free radical production (**Brand M et al., 2004**).

ROS production from either mitochondrial and/or non-mitochondrial sources can generate the following products, i.e. the superoxide radical (O₂^{•-}), hydrogen peroxide (H₂O₂), and hydroxyl radical (OH[•]). The hydroxyl radical can be formed in the presence of copper or iron by the amalgamation of O₂^{•-} and H₂O₂ (**Argirova M and Ortwerth B., 2003; Eaton J et al., 2002**). Increased ROS levels are not only the consequence from its overproduction, but may also manifest due to reduced efficacy of inhibitory scavenger systems (**Argirova M and Ortwerth B., 2003**). The endogenous oxidative stress defense mechanisms are stunted during extreme conditions such as hyperglycemia and/or

ischemia-induced oxidative stress (Dhar A *et al.*, 2008). Such antioxidant mechanisms include both glutathione reductase and peroxidase that are involved in the recycling of glutathione (GSH) in its reduced and oxidized form glutathione disulfide (GSSG) (McCord J and Edeas M., 2005). Glutathione peroxidase eliminates hydrogen peroxide by oxidizing GSH into GSSG, which acts as an antioxidant by changing GSSG to GSH (McCord J and Edeas M., 2005). SOD is another antioxidant that converts superoxide to H_2O_2 . SOD can present in three isoforms, i.e. SOD1; a dimer occurring in the cytoplasm, while SOD2 and SOD3 are tetramers found in the mitochondrion and extracellular matrix, respectively (McCord J and Edeas M., 2005). The generation of superoxide leads to events resulting in the inhibition of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), causing increased flux of glycolytic metabolites through the non-oxidative glucose pathways which will be discussed below.

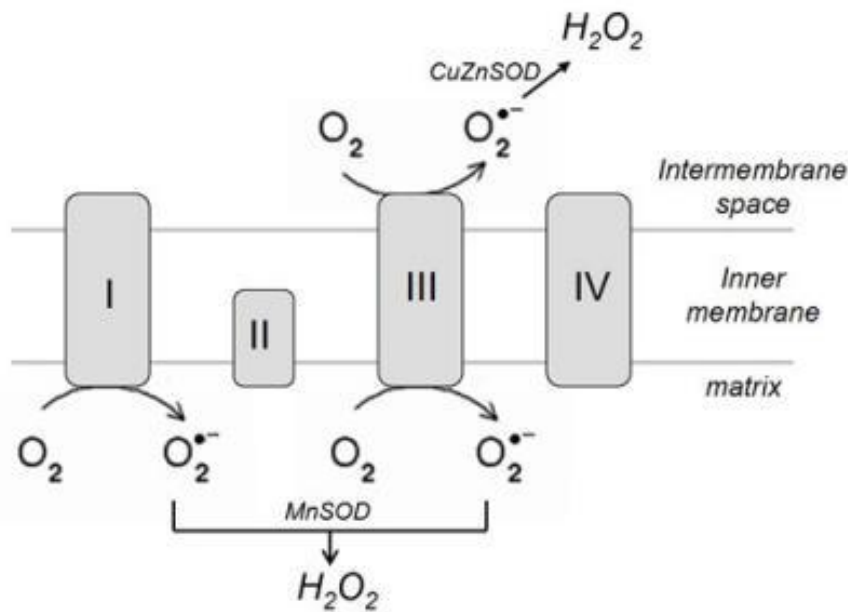


Figure 1.4 The production of superoxide ($O_2^{\bullet-}$) by mitochondrial complex I and III. Free electrons leaked from the electron transport chain during oxidative respiration, allow for the formation of $O_2^{\bullet-}$ from molecular oxygen in complex I and III. $O_2^{\bullet-}$ generated from complex I get converted to hydrogen peroxide (H_2O_2) by manganese superoxide dismutase (MnSOD) in the mitochondrial matrix. $O_2^{\bullet-}$ generated by complex III undergoes a similar fate, but also gets translocated to the cytosol where it is converted to H_2O_2 by copper/zinc superoxide dismutase (Cu/Zn SOD). SOD – superoxide dismutase modified from An D and Rodrigues B., 2003

1.10.2 Hyperglycemia-induced non-oxidant pathway activation

Several animal and human studies have shown that with hyperglycemia there is a decrease in the activity of the key glycolytic enzyme GAPDH due to ROS production (**Boudina S *et al.*, 2007**). As shown in Figure 1.5, the intracellular levels of several glycolytic intermediates (upstream of GAPDH) consequently increase. This in turn can activate two of the four pathways, i.e. the 1) AGE pathway because the major intracellular AGE precursor methylglyoxal is formed from glyceraldehyde-3 phosphate, and the 2) PKC pathway, since the activator of PKC, DAG, is also formed from glyceraldehyde-3 phosphate. However further upstream, levels of the glycolytic metabolite fructose-6 phosphate increase thereby enhancing flux through the 3) hexosamine pathway, where fructose-6 phosphate is converted by the enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT) to uridine diphosphate acetylglucosamine (UDP-GlcNAc) (**Du X *et al.*, 2003**). Finally, inhibition of GAPDH increases intracellular glucose levels that elevates flux through the 4) polyol pathway, where the enzyme aldose reductase converts glucose to sorbitol, consuming NADPH in the process. These pathways are briefly explained below as they are not the focus of this project.

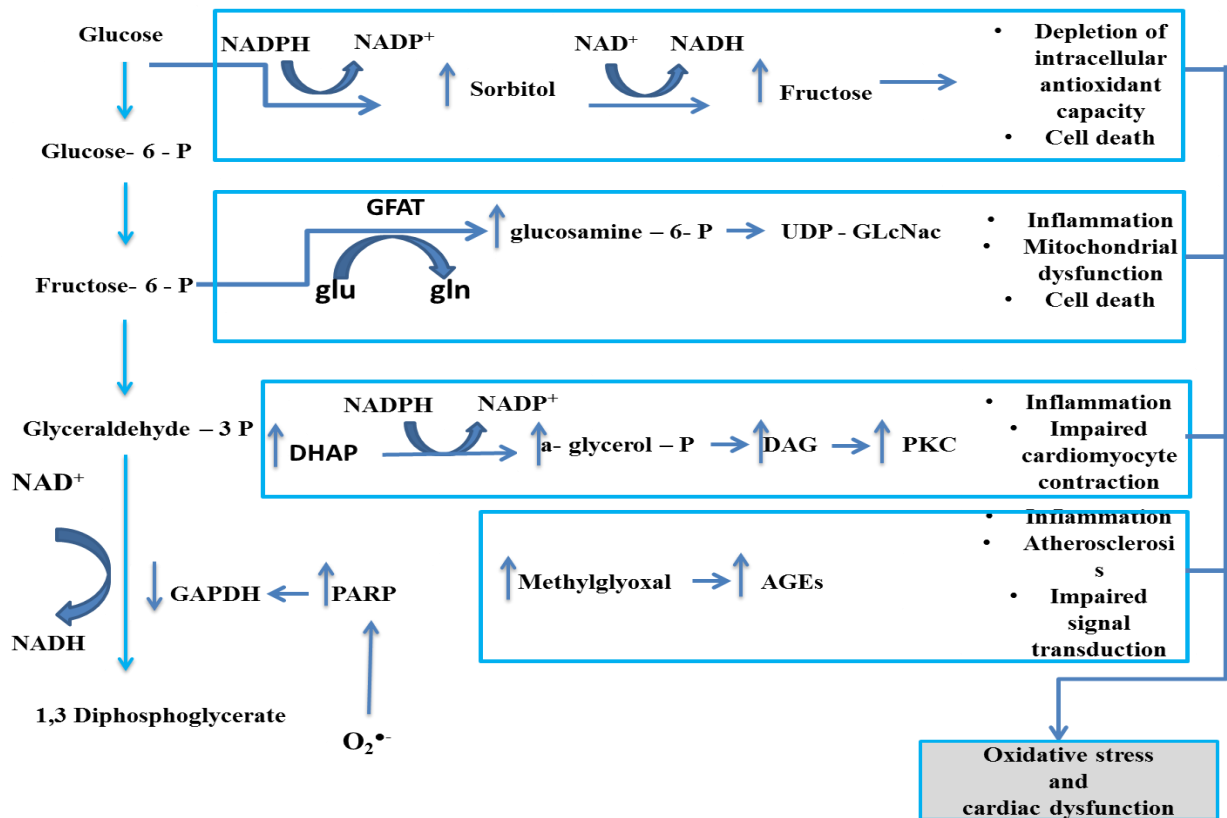


Figure 1.5 Mitochondrial overproduction of superoxide activates four major pathways of hyperglycemic damage by inhibiting GAPDH. NADH (nicotinamide adenine dinucleotide hydrogen); NAD⁺ (oxidized nicotinamide dinucleotide); GAPDH (glyceraldehyde-3-phosphate dehydrogenase); PARP (poly(ADP)ribose polymerase); O₂^{•-} (superoxide radical); NADPH (nicotinamide adenine dinucleotide phosphate hydrogen); NADP (oxidized nicotinamide adenine dinucleotide phosphate); GFAT (glutamine:fructose-6-phosphate amidotransferase); UDP (uridine diphosphate); AGEs (advanced glycation end products); DHAP (dihydroxyacetone phosphate); DAG (diacylglycerol); PKC (protein kinase C). American Diabetes Association. *Diabetes*, 2005

1.10.2.1 Polyol pathway

Here the enzyme aldose reductase reduces the aldehyde form of glucose to sorbitol. Sorbitol is then oxidized to fructose by sorbitol dehydrogenase and enters glycolysis. However, with hyperglycemia there is an increase in sorbitol levels which may exert various detrimental effects. These include deregulation of the cellular osmotic status, reduction of sodium/potassium (Na⁺/K⁺) -ATPase activity, increased cytosolic NADH/NAD⁺, and decreased cytosolic NADPH leading to increased ROS production. Ultimately vascular complications occur through the action of aldose reductase (Lou MF *et al.*, 1988; Obrosova IG *et al.*, 1999).

1.10.2.2 Hexosamine pathway

With the hexosamine pathway, fructose 6-phosphate is converted into N-acetylglucosamine a substrate for proteoglycan synthesis and results in the formation of O-linked glycoprotein. N-acetylglucosamine is implicated in the activation of the transcriptional factor specificity protein 1 (Sp1) which increases synthesis of growth factor-[beta] 1 and plasminogen activator inhibitor-1 associated with development of vascular complications as well as increased oxidative stress (**Han I & Kudlow J., 1997**).

1.10.2.3 DAG-PKC pathway

Hyperglycemia can stimulate *de novo* synthesis of diacylglycerol (DAG), followed by the activation of PKC. This in turn induces changes in endothelial permeability, vasoconstriction, increased synthesis of extracellular matrix, and stimulation of cytokine synthesis, cell growth, angiogenesis, and leukocyte adhesion (**Koya D et al., 2000**). PKC modulates various enzymes including, phospholipase A2, Na⁺/K⁺ -ATPase (**Koya D et al., 2000**).

1.10.2.4 Advanced glycation end-products and associated receptors

Proteins and lipids may become non-enzymatically glycated and oxidized when in subsequent contact with aldose sugars, resulting in AGEs formation as a product of the Maillard reaction - (**Singh R et al., 2001**). The reducing sugars in the Maillard reaction react with amino groups of proteins to produce a Schiff base. Molecular rearrangements result in more stable Amadori products (ketoamine). Further rearrangements, condensations and dehydrations result in the formation of intermediate α -oxoaldehydes such as methylglyoxal (MG) and 3-deoxyglucosone (**Gkogkolou P and Böhm M., 2012**). MG can also be made via non-oxidative mechanisms in anaerobic glycolysis (**Thornalley PJ., 1996**), and the oxidative breakdown of polyunsaturated FAs inducing oxidative stress and cell death (**Okado A et al., 1996; Zarubin T and Han J., 2005**).

AGE-related injuries can occur via three possible mechanisms: firstly, AGEs can accumulate in the extracellular matrix resulting in cross-link formation, causing blood vessels to become narrower and stiffen (as in atherosclerosis) (**Yamagishi S and Imaizumi T., 2005**). Secondly, AGEs can have an effect on signaling pathways through AGE-mediated glycation of intracellular proteins, e.g. intracellular AGEs decrease

endothelium nitric oxide synthase (eNOS) activity resulting in defective vasodilation (**Bucala R et al., 1991**). MG is also a precursor of intracellular AGEs which alter antioxidant systems causing increased oxidative stress (**Bucala R et al., 1991**). Lastly, the interaction between AGEs and its receptors (RAGEs), leads to the stimulation of downstream signaling cascades, resulting in increased ROS generation via NADPH oxidase (NOX) activation (**Basta G et al., 2004**). The enhancement of oxidative stress and stimulation of cytokines and growth factors lead to the acceleration of chronic inflammation and endothelial dysfunction as well as development of vascular diabetes complications (**Basta G et al., 2004**).

Together this indicates that mitochondrial and non-mitochondrial ROS are key players in the manifestation of cardio-metabolic pathophysiologies. Here the initiating event is mitochondrial superoxide production that triggers further ROS generation (other sources) thereby creating a vicious cycle. Subsequently, ROS can induce cellular damage by various mechanisms. Therefore it is important to understand the molecular mechanisms of events which occur during acute myocardial infarction (AMI) under hyperglycemic conditions and how this can result in myocardial damage.

1.11 Metabolic changes during ischemia and reperfusion

As stated before, most of the heart's energy requirements are derived from the oxidative phosphorylation of metabolic fuel substrates. Thrombosis or atherosclerosis causes myocardial ischemia resulting in minor or severe deficiency of coronary blood flow from the large arteries to the myocardium thus leading to an oxygen-deprived myocardium, fuel substrates and the build-up of metabolites (**Park J and Lucchesi B, 1999**) and ultimately triggering cell death within the infarcted area (**Mochizuki S and Neely R, 1979; Solaini G and Harris DA, 2005**). Ischemic conditions also alter cardiac structure, function and perturbations in energy substrate metabolism influencing FA and GO. There is a rapid loss of contractile force and disturbances in ionic homeostasis due to lack of blood supply to the heart. Alterations in oxygen and substrate availability in turn change mechanisms regulating substrate metabolism hence affecting cardiac efficiency (**Kim AS and Johnston SC, 2011**). Prominent alterations in energy metabolism occur in the setting of myocardial ischemia, depending on the severity (mild to severe) and reperfusion resulting in a decrease in cardiac efficiency. The reduced oxygen availability attenuates both GO and FAO (**Jaswal JS et al, 2011; Seifert EL et al, 2008**).

Interestingly, during mild ischemia FAO dominates as the source of myocardial oxidative metabolism. It is important to note that unlike the normal heart, exposure of the ischemic heart to FAs does not hinder glycolysis. The glycolytic rates are raised in the ischemic heart (Lopaschuk GD., 1993); however chronic hyperglycemia can result in amplified ischemic metabolic changes due to the manifestation of altered metabolic dysfunction (Kannel WB and McGee DL., 1979). This implies that the FA surplus (and intermediates e.g. fatty acyl-CoAs, DAG and ceramides) and attenuated GO found with diabetes or the metabolic syndrome (Lewandowski E and White L., 1995, Liedtke A *et al.*, 1988) is further exacerbated during AMI since it manifests in the same way. Nevertheless there is a rise in glycolysis and diminished GO in the ischemic heart. This occurs due to elevation of glucose uptake by the myocardial cell membrane (Sun D *et al.*, 1994) that occurs by increased GLUT1/4 translocation and activity (Brosius III F *et al.*, 1997, Chen T *et al.*, 1997) secondary to increased AMPK activation (Misra P and Chakrabarti R., 2007). The increase in glucose transport with decreased oxygen availability may be of physiological importance, i.e. allowing the heart to obtain more energy from glycolysis under conditions where FAO is predominant (Stanley WC *et al.*, 1997). However, increased FAO inhibits GO, resulting in an imbalance between glycolysis and GO (Saddik M and Lopaschuk G., 1991) resulting in lactate and proton accumulation in the ischemic myocardium.

1.12 Ionic imbalances during ischemia and reperfusion

The uncoupling of glycolysis from GO largely explains the acidosis observed in the severely ischemic heart. Increased FAO results in a scenario where there is an accumulation of protons that can lead to sodium (Na^+) and calcium (Ca^{2+}) overload in the heart, resulting in decreased cardiac efficiency as ATP is used to restore ionic homeostasis (Ashrafian H., 2011). Furthermore, the decrease in intracellular pH is associated with a loss of troponin C sensitivity to PFK and Ca^{2+} resulting in contractile arrest and cellular bulging (Abe T *et al.*, 2002; Belke DD *et al.*, 2004, Choi KM *et al.*, 2002). Thus Na^+ overload occurs as the myocardium tries to compensate for the fall in pH by excessively excreting hydrogen ion (H^+) in exchange for Na^+ influx by the sodium/hydrogen (Na^+/H^+) exchanger (Pike MM., 1993). On the other hand, intracellular ATP reduction gradually inactivates ATPases, such as the Na^+/K^+ ATPase, ATP-dependent Ca^{2+} reuptake, and activates Ca^{2+} excretion, resulting in Ca^{2+} overload. This

causes a fragile cellular structure, hypercontracture, which leads to contraction band necrosis, and the initiation of apoptotic cascades (**Schäfer C., 2001**) due to the uncoupling of glycolysis under ischemic events.

Upon reperfusion, speedy normalization of the extracellular pH will promptly create an extreme H^+ gradient across the plasma membrane that generates a robust Na^+/H^+ exchange and a massive Na^+ influx, leading to Ca^{2+} overload. Meanwhile, rapid normalization of intracellular pH disinhibits a low pH-derived inhibition of the Ca^{2+} -dependent protease calpain, hypercontracture, and the mitochondrial permeability transition all at once and promptly hastens myocardial damage in the initial stages of reperfusion (**Hausenloy D., 2012**). Although there is no doubt that reperfusion does indeed salvage the ischemic myocardium, it can paradoxically also induce some detrimental effects, i.e. a phenomenon referred to as “ischemia/ lethal reperfusion injury” (**Sanada S et al., 2011; Hausenloy D., 2012**) discussed below

1.13 Myocardial reperfusion injury

Reperfusion injury refers to myocardial, vascular, or electrophysiological dysfunction that is induced by the restoration of blood flow to previously ischemic tissue and was first postulated by Jennings et al. in 1960.

He proposed that during myocardial reperfusion, the acute ischemic myocardium is subjected to numerous unexpected biochemical and metabolic changes, which compound the fluctuations generated during the period of myocardial ischemia. These changes include mitochondrial re-energization, the generation of ROS, intracellular Ca^{2+} overload, the rapid restoration of physiologic pH, and inflammation, all of which cross-interacts with each other to mediate cardiomyocyte death through the opening of the mitochondrial permeability transition pore (PTP) and the induction of cardiomyocyte hypercontracture. During myocardial reperfusion, ROS is generated by xanthine oxidase (mainly from endothelial cells) and the re-energized ETC in cardiomyocyte mitochondria. Free radicals damage myocytes directly by altering membrane proteins and phospholipids (**Zweier JL., 1988**). Because these membrane constituents play crucial roles as receptors, enzymes, and ion channels, free radical injury can lead to fatal metabolic and structural derangements. For example, radicals injure the sarcolemma and may impair contractile function of the myocyte (**Hearse DJ et al., 1973**). The role for free radicals as a source of significant

myocardial damage is further supported by studies showing that free radical scavengers, such as SOD, administered during thrombolytic therapy, help preserve myocardial function (**Flaherty JT et al., 1994**). However, several hours later NADPH oxidase (source of ROS mainly from neutrophils) stimulates leukocyte activation, chemotaxis, and leukocyte-endothelial adherence (**Zweier JL., 1988; Hearse DJ et al., 1973**).

The neutrophil accumulation in non-perfused microvessels and complement activation may contribute to the "no-reflow" phenomenon (**Ito H., 2003; Piper HM et al., 1988**). Once in the extravascular space, leukocytes can release proteases and elastases that destroy the cell membrane and cause cell death (**Piper HM et al., 1988; Keeley EC et al., 2003**). However, the roles of the various inflammatory mediators are complex and incompletely understood. Although they play a role in reperfusion injury, some also appear to have cardioprotective effects (**Vinten-Johansen J., 2004; Zhao ZQ et al., 1997**). Terminal complement cascade species directly injure the endothelium, rendering it incapable of producing vasodilatory compounds such as NO. This perpetuates a cycle of vasoconstriction and reduction in microvascular perfusion, leading to apoptosis not only within the infarct area, but also in the border zone (**Zweier JL., 1988; Ly HQ et al., 2005**).

ROS also mediates myocardial injury by inducing mitochondrial permeability transition pore (mPTP) opening already, acting as neutrophil chemoattractants, mediating dysfunction of the sarcoplasmic reticulum and contributing to intracellular Ca^{2+} overload, damaging the cell membrane by lipid peroxidation, inducing enzyme denaturation, and causing direct oxidative damage to DNA (**Bär F;W et al., 2006**). Furthermore, the restoration of the mitochondrial membrane potential drives the entry of Ca^{2+} into mitochondria that in conjunction with the loss of the inhibitory effect of the acidic pH on the mPTP and the generation of ROS, act in concert to mediate the opening of the mPTP (**Idem., 2003; Gumina RJ., 1999**). This opening induces cardiomyocyte death by uncoupling oxidative phosphorylation and inducing mitochondrial swelling.

During myocardial reperfusion, the rapid washout of lactic acid together with the function of the Na^+-H^+ and sodium bicarbonate (Na^+-HCO_3) transporters mediate the rapid restoration of physiologic pH, and also facilitating mitochondrial PTP opening and cardiomyocyte hypercontracture (**Zeymer U et al., 2001**). Several hours after the onset of myocardial reperfusion, neutrophils accumulate in the infarcted myocardial tissue in

response to the release of the chemoattractants ROS, cytokines, and the activated complement pathway. The up-regulated cell-adhesion molecules P-selectin and complement receptors consisting of CD18 (integrin β_2) and CD11b (integrin α_M), and intracellular adhesion molecule 1 (ICAM-1) then facilitate the migration of neutrophils into the myocardial tissue, where they mediate cardiomyocyte death by causing vascular plugging, releasing degradative enzymes, and generating ROS (Mertens P *et al.*, 2006; Tanguay JF *et al.*, 2004).

1.14 Various modes of cell death in CVDs

While ischemic damage occurs principally through necrosis, reperfusion injury may also manifest as a result of apoptosis (Kajstura J *et al.*, 1996). For example, markers of apoptosis found in reperfused tissue were not present in normal tissue or tissue injured solely by ischemia (Fliss H and Gattinger D., 1996). Others have reported the appearance of apoptotic cells in the peri-necrotic zone during reperfusion (Lee P *et al.*, 2003) and suggested that understanding the different contributions of necrosis and apoptosis to reperfusion injury may help identify novel treatment strategies (Bhuiyan MS and Fukunaga K., 2007). A link between reperfusion and apoptosis is supported by studies demonstrating the role of magnesium-dependent SOD and $O_2^{\bullet-}$ formation in the activation of pro-apoptotic factors. An apoptosis repressor is capable of inhibiting this process and reducing the extent of myocardial infarction (Fliss H and Gattinger D., 1996). Cardiomyocytes respond to stress by the accumulation of protective proteins that may counteract damage, thereby temporarily increasing tolerance to such damage. On the other hand, it may trigger programmed cell death (apoptosis) to remove terminally damaged cells (Anversa P *et al.*, 1998, Bhuiyan MS and Fukunaga K., 2007) which can be eliminated by the ubiquitin proteasome system and/or lysosomal-autophagal system. We will next discuss the various modes of cell death that may occur in various cardiac diseases e.g. HF, diabetic cardiomyopathy, AMI, and I/R. The modes of cell death discussed are autophagy, apoptosis, and necrosis as they are present in various CVDs of interest to us AMI; however, for this thesis apoptosis is of particular interest.

1.14.1 Necrosis

Necrosis is recognized by distinctive morphological changes, including cell swelling, plasma membrane damage, loss of ATP, and organelle swelling. The disturbance of cell integrity and release of cellular content leads to a secondary inflammatory response, with potential pathological consequences (**Whelan RS *et al.*, 2010**). Necrosis is usually activated by physical or chemical distress to the cell and has been thought to be a passive and accidental form of cell death (**Vanlangenakker N *et al.*, 2008**). In recent times, growing evidence suggests that a portion of necrosis is regulated by serial signaling events in an exact and orchestrated manner. Numerous terms have been introduced to describe this form of necrosis, such as programmed necrosis, caspase-independent cell death, and necroptosis (**Henriquez M *et al.*, 2008**). Numerous mechanisms have been linked to describe the initiation and execution of necrosis, including death receptors, ROS, Ca^{2+} , and mPTP opening (**Kroemer G *et al.*, 2007**; **Vanlangenakker N *et al.*, 2008**).

The ionic imbalances that occur in certain pathologies such as I/R (refer to section 1.12) and the associated mitochondrial swelling and membrane rupture (**Schäfer C., 2001**) result in necrotic cell death (**Whelan RS *et al.*, 2010**). Moreover, increased H^+ in the cytoplasm and inactivation of H^+ pumps elicit declines in lysosomal pH, which results in over-activation of proteases such as cathepsins. The substantial entry of water into lysosomes causes swelling, membrane rupture and the release of proteases into the cytoplasm. Together with other activated proteases, such as calpains, this can digest different substrates, including cytoskeletal proteins, contributing to necrosis (**Henriquez M *et al.*, 2008**). The activation of death receptors, such as the tumour necrosis factor alpha (TNF- α) receptor, represents other necrotic pathways in cardiac myocytes under certain conditions such as HF. The activation of such receptors could lead to the activation of receptor-interacting protein (RIP), increased ROS, and necrosis. However, with myocardial infarction there remains disagreement concerning the leading mode of cell death i.e. necrosis, apoptosis, and autophagy (**Baines C *et al.*, 2005**; **Gottlieb R *et al.*, 1994**).

1.14.2 Apoptosis

Apoptosis is an active evolutionarily conserved form of cell self-destruction. Cells undergoing apoptosis become fragmented and form apoptotic bodies. These are then recognized by macrophages and cleared from the tissue to avoid inflammatory responses. Apoptosis is facilitated in two ways, the extrinsic and the intrinsic pathways, and both have been described in cardiac myocytes (**Kerr J *et al.*, 1972; Kroemer G *et al.*, 2005**).

1.14.2.1 Extrinsic /external/death receptor pathway

The external pathway is triggered by binding of death ligands to cell surface receptors (endonucleases). The best recognized receptors are those for TNF- α and Fas also named apoptosis antigen 1 (APO-1) ligand receptor (**Peter M and Krammer P., 2003**). The stimulated death receptor then transfers signals to the Fas-associated death domain protein (FADD). The Fas and TNF- α receptors are then expressed in cardiac myocytes and implicated in cardiovascular pathology. Since TNF- α signaling is more complex due to the fact that it can either promote survival or death; events following binding of Fas ligand are defined. The death receptor activation by a death ligand induces death-inducing signaling complex (DISC) formation and caspase-8 activation, which in turn activates caspase-3 (**Ashkenazi A., 2002**).

1.14.2.2 The intrinsic/mitochondrial pathway

As its name suggests, the intrinsic (or mitochondrial) pathway is initiated from within the cell. It is activated in response to signals resulting from DNA damage, loss of cell-survival factors, or other types of severe cell stress. Because mitochondria can also contribute to cell death in response to multiple stresses, cardiac myocytes have developed special strategies to achieve strict control over the intrinsic apoptotic pathway (**Whelan RS *et al.*, 2010; Lee Y and Gustafsson AB., 2009**). Normally, pro-apoptotic proteins are released from mitochondria to activate caspase proteases and trigger apoptosis (**Cotter T.G., 2009**) (Figure 1.6). The intrinsic pathway is activated by the proteolysis of B cell leukemia/lymphoma-2 (Bcl-2) - homology domain 3 (BH3) -only interacting protein domain (BID) to truncated BID (t-BID) by caspase-8 and interaction of t-BID with Bcl-2-associated X protein (BAX) in the mitochondria. Pro-apoptotic Bcl-2-associated X protein/ Bcl-2 homologous antagonist /killer (BAX/ BAK) induces cytochrome c, second

mitochondria-derived activator of caspase/direct inhibitor of apoptosis (IAP)-binding protein with low PI (Smac/DIABLO), apoptosis inducing factor (AIF), and Endo G release from the mitochondria. Cytochrome c together with adaptor protein apoptotic protease activating factor-1 (Apaf1) and caspase-9 form the apoptosome with the activation of caspase-9. Caspase activity, however, is controlled by the endogenous caspase inhibitor X-linked inhibitor of apoptosis protein (XIAP). Cardiac myocytes are naturally less vulnerable to apoptosis because of their low-level expression of Apaf1 and caspases and high levels of XIAP (Ashkenazi A., 2002) (Figure 1.6).

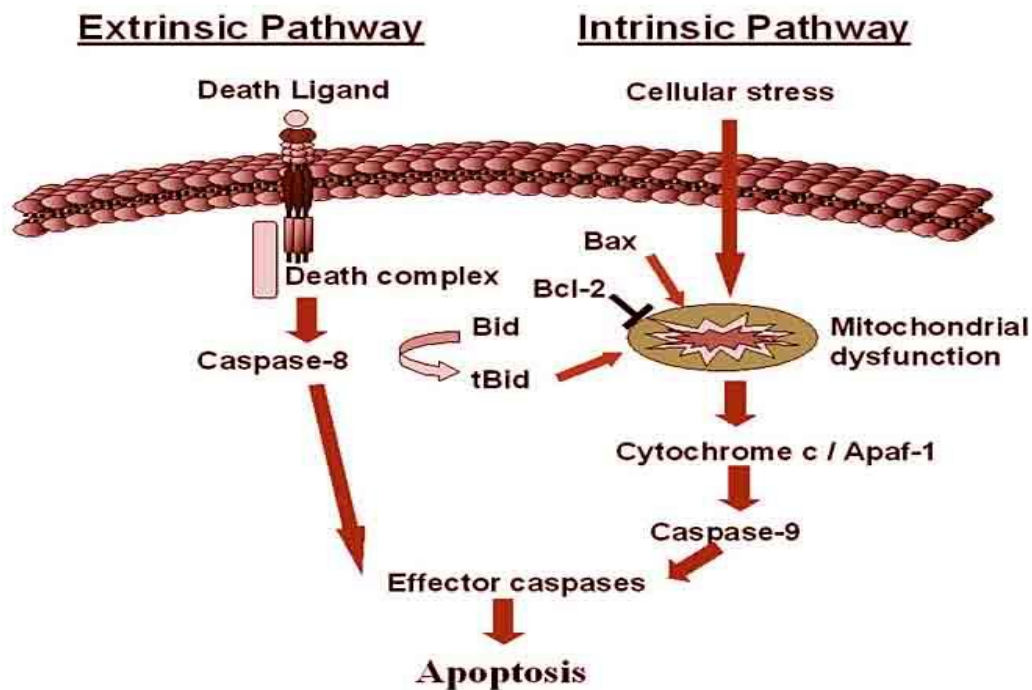


Figure 1.6. Apoptotic pathway in cardiomyocytes. Bax (Bcl-2-associated X protein), Bcl-2 (B cell leukemia/lymphoma-2), Bid (BH3 -only interacting protein domain), tBid (truncated BID), Apaf-1 (adaptor protein apoptotic protease activating factor-1) modified from Russell S et al., 2010

When such pro-apoptotic signals are not released, cells are unable to die (Cotter TG., 2009). The intrinsic pathway hinges on the balance of activity between pro- and anti-apoptotic signals of the Bcl-2 family (Vogler M et al., 2009). Pre-clinical studies indicate that members of the Bcl-2 family regulate the permeability of the mitochondrial

membrane and determine whether a pro- or anti-apoptotic signal will be released inside the cell (**Mayer B and Oberbauer R., 2003**). Research implies that in this cascade, the anti-apoptotic proteins [e.g. B cell leukemia/lymphoma-x-isoform (Bcl-x_L)] antagonize Bax and Bak by binding to their BH3 domains. This antagonism is relieved by BH3-only, pro-apoptotic proteins [e.g., Bcl-2 like protein 11 (BIM), BH3-only interacting protein domain (BID), Bcl-2 associated death promoter (BAD)], which alternately bind to anti-apoptotic proteins, like Bcl-x_L. In normal cells, cellular stress would result in an upregulation of these BH3-only proteins in order to initiate apoptosis via the intrinsic apoptotic pathway (**Lessene G et al., 2008**).

Activation of the mitochondrial apoptotic pathway leading to executioner caspase activation is relevant with heart injuries (**Whelan RS et al., 2010**), emerging from studies using immunofluorescence microscopy (**Lee Y, Gustafsson AB., 2003**). Bae et al. 2010 reported that apoptosis can be induced in the heart lacking caspase activation via caspase-independent pathways, probably through apoptosis inducing factor (AIF). Although it has been described that AIF is released from cardiac myocyte mitochondria during I/R, its contribution to I/R-induced apoptosis was discounted (**Bahi N et al., 2006**). However, AIF has been implicated in cardiac myocyte death induced by oxidative stress and HF (**Chen M et al., 2004**). Both the intrinsic and extrinsic pathways can be inhibited by the cytoprotective protein apoptosis repressor with caspase recruitment domain (ARC) (**Gustafsson AB et al., 2004**). Because of the intrinsically low levels of Apaf1 and caspases in cardiac myocytes, non-myocyte cells within the heart may be more susceptible to apoptosis (**Bahi N et al., 2006, Gustafsson AB et al., 2004**).

The central role of intrinsic apoptosis in cardiac dysfunction following I/R has been reported in several studies. For example, cardiac myocyte-specific overexpression of Bcl-2 substantially reduces infarct size, cardiac myocyte apoptosis, and cardiac dysfunction following I/R (**Chen Z et al., 2001**); Bax deficiency reduces infarct size and cardiac dysfunction following I/R and following MI in mice (**Hochhauser E et al., 2007**) and targeted deletion of PUMA, which is a BH3-only protein, reduced infarct size in an *ex vivo* Langendorff I/R model (**Toth A et al., 2006**). With regards to hyperglycemia, oxidative stress has been suggested to play a more central role in causing apoptosis, particularly when linked with mitochondrial dysfunction (**Bhat N and Zhang P., 1999**;

Buttke T and Sandstrom P., 1994). The last form of cell death is autophagy which will be discussed below.

1.14.3 Autophagy

The term autophagy means from the Greek “auto” or oneself and “phagy” or to eats. Involves the degradation of cellular components, either because they are deleterious (e.g., damaged organelles and microbial invaders) or because the resulting breakdown products are needed to support metabolism (**Levine B, Kroemer G *et al.*, 2008**). Autophagy is one of the key cellular pathways that mediate’s stress-induced adaptation and damage control. It is a highly conserved process of delivering intracellular components (including mitochondria and long-lived macromolecules) to lysosomes for degradation via a double membrane structure called autophagosome (**Levine B, Kroemer G *et al.*, 2008**). The expansion of autophagosome membranes involves Atg12 and Atg8 (called LC3 in mammals). Atg7 and E2-like Atg10 covalently link Atg12 with Atg5, which together bind Atg16L1 to produce pre-autophagosomal structures. LC3 is then cleaved by the protease Atg4. Phosphatidylethanolamine is conjugated to cleaved LC3 by Atg7, Atg3, this lipidated LC3-II associates with newly forming autophagosome membranes. LC3-II remains on mature autophagosomes until after fusion with lysosomes (**Mizushima N., 2007**). Autophagy of depolarized mitochondria is initiated by Pink1-dependent mitochondrial translocation of Parkin. This is followed by ubiquitination of the mitochondrial proteins and recruitment of p62 to direct mitochondria to be autophagocytosed which can lead to cell death (**Mizushima N., 2007**). In eukaryotic cells and in cardiac myocytes, starvation/nutrient deprivation, hypoxia, ROS, damaged organelles, and protein aggregates have been shown to induce autophagy in a mammalian target of rapamycin (mTOR)-dependent process (**Levine B, Kroemer G *et al.*, 2008; Marambaio P *et al.*, 2010**). Similarly, mTOR-independent autophagy has been reported; here, cytokines which do not exist in yeast, converge on type III phosphatidylinositol 3-kinase to induce autophagy (**Lipinski MM *et al.*, 2010**). Autophagy is a pro-survival mechanism that replenishes energy under stress conditions.

The two pathways responsible for ischemia/hypoxia-induced autophagy are (Bcl2)/adenovirus E1B interacting protein 3 (BNIP3) or AMPK (**Bahi N *et al.*, 2006; Russell RR *et al.*, 2004**). In a mouse model conveying dominant-negative AMPK in cardiac myocytes, the autophagic response to ischemia was reduced, resulting in a larger

MI and worse cardiac function (**Russell RR *et al.*, 2004**). During reperfusion, autophagy is further upregulated, even though the delivery of oxygen and nutrients is restored and AMPK is rapidly inactivated (**Matsui Y *et al.*, 2004**; **Hamacher-Brady A *et al.*, 2006**). The continued activation of autophagy during reperfusion is qualitatively different to the ischemic context, especially in terms of mechanisms of induction (**Hamacher-Brady A *et al.*, 2006**). For example, oxidative stress, mitochondrial damage/BNIP3, endoplasmic reticulum stress, and calcium overload, are likely to have more important roles in maintaining autophagy at a higher level during reperfusion (**Russell RR *et al.*, 2004**) whereas with ischemia the main stimulators of autophagy are from different pathways such as Beclin-1-mediated (**Valentim L *et al.*, 2006**). Upregulation of autophagy can be either beneficial or detrimental within the context of I/R (**Matsui Y *et al.*, 2004**; **Hamacher-Brady A *et al.*, 2006**). One reason why this is the case is that during ischemia there is a decline in the levels of lysosome-associated membrane protein 2 (LAMP2), a protein critical for autophagosome–lysosome fusion, mediated by ROS-induced activation of serine and cysteine proteases; reperfusion induces upregulation of Beclin-1, which further impairs autophagosome processing, culminating in increased ROS generation, mitochondrial permeabilization, and cardiomyocyte death (**Ma X *et al.*, 2011**). More investigation is needed to clarify when and how elevated autophagy may be pro-survival to cardiac myocytes subjected to reperfusion injury. As a result, additional studies are essential to resolve such conflicting findings.

1.14.4 Cross talk between the ubiquitin–proteasome system and autophagy

A connection between the proteasome and autophagy systems has been established and is well documented, with studies establishing that ubiquitinated protein aggregates can be substrates for autophagic degradation (**Komatsu M *et al.*, 2007**; **Korolchuk VI *et al.*, 2009**; **Kirkin V *et al.*, 2009**). Various studies demonstrated that when the UPS is inhibited it leads to increased autophagy and *vice versa* (**Ding WX *et al.*, 2007**; **Hara T *et al.*, 2006**; **Komatsu M *et al.*, 2006**). This is largely due to p62/SQSTM1 also known as sequestosome 1 that is an ubiquitin-binding scaffold protein that co-localizes with ubiquitin protein aggregates in many neurodegenerative diseases (**Bjorkoy G *et al.*, 2009**). It polymerizes via N-terminal polymerase basic 1 (PB1) domain and can interact with ubiquitinated proteins via the C-terminal ubiquitinated-associated domain (UBA)

domain (Bjorkoy G *et al.*, 2009). Furthermore, it is able to bind directly with light chain 3 (LC3) through specific motifs. It can be degraded by autophagy and may serve as a link for ubiquitinated proteins to the autophagic machinery; to eventually be degraded by the lysosome (Bjorkoy G *et al.*, 2009). Evidence also exists that although the synchronized activity of these two systems are controlled by similar transcription factors, it is still functionally independent of one another (Zhao J *et al.*, 2008; Zhao J *et al.*, 2007). *In vitro* studies demonstrated that the accumulation of polyubiquitinated proteins was adequate enough to induce cardiomyocyte autophagy (Komatsu M *et al.*, 2007; Korolchuk VI *et al.*, 2009; Kirkin V *et al.*, 2009). The ubiquitin-binding protein nucleoporin p62 (p62) controls the formation of protein masses and is removed by autophagy. Neighbor of BRCA1 gene 1 protein (NBR1) is also recruited to ubiquitin-positive protein aggregates and degraded by autophagy (Yamaguchi O *et al.*, 2012). NBR1 and p62 can act as receptors for selective autophagosomal degradation of ubiquitinated targets (Yamaguchi O *et al.*, 2012). These results propose a connection between the ubiquitin–proteasome system and autophagy. Even though cross-talk exists between autophagy and the lysosomal protein degradation, our attention is on the non-lysosomal UPS and the part it plays in the degradation of proteins in the heart under hyperglycemic conditions with I/R.

1.15 Structure and general function of the UPS

Proteasomes are very large protein complexes found inside all eukaryotes, and their main function is to degrade surplus or damaged proteins by proteolysis, a chemical reaction that breaks peptide bonds (Lodish, 2004). The degradation process yields peptides of about seven to eight amino acids long, which can then be further degraded into amino acids and used for the synthesis of new proteins (Lodish, 2004). Therefore this process is vital for normal cellular function. The UPS also partakes in the degradation of misfolded and dislocated proteins from the endoplasmic reticulum (ER) and therefore plays a key role in the cells' response to ER stress and the removal of damaged proteins by oxidative stress (Grune T *et al.*, 1997; Hershko A., 1991). The system is present within the nucleus and the cytosol, and here the proteins are marked for degradation by covalent linkage to multiple ubiquitin molecules, which is a multistep process (Zolk O *et al.*, 2006) (Figure 1.7).

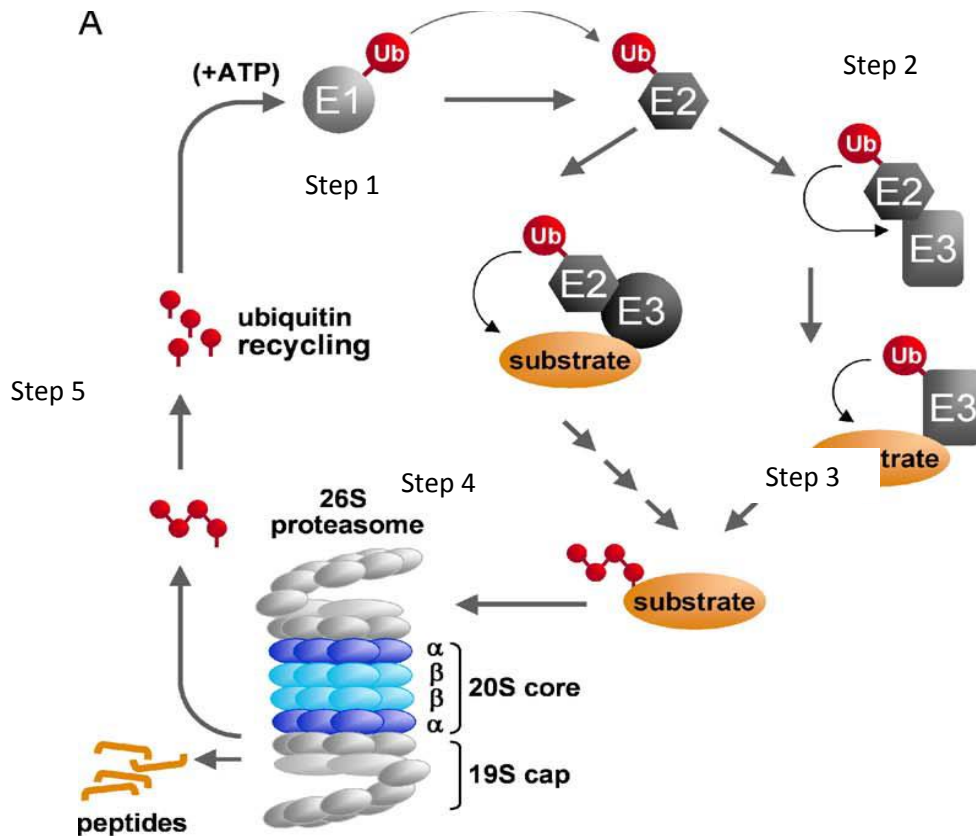


Figure 1.7. *The ubiquitin-proteasome pathway. Step 1: activation of ubiquitin by the ubiquitin activating enzyme E1, an ubiquitin-carrier protein, E2, and ATP, producing a high energy E2ubiquitin thiol ester intermediate. Step 2: binding of the protein substrate to a specific ubiquitin-protein ligase, E3. Step 3: multiple (n) cycles of ubiquitin conjugation, resulting in a polyubiquitin chain. Step 4: degradation of the substrate by the 26S proteasome complex with release of short peptides. Step 5: recycling of ubiquitin is recycled via deubiquitinating enzymes (DUBs) Zolk et al, 2006.*

Polyubiquitin chains are assembled via an isopeptide linkage between the lysine residue of the previous ubiquitin and the C-terminal glycine residue of the subsequent ubiquitin moiety. Once marked by polyubiquitin chains, proteins are swiftly degraded by the 26S proteasome, which is a 2000-kDa ATP-dependent proteolytic complex (Zolk O *et al.*, 2006). The 20S proteasome is a C₂-symmetric cylindrical complex consisting of a "core" of four stacked rings around a central pore. Each ring is composed of seven individual proteins. The inner two rings are made of seven β subunits that contain the six protease active sites. These sites are situated on the interior surface of the rings, so that the target protein must enter the central pore before it is degraded (Mearini G *et al.*, 2008). However, eukaryotes lack enzymatic activity of the β ₃, β ₄, β ₆ and β ₇ but this loss is offset by the diverged substrate specificity of the remaining subunits. Of these, β ₁ also

known as 'caspase-like' because it recognizes and processes substrates having acidic residues at position 1 (the amino acid occupying in the proteasome active site the position containing the scissile, a covalent chemical bond that can be broken by an enzyme, amide bond) (**Jung T *et al.*, 2009**). The β 2-subunit preferentially cleaves the C-terminus of basic amino acids and is also referred to 'trypsin-like', whereas β 5 prefers hydrophobic residues and is referred to as 'chymotrypsin-like'. The 19S proteasome contains two outer rings, each comprising seven α subunits whose function is to maintain a "gate" through which proteins enter the barrel. The α subunits are controlled by binding to "cap" structures or regulatory particles that identify the polyubiquitin tags attached to the protein substrates and initiate the degradation process. The overall system of ubiquitination and proteasomal degradation is known as the ubiquitin-proteasome system (**Mearini G *et al.*, 2008; Jung T *et al.*, 2009**).

The 19S regulatory particle is responsible for stimulating the 20S unit to degrade proteins. A primary function of the 19S regulatory ATPases is to open the "gate" within the 20S that usually blocks the entry of substrates into the degradation chamber. The mechanism by which the proteasomal ATPase opens this has recently been elucidated. Here the 20S gate opening, and thus substrate degradation, requires the C-termini of the proteasomal ATPases, which contains a specific motif (i.e. HbYX motif) (**Jung T *et al.*, 2009**). The ATPases C-termini bind into pockets at the top of the 20S, and tether the ATPase complex to the 20S proteolytic complex thus joining the substrate unfolding equipment with the 20S degradation machinery. Binding of C-termini into 20S pockets by themselves excites the opening of the gate much like a "key-in-a-lock" opens a door. The precise mechanism by which this "key-in-a-lock" mechanism functions has been structurally revealed (**Jung T *et al.*, 2009**).

The conjugation of ubiquitin (Ub) to substrates usually involves three steps (refer Figure 1.7): an initial activation step catalyzed by E1; an intermediate step in which the Ub is covalently linked to a conjugating enzyme, E2; and a final step where the Ub reaches its final destination of the substrate amino group (**Zolk O *et al.*, 2006**). The last step is assisted by the E3 ligase enzyme family. Some types of E3s facilitate Ub conjugation to the substrate directly from E2 by acting as a bridging factor. Another type of E3 forms an Ub–thiol–ester intermediate before the ubiquitin is transported to the substrate. After the linkage of a polyubiquitin chain onto the protein that is to be degraded, its recognition occurs at the 19S cap of the 26S proteasome (**Powell SR *et al.*, 2006**). The mechanism by

which a polyubiquitinated protein is targeted to the proteasome is not completely understood. However, the ubiquitin-receptor proteins contains an N-terminal ubiquitin-like (UBL) domain and one or more UBA domains that are recognized by the 19S proteasome caps and bind ubiquitin via three-helix bundles, respectively (**Herrmann J *et al.*, 2004**). These receptor proteins may aid the polyubiquitinated proteins towards the proteasome, though the specifics of this interaction and its regulation are uncertain. Degradation is brought about by the proteolytic activity of a pair of three different h-subunits, h1, h2, and h5, each located in the cylinder of the 20S core unit of the 26S proteasome (**Zolk O *et al.*, 2006; Herrmann J *et al.*, 2004**).

1.16 The cardiac 26S proteasome

Cardiac proteins are in a dynamic state of constant degradation and re-synthesis. The cardiac proteasome has distinct properties with a specific molecular composition and post-translational modifications (**Toth A *et al.*, 2006**), with substrate specificity mainly restin in the E3 ubiquitin ligases. Numerous cardiac E3 ligases including atrogin-1/muscle atrophy F-box (MAFbx), muscle RING finger (MuRF), Hsp70-interacting protein (CHIP), and murine double minute 2 (MDM2) have already been implicated in the onset of cardiac diseases (**Toth A *et al.*, 2006**). Cardiac proteasome activity has also been found to decrease with age, as validated by increased oxidized and Ub proteins together with decreased 20S proteasome content and loss of specific activities. Loss of proteasome function may damage the ability of myocytes to elicitan appropriate response to stress, hence placing the ageing heart at risk for the development of cardiovascular diseases (**Yu X and Kem DC., 2010**). In the heart the UPS regulates cardiomyocyte receptors and ion channels that play important roles in both health and disease (**Yu X and Kem DC., 2010**).

The UPS regulates cardiac membrane channels and receptors such as Connexin 43 and β 2-adrenergic signaling (**Hare JF *et al.*, 1991; Li HH *et al.*, 2004**). Connexin 43 is a gap junction protein in ventricular myocardium. The specifics of the molecular mechanisms by which gap junctions are targeted for degradation have not yet been clarified (**Hare JF *et al.*, 1991**). The UPS also plays an essential role in modulating β -adrenergic receptors and G protein (heterotrimeric guanine nucleotide-binding protein)-coupled-receptor (GPCR) signaling pathways which stimulate guanine nucleotide binding protein G's which control the L-type Ca^{2+} channels, TnI, phospholamban, and ryanodine receptors (**Li**

HH et al., 2004). Stimulation of β 2-adrenergic receptors (β 2ARs) leads to quick ubiquitination of the β 2AR receptor and the receptor regulatory protein, β -arrestin. Studies have found that proteasome inhibitors decrease receptor internalization and degradation, hence implicating a role for proteasome-dependent degradation in the exchange of the β 2AR (**Li HH et al., 2004**).

While most proteasomal substrates must be ubiquitinated before being degraded, there is some exclusion to this general rule, especially when the proteasome plays a normal role in post-translational processing (**Young GW et al., 2008**). The proteasomal activation of nuclear factor-kappa beta (NF- κ B) by processing p105 into p50 via internal proteolysis and degradation of the enzyme ornithine decarboxylase are two major examples (**Young GW et al., 2008**). With this background in mind, it is important – in the context of this thesis - to discuss the role of proteasome inhibitors as it is the main focus of this project, particularly as novel cardioprotective agents.

1.17 Proteasome inhibitors

Many different PIs have been described over the past decades, including those derived from natural sources and through organic synthesis. Both covalent reversible, covalent irreversible and non-covalent inhibitors have been described and employed. There are five different classes of covalent inhibitors, i.e. 1) peptide aldehydes 2) epoxyketones 3) boronic acids 4) vinyl sulfones 5) β -lactones (**Beck P et al., 2012; Kisselev AF et al., 2012**). However, for the purposes of this thesis we will be focusing on peptide aldehydes (MG-132) and β -lactones (lactacystin).

1.17.1 Peptide aldehydes

The first class are the **peptide aldehydes**, with MG-132 (Z-Leu-Leu-Leucinal-) as its most widely used member (Rentsch A et al., 2013). Aldehydes form covalent, reversible bonds within the proteasome active sites and inactivate the catalytic activities by hemiacetal formation with the N-terminal threonine of the proteasome subunits (Rentsch A et al., 2013).

1.17.2 Epoxyketones

A well-known class belongs to the **epoxyketones** family, with epomoxcin the most recognized. They are highly selective for the proteasome, and no other off targets have been found. They form active sites between threonine and epoxketone in which both the γ -hydroxyl and the free amine of the n-terminal threonine participate (**Groll M *et al.*, 2000**).

1.17.3 Boranic acids

The next class are the **boranic acids**, with bortezomib as the most renowned example. It forms tetrahedrals adducts with active site threonines, which are stabilised by hydrogen bonds. Bortezomib has been approved for the treatment of multiple myeloma patients (**Groll M *et al.*, 2006**).

1.17.4 Vinyl sulfones

Vinyl sulfones are more readily synthesized and have been used in many peptide inhibitors and activity base probes. It can form covalent adducts by conjugate addition of the hydroxyl-group of the active site threonine (**Groll M *et al.*, 2002**).

1.17.5 β -lactones

The last class are **β -lactones**, which form covalent and stable adducts to the proteasome by the attack of the catalytic threonine to lactone, thereby forming an ester bond. The most used form of this class is lactacystin (**Groll M *et al.*, 2006**) that is a microbial natural product that inhibits cell proliferation and induces neurite outgrowth in a murine neuroblastoma cell line. It has become a widely used reagent in functional studies of the proteasome. This small molecule is currently the only known compound that specifically inhibits the proteasome without blunting other proteases thus far tested *in vitro*. It also does not inhibit intracellular lysosomal protein degradation in contrast to other commonly used proteasome inhibitors, such as peptide aldehydes and 3, 4-dichloroisocoumarin, which inhibit a wide range of proteases. Furthermore, it has chymotrypsin-like and trypsin-like activity. Lactacystin enters cells and inhibits the $\beta 5$ subunit of the 20 proteasome core, as a β -lactone derivative. Recently, a novel, small molecular-weight analog of lactacystin, clasto-lactacystin β -lactone (called PS519), was synthesized and shown to elicit cardioprotective effects following ischemia/reperfusion in isolated

perfused rat hearts (Pye *et al.*, 2003) and to exhibit cerebroprotective properties in a rat model of focal cerebral ischemia. Lactacystin also has the ability to inhibit the system's functionality more rapidly and without resulting in a stress response. Its use is highly portable, even to systems that are less tractable to molecular biology, such as tissues and multicellular organisms.

The effects of PI during myocardial ischemia on cardiac function have been controversial, with both advantageous and damaging effects described. However, short-term (acute) PI under certain conditions has shown early promise as a novel therapeutic approach for myocardial ischemia and reperfusion. Several mechanisms involved in cardioprotection by proteasome inhibition have been suggested, including inhibition of NF- κ B inflammatory pathway activation, prevention of ventricular tachyarrhythmia inactivation of G-protein-coupled receptor kinase 2 (GRK2) and inhibition of cardiomyocyte apoptosis (Pye *et al.*, 2003).

1.18 Proteasomal involvement during myocardial ischemia and reperfusion

Myocardial infarction results from irreversible myocardial necrosis caused by extensive ischemia and hypoxia. Early restoration of blood flow during myocardial ischemia is crucial for recovering the myocardium; unfortunately reperfusion itself can paradoxically intensify myocardial damage, this is known as reperfusion injury. Reperfusion injury involves the production of ROS, intracellular Ca^{2+} overload, microvascular and endothelial dysfunction, altered myocardial metabolism, activation of neutrophils, platelets, and complement (Hedhli N *et al.*, 2007). Current research of the proteasome pathway in animal models of myocardial ischemia suggests that compromised proteasome function is linked with the pathophysiology of myocardial I/R. Proof for proteasome dysfunction has been established in various models by measurements of the build-up of ubiquitinated proteins and/or by demonstrating altered proteasome activity. These irregularities in proteasome function and changes within UPS components have been observed to variable degrees with myocardial ischemia (Table 2) (Yu X and Kem DC, 2010). For example, CHIP-deficient mice are more inclined to I/R injury and exhibit an increase of reperfusion arrhythmias and increased infarct size compared to wild-type controls (Zhang C *et al.*, 2005). This therefore provides evidence for the cardioprotective function of CHIP, with the mechanism CHIP's ability to bind to damaged proteins with

molecular chaperone heat shock protein 70 (Hsp70) or Hsp90, to direct their re-folding or facilitate their degradation through ubiquitination (**Zhang C *et al.*, 2005**). Likewise, hearts with reduced MDM2 (genetic mouse model) were more sensitive to I/R injury, while overexpression of MDM2 protected it against hypoxia/reoxygenation-induced apoptosis (**Toth A *et al.*, 2006**). Therefore, MDM2 is believed to be a pre-requisite for conserving myocardial function and survival with I/R injury. MDM2 is also a key regulator of the pro-apoptotic transcription factor p53. It is responsible for p53 ubiquitination and proteasome degradation, thus triggering cardioprotective effects as a result of the inhibition of p53-induced apoptosis (**Toth A *et al.*, 2006**). In addition, mice deficient in MuRF3 are pre-disposed to cardiac rupture after acute MI and this modulator is therefore vital for conservation of ventricular integrity and function after myocardial infarction. Moreover, Foo *et al.*, (2008) established that oxidative stress-induced apoptosis was linked with decreased ARC (apoptosis repressor with caspase recruitment domain) and similar to the up-regulation of MDM2. Thus MDM2 endorses ARC protein turnover via ubiquitination and proteasome dependent degradation. Loss of ARC increased myocardial infarct size following ischemia/reperfusion (**Foo RS *et al.*, 2008**), thus MDM2-mediated loss of ARC can increase cardiac damage.

Table 1.2 Regulation of the cardiac UPS in myocardial ischemia

Experimental model	UPS alterations	References
Rat model of myocardial I/R	Increased ubiquitinated proteins; decreased 20S proteasome activities; oxidative modification of 20S subunits	Bulteau et al.
I/R in isolated perfused rat heart	Increased ubiquitinated proteins; decreased 20S/26S proteasome activities	Powell et al.
I/R in isolated perfused rat heart	Decreased 20S/26S proteasome activities	Das et al.
I/R in isolated perfused rat heart	Selective inhibition of proteasome activity	Gurusamy et al.
Dog model of myocardial I/R	Increased ubiquitinated proteins; decreased 26S proteasome activities	Asai et al.
Rat model of chronic myocardial infarction	Increased ubiquitinated proteins; increased E3 ligase (MuRF 1/MAFbx)	Adams et al.
Mouse model of chronic myocardial infarction	Increased proteasome activity; increased 11S/19S/20S subunits	Hedhli et al.
Rat H9c2 cardiomyocytes exposed to oxidative stress	Increased E3 ligase (MDM2)	Foo et al.

Table 1.2, ischemia/reperfusion; *MuRF-1*, muscle ring finger 1; *MAFbx*, atrogin-1/muscle atrophy F-box; *MDM2*, murine double minute 2 adapted from Yu and Kem, 2010

1.19 UPS and inflammation - hyperglycemia and I/R

The most significant link between the UPS and inflammation is the transcription factor NF κ B that triggers expression of inflammation-associated target genes (Evans JL *et al.*, 2002). The proteasome is necessary for activation of NF κ B a vital transcription factor that controls inflammatory genes by degradation of its inhibitory I κ B proteins (Karin M and Delhase M., 2000). NF κ B activation also leads to the expression of various cell survival- and proliferation-related genes (Evans JL *et al.*, 2002). Latest findings propose that proinflammatory cytokines (PIC), like TNF- α , interleukin-1 beta (IL-1 β) and IL-6, play central roles in the pathophysiology of heart disease (Sriramula S *et al.*, 2008; Kang YM *et al.*, 2008). Diabetes is usually associated with a chronic inflammatory state and animals with diabetes display increased NF- κ B p50 gene expression and NF- κ B p65 activity (Mariappan N *et al.*, 2010). Furthermore, chronic TNF- α administration induces cardiac damage and mitochondrial dysfunction (Mariappan N *et al.*, 2010). NF- κ B p50 is

also amplified within mitochondria of obese db/db mice and has been linked with increased oxidative stress. Blockade of NF- κ B in obese mice led to protection of such hearts against oxidative stress, restored mitochondrial integrity, and improved cardiac function (**Mariappan N *et al.*, 2010**). Moreover, genes which are controlled by NF κ B e.g. the IAP proteins, includes c-IAP1, c-IAP2, and XIAP. IAPs have been recognized as ubiquitin ligases, facilitating functional modification and degradation of effector molecules in apoptotic cells such as caspase-3 (**Yang Y *et al.*, 2000**). The UPS is also involved in negatively regulating the pro-apoptotic transcription factor p53 (**Yang Y *et al.*, 2000**). MDM-2 facilitates the relocation of p53 out of the nucleus and endorses the ubiquitination and degradation of p53. Auto-Ub of IAPs and their succeeding degradation by the proteasome is essential for apoptosis, as it eliminates their potent inhibitory impact upon caspases and therefore the advancement of cell death (**Yang Y *et al.*, 2000**).

Atherosclerosis is initiated as an inflammatory-proliferative reaction to an injurious stimulus, established by cardiovascular risk factors such as hypercholesterolemia, hypertension, smoking, diabetes, and age. Here endogenous oxidative stress causes modification of lipids, proteins, and DNA (**Ames BN *et al.*, 1993**), leading to structural and functional alterations within the vascular wall. Furthermore, the reduction in bioavailability of NO as a result of increased assembly of ROS is another important pathophysiologic element in atherogenesis (**Palombella VJ *et al.*, 1944**). Under normal conditions NO is produced by endothelial cells antagonizing pro-atherosclerotic processes and suppressing NF κ B transcriptional activity, a pivotal mediator of the inflammatory-proliferative process of atherogenesis. Decreased NO bioavailability together with an alteration of intracellular signaling pathways (due to increased oxidative stress) subsequently leads to the initiation of the NF κ B cascade (**Palombella VJ *et al.*, 1944**) (**Figure 1.8**).

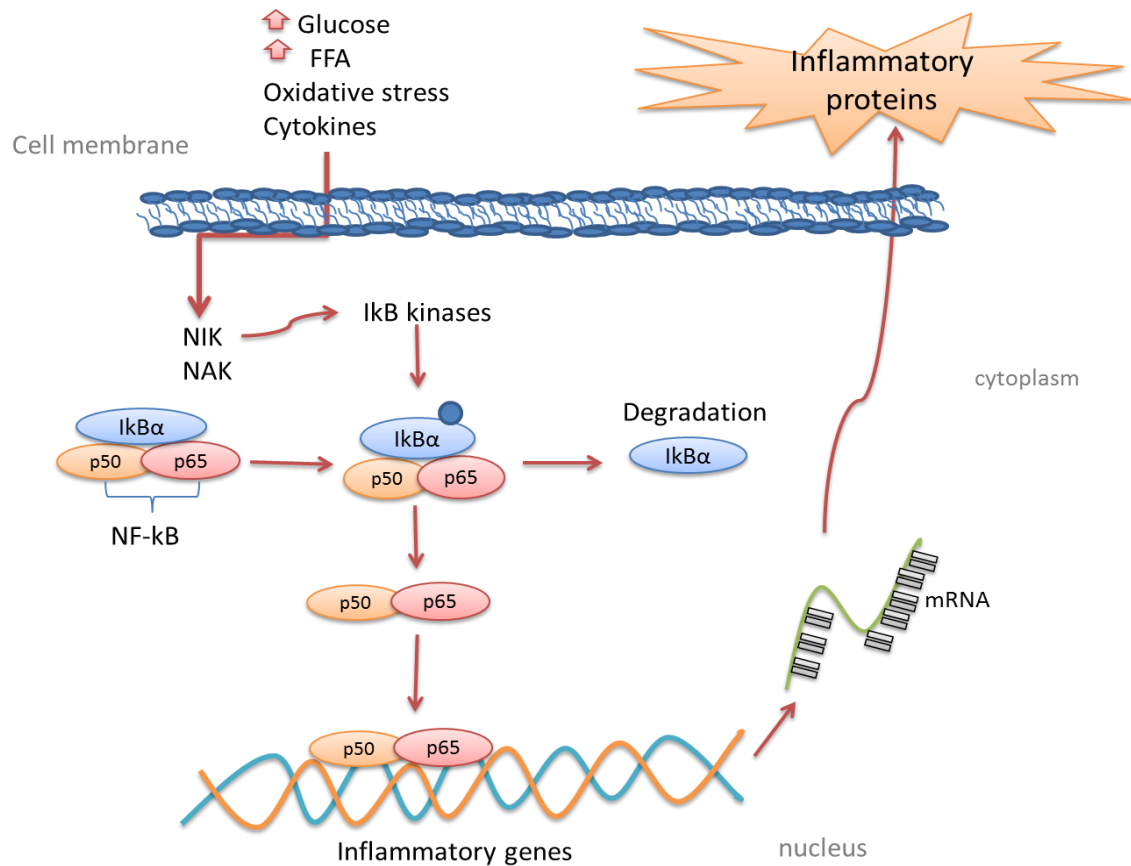


Figure 1.8 Model of NF-κB activation by hyperglycemia. NFκB (nuclear factor kappa beta), IκBα (interleukin kappa beta alpha), FFA (free fatty acids) modified from Evans et al, 2002

Depending on PI class and experimental systems employed, the role of the UPS with hyperglycemia is contentious and it remains unclear whether decreased/increased activity is advantageous or not. For example, some studies found that with PIs, a beneficial effect was found (Marfella R et al., 2009; Powell SR et al., 2008), while others observed the converse, i.e. triggering damaging effects on the heart (Tsukamoto O et al., 2010; Tsukamoto S and Yokosawa H., 2009). For example, the 26S ATP-dependent activity reduced in response to prolonged hyperglycemia, while the 11S activity increased together with higher oxidative stress and impaired cardiac function (Powell SR and Divald A., 2010). This shows that increased 11S activity may be a compensatory mechanism to reduce the effects of oxidative stress in response to chronic hyperglycemia.

Moreover, others found that an elevated level of ubiquitinated proteins indicates a dysfunctional UPS (**Powell SR and Divald A., 2010; Powell SR *et al.*, 2008**).

These discrepancies in the activity of the UPS may arise from differences in the type and period of ischemia, the nature of the proteasome assays, the specificity and dosage/frequency of proteasome inhibitors, and the experimental model (cultured cardiomyocytes, *ex vivo* or *in vivo* heart models) used in different studies. A firm distinction must be made between studies using cultured cardiomyocytes, *ex vivo* (Langendorff with buffered perfusate) and *in vivo* heart models where the full complement of circulating metabolites and hormones are available.

1.20 Conclusion

Although the elucidation of the UPS is still in its infancy in terms of its role in cardiac disease, there is some evidence suggesting its use as a therapeutic strategy in the case of diabetes/hyperglycemia presenting together with myocardial ischemia and reperfusion. It is known that hyperglycemia increases ROS, and that altered metabolic factors present during diabetes can cause glycation, oxidation, and cross-linking of proteins thereby altering proteasome function. An in-depth study on proteasome alterations in diabetic/hyperglycemic hearts has not been reported, however, increased proteasome function has been implicated in diabetes. Increased ROS may alter proteasome function through oxidative modification of the structural subunits. Despite such progress, the role of proteasome dysfunction with hyperglycemia/ diabetic cardiovascular disease remains to be fully determined and understood. As such this study focused on investigating proteasome inhibitors as novel cardioprotective agents that may blunt hyperglycemia-induced contractile dysfunction during ischemic-reperfusion. The main aims of this thesis were to:

1. Assess heart function (functional recovery and infarct sizes) *ex vivo* of rat hearts in response to ischemia-reperfusion under low and high glucose exposure with or without UPS inhibitors.
2. Investigate the role of UPS inhibitors in hyperglycemia-mediated cardiac dysfunction with or without UPS inhibitors.
3. Determine the role of oxidative stress in hyperglycemia-mediated cardiac dysfunction with or without UPS inhibitors.
4. Determine the role of inflammation in hyperglycemia-mediated cardiac dysfunction with or without UPS inhibitors.
5. Evaluate the overall status of candidate apoptotic signaling proteins under hyperglycemic conditions with or without UPS inhibitors.

Chapter 2

Methods and Materials

This chapter describes the methods that were carried out to pursue the hypothesis and aims described previously. The methods are divided into three sections i.e. *ex vivo* perfusions; Western blotting and the assessment of UPS activity.

2.1 Animals and ethics statement

All animals were treated in accordance with the Guide for the Care and use of Laboratory Animals of the National Academy of Sciences (NIH publication No. 85-23, revised 1996). Studies were performed with the approval of the Animal Ethics Committee of Stellenbosch University (**refer Appendix 1**). Male Wistar rats (180-220 g) were used throughout the study and were allowed to acclimatize for a day at Stellenbosch University, after the arrival from the Tygerberg Campus. They were allowed access to food and water *ad libitum*. A standard light/dark cycle (12h - 12h) was employed.

2.2 Drugs and chemicals used

All reagents used were of analytical grade and these include: NaCl, KCl, MgSO₄.7H₂O, CaCl₂.2H₂O, KH₂PO₄ and NaHCO₃. The drugs used in the perfusions were from Sigma Aldrich (St. Louis, MO, USA) and included: Lactacystin, MG132 (Z-Leu-Leu-Leu-al), Ibuprofen sodium salt (referred to as ibuprofen in this thesis) and N- acetylcystine (NAC).

2.3 *Ex-vivo* perfusions global ischemia during simulated acute hyperglycemia

In our initial experiments we evaluated the effects of MG132. To validate our findings we further investigated whether lactacystin elicited the same effects on the rat heart under high glucose conditions following ischemia and reperfusion. Rats were anesthetized (pentobarbitone, 100 mg/kg i.p) and hearts rapidly excised and perfused in a modified Langendorff model with Krebs-Henseleit buffer (**refer Appendix 2 for preparation**) equilibrated with 95% O₂-5% CO₂ (37°C, pH 7.4) at a constant pressure (100 cm). The Krebs-Henseleit buffer contained (in mM) 11 Glucose, 118 NaCl, 4.7 KCl, 1.2 MgSO₄.7H₂O, 2.5 CaCl₂.2H₂O, 1.2 KH₂PO₄, and 25 NaHCO₃. Hearts were randomly distributed into the following experimental groups:

Table 2.1 Investigational heart grouping

11 mM glucose n= 8/group	33 mM (High) glucose n= 8/group
Untreated	Untreated
5 μM Lactacystin (treated)	5 μ M Lactacystin (treated)
10 μM MG132 (treated)	10 μ M MG132 (treated)
29 μM Ibuprofen (treated)	29 μ M Ibuprofen (treated)
0.4 mM NAC (treated)	0.4 mM NAC (treated)
NAC + Ibuprofen (treated)	NAC + Ibuprofen (treated)

The Krebs-Henseleit buffer mimics the key ionic content of rat plasma or blood (**Hearse D and Sutherland F., 2000; Skrypiec-Spring M *et al.*, 2007**) and led to no hemodynamic dysfunction in the *ex vivo* heart perfusion system (**Cunningham MJ *et al.*, 1990**). The use of the high glucose perfusions, attempts to simulate acute hyperglycemia within the clinical setting. Moreover, since *ex vivo* Langendorff perfusions are typically performed with 11 mM glucose at baseline, we are of the opinion that the 33 mM concentration is representative of a three-fold elevation of glucose levels (above normal) within the clinical setting.

The protocol was divided into two parts, i.e. perfusions a) with global ischemia and b) regional ischemia followed by reperfusion for cardiac functional assessment and infarct size determination, respectively. Various drugs were employed for this study: the effect of UPS inhibition, (5 μ M lactacystin, 10 μ M MG132), the role of inflammation (29 μ M Ibuprofen), and the role of oxidative stress (0.4 mM NAC) was administered during the first 20 min of reperfusion (Figure 2.1A). Lactacystin and MG132 were directly dissolved in the buffer; however, ibuprofen was initially heated in deionized water (**Ghorab MK and Adeyeye MC., 2003**) and NAC in 100% ethanol (**Llu Y *et al.*, 2007**). Less than 0.0005% (v/v) DMSO and 0.001% (v/v) ethanol was present during perfusion experiments. The doses used for these drugs were based on perfusion studies (**Yew *et al.*, 2005; Meiners S *et al.*, 2002; Use of NSAID for Relief of Pain in Rodents and Rabbits” Lab Animal 26: p. 241-255; Peng Y *et al.*, 2011**).

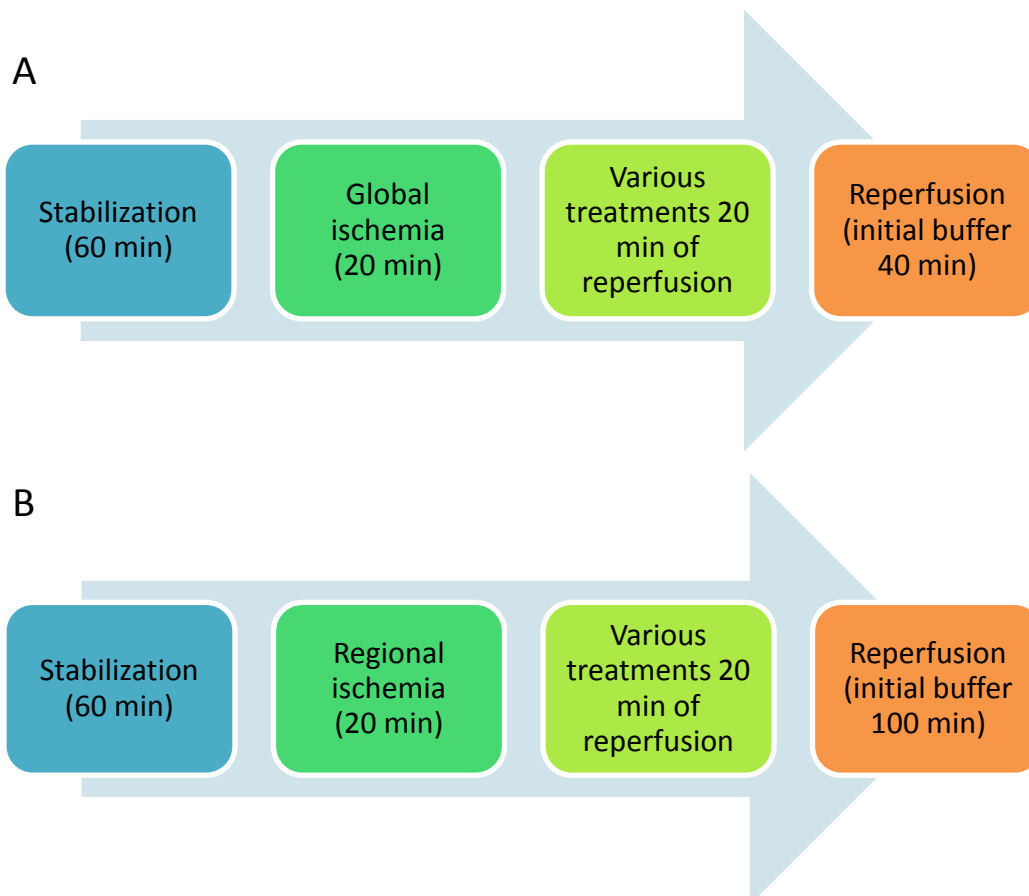


Figure 2.1 Schematic diagrams showing the perfusion protocols for evaluation of the effects of various drug treatments (Lactacystin, MG132, Ibuprofen and NAC) on (A) heart contractile function following global ischemia and (B) infarct size following regional ischemia.

During perfusion, a latex balloon attached to a pressure transducer (Stratham MLT 0380/D, AD Instruments Inc., Bella Vista, NSW, Australia) compatible with the PowerLab System ML410/W (AD Instruments Inc., Bella Vista, NSW, Australia), was inserted into the left ventricle and inflated to produce a diastolic pressure of 4-12 mm Hg. The protocol included a 60 min stabilization period, 20 min of global ischemia, followed by reperfusion for 60 min. Contractile parameters assessed included heart rate (HR), left ventricular developed pressure (LVDP; $\text{LVDP} = \text{systolic pressure} - \text{diastolic pressure}$), and rate-pressure product (RPP; $\text{RPP} = \text{HR} \times \text{LVDP}$). Coronary flow (CF) was measured by collection of the effluent at regular timed intervals. Both left and right ventricular tissues were collected after 60 min of reperfusion. Collected tissues were freeze-clamped in liquid nitrogen and stored at -80°C for further analysis.

2.4 *Ex-vivo* regional ischemia and reperfusion during simulated acute hyperglycemia

To further strengthen our Langendorff functional data, we also evaluated the effects of Lactacystin and MG132 by infarct size determination. This was performed as described before but with slight modifications (Kelly RF *et al.*, 2010), i.e. we employed regional ischemia with a reperfusion time of 2 hr. (see Figure 2.1B). Here a 3/0 silk suture was placed on the proximal portion of the left anterior descending coronary artery and passing the ends through a plastic tube. For induction of regional ischemia, the ends were tightened by pressing the plastic tube against the surface of the heart (above the artery) for 20 min. The snare was released during the reperfusion period. The efficacy of ischemia was confirmed by regional cyanosis and a substantial decrease in coronary flow as well as in LVDP.

2.5 Determination of infarct size

After completion of each regional ischemia and reperfusion experiment the snare was re-tightened and 2.5% Evans Blue dye (in Krebs buffer) was perfused through the hearts for infarct development. Hearts were subsequently removed from the Langendorff apparatus, blotted dry, suspended within 50 ml plastic tubes (using suture) and frozen at -20°C for 3 days. Thereafter, frozen hearts were sliced into 2 mm transverse sections and incubated with 1% 2,3,5-triphenyl tetrazolium chloride (TTC) in phosphate-buffered saline for 20 min at 37°C to identify non-infarcted (stained) from infarcted (nonstained) tissues. The area that was not stained with Evans Blue was defined as the area at risk (AAR). The area which demonstrated neither blue nor red was defined as the infarct site. Slices were then fixed in 10% formalin for 24 hr at room temperature before being placed between glass plates for scanning (both sides). The infarct area (IA) size and the area at risk (AAR) were calculated using Image J software (v1.46p, National Institutes of Health, USA) (see Appendices 3 and 4). Values of tissue slices were added together in order to obtain the total IA and AAR for each heart analyzed. We expressed the infarct size as the ratio of IA versus the AAR (%IA/AAR) [see appendix 3 and 4]. Coronary flow was decreased by $40\% \pm 3.9$ under control conditions and $35\% \pm 2.6$ under hyperglycemic conditions this was together seen with a drop in LVDP by 5mm/hg respectively thereby proving ligation consistency.

2.6 Western blot analysis

Protein isolation was performed as previously described in our laboratory (**Rajamani U and Essop MF., 2010**). Briefly, collected heart tissues were homogenized with modified RIPA buffer (**see Appendix 5**); the supernatant was centrifuged twice at 4, 300 g for 10 min at 4°C then stored at -80°C until further use. Protein concentration was determined by employing the Bradford technique, with BSA as the protein standard (**see Appendix 5**).

Samples for Western blotting were prepared by addition of a Laemmli sample buffer containing β -mercaptoethanol to a final protein concentration of 50 μ g. Samples were boiled at 95°C to ensure denaturation of proteins into a linear formation, capable of migrating through the SDS-PAGE gel. Briefly, prepared samples were then loaded in commercially available 10-well precast Mini-PROTEAN® TGX Stain-Free™ polyacrylamide gels (Bio-Rad, Hercules CA, USA) and subjected to electrophoresis in a 1 \times running buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS, and at pH 8.3.

Gels were electrophoresed at 250 V until adequate separation of proteins was achieved. Hereafter transfer of proteins from the gel to the membrane was performed by the employment of commercially available transfer packs in conjunction with the Trans-Blot® Turbo™ Transfer system (Bio-Rad, Hercules CA, USA). The system makes use of semi-dry transfer, ensuring the migration of separated proteins from the gel to the pre-wet polyvinylidene fluoride (PVDF) membrane contained in the transfer pack, in 7 min (**see Appendix 6**). The membrane was then subjected to various washing steps in Tris-buffered saline solution containing Tween-20 (2 L TBS-T: 20 mM Tris-HCl, 500 mM NaCl, 2 mL Tween-20). Blocking for the primary antibody was performed using 5% fat-free milk solution (5 g fat-free dry milk powder dissolved in 100 mL TBS-T solution). The membrane was incubated to settle with the primary antibody (1:1,000) at 4°C, overnight (Table 1) Incubation in secondary antibody, 1:4,000 or 1:10,000 concentrations, was performed at room temperature (**see Appendix 7**).

Table 2.2 Antibodies employed for Western blotting analysis

	Primary antibody	Supplier
Cell death		
ARC	α -rabbit ARC	Sigma-Aldrich, MO, USA
PARP	α -rabbit PARP	Cell Signaling Technology, MA, USA
Total Bad	α -rabbit Bad	Santa Cruz Biotechnologies, CA, USA
Bcl-2	α -rabbit Bcl-2	Santa Cruz Biotechnologies, CA, USA
Caspase-3	α -rabbit Caspase-3	Cell Signaling Technology, MA, USA
Cytochrome C	α -rabbit Cytochrome C	Cell Signaling Technology, MA, USA
pBad	α -rabbit pBad	Santa Cruz Biotechnologies, CA, USA
Inflammation		
IκB-α	α -rabbit I κ B- α	Sigma-Aldrich, MU, GER
TNF-α	α -rabbit TNF- α	Sigma-Aldrich, MU, GER
IL-6	α -rabbit IL-6	Cell Signaling Technology, MA, USA
ROS		
eNOS	α -rabbit eNOS	Santa Cruz Biotechnologies, CA, USA
Sod1	α -rabbit Sod1	Santa Cruz Biotechnologies, CA, USA
Sod2	α -rabbit Sod2	Santa Cruz Biotechnologies, CA, USA
Autophagy		
P62	α -rabbit P62	Cell Signaling Technology, MA, USA
LC3 I/II	α -rabbit LC3 II	Cell Signaling Technology, MA, USA
Others		
GR2K	α -rabbit GR2K	Sigma-Aldrich, MU, GER
Ubiquitin	α -rabbit Ubiquitin	Cell Signaling Technology, MA, USA
β-actin	α -rabbit β -actin	Cell Signaling Technology, MA, USA

Visualization of the membrane was performed with enhanced chemiluminescence (ECL). Briefly, as a result of the conversion of luminol to 3-aminophthalae, catalyzed by the horseradish peroxidase (HRP) enzyme linked to secondary antibody, light emission at 428 nm is detected via a digital camera by the ChemiDoc™ XRS+ system with Image Lab™ Software v2.0 (Bio-Rad, Hercules CA, USA) and a digital image is produced on a computer.

Protein expression was determined by the adjusted percentage volume, (intensity units of pixels of band \times mm²), after background subtraction (Quantity One Software v4.6.9, BioRad, Hercules CA, USA), and normalized to β -actin to correct for loading variations. The adjusted volume for the control group was represented as 100%, and corresponding values for vehicle and treatment groups were expressed as a percentage of the average of the control. This method was performed for each experiment separately before all values were combined in the statistical program. An n=8 was used for each experimental group.

2.7 Proteasome activity measurements

Proteasome activity measurements Chymotrypsin-like, trypsin-like, and caspase-like activities of the proteasome were assayed using fluorogenic peptides (Sigma-Aldrich, St Louis, MO, USA): Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-MCA at 25 μ M), N-t-Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin (LSTR MCA at 40 μ M) and N-Cbz-Leu-Leu-Glu-b-naphthylamide (LLE-NA at 150 μ M), respectively (28). Assays were performed with \sim 50 μ g of protein lysate (in 25 mM Tris-HCl, pH 7.5) and the appropriate substrate that were incubated together for 0-30 min at 37°C. Aminomethylcoumarin and β -naphthylamine fluorescence were measured at excitation/emission wavelengths of 350/440 and 333/410 nm, respectively, using a Fluostar fluorometric microplate reader (BMG Labtech, Ortenberg, Germany). Peptidase activities were measured in the absence/presence of 20 μ M of the proteasome inhibitor, MG-132 (N-Cbz-Leu-Leu-leucinal), and the difference between the two values was attributed to proteasome activity. Data were normalized to protein concentrations.

2.8 Glutathione assay Kit

Glutathione (GSH) was measured using the glutathione assay kit (Sigma-Aldrich, St Louis, MO, USA) in heart tissue extracts. The samples were first deproteinized with 5% 5-sulfosalicylic acid solution. Glutathione content of the sample was then assayed using a kinetic assay in which catalytic amounts of glutathione cause a continuous reduction of 5, 5'-dithiosbis-(2-nitrobenzoic) acid (DTBN) to TBN. Oxidized glutathione is formed and recycled by glutathione reductase and NADPH. TBN is assayed colorimetrically at 421nm.

2.9 Statistical analysis

Data are presented as mean \pm standard error of mean (SEM). Differences between treatment groups and time points were analyzed using one way analysis of variance (ANOVA) followed by Tukey –Kramer post hoc. Mann-Whitney unpaired t-test was used when comparisons were made between only two groups. All statistical analyses were performed using GraphPad Prism version 5.01 (Graphpad Software, Inc, San Diego, USA). Values were considered significant when $p < 0.05$.

Chapter 3: Results

Diabetes represents a foremost health challenge, which causes cardiovascular complications thus further increasing the overall burden of the disease. Moreover, stress-induced hyperglycemia in non-diabetic individuals with AMI is linked with higher in-hospital mortality. Prior studies associate oxidative stress, inflammation and a dysfunctional UPS as potential mediators of this process. Thus far, PIs such as lactacystin and MG-132 have yielded mixed results but with the promise of offering potential benefits as novel therapeutic interventions in this case. However, additional studies are required to determine the mechanisms underlying dysfunctional UPS in the heart under hyperglycemic conditions. We therefore hypothesized that PIs reduce acute hyperglycemia-mediated pathophysiologic molecular events such as, oxidative stress, apoptosis, inflammation, UPS, and thereby improve contractile function in response to I/R.

3.1 Evaluation of *ex vivo* heart function during global ischemia and reperfusion (simulated acute hyperglycemia) \pm MG-132

Our results indicate that acute hyperglycemia during the onset of I/R elicits detrimental effects on the heart as shown by both the reduced LVDP (Figure 3.1A, $p < 0.01$ vs. control) and RPP recovery (Figure 3.1B, $p < 0.05$ vs. control). LVDP improved to $26 \pm 3.9\%$ for control hearts whereas high glucose perfused hearts recovered to only $12.3 \pm 2.6\%$ after 60 min of reperfusion. Similarly RPP recovery improved in control hearts after 60 min of reperfusion versus hyperglycemic hearts (Figure 3.1B, $25.5 \pm 1.7\%$ vs. $13.0 \pm 1.6\%$, $p < 0.05$).

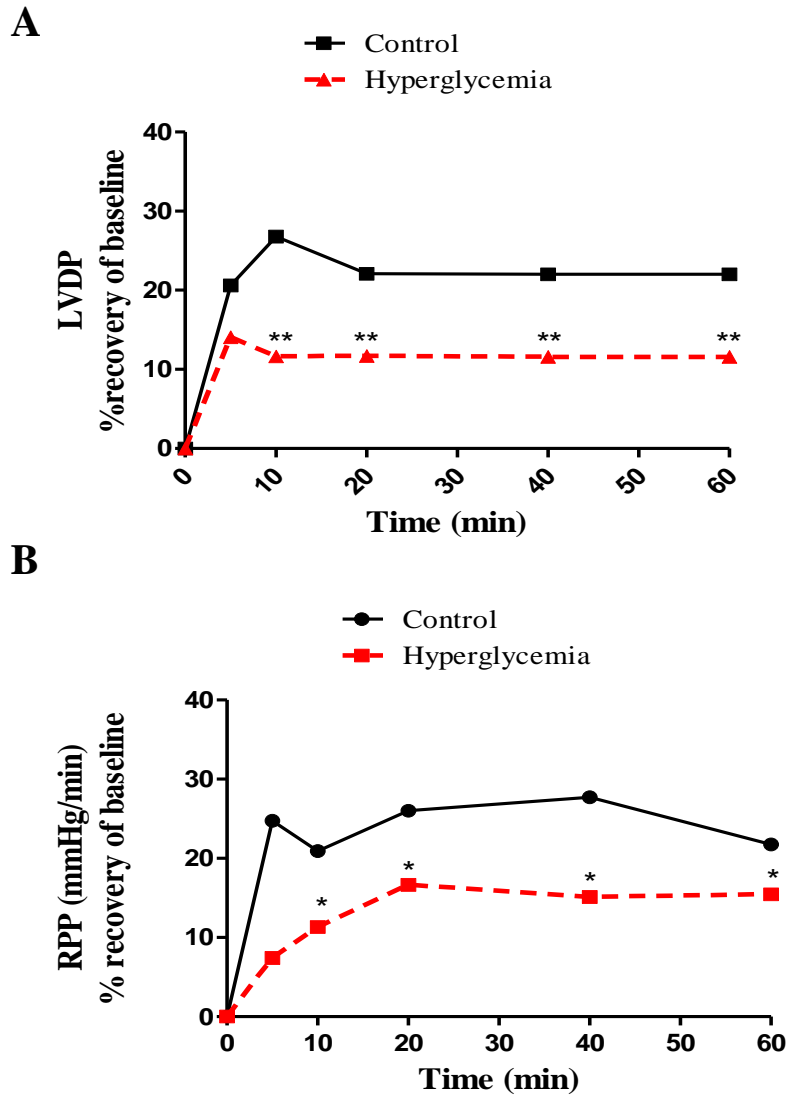


Figure 3.1 High glucose-induced cardiac contractile dysfunction in rat hearts exposed to high glucose conditions vs. baseline glucose concentrations. Isolated rat hearts were perfused under high glucose conditions (33 mM glucose) vs. controls (11 mM glucose) for 60 min and subjected to 20 min of global ischemia, followed by 60 min of reperfusion. (A) Left ventricular developed pressure (% recovery) and (B) rate pressure product (RPP) (% recovery) between control and high glucose perfused groups. Values are expressed as mean \pm SEM (n=8). *p<0.05, **p<0.01 vs. respective controls.

To test our hypothesis, we next performed similar experiments but in the presence of MG-132, a specific UPS inhibitor. Our findings show that acute MG-132 treatment (20 min) significantly improved LVDP at baseline (11 mM glucose) and for the high glucose group (Figures 3.2A and B). Changes in rate pressure product (RPP) reflected a similar trend (Figures 3.2C and 3.2D), while no differences were found for the HR and dp/dt_{max} (Figure 3.3).

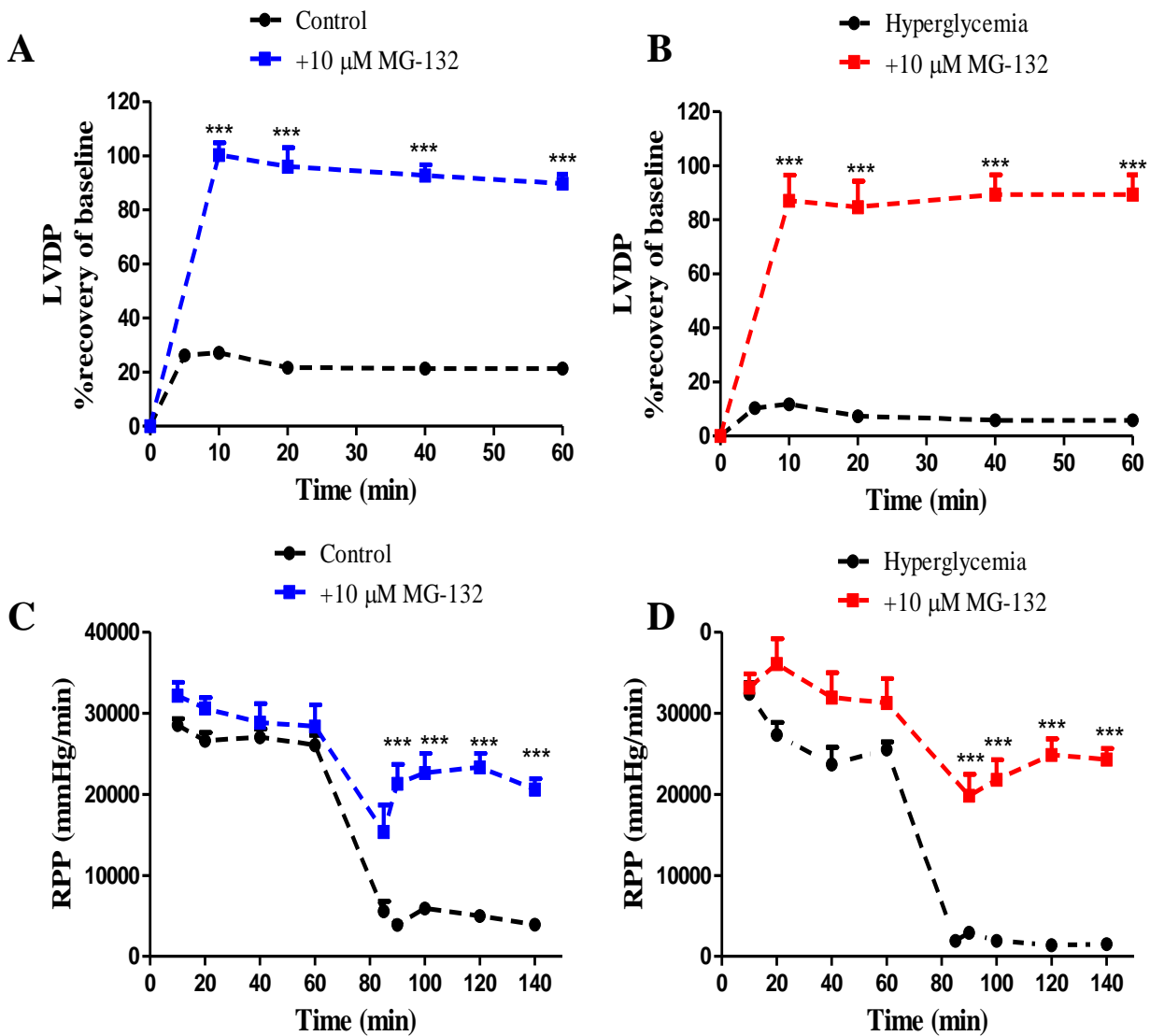


Figure 3.2 Acute MG-132 treatment increases cardiac contractile function under baseline glucose and high glucose perfusion conditions reflected by improved LVDP and RPP vs. untreated hearts. Isolated rat hearts were perfused under simulated hyperglycemic conditions (33 mM glucose) vs. controls (11 mM glucose) and subjected to 20 min of global ischemia, followed by 60 min of reperfusion. 10 μ M MG-132 were added to the treatment group during the first 20 min of reperfusion. (A) Left ventricular developed pressure (% recovery) at baseline glucose levels (11 mM), and (B) with high glucose (33 mM). Rate pressure product (RPP) at baseline glucose levels (C), and (D) under high glucose conditions. Values are expressed as mean \pm SEM (n=8). ***p<0.001 vs. respective controls.

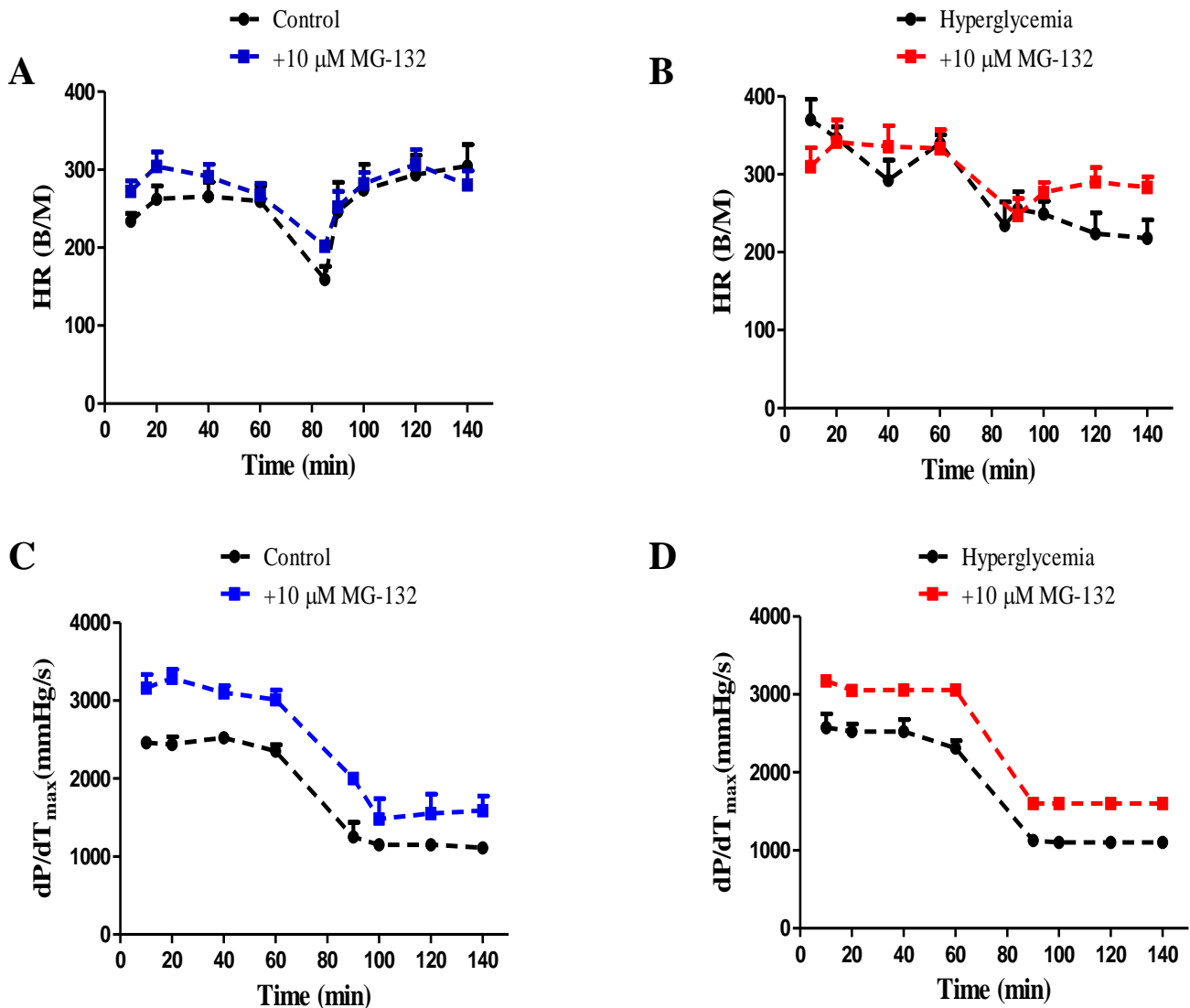


Figure 3.3 MG-132 treatments had no effect on HR and dP/dt_{max} . Isolated rat hearts were perfused under simulated hyperglycemic conditions (33 mM glucose) vs. controls (11 mM glucose) and subjected to 20 min of global ischemia, followed by 60 min of reperfusion. 10 μ M MG-132 were added to the treatment group during the first 20 min of reperfusion. (A) Heart rate (HR) at baseline glucose levels (11 mM), and (B) with high glucose (33 mM). Velocity of contraction (dP/dt_{max}) at baseline glucose levels (C), and (D) under high glucose conditions. Values are expressed as mean \pm SEM (n=8).

Additionally, MG-132 treatment enhanced coronary flow vs. untreated group significantly ($p < 0.05$) under high glucose perfusion condition (Table 3.1). This was accompanied by a decrease in end diastolic pressure in hearts exposed to high glucose conditions though there were no changes in the control group.

Table 3.1. Coronary flow and end-diastolic pressure (EDP) under high glucose vs. baseline (11 mM) glucose conditions during the first ten min of stabilization (“Pre-ischemia”) and at the end of reperfusion (“Post-ischemia”).

	Coronary Flow (ml/min)		EDP (mmHg)	
	Pre-ischemia (1 st 10min)	Post-ischemia (140min)	Pre-ischemia	Post-ischemia
Control (11 mM)	10 ± 2.3	8.2 ± 1.3	14 ± 0	33 ± 5
10 µM MG-132	12 ± 1	8 ± 2.4	11 ± 2	31 ± 2
High Glucose (33 mM)	11.2 ± 1.8	6 ± 1.5	8 ± 3	65 ± 7*
10 µM MG-132	7 ± 1	11 ± 1*	14 ± 2	22 ± 5*

Values are expressed as mean ± SEM. *p<0.05vs. respective control, (n=8 per group).

3.2 Assessment of infarct size following regional ischemia and reperfusion (simulated acute hyperglycemia ± MG-132)

We next proceeded to strengthen our findings by testing the effects of MG-132 on infarct size following regional ischemia. The data demonstrate that the infarct size was noticeably higher in the high glucose compared to the control group (Figure 3.4). However, MG-132 treatment decreased infarct sizes following regional ischemia at baseline (11 mM glucose) and under high glucose conditions (Figure 3.4A), diminishing infarct sizes to 40.3 ± 2.5% (11 mM) and 49.7 ± 7.4% (33 mM) versus 56.3 ± 2.8% (11 mM) and 73.5 ± 4.9% (33 mM) at baseline and high glucose perfusions, respectively. There were no differences in the area at risk amongst all the groups (Figure 3.4B).

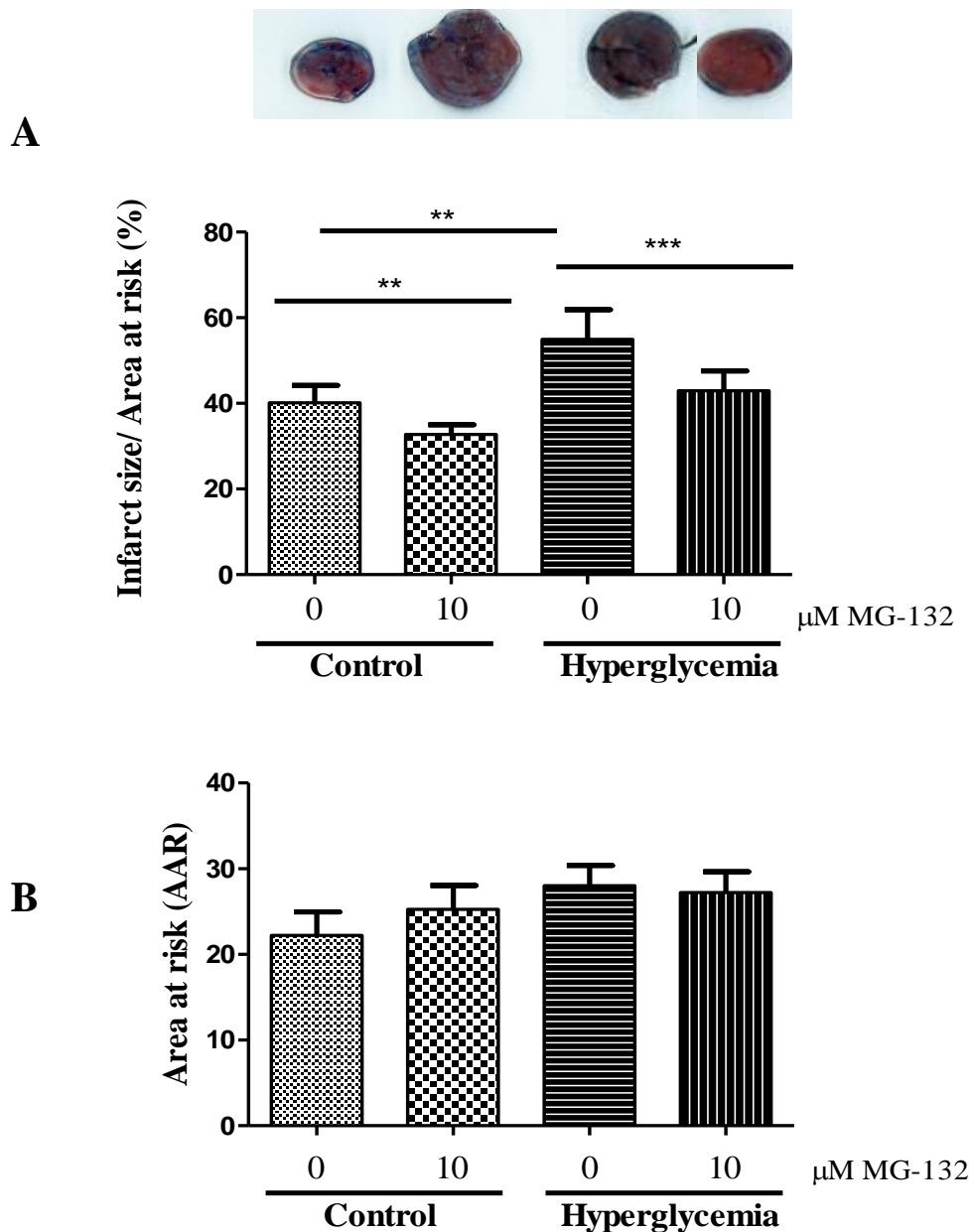


Figure 3.4 MG-132 administration exhibited cardio-protective properties following regional I/R under high glucose perfusion conditions. Isolated rat hearts were perfused under high glucose conditions (33 mM) vs. controls (11 mM) and subjected to regional ischemia. For MG-132 treated groups, 10 μ M MG-132 was given during the first 20 min of the two hour reperfusion period. (A) Infarct size/area at risk (%) and (B) area at risk under baseline (simulated normoglycemia) vs. high glucose perfusion conditions (simulated acute hyperglycemia). Evans blue dye and TTC staining allowed visualization of viable tissue (blue), infarcted area (white) and the area at risk (red). Values are expressed as mean \pm SEM (n=8). **p<0.01, ***p<0.001 vs. respective controls.

3.3 The effects of MG-132 treatment on myocardial proteasomal activity in hearts exposed to ischemia and reperfusion under high glucose settings

We subsequently assessed post-ischemic proteasomal activity in our experimental system. These data revealed that hyperglycemia induced a marked increase in chymotrypsin-like, trypsin-like proteasomal activity following I/R (Figure 3.5). The 11 mM glucose perfusions MG-132 treatment decreased chymotrypsin-like proteasomal activity compared to the untreated control significantly ($p < 0.01$), but did not result in any significant effects on trypsin-like and caspase-like proteasomal activities (Figure 3.6B). Moreover, MG-132 treatment considerably reduced chymotrypsin-like proteasomal activities in high glucose exposed rat hearts (Figure 3.6B). Conversely, no effects were observed on caspase-like activity in such hearts (Figure 3.6C and 3.6D). Total ubiquitinated protein was unexpectedly decreased (Figure 3.6A).

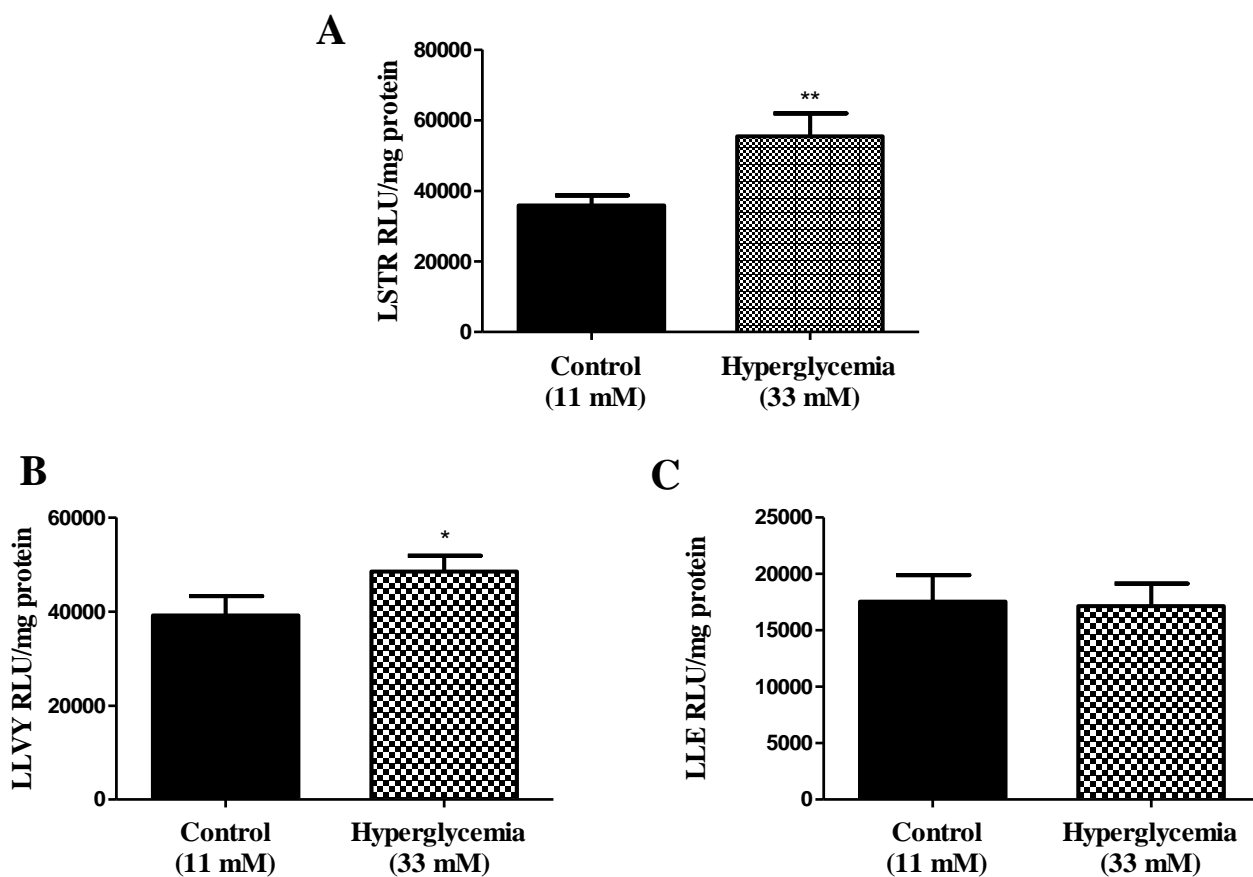


Figure 3.5 Increased proteasomal activity in hearts subjected to I/R under high glucose conditions. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to ischemia and reperfusion. A) Chymotrypsin-like proteasomal, (B) trypsin-like, and (C) caspase-like activities after 60 min of reperfusion under high glucose conditions (33 mM) vs. control (11 mM). Values are expressed as mean \pm SEM (n=8). * $p < 0.05$, ** $p < 0.01$ vs. respective controls.

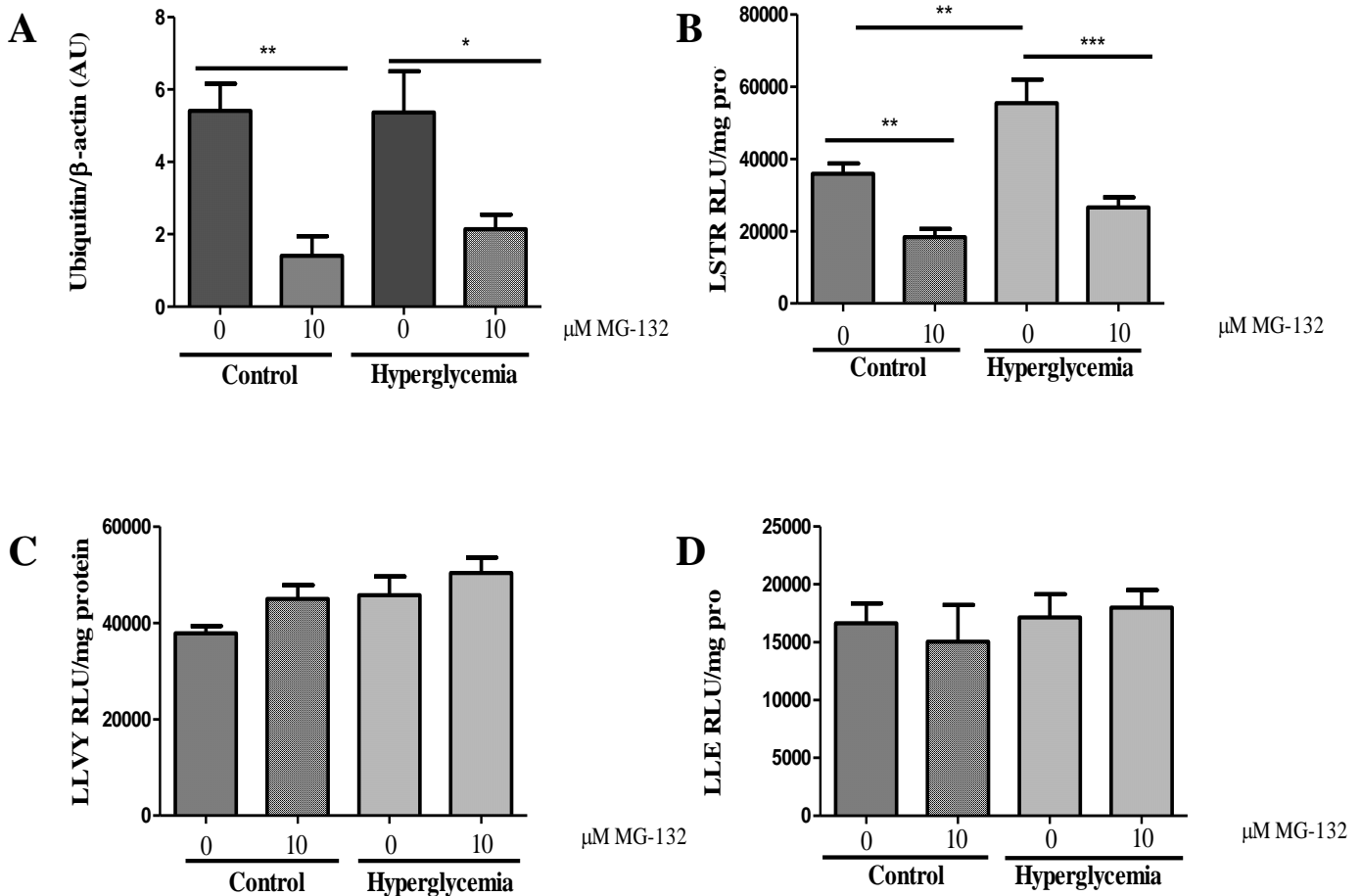


Figure 3.6 MG-132 reduces high glucose-induced chymotrypsin-like proteasomal activity following I/R. Isolated rat hearts were perfused under high glucose (33 Mm) conditions vs. controls and subjected to I/R. For MG-132 treatment groups, 10 μM MG-132 was added during the first 20 min of reperfusion. (A) Total ubiquitinated proteins (B) chymotrypsin-like proteasomal, (C) trypsin-like, and (D) caspase-like activities after 60 min of reperfusion at baseline (11 mM glucose). Values are expressed as mean \pm SEM (n=8). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. respective controls.

In line with our hypothesis, we evaluated the roles of ROS and inflammation and links to increased UPS activity following ischemia and reperfusion under high glucose condition. Hence N-acetylcystine (NAC) was employed as an antioxidant, and Ibuprofen as an anti-inflammatory agent. These data demonstrated that Ibuprofen and NAC both improved LVDP recovery under control (11 mM) and high glucose (33 mM) conditions significantly (Figures 3.7A and 3.7B $p < 0.001$, $p < 0.05$ vs. controls respectively). Ibuprofen exhibited a similar trend for RPP recovery under both low and high glucose conditions significantly (Figure 3.7C, $p < 0.01$ and 3.7D $p < 0.001$). Furthermore, the

combination of NAC and Ibuprofen displayed an additive cardioprotective effect under high glucose conditions (Figures 3.7A and 3.7B $p < 0.001$ and C/D $p < 0.01$).

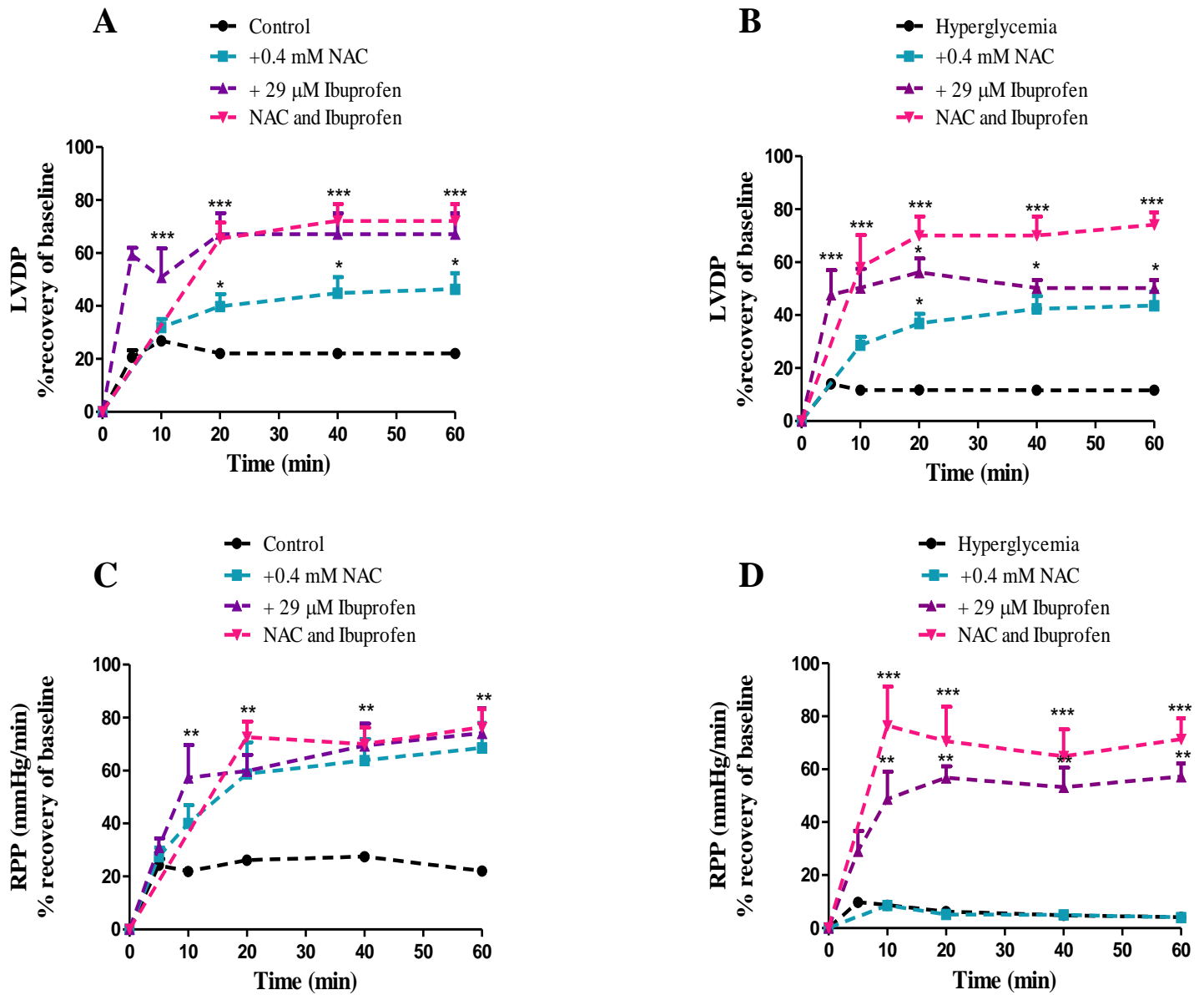


Figure 3.7 NAC and Ibuprofen treatment elicit increased cardiac contractile function under baseline and high glucose conditions. Isolated rat hearts were perfused under simulated hyperglycemic conditions (33 mM glucose) vs. controls (11 mM glucose) and subjected to 20 min of global ischemia, followed by 60 min of reperfusion. We added a) 0.4 mM NAC, or b) 29 μ M Ibuprofen, or c) NAC and Ibuprofen during the first 20 min of reperfusion. (A) Left ventricular developed pressure (% recovery) at baseline glucose levels (11 mM), and (B) with high glucose (33 mM). Rate pressure product (RPP) at baseline glucose levels (C), and (D) under high glucose conditions. Values are expressed as mean \pm SEM (n=8). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. respective controls.

Changes in coronary flow and end-diastolic pressure under baseline (11 mM) and with simulated hyperglycemia \pm NAC and Ibuprofen concentrations are shown in Table 3.2. Ibuprofen treatment increased coronary flow vs. untreated group ($p < 0.05$) under high glucose perfusion conditions. This was accompanied with a decrease in end diastolic pressure in hearts exposed to high glucose conditions ($p < 0.001$) though there were no changes in the control group. The combination treatment group followed the same trend with significant changes found in coronary flow vs. untreated group ($p < 0.05$) under high glucose conditions as well as a reduction in end diastolic pressure ($p < 0.001$). However, no changes were observed in heart rate and dP/dt_{max} in either groups of treatment following I/R. Infarct size however, was not measured for both Ibuprofen and NAC.

Table 3.2 Coronary flow, end-diastolic pressure, heart rate and velocity of contraction for hearts perfused under high glucose (33 mM) vs. control (11 mM) \pm NAC and Ibuprofen treatments as discussed.

	Coronary Flow (ml/min)	EDP (mmHg)	HR (beats/min)	dP/dt max (mmHg/s)
Control (11 mM)	8.2 \pm 1.3	33 \pm 5	282 \pm 24	1112 \pm 63
0.4 mM NAC	6 \pm 3	28 \pm 3	326 \pm 8	1062 \pm 56
29 μM Ibuprofen	9 \pm 1	31 \pm 4	302 \pm 14	1500 \pm 16
NAC + Ibuprofen	6 \pm 3	32 \pm 1	274 \pm 12	1333 \pm 78
High Glucose (33 mM)	6 \pm 1.5	65 \pm 7*	361 \pm 18	1133 \pm 45
0.4 mM NAC	9 \pm 1	48 \pm 2	242 \pm 12	1237 \pm 47
29 μM Ibuprofen	12 \pm 1.5 *	31 \pm 4**	215 \pm 30	1600 \pm 33
NAC + Ibuprofen	11 \pm 2*	29 \pm 4**	243 \pm 9	1000 \pm 54

EDP- end diastolic pressure; dP/dt_{max} - maximal velocity of contraction. 29 μ M Ibuprofen (anti-inflammatory); 0.4 nM NAC (antioxidant). Values are expressed as mean \pm SEM. * $p < 0.05$; ** $p < 0.01$ vs. respective control. (n=8 per group). Values shown at the end of reperfusion

To sum up, the MG-132 yielded very promising data and to confirm such effects we performed additional experiments by employing lactacystin, a more potent and specific PI.

3.4 Evaluation of *ex-vivo* heart function during global ischemia and reperfusion (simulated acute hyperglycemia) \pm lactacystin

Lactacystin treatment improved functional recovery of the control and high glucose groups, i.e. to $36 \pm 4\%$ and $34 \pm 2.3\%$, respectively (Figures 3.8A and 3.8B). Lactacystin treatment also improved RPP for the high glucose perfused group significantly but showed no significant effects for baseline treated hearts (Figures 3.8C and 3.8D), while dP/dt_{max} (Figures 3.9A and 3.9B) and HR (Figure 3.9C and 3.9D) remained unaltered.

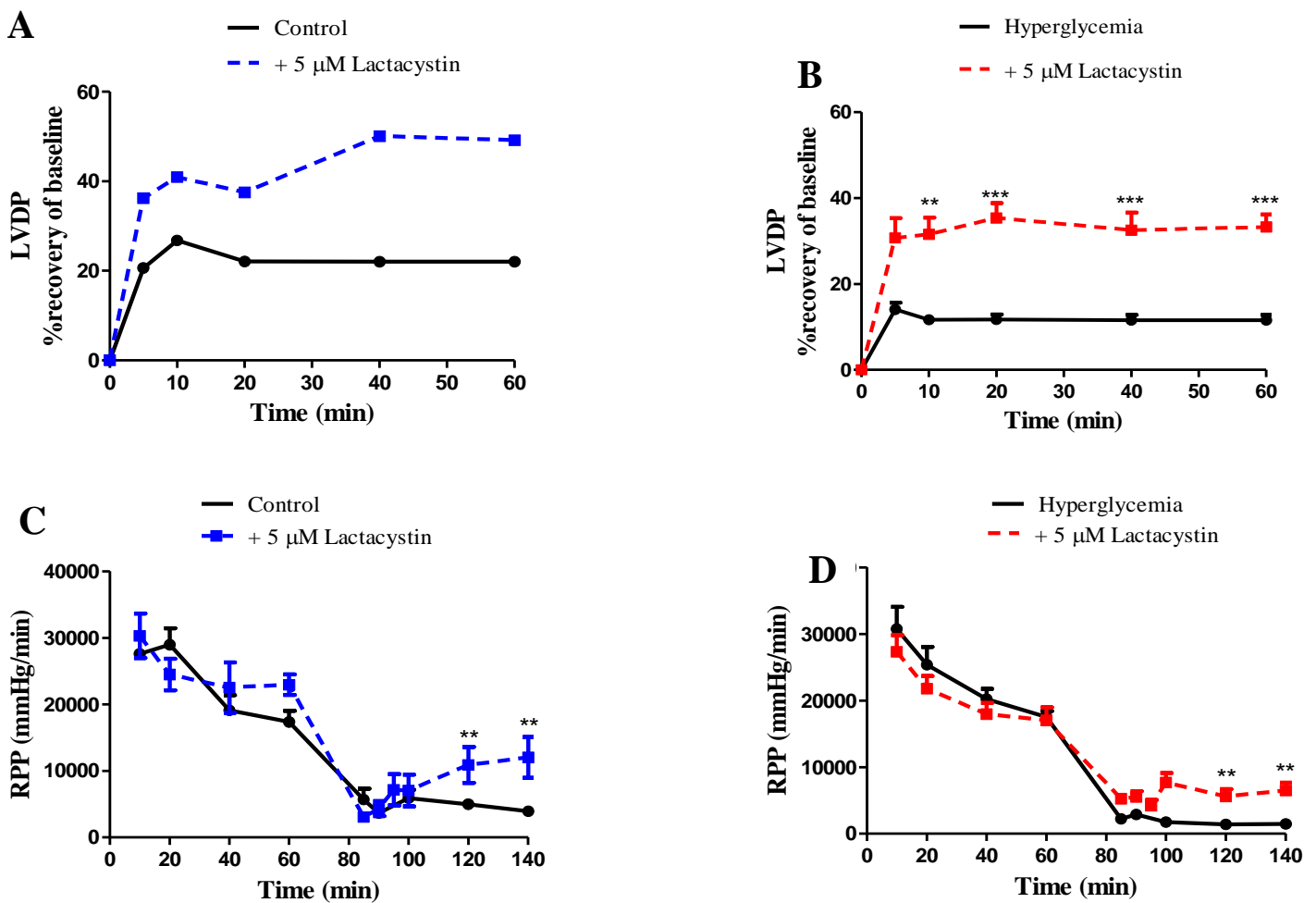


Figure 3.8 Lactacystin treatment during reperfusion improves contractile function at baseline and under high glucose condition following I/R. Isolated rat hearts were perfused under simulated hyperglycemic conditions (33 Mm glucose) vs. controls (11 mM glucose) and subjected to 20 min of global ischemia, followed by 60 min of reperfusion. 5 μ M lactaystin were added to the treatment group during the first 20 min of reperfusion. (A) Left ventricular developed pressure (% recovery) at baseline glucose levels (11 mM), and (B) with high glucose (33 mM). Rate pressure product (RPP) at baseline glucose levels (C), and (D) under high glucose conditions. Values are expressed as mean \pm SEM (n=8). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. respective controls.

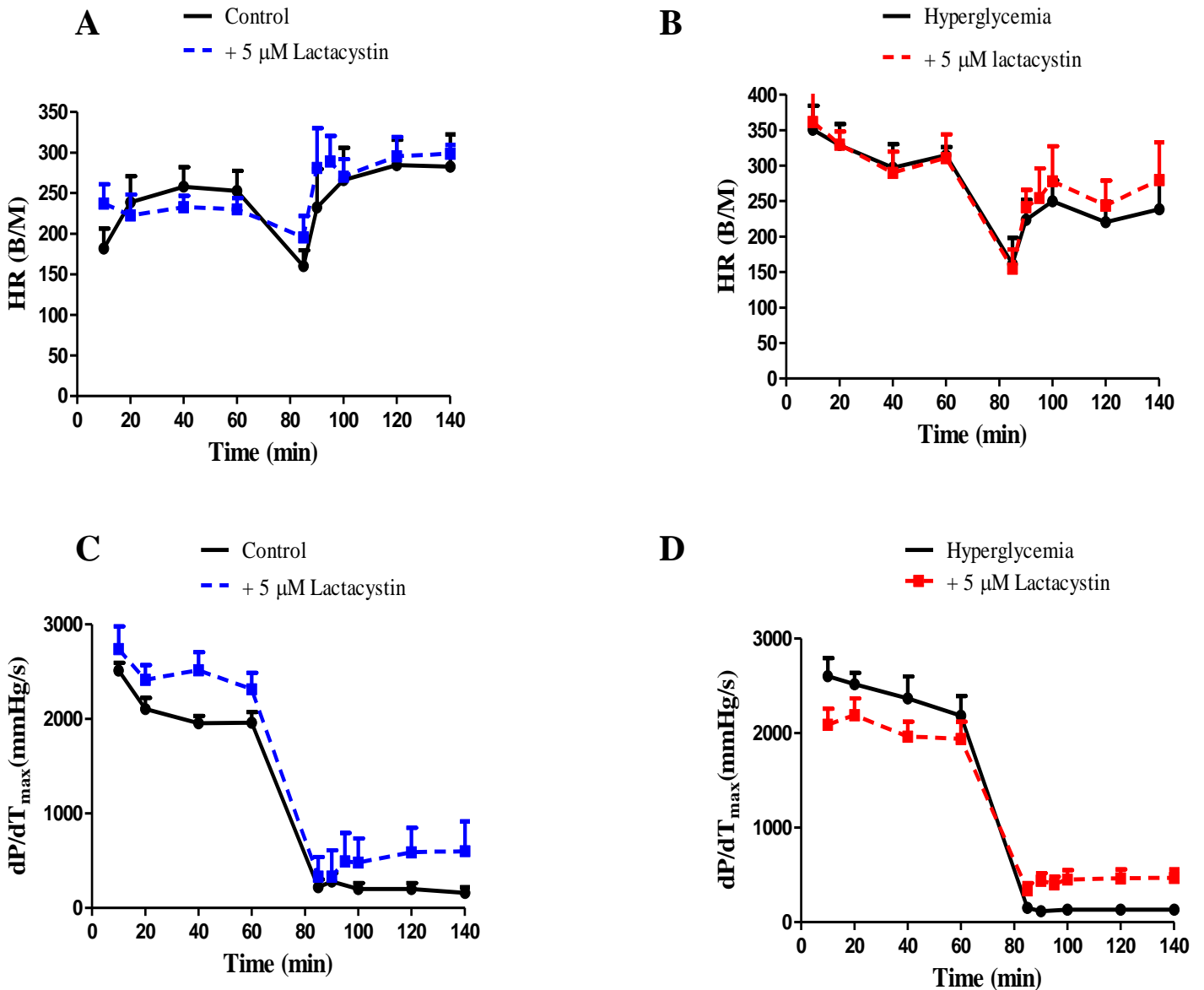


Figure 3.9 The effects of lactacystin treatment on HR and dP/dt_{max} . Isolated rat hearts were perfused under simulated hyperglycemic conditions (33 mM glucose) vs. controls (11 mM glucose) and subjected to 20 min of global ischemia, followed by 60 min of reperfusion. 5 μM lactacystin were added to the treatment group during the first 20 min of reperfusion. (A) Heart rate (HR) at baseline glucose levels (11 mM), and (B) with high glucose (33 mM). Velocity of contraction (dP/dt_{max}) at baseline glucose levels (C), and (D) under high glucose conditions. Values are expressed as mean \pm SEM (n=8).

Lactacystin treatment also significantly improved coronary flow vs. untreated group under high glucose perfusion setting. This was accompanied by a reduction in end-diastolic pressure in hearts exposed to high glucose conditions, though there were no differences for the control group (Table 3.3).

Table 3.3. Effects of lactacystin on *ex vivo* coronary flow and end-diastolic pressure during the first ten min ('Pre-ischemia') of stabilization and at the conclusion of reperfusion ('Post-ischemia').

	Coronary Flow (ml/min)		EDP (mmHg)	
	Pre-ischemia	Post-ischemia	Pre-ischemia	Post-ischemia
Control (11 mM)	10 ± 2.3	8.2 ± 1.3	14 ± 0	33 ± 5
5 µM Lactacystin	8.4 ± 1	7.5 ± 1.5	10 ± 1	29 ± 7
High Glucose (33 mM)	10.2 ± 1.8	6 ± 1.5	8 ± 3	65 ± 7*
5 µM Lactacystin	9 ± 2	11.2 ± 0.6*	15 ± 3	26 ± 7**

Values are expressed as mean ± SEM. *p<0.05; **p<0.01 vs. respective controls, (n=8 per group).

3.5 Assessment of infarct size following regional ischemia and reperfusion (simulated acute hyperglycemia) ± lactacystin

The regional I/R experiments demonstrated lactacystin treatment of high glucose perfused hearts attenuated the infarct size from 66.4 ± 3.4% to 48 ± 3.9% (p<0.05 vs. high glucose untreated) with no variations for the area at risk between the groups (Figure 3.10).

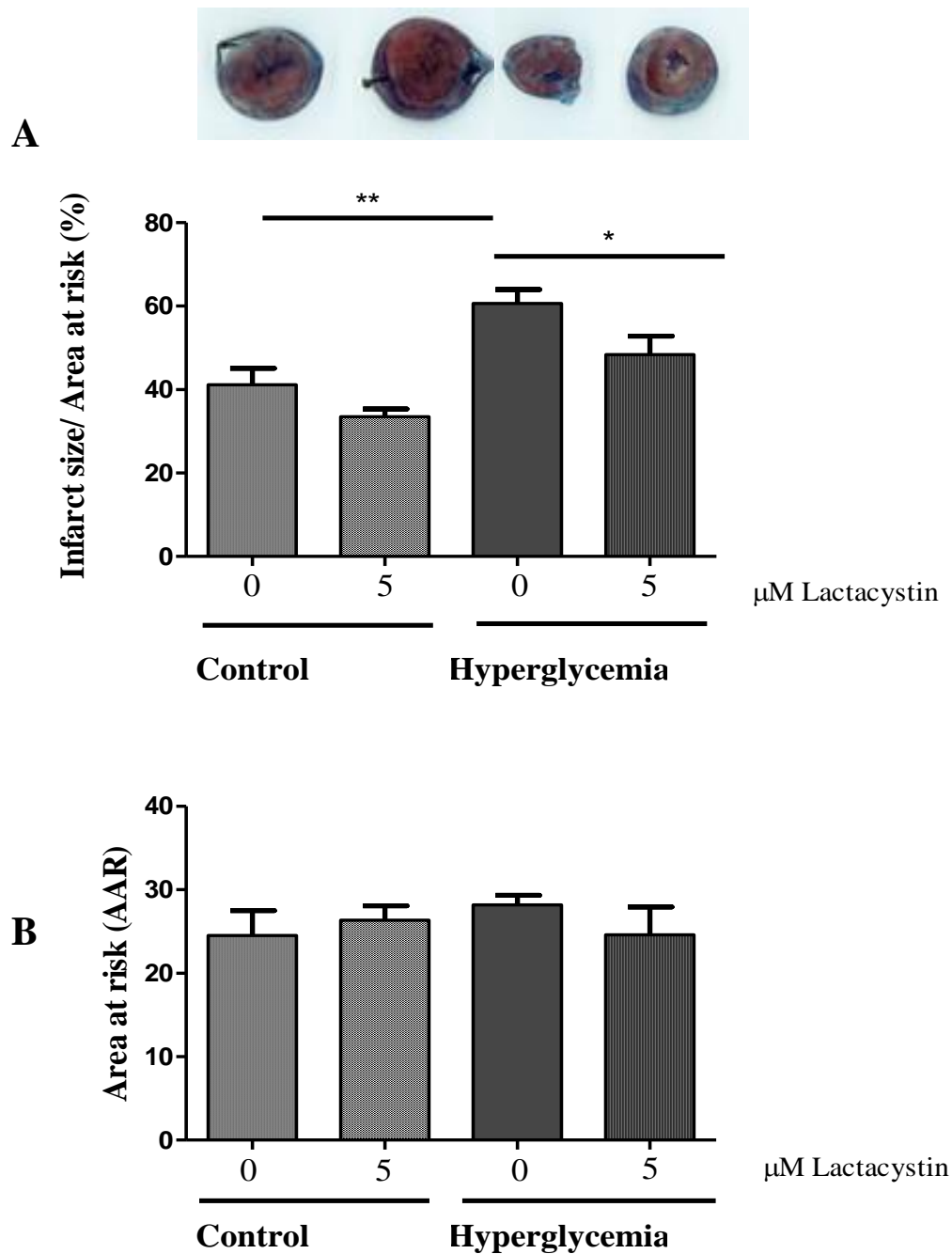


Figure 3.10 Lactacystin administration leads to cardio-protection following regional I/R. Isolated rat hearts were perfused under high glucose conditions (33 mM) vs. controls (11 mM) and subjected to regional ischemia. For lactacystin treated groups, 5 μM was given during the first 20 min of the two hour reperfusion period. (A) Infarct size/area at risk (%) and (B) area at risk under baseline (simulated normoglycemia) vs. high glucose perfusion conditions (simulated acute hyperglycemia). Evans blue dye and TTC staining allowed visualization of viable tissue (blue), infarcted area (white) and the area at risk (red). Values are expressed as mean ± SEM (n=8). *p<0.05, **p<0.01 vs. respectively controls.

3.6 Lactacystin treatment diminishes proteasomal activity

Our data revealed that there was a significant reduction in post-ischemic chymotrypsin-like activities under both low and high glucose conditions with the treatment of lactacystin (Figure 3.11B). However, there was no effect on post-ischemic trypsin and caspase-like proteasomal activity under baseline conditions or high glucose conditions (Figure 3.11C and 3.11D). General ubiquitinated proteins were lower with lactacystin under control and high glucose perfusion conditions (Figure 3.11A).

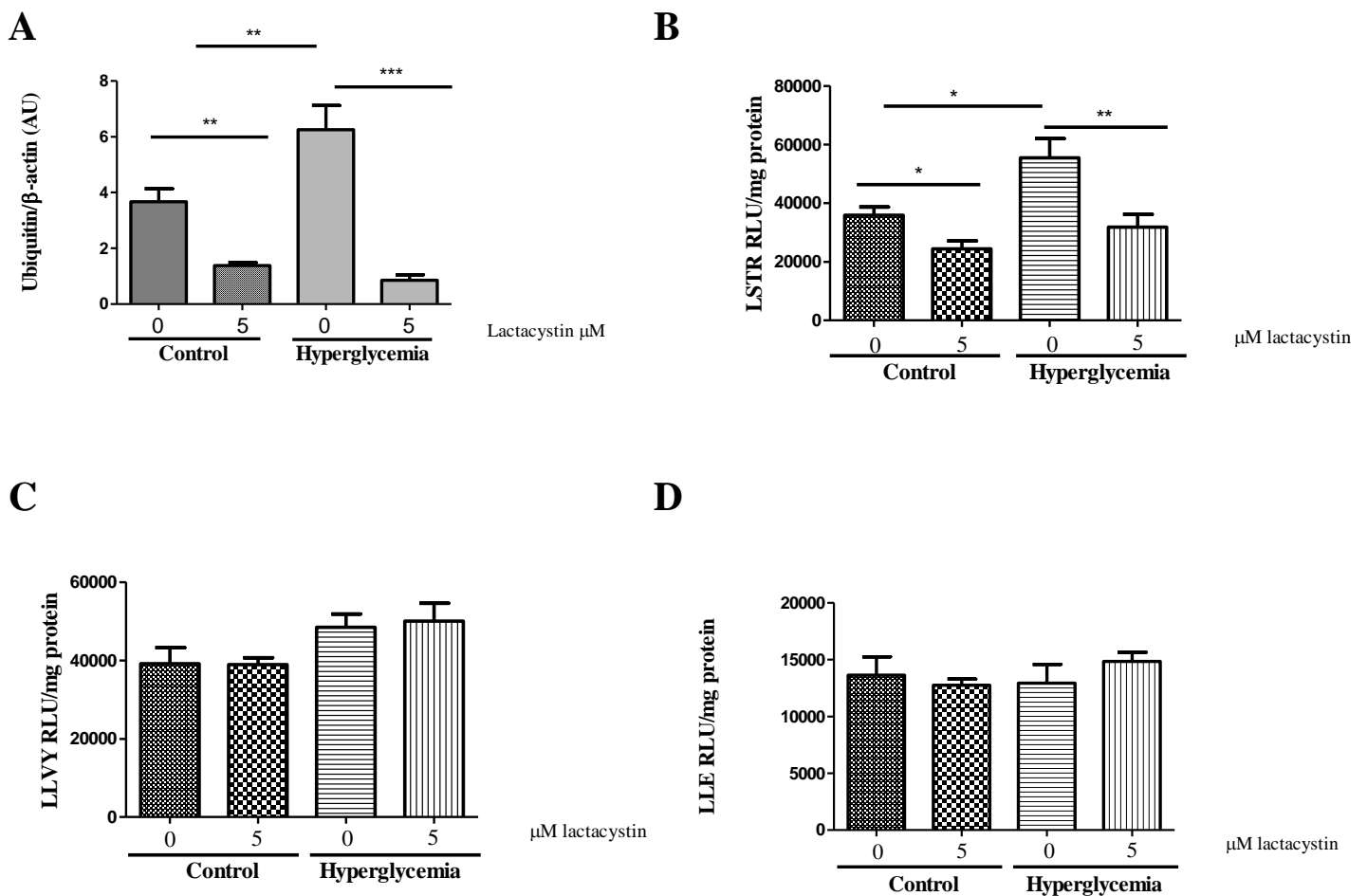


Figure 3.11 Lactacystin decreases high glucose-induced chymotrypsin-like proteasomal activity after I/R. Isolated rat hearts were perfused under high glucose (33 mM) conditions vs. controls and subjected to ischemia and reperfusion. For lactacystin treatment groups, 10 μM MG-132 was added during the first 20 min of reperfusion. (A) Total ubiquitinated proteins (B) chymotrypsin-like proteasomal, (C) trypsin-like, and (D) caspase-like activities after 60 min of reperfusion at baseline (11 mM glucose). Values are expressed as mean ± SEM (n=8). *p<0.05, **p<0.01, ***p<0.001 vs. respective controls.

3.7 Comparative analyses of treatment modalities

Our data established that MG-132 treatment triggered robust cardio-protection at baseline and under high glucose, to a much greater extent than lactacystin (Figure 3.12). Ibuprofen provided the next best treatment option under high glucose conditions, followed by NAC (Figure 3.13). Infarct sizes were also attenuated by both treatments; however MG-132 elicited a greater effect than lactacystin (Figure 3.14). Chymotrypsin-like proteasomal activities were lower with either PI with no effects found with trypsin and caspase-like proteasomal activity (Figure 3.15).

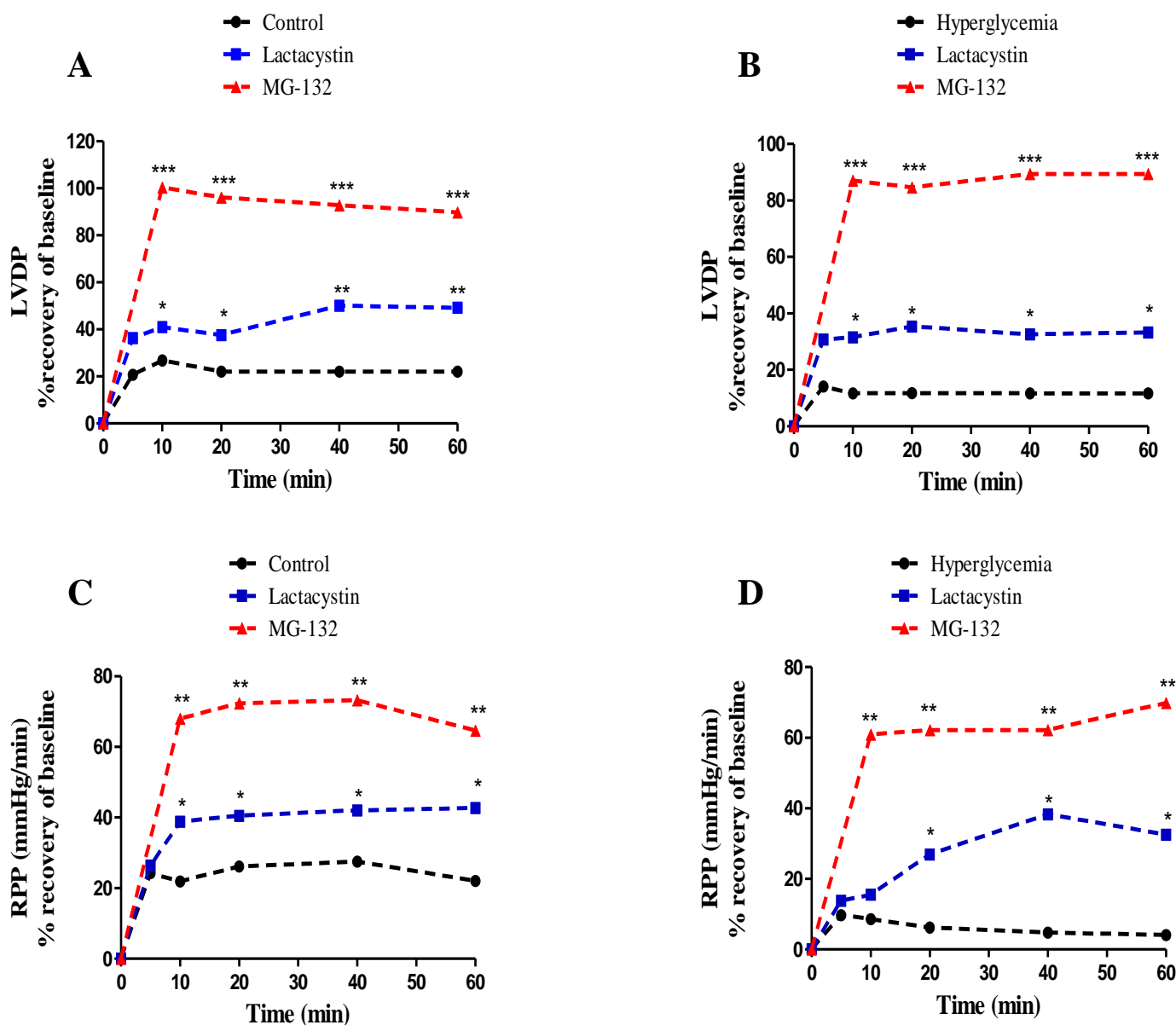


Figure 3.12 Comparative assessment of PI treatment during reperfusion improves function recovery at baseline and under high glucose conditions following I/R.

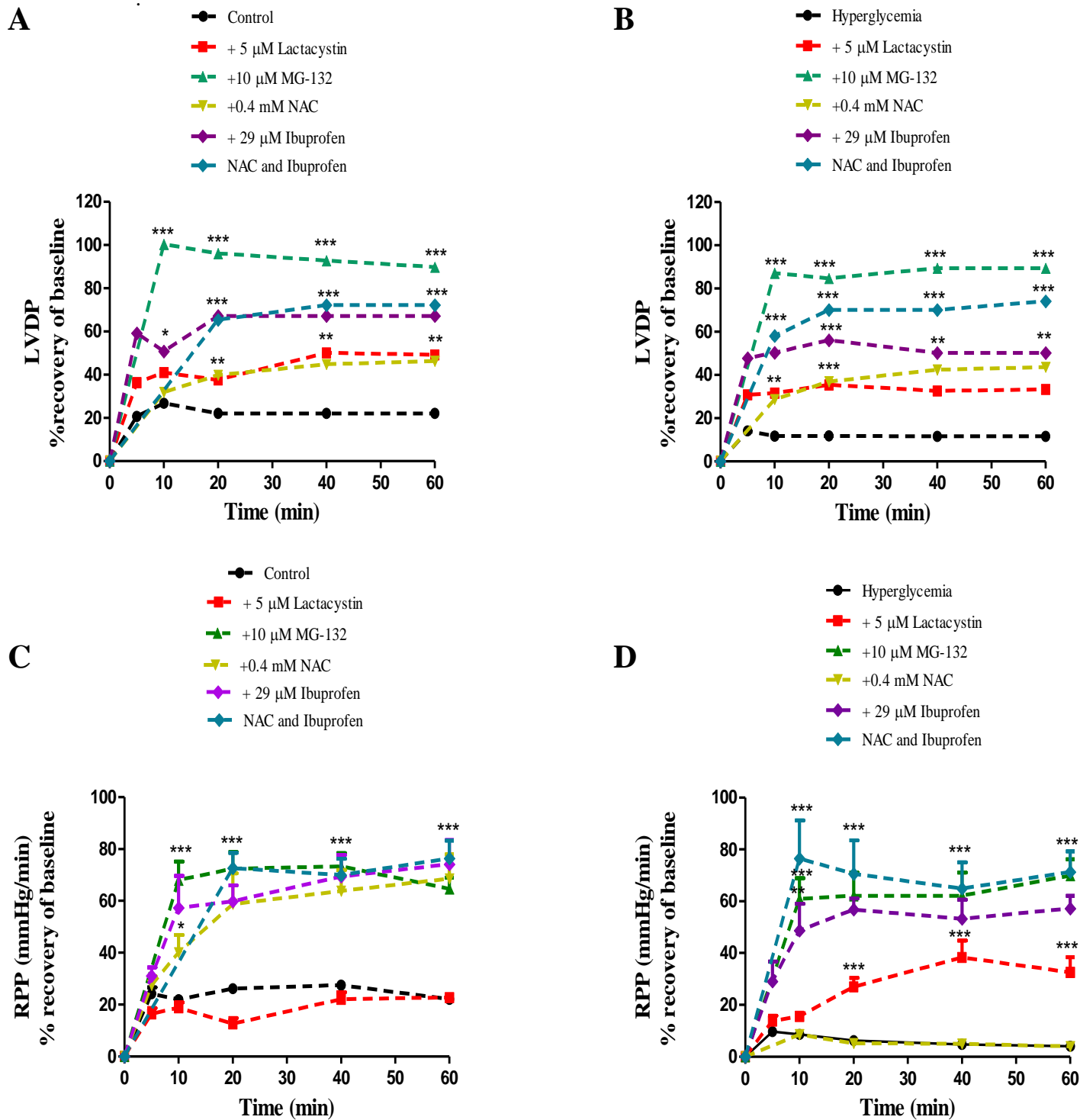


Figure 3.13 Comparison of treatment modalities with I/R under high glucose conditions.

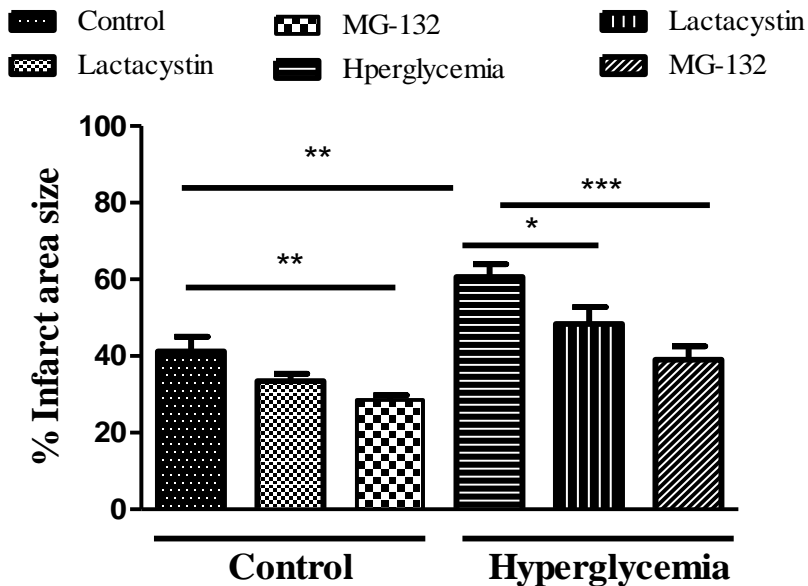


Figure 3.14 Comparison infarct size percentage decreased for MG-132 vs. lactacystin.

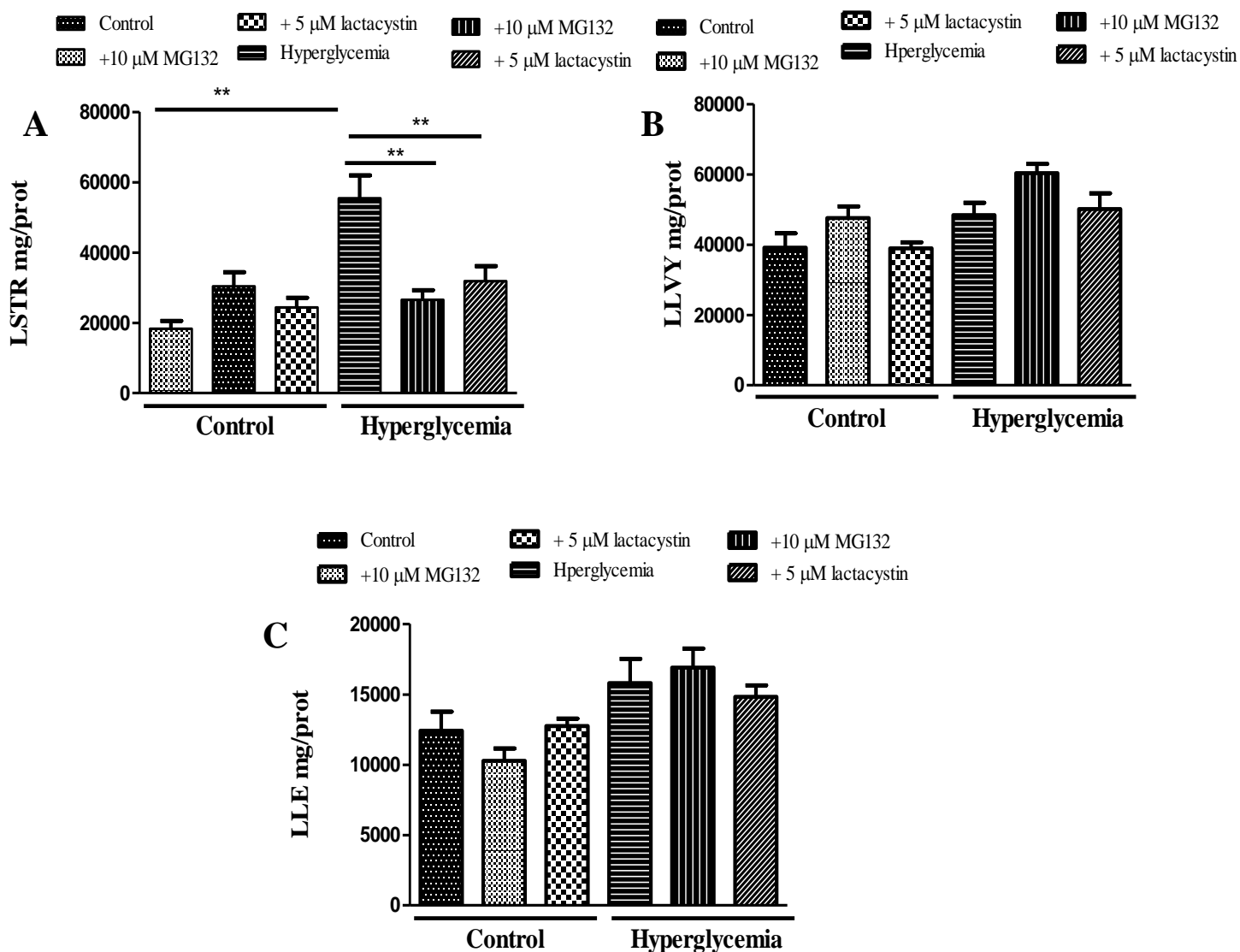


Figure 3.15 Comparison of proteasomal activity between MG-132 vs. lactacystin.

For the remainder of this study we employed lactacystin as the PI of choice. This was done for several reasons: a) lactacystin is considered a more specific inhibitor of the UPS, while MG-132 may have additional, nonspecific outcomes. These include interaction with MAPK as well as JNK 1, which is activated during the hyperglycemia and may have direct or indirect effects on certain markers we are investigating. (Powell SR., 2006; Tsukamoto O *et al.*, 2010; Zolk O *et al.*, 2006), and b) assessment of one inhibition is more cost-effective approach in terms of resources available for this study.

3.8 Effects of lactacystin treatment on myocardial ROS levels and apoptosis

We next assessed whether lactacystin cardio-protection is associated with anti-oxidant and anti-apoptotic properties in heart tissues collected from our experimental system. Here acute hyperglycemia during the onset of I/R increased cleaved PARP (Figure 3.16A $p < 0.05$ vs. untreated control) and this effect was blunted with lactacystin treatment ($p < 0.01$ vs. untreated high glucose). Moreover, lactacystin treatment significantly enhanced SOD1 and SOD2 peptide levels under control and high glucose conditions vs. controls (Figure 3.16B $p < 0.05$, $p < 0.001$, Figure 3.16C $p < 0.01$, $p < 0.001$). The GSH was increased with lactacystin for the control group (Figure 3.16D $p < 0.05$). Conversely, under high glucose conditions no change was found with treatment.

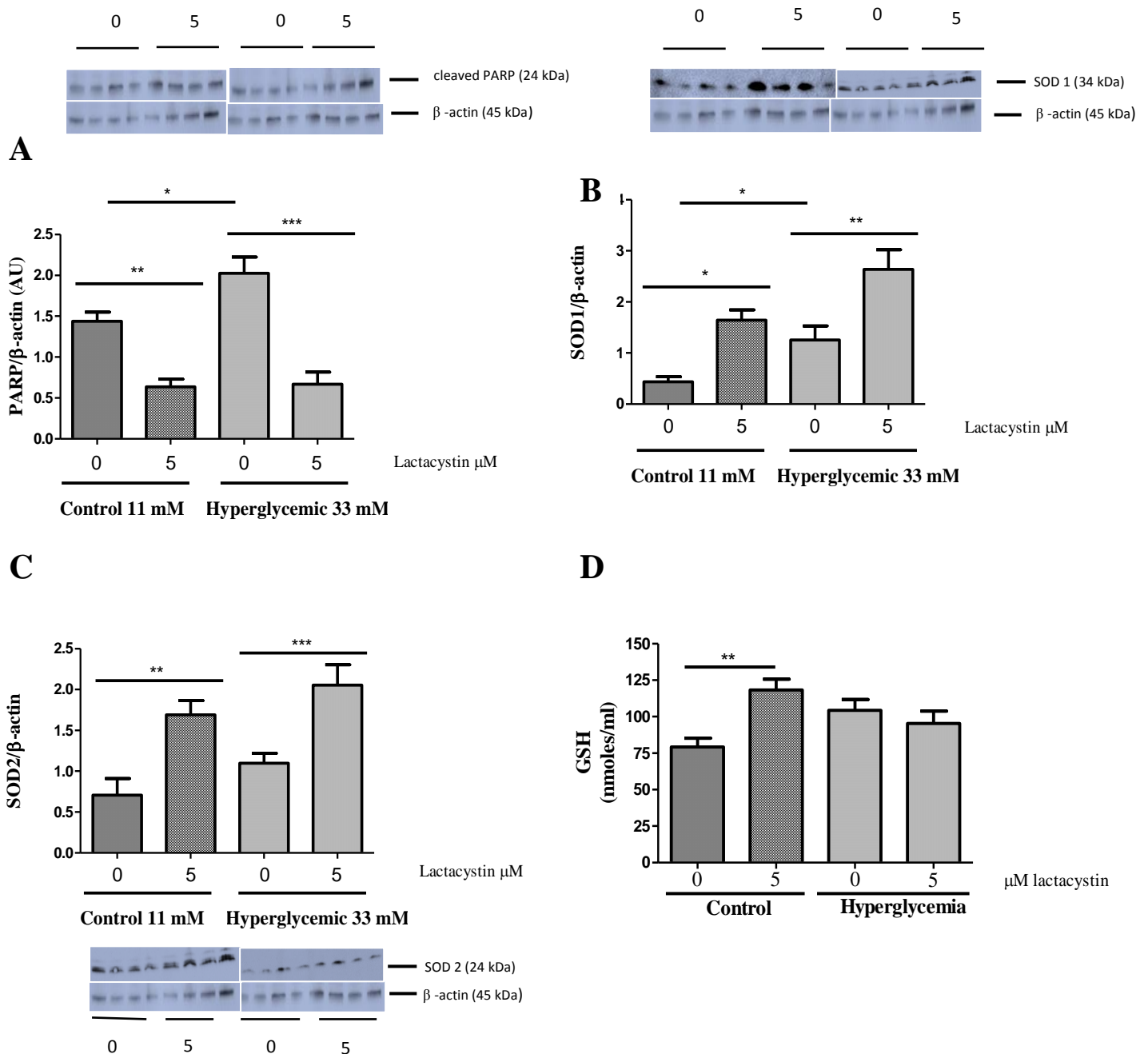


Figure 3.16 Anti-oxidant effects of lactacystin in hearts subjected to I/R under baseline and high glucose conditions. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to I/R \pm lactacystin treatment during reperfusion. Western blot analysis for A) PARP; B) SOD1; C) SOD2 peptide levels is shown with β -actin as loading control; D) GSH/GSSG ratio. Values are expressed as mean and \pm SEM (n=8). *p<0.05, **p<0.01, ***p<0.001 vs. respective controls.

In terms of apoptotic markers evaluated, lactacystin treatment reduced cleaved caspase-3 and cytochrome c peptide levels under control and high glucose conditions (Figure 3.17A p<0.01 and 3.17B p<0.001). Anti-apoptotic markers such as p-BAD/BAD, Bcl-2 (Figure 3.17C and 3.17D) and ARC (Figure 3.18) were diminished under high glucose conditions

($p < 0.05$, $p < 0.001$, $p < 0.01$ correspondingly) nonetheless this effect was reversed with lactacystin treatment under high glucose perfusion conditions.

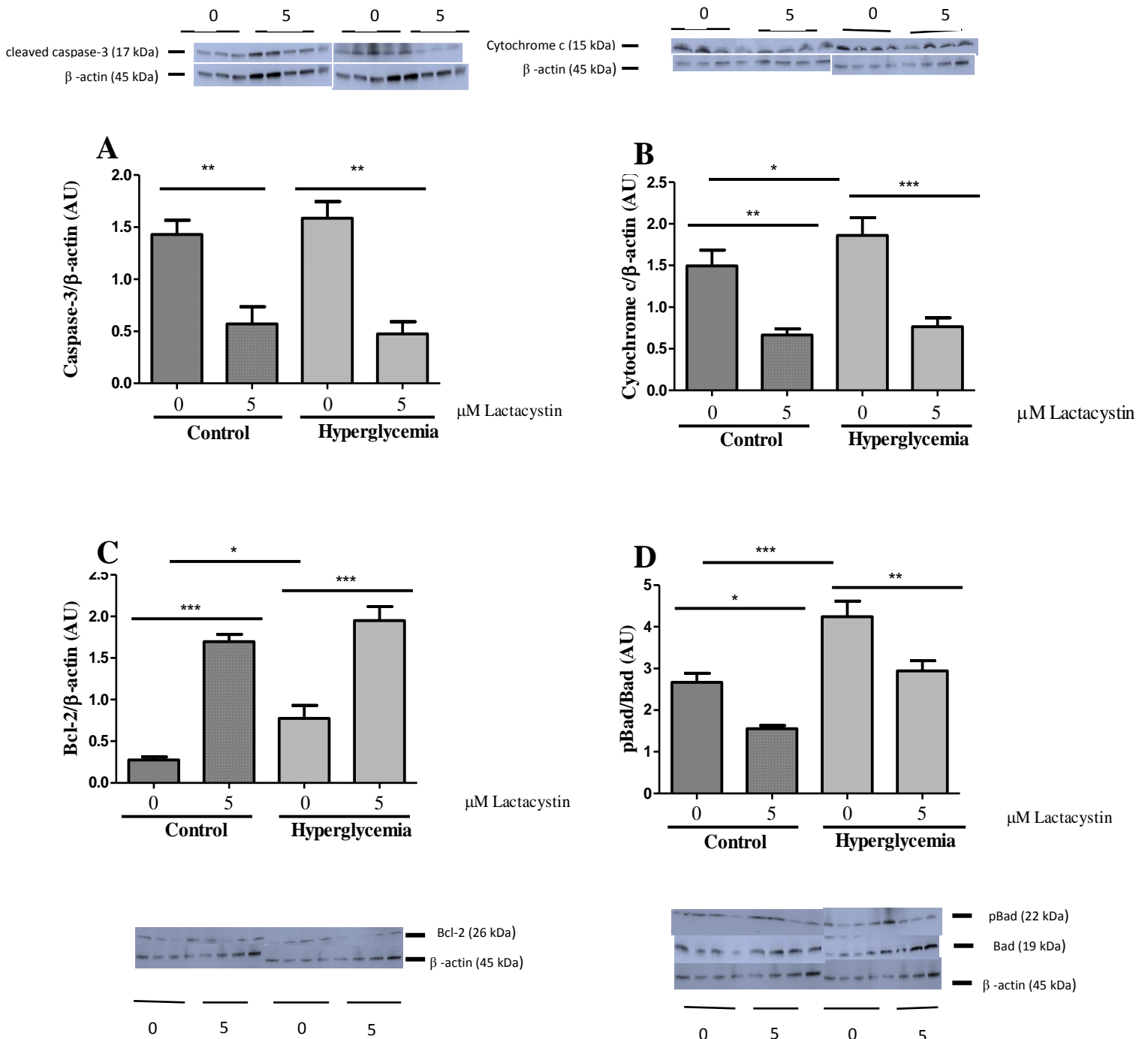


Figure 3.17 Anti-apoptotic effects of lactacystin in hearts subjected to I/R under high glucose conditions. Isolated rat hearts were perfused under high glucose conditions vs. controls

and subjected to ischemia and reperfusion \pm lactacystin treatment during reperfusion. Western blot analysis for A) caspase- 3; B) cytochrome c; C) Bcl-2 and D) pBad/Bad peptide levels is shown with β -actin as loading control. Values are expressed as mean \pm SEM (n=8). * p <0.05, ** p <0.01, *** p <0.001 vs. respective controls.

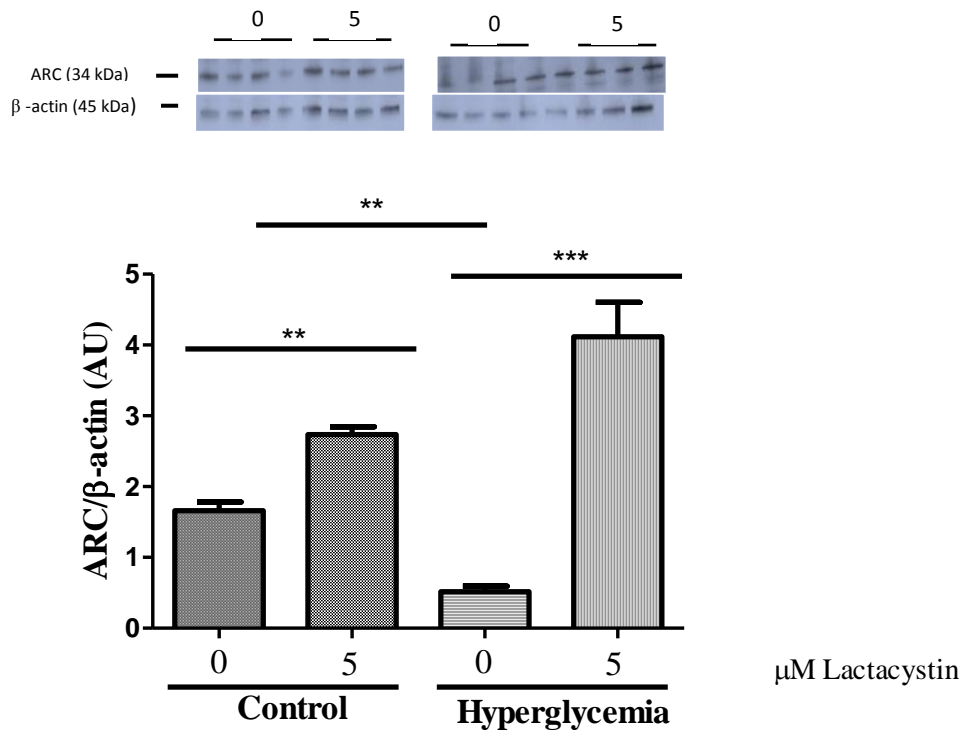


Figure 3.18 Anti-apoptotic effects of lactacystin in hearts subjected to I/R under control and high glucose conditions. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to ischemia and reperfusion \pm lactacystin treatment during reperfusion. Western blot analysis of ARC peptide levels is shown with β -actin as loading control. Values are expressed as mean \pm SEM (n=8). ** p <0.01, *** p <0.001 vs. respective controls.

3.9 Lactacystin blunts inflammation in hearts exposed to ischemia and reperfusion under high glucose conditions

The effects of proteasome inhibition on NF κ B activation were studied by analyzing I κ B α , NF κ B and IL-6 peptide levels. Here the data demonstrate that I κ B α levels were lower with lactacystin treatment under control (p <0.01) and high glucose perfusion conditions (p <0.001) (Figure 3.19A). Likewise, higher NF κ B and IL-6 peptide levels usually found under high glucose conditions and lactacystin treatment blunted this (Figure 3.19B p <0.001 and 3.19C p <0.001).

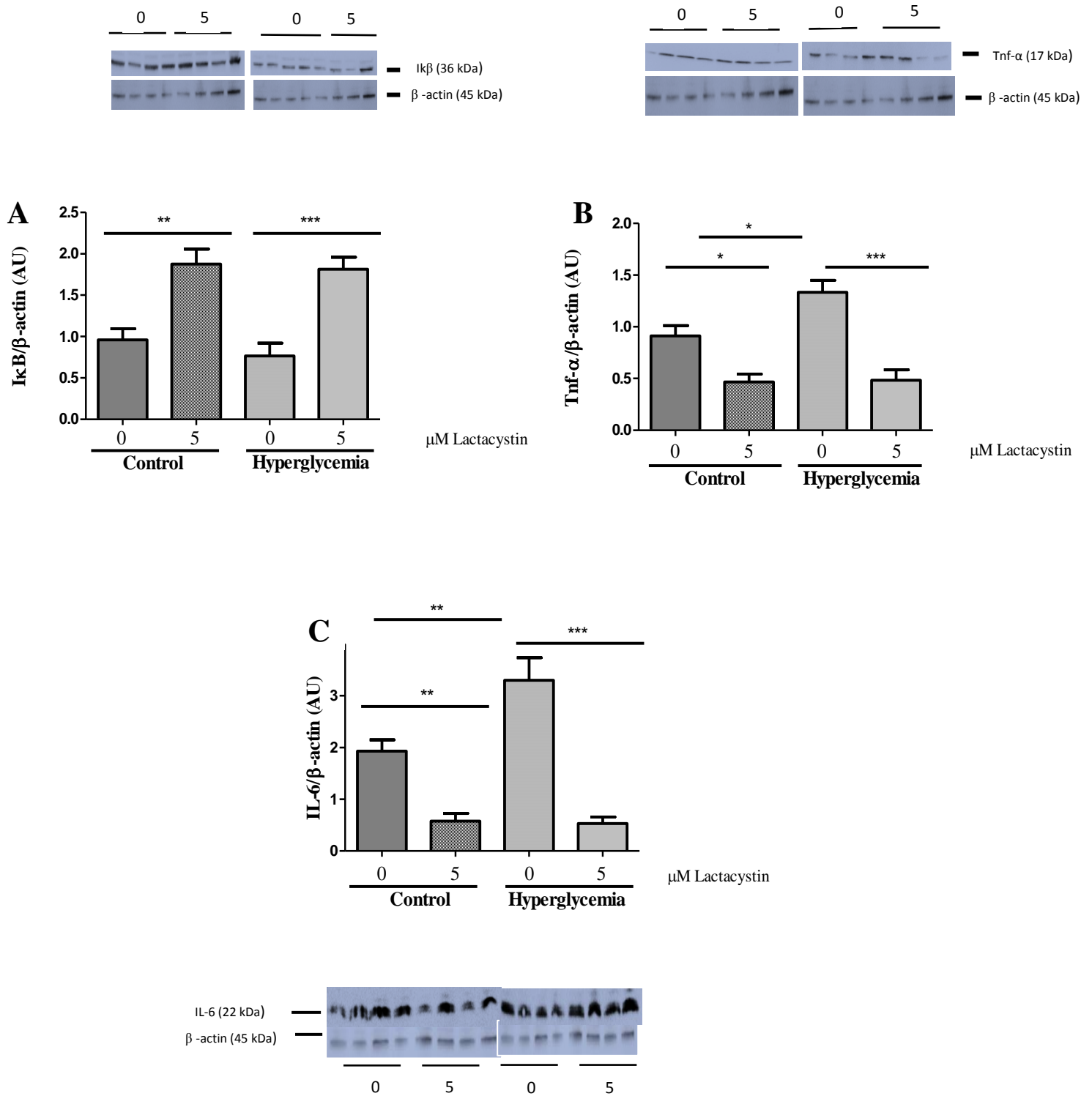


Figure 3.19 Anti-inflammatory effects of lactacystin in hearts subjected to I/R under control and high glucose conditions. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to ischemia and reperfusion ± lactacystin treatment during reperfusion. Western blot analysis for A) IkB α ; B) NFk β and C) IL-6 peptide levels is shown with β -actin as loading control. Values are expressed as mean \pm SEM (n=8). *p<0.05, **p<0.01, ***p<0.001 vs. respective controls.

3.10 Autophagy upregulated in lactacystin-treated hearts subjected to ischemia and reperfusion

Due to the interplay between the UPS and autophagy we also investigated the effects of PI treatment on the autophagic system under high glucose perfusion conditions. These data show that the LC3 ratio and p62 (both markers of autophagy), were augmented under high glucose conditions (Figure 3.20). Lactacystin treatment increased the LC3 ratio (Figure 3.20A) under control ($p < 0.05$) and high glucose conditions ($p < 0.001$), while it decreased p62 levels (Figure 3.20B) under control ($p < 0.05$) and high glucose conditions ($p < 0.001$).

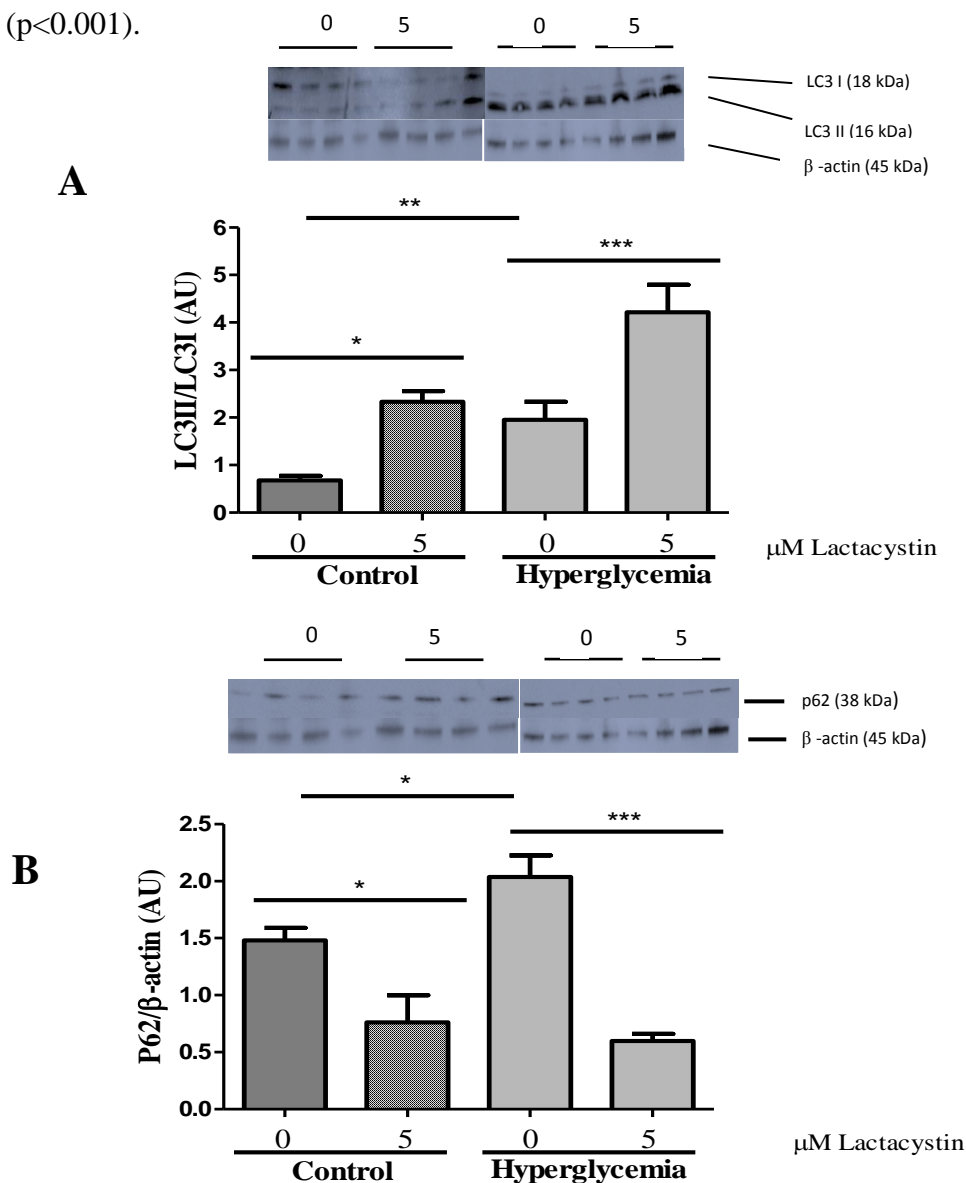


Figure 3.20 Lactacystin increases autophagy in hearts subjected to I/R under control and high glucose conditions. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to I/R \pm lactacystin treatment during reperfusion. Western blot analysis for

A) LC3I/LC3II ratio and B) P62 peptide levels is shown with β -actin as loading control. Values are expressed as mean \pm SEM (n=8). *p<0.05, **p<0.01, ***p<0.001 vs. respective controls.

3.11 Reduction in eNOS activation due to lactacystin under high glucose conditions

We also assessed whether eNOS peptide levels could be correlated with our coronary flow results (perfusion data). Here eNOS levels were lowered by lactacystin treatment under control (p<0.01) and high glucose perfusion conditions (p<0.001) (Figure 3.21).

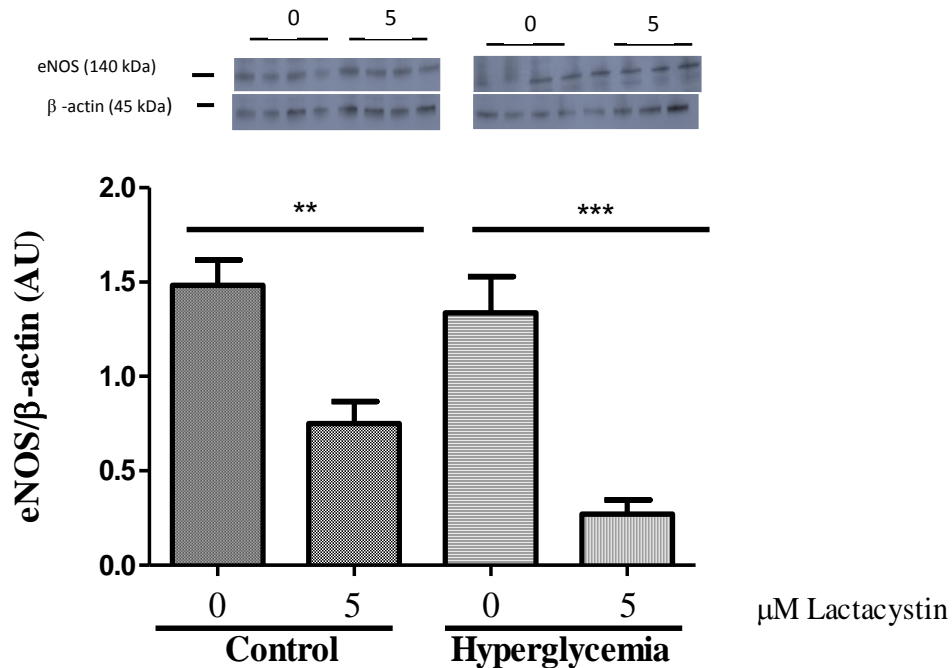


Figure 3.21 Lactacystin blunts eNOS in hearts subjected to I/R under control and high glucose conditions. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to I/R \pm lactacystin treatment during reperfusion. Western blot analysis eNOS peptide levels are shown with β -actin as loading control. Values are expressed as mean \pm SEM (n=8). **p<0.01, ***p<0.001 vs. respective controls.

3.12 Lactacystin blunts GR2K under high glucose conditions

To gain additional insight into our functional data we evaluated GR2K peptide levels as it is linked with increased arrhythmias after an ischemic insult. These data revealed that GR2K levels were increased with lactacystin treatment under control (p<0.05) and high glucose condition (p<0.001) (Figure 3.18).

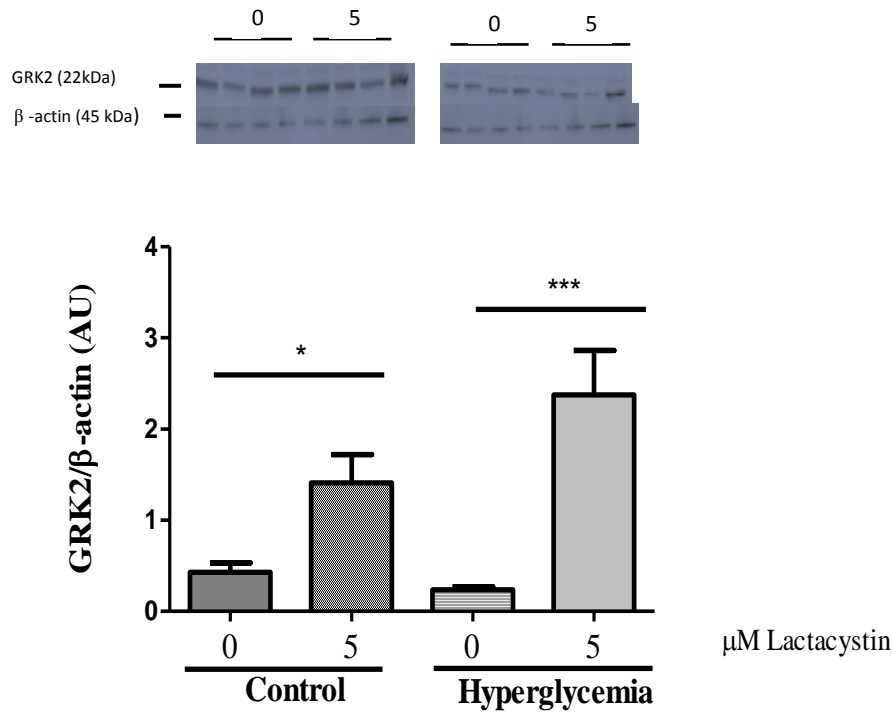


Figure 3.22 Lactacystin blunts GR2K in hearts subjected to I/R under control and high glucose conditions. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to ischemia and reperfusion \pm lactacystin treatment during reperfusion. Western blot analysis GR2K peptide levels are shown with β -actin as loading control. Values are expressed as mean \pm SEM (n=8). * p <0.05, *** p <0.001 vs. respective controls.

Chapter 4: Discussion

4.1 Introduction

The dramatic surge in diabetes during the past few decades constitutes a major threat to human health in developed and developing nations (Wild S et al., 2004; Bradshaw D et al., 2007). CVD frequently seen in diabetic patients, and here chronic hyperglycemia presents as a significant risk factor for acute myocardial infarction (AMI) (Cai L et al., 2002). Moreover, stress-induced, acute hyperglycemia in non-diabetic individuals with AMI is associated with increased in-hospital deaths (Capes SE et al., 2003). Thus, acute and hyperglycemia are able to trigger biochemical and electrophysiological perturbations which contributes to contractile dysfunction. Acute hyperglycemia (AHG) co-presenting with myocardial infarctions is associated with poor prognosis. Although AHG induces oxidative stress that may dysregulate the ubiquitin-proteasome system (UPS), it is unclear whether increased/decreased UPS is detrimental with ischemia-reperfusion under such conditions. As our earlier studies implicated the UPS as a mediator of cardiac damage we therefor explored our hypothesis by employing *ex vivo* rat heart perfusion models (global and regional ischemia), Western blot analysis and various biochemical assays. *The central discovery of this study is that PI treatment elicits cardio-protection in rat hearts by reducing hyperglycemia-induced oxidative stress, apoptosis, inflammation and proteasomal activity following I/R.*

4.2 Cardio-protection using PIs after ischemia-reperfusion under simulated hyperglycemic conditions

We initially examined our hypothesis by exposing isolated rat hearts to I/R and found a noticeable decline in contractile function under high glucose perfusion settings (simulated acute hyperglycemia). On the contrary, PI treatment with either MG-132 or lactacystin resulted in a prominent increase in functional recovery and shows that it elicits cardio-protection under both normal and high glucose perfused rat hearts. The protective effect under baseline condition can be accounted by the fact that I/R *per se* can also lead to inflammation and other forms of oxidative stress which causes modification of lipids, proteins, and DNA (Ames et al., 1993, Evans J.L et al., 2002, Toth A et al., 2006). Furthermore, NO bioavailability is reduced with I/R along with alteration in cell signaling pathways due to increased oxidative stress, resulting in the activation of NF κ B and cardiomyocyte death. (Palombella et al., 1944). However, PI administration at baseline may inhibit this sequence of events by increasing GSH levels and decreasing inflammation at baseline.

Cardiac muscle contains 70% of 20S trypsin-like activity. Several studies found that loss of the 20S trypsin-like activity linked with oxidative modification (4-hydroxynonenal) of numerous proteasome subunits results in an increase in oxidized and ubiquitinated proteins and cardiomyocyte death (Powell et al., 2006, Powell SR et al., 2005, Xichun Yu and David C. Kem, 2010). However, our PIs did not result in the inhibition of trypsin-like proteasome activity and thus it may (in part) contribute to the cardio-protection observed. Infarct size data from both PIs further validated these, while MG-132 post-ischemic-treated hearts also showed improved coronary flow and reduced end diastolic pressure. However, in this study it is unclear which heart cells are targeted by such treatment but the endothelial cells are likely to be primary targets for example, the inhibition of the UPS triggers significant effects on endothelial cells, i.e. higher eNOS expression and activity, that can lead to improved endothelial function (Stangl V., Lorenz, M et al., 2004). This may therefore lead to increased oxygen supply to the myocardium resulting in improved post ischemic function (Kassiotis C et al., 2008). Therefore further studies on especially the endothelium are needed to test whether this is the case in our experimental model.

When comparing the effects of the two PIs, MG-132 proved to be more effective than lactacystin. Various reasons can account for this, e.g. an artificial peptide substrate like lactacystin that can be cleaved by the proteasome particle might over-emphasize the role of the UPS resulting in it being less efficient (**Liu Z et al., 2000**). Secondly, the dosage of PIs plays a major role in its efficacy and therefore a lower dose of lactacystin may result in an improved recovery (**Gurusamy N et al., 2008, Shirley RB et al., 2005**). In addition, MG132 can protect cardiomyocytes from injury through induction of Hsp72 as well as Hsp32 and/ or activation of p38 MAP kinase (**Lüss H et al., 2002**). MG-132 also has other properties and mechanisms that may exert additional effects on the systems such as calpain and cathepsins (**Kisselev AF and Goldberg AL., 2001**). Together these data suggest that MG-132 is likely to exert non-UPS effects that may also result in cardio-protection. However, our findings strongly support a role for UPS inhibition as a novel cardio-protection agent.

To gain a better understanding of the core mechanisms whereby PIs result in cardio-protection, we evaluated whether it exhibits anti-oxidant and/or anti-apoptotic properties in our experimental systems. It is well established that myocardial ischemia-reperfusion is associated with increased ROS resulting in apoptotic and necrotic cell death (**Buja LM and Entman ML., 1998, Murriel CL., 2004, Nabel EG., 2003**). Moreover, glucose-mediated toxicity results in oxidative stress due to an imbalance in terms of production of free radicals and intracellular antioxidant defenses (**Kohner EM et al., 1998, Baynes JW., 1991**). For this study we found that NAC improved functional recovery under high glucose conditions. In support, PARP was significantly raised together with decreased SOD activity and a reduced GSH levels under baseline.

The ROS generated in our model may stem from numerous sources in the heart, including the mitochondrial ETC, glucose auto-oxidation and uncoupled nitric oxide synthase (ucNOS) (**Chen Q et al., 2003, Brownlee M., 2001**). Other sources may include the NADPH oxidases as shown before by us (**Joseph D et al., 2014**). Such oxidative stress can lead to particular ubiquitin genes being upregulated to assist to clear damaged proteins (**Sun L et al., 2002, Liu Z et al., 2000, Price SR et al., 1996**). Thus PI-induced upregulation of cardiac SOD-1 may be a key mechanism to detoxify ROS in our model and may also reverse downstream effects. We are unclear regarding the transcriptional

modulators driving this system. However, the basic leucine zipper transcription factor, nuclear factor erythroid 2 related factor 2 (Nrf2), is strong candidate (**Holtzclaw WD et al., 2004, Kobayashi A et al., 2004, Kwak MK et al., 2004**). Nrf2 activates the expression of genes responsible for cytoprotection such as NAD (P) H dehydrogenase [quinone] 1 (NQO1) and glutathione S-transferase (GST) via an antioxidant/electrophile responsive gene promote element (ARE/EpRE) (**Kobayashi M and Yamamoto M., 2005, McMahon M et al., 2003, Motohashi H and Yamamoto M., 2004**). Kelch ECH associating protein 1 (Keap1) acts as a stress sensor that suppresses Nrf2 activity and accelerates Nrf2 degradation by the UPS, thereby preventing Nrf2-individual transcription of target genes (**Itoh K et al., 2003, McMahon M et al., 2003**). UPS inhibition thus leads to Nrf2 accumulating in the nucleus and resulting in the activation of target genes. We propose it is likely the route whereby PIs may act as anti-oxidants with I/R under acute hyperglycemic conditions. However, further studies are needed to confirm this.

The improved functional effects in our experimental model with PI treatment can also be attributed to its anti-apoptotic effects. This is likely to be a downstream effect, subsequent to elevated oxidative stress. Hyperglycemia leads to surplus free-radical generation and oxidative stress such as superoxide and NO which favors the production of peroxynitrite (ONOO^-) (**Brownlee M., 2001**). Peroxynitrite can in turn activate caspases such as caspase-3, essential for apoptosis (**Virag L et al., 1998, Zhuang S et al., 2002**). Here oxidative stress-induced apoptosis is initiated via Bax-mediated mitochondrial permeability and cytochrome c release resulting in the formation of an apoptosome with subsequent caspase-3 activation (**Vincent AM et al., 2005**). Moreover, high glucose levels attenuate the phosphorylation of Bad, which is associated with increased Bax/Bcl-2 levels and cytochrome c release (**Verzola D et al., 2002, Fernandez M et al., 2004**). Our data are in agreement, i.e. various cell death markers were elevated with ischemia-reperfusion under high glucose conditions. We also propose that p53 - a pro-apoptotic transcript regulated by MDM2 (which in turn is degraded by the UPS) - may play a role in this instance. MDM2 functions as an E3 ubiquitin ligase that can regulate p53 degradation by the UPS. However, if the UPS is attenuated, this will result in greater intracellular p53 levels and enhanced anti-apoptotic effects (**Toth et al., 2006**). By contrast the ARC antagonizes: a) extrinsic-mediated apoptosis by non-homotypic death-fold interaction with Fas (CD95/Apo-1) and FADD (Fas-associated death domain protein)

and b) intrinsic pathways of apoptosis via interactions between ARC and the C-terminal regulatory domain of Bax (Bcl-2-associated X protein) thereby preventing apoptotic stimuli (Wang M et al., 2005, Nam YJ et al., 2004, Gustafsson AB et al., 2004). This was reflected in our data where PI treatment triggered an attenuation of cell death markers together with increased anti-apoptotic markers (Figures 3.17 and 3.18).

4.3 PI blunts inflammation in hearts exposed to ischemia-reperfusion under high glucose conditions

Disproportionately elevated UPS activation can lead to the activation of harmful signaling pathways, e.g. NF κ B (Marfella R et al., 2009). The most significant association between the UPS and inflammation is linked to NF κ B. While, NF κ B is actively inhibited when bound to I κ B, the latter's degradation via the UPS can lead to its activation. The UPS can also control inflammatory pathways by regulation of macrophage function (Qureshi N et al., 2005). Various studies have linked pro-inflammatory cytokines, like TNF- α , IL-1 β and IL-6, to the pathophysiology of heart disease (Sriramula S et al., 2008, Kang YM et al., 2008). Moreover, increased NF- κ B p50 sub-unit gene expression and NF- κ B p65 sub-unit activity were found to be elevated under hyperglycemic conditions (Marfella R et al., 2009, Qureshi N et al., 2005). In agreement, our data revealed that Ibuprofen improved heart recovery after I/R under high glucose conditions, together with a lower inflammatory response. In support, others found that blockade of NF- κ B in obese db/db mice shields the heart against oxidative stress by restoring mitochondrial integrity and improving cardiac function (Schreck R et al., 1992, Schreck R et al., 1991, Mariappan N et al., 2007, Mariappan N et al., 2009,). Furthermore, PI inhibition of inflammation resulted in enhanced cardiac function in response to ischemia-reperfusion (Evans J.L et al., 2002, Toth A et al., 2006). This was supported by another study where PS-519, a synthetic analog of lactacystin, which is a highly selective and potent proteasome inhibitor, resulted in a decrease of NF- κ B activation, attenuated the size of myocardial infarction, reduced reperfusion injury, and preserved regional myocardial function with ischemia and reperfusion (Pye J et al., 2003). Moreover, a decrease in p65 and TNF- α expression preserved I κ B expression as PS-519 inhibited the NF- κ B inflammatory pathway activation (Figure 1.9) (Pye J et al., 2003). We do however, take into consideration that this data might be different when compared to other models such as *in-vivo*. Nonetheless, together these findings show that glucose-mediated inflammation plays a detrimental role with ischemia and reperfusion. Though by, blunting the UPS we are

able to attenuate pro-inflammatory responses and this is linked with cardio-protection under such conditions.

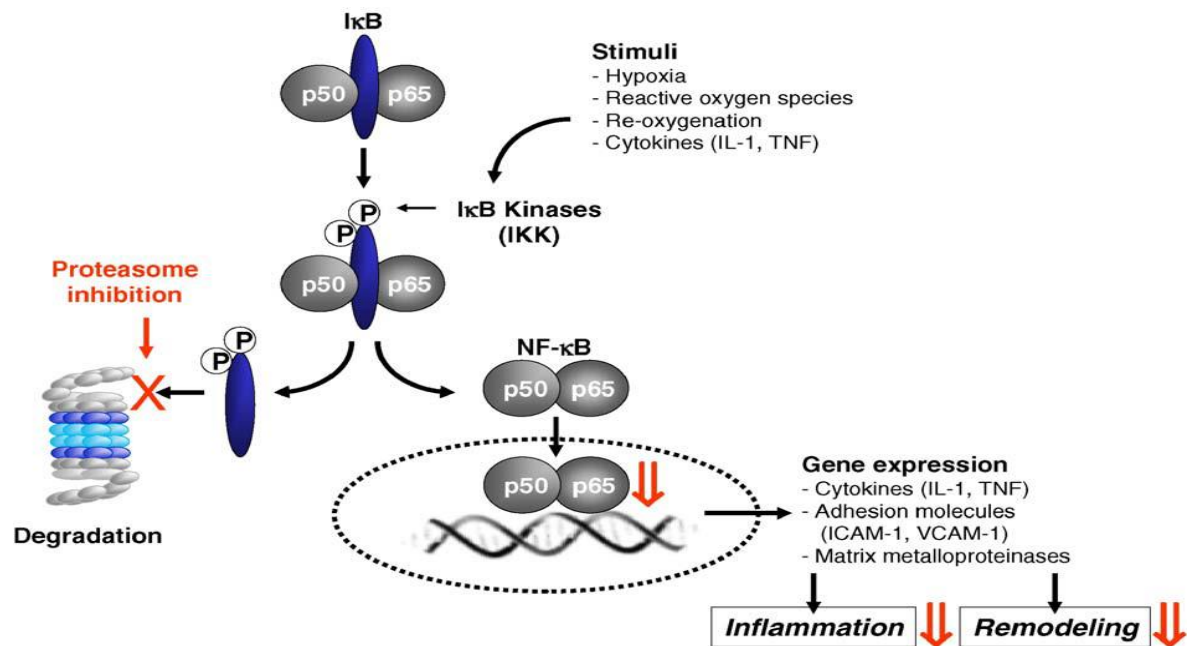


Figure 1.9. A schematic presentation of the activation of the NF- κ B Zolk et al., 2006.

4.4 Other mechanisms involved in proteasome inhibition-mediated cardio-protection

Normally with the inhibition of the UPS an increase in Ub proteins would be expected (Baracos VE et al., 1995, Powell SR., 2006, Pye J et al., 2003, Kastle M and Grune T., 2001). However, our findings showed the exact opposite where a decrease was observed. Enhanced autophagy was similarly detected in mouse hearts and isolated cardiomyocytes exposed to simulated I/R (Matsui Y et al., 2007, Valentim L et al., 2006) and the functional role of autophagy in these conditions was examined. Matsui et al. revealed that reduced autophagy enhanced myocardial injury in acute myocardial ischemia (Matsui Y et al., 2007). Another study found that an autophagic marker in pig hearts with repetitive myocardial ischemia was prevalent in areas of sub-endocardium where apoptosis was lacking (Yan L et al., 2005). Therefore autophagy may contribute to the survival of cardiomyocytes in response to both acute and chronic MI. However, in our study we are unclear why this may be the case but recent research reveals an association of K63-linked poly-ubiquitin chains to the autophagy pathway (Kirkin V et al., 2006). Here adaptor molecules such as p62/SQSTM1 (referred to as p62 in this thesis) and

NBR1 enables the binding, by K63-linked damaged proteins, to both ubiquitin and autophagy machinery thereby linking the two pathways together (**Bjorkoy G et al., 2005**). P62 can bind poly-ubiquitinated substrates through its ubiquitin-associated domain and directly binds to LC3 by the LC3 interacting region motif (**Pankiv S et al., 2007**) or via its PB1 domain and interacts with the proteasome by an UBL domain (**Seibenhener ML et al., 2004**). Thus the activation of autophagy in our system may help alleviate proteolytic stress (**Kuusisto E et al., 2001**) and could potentially explain our results. Taken collectively, p62 plays a crucial role in the formation of ubiquitin-positive inclusions, degradation of Ubproteins under stress, and is a perfect candidate for a messenger between the UPS and autophagy. However, further studies are required to confirm this.

Another mechanism involved in PI-induced cardio-protection maybe via the (GR2K. Various studies reported a robust reduction in GR2K levels with CVD leading to ventricular tachyarrhythmias and sudden cardiac death (SCD) within minutes or hours following an acute myocardial infarction (**Opitz CF et al., 1995, Patterson E et al., 1991, Patterson E et al., 1991, Yu X et al., 2005**). However, UPS inhibition leads to GR2K preservation (Figure 3.18) and would be expected to result in less/reduced occurrence of ventricular tachyarrhythmias and SCD. However, no SCD occurred in our treatment groups compared to controls, therefore it remains unclear whether this situation applies in our model or not. Further investigations are required to evaluate this effect in this and/or amended models.

4.5 Conclusion

This study examined the damaging effects of hyperglycemia in the setting of ischemia and reperfusion. Numerous deductions can be drawn from our work. We saw that hyperglycemia led to a poor cardiac function. UPS activity was increased in our model and can be attributed to the cardiac dysfunction. Our data revealed that oxidative stress, apoptosis and inflammation was increased under hyperglycemic conditions.

Another important objective was to identify novel therapeutic agents that are able to blunt the harmful effects of hyperglycemia in the context of ischemia and reperfusion. Our findings demonstrate that MG-132 and lactayctin can be used to treat acute hyperglycemia-induced cardiac contractile dysfunction. The PI's were found to decrease

oxidative stress as well as apoptosis and limiting inflammation. These data are encouraging since it may ultimately result in novel therapeutic interventions to treat stress-induced acute hyperglycemia and diabetic patients with associated CVD complications such as MI.

4.6 Future directions

4.6.1 Limitations of the study

The present study has certain limitations that need to be taken into account. The drug doses to prove anti-inflammatory and anti-oxidant activity of the PIs were based on non-rodent models (**Yew et al., 2005; Meiners S et al., 2002; Use of NSAID for Relief of Pain in Rodents and Rabbits” Lab Animal 26: p. 241-255; Peng Y et al., 2011**). Evaluations of the PIs under non-ischemic conditions are required to assess the effects of the PIs on normal cardiac tissue. Furthermore, even though ROS was linked by measuring SOD1/2 and GSH anti-oxidant levels, we acknowledge that other ROS regulators/mediators may also be implicated in the damaging effects of hyperglycemia and also impacting on the UPS, therefore other assessments would be useful such as ORCA, superoxide levels or GSH/GSSG ratio. Additionally, stronger evidence is also needed to link the cross-talk between the UPS and autophagy by assessing ubiquitin markers such as K48 as well as Nrf2 expression levels and/or its activation. These experiments will further strengthen our existing data.

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Appendix 1

4 June 201

Prof. M. F. Essop
Physiological Sciences
Stellenbosch University
Stellenbosch

Dear Prof Essop

Application for Ethical Clearance:
BENFOTIAMINE ATTENUATES HYPERGLYCEMIA-MEDIATED DECREASE IN
MYOCARDIAL FUNCTION IN RESPONSE TO ISCHEMIA-REPERFUSION
Ref: 10NF_ESS01

Your application for ethical clearance has been approved by the SU ACU committee. Please note that this clearance is only valid for a period of twelve months. Ethical clearance of protocols spanning more than one year must be renewed annually through submission of a progress report, up to a maximum of three years.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document will be available on the Division for Research Development's website www.sun.ac.za/research , shortly.

Please feel free to contact Mr. Winston Beukes if any additional information is needed.

Kind regards



Ms M Foucho (Manager: Research Support)

Appendix 2

Krebs-Henseleit stock solutions

1. NaCl (279g/ 2L)
2. NaHCO₃ (83.6g/ 2L)
3. KCl (17.6g) + K H₂PO₄ (8.1 g) in 2 L
4. MgSO₄. 7H₂O (7.4g) + NaSO₄ (4.2g) in 1L
5. CaCl₂.2H₂O (18 g) in 1L

To make up the 5 L working Krebs-Henseleit buffer mix 250ml of 1 and 2; add 100ml of 3 and 4 then add glucose and finally # 5. Filter and p.H to 6.7 with HCl or NaOH. To make up 11 mM glucose add 9.9 g and increase twice or thrice for 22 mM and 33 mM buffer solutions, respectively.

Appendix 3

Preparation of the triphenyl-tetrazolium chloride (TTC) solution

Make up a high pH phosphate buffer by dissolving 14.2 mg of Na₂HPO₄ in a liter of distilled water and a low pH phosphate buffer by dissolving 12 mg of NaH₂PO₄ in a liter of distilled water. Mix in a ratio of 8 parts of the high pH buffer to 2 for the low pH buffer. pH to 7.4. For every heart use 5 ml of the mixed phosphate buffers and weigh 10 mg of the TTC salt for every 1 ml of the solution. Incubate the cut heart slices at 37 °C for 20 min shaking at least once.

Appendix 4

Determination of infarct size and area at risk using Image J software

Select the heart slices using the third tool for polygon selections in the tools bar and pressing down on the Shift key of the keyboard. Determine the total area by clicking the Edit tab then select clear outside, thereafter click the Analyze tab and select Measure. A table with the first set of results shows and for infarct size use the area values. Thereafter click on the Image tab, select Split channels. Here 3 windows will open i.e. green, red, and blue channels. Close the

blue channel, use values from the green channel will be used for calculating infarct size and from the red channel area at risk. Here, it is advisable to do the analysis with a picture of the heart alongside. In either of the green or red channel, click on the Image tab, select Adjust threshold and using the eighth tool select the respective highlighted area in the heart slices corresponding to the infarct size and area at risk, respectively. Thereafter click on the Analyze tab and select measure. At the end three readings should appear for total area, infarct size and area at risk. For the calculations subtract the second and third readings from the total area to obtain infarct size and area at risk values, respectively. These are calculated for both sides of the heart slices and expressed as infarct size/area at risk.

Appendix 5

Modified RIPA Buffer:

A 100 ml modified RIPA buffer contains:

- 50 mM Tris-HCl (790 mg of Tris in 75 ml distilled water and 900 mg of NaCl and pH made 7.4 using HCl)
- 10 ml of 10% NP-40 [final concentration 1%]
- 2.5 ml of 10% sodium deoxycholate [final concentration 0.25%]
- 1 ml of 100 mM EDTA pH 7.4 [final concentration 1 mM]

Protease inhibitors

- 500 μ L of 200 mM phenylmethylsulfonyl fluoride (PMSF) [final concentration 1 mM]
- 100 μ L of Leupeptin (1 mg/ml water) [final concentration 1 μ g/ml]
- 80 μ L of SBT1 (5 mg/ml water) [final concentration 4 μ g/ml]
- 100 μ L of Benzamidine (1 M) [final concentration 1 mM]

Protein phosphatase inhibitors

- 500 μ L of 200 mM activated sodium orthovanadate (Na_2VO_3) [final concentration 1 mM]
- 500 μ L of 200 mM NaF [final concentration 1mM]
- 1 ml Triton X-100

This buffer is then made up to a final volume of 100 ml with distilled water and stored at -20°C.

Appendix 6

Bradford protein quantification method:

Bradford reagent

- 500 mg of Coomassie Brilliant Blue G in 250 ml of 95% ethanol
- 500 ml of phosphoric acid

This is made up to 1 litre using distilled water, filtered and stored at 4°C.

Working solution:

The Bradford stock is diluted in 1:5 ratio using distilled water, filtered and used for protein quantification.

Bradford method:

BSA (1 mg/ml) is diluted in a 1:4 ratio using distilled water. A protein standard with varying protein concentrations is prepared as follows:

BSA (μL)	BSA concentration (μg)	Volume of distilled water (μL)
0	Blank	100
10	2	80
20	4	60
40	6	40
60	8	20
80	12	10
100	14	0
5	Unknown protein sample	95

To all these protein and BSA standards, 900 μL of Bradford working solution is added and vortexed gently. Samples were allowed to stand for ~ 5 minutes. The absorbancies of each

sample was read using a spectrophotometer at 595 nm. (If the protein absorbancies fall outside the protein standard, the proteins must be diluted with RIPA buffer and reading taken again). The absorbancies were then plotted to construct a linear plot for the standards. Thereafter the amounts of protein in unknown samples were quantified in relation to the linear standard plot.

Sample preparation (tissue lysates) Western blotting- SDS-PAGE:

Set heating block temperature to 99 °C and keep samples on ice, and allow thawing. A stock solution of sample buffer containing 850 µL sample buffer and 150 µL mercaptoethanol is prepared. A volume of sample buffer equivalent to 1/3 final volume of the sample must be added (under the fume hood). The appropriate amounts of proteins are added to each tube calculated previously. Tiny holes are punched on the lids of microfuge tubes (containing the prepared sample) and placed in boiling water for about 5 minutes. The tubes are then briefly spun in a table top centrifuge. Samples can now be used for Western blot analysis or alternatively be stored at -80°C for later use.

SDS = denature proteins, constant anionic charge-to-mass ration

Glycerol = give sample a higher density than buffer to “sink” to the bottom of the well

Mercaptoethanol = reduce disulfide bonds present in protein sample

SDS-PAGE -Western blotting

Insert commercially available 10-well precast Mini-PROTEAN® TGX Stain-Free™ polyacrylamide gels stand and place them in the U-shaped adaptor cassette with the small plates facing inward. Retrieve prepared samples from the -80 C freezer and allow it to thaw on ice. Once thawed, vortex each sample briefly before denaturing on the heating block for 5 minutes. Place the U-shaped adaptor into the loading system and push the latches closed, away from your body. Carefully pour running buffer into the middle compartment between the gel plates, allowing the buffer to flow over the wells. Add 10 µl of pre-stained weight marker (peqGOLD, PEQLAB Biotechnologie GMBH, Germany) into the first well on the left of each gel for orientation and electrophoretic determination of molecular weights of specific bands. Hereafter add 50 µg of protein samples into each well in the desired order using a micropipette and clean loading tips for every sample. Place the system into the outer running chamber; add the running buffer until ~1cm below the wells. Place the green lid with

electrical leads onto the cell system, making sure to attach the electrodes correctly i.e. red to red and black to black. Run gels were run for 60 minutes at 100 V (constant) and 400 mA (Mini Protean System, Bio-Rad, USA) for 10 minutes then the voltage was increased to 200 V with the same current for 60 minutes.

Electrotransfer of proteins:

Cut two chromatography filter papers and one 0.2 micron polyvinylidene fluoride (PVDF) membranes (Immunibilon, Millipore, USA). Soak filter papers in transfer buffer and for the membrane first soak in methanol for 15 seconds, thereafter wash with distilled water before soaking in transfer buffer. Place on filter paper onto the semi-dry apparatus (Bio-Rad, USA) and carefully place the PVDF membranes on top. Roll with a wet tube to remove any bubbles. Hereafter place gels onto membranes making sure to get rid of any bubbles with a wet tube. To complete the sandwich place filter paper on top of the gel, close the system and run at limit 0.5 A and 15 V for ~ 1 h.

Detection of proteins (Western blots):

Probing the membrane In order to prevent non-specific binding membranes was blocked in 5 % (weight/volume) fat-free milk in 0.1 % Tris Buffered Saline-Tween 20 (TBS-T) for 2 hours at room temperature. Membranes were then incubated with the respective primary antibody diluted in 5 % (w/v) fat-free milk in 0.1 % TBS-T (1: 1000), overnight at 4 °C. Hereafter on the following day, membranes were washed a further three times in TBS-T (3x5 minutes) before being incubated in the secondary anti-mouse/rabbit/sheep/goat HRP monoclonal antibody (1:4000) for 1 hour at room temperature with gentle agitation.

For detection of the antibodies, membranes were treated with 2 ml ECL LumiGLO Reserve™ chemiluminescent substrate kit (KPL, Inc., USA) as per manufacturer's instructions. It was then dried on tissue paper, placed between transparencies and was then developed in a dark room where bands were exposed to autoradiography film (Hyperfilm, Amersham Biosciences, UK). The film was then developed by placing it in developing solution until bands appeared followed by 15 seconds in a fixative. This film was then visualised and quantified by densitometry using the UNSCAN-IT© densitometry software (Silk Scientific Corporation, Utah, USA). All bands were expressed as optical density readings relative to a control present on the same blot.

Appendix 7

Buffers used during Western blotting

10 x TBS (STOCK – store in fridge)

- 48.4 g Tris
- 160 g NaCl

Dissolve in 500 ml distilled water, set pH to 7.6 with concentrated HCl and then make up to 2 L.

10X running buffer (STOCK – store in fridge)

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

Dissolve in 2 liter distilled water.

Transfer buffer (ready to use)

In a 1L bottle add :

- 100 ml Biorad 10X transfer buffer
- 200 ml methanol
- 700 ml dH₂O