THE EFFECT OF

CREATINE SUPPLEMENTATION

ON MYOCARDIAL METABOLISM AND

FUNCTION IN SEDENTARY AND EXERCISED

RATS

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Prof B Huisamen

Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Background: There has been a dramatic increase in the use of dietary creatine supplementation among sports men and women, and by clinicians as a therapeutic agent in muscular and neurological diseases. The effects of creatine have been studied extensively in skeletal muscle, but knowledge of its myocardial effects is limited.

Objectives: To investigate the effects of dietary creatine supplementation with and without exercise on 1) basal cardiac function, 2) susceptibility to ischaemia/reperfusion injury and 3) myocardial protein expression and phosphorylation and 4) mitochondrial oxidative function.

Methods: Male Wistar rats were randomly divided into control or creatine supplemented groups. Half of each group was exercise trained by swimming for a period of 8 weeks, 5 days per week. At the end of the 8 weeks the open field test was performed and blood corticosterone levels were measured by RIA to determine whether the swim training protocol had any effects on stress levels of the rats. Afterwards hearts were excised and either freeze-clamped for biochemical and molecular analysis or perfused on the isolated heart perfusion system to assess function and tolerance to ischaemia and reperfusion. Five series of experiments were performed: (i) Mechanical function was documented before and after 20 minutes global ischaemia using the work heart model, (ii) A H_2O filled balloon connected to a pressure transducer was inserted into the left ventricle to measure LVDP and ischaemic contracture in the Langendorff model, (iii) The left coronary artery was ligated for 35 minutes and infarct size determined after 30 minutes of reperfusion by conventional TTC staining methods. (iv) Mitochondrial oxidative capacity was quantified. (v) High pressure liquid chromatography (HPLC) and Western Blot analysis were performed on blood and heart tissue for determination of high energy phosphates and protein expression and phosphorylation.

Results: Neither the behavioural studies nor the corticosterone levels showed any evidence of stress in the groups investigated. Hearts from creatine supplemented sedentary (33.5 ± 4.5%), creatine supplemented exercised rats (18.22 ± 6.2%) as well as control exercised rats (26.1 ± 5.9%) had poorer aortic output recoveries than the sedentary control group (55.9 ± 4.35% $p < 0.01$) and there was also greater ischaemic contracture in the creatine supplemented exercised group compared to the sedentary control group (10.4 ± 4.23 mmHg vs 31.63 ± 4.74 mmHg). There were no differences in either infarct size or in mitochondrial oxygen consumption between the groups. HPLC analysis revealed elevated phosphocreatine content (44.51 ±14.65 vs 8.19 ±4.93 nmol/gram wet weight, $p < 0.05$) as well as elevated ATP levels (781.1 ±58.82 vs 482.1 ±75.86 nmol/gram wet weight, $p<0.05$) in blood from creatine supplemented vs control sedentary rats. These high energy phosphate elevations were not evident in heart tissue and creatine tranporter expression was not altered by creatine supplementation. GLUT4 and phosphorylated AMPK and PKB/Akt were all significantly higher in the creatine supplemented exercised hearts compared to the control sedentary hearts.

Conclusion: This study suggests that creatine supplementation has no effects on basal cardiac function but reduces myocardial tolerance to ischaemia in hearts from exercise trained animals by increasing the ischaemic contracture and decreasing reperfusion aortic output. Exercise training alone also significantly decreased aortic output recovery. However, the exact mechanisms for these adverse myocardial effects are unknown and need further investigation.

OPSOMMING

Agtergrond: Die gebruik van kreatien as dieetaanvulling het in die afgelope aantal jaar dramaties toegeneem onder sportlui, sowel as mediese praktisyns wat dit as ‘n terapeutiese middel vir die behandeling van spier- en neurologiese siektes aanwend. Die effekte van kreatien op skeletspier is reeds deeglik ondersoek, maar inligting aangaande die miokardiale effekte van die preperaat is beperk.

Doelwitte: Om die effekte van kreatien dieetaanvulling met of sonder oefening ten opsigte van die volgende aspekte te ondersoek: 1) basislyn miokardiale funksie, 2) vatbaarheid vir iskemie/herperfusie besering, 3) proteïenuitdrukking en -fosforilering in die miokardium en 4) mitochondriale oksidatiewe funksie.

Metodes: Manlike Wistar rotte is ewekansig in kontrole of kreatien aanvullings groepe verdeel. Helfte van elke groep is aan oefening in die vorm van swemsessies, vir ‘n periode van 8 weke, 5 dae per week blootgestel. Gedrags- en biochemiese toetse is aangewend om die moontlike effek van die swemprotokol op die rotte se stres vlakke te bepaal. In hierdie verband is die oop area toets gebruik, asook bloed kortikosteroon vlakke gemeet deur radioaktiewe immuunessais. Harte is daarna uit die rotte gedissekteer en gevriesklamp vir biochemiese en molekulêre analise, of geperfuseer op die geïsoleerde werkhart perfusiesisteem om sodoende funksie en weerstand teen iskemie en herperfusie beskadeging te bepaal. Vyf eksperimentele reekse is uitgevoer: (i) Meganiese funksie is noteer voor en na 20 minute globale isgemie in die werkhart model; (ii) ‘n Water gevulde plastiek ballon, gekoppel aan ‘n druk omsetter, is in die linker ventrikel geplaas om sodoende linker ventrikulêre ontwikkelde druk (LVDP), asook iskemiese kontraktuur te meet; (iii) Linker koronêre arterie afbinding is vir ‘n periode van 35 minute toegepas en die infarktgrootte bepaal na 30 minute herperfusie deur gebruik te maak van standaard kleuringsmetodes; (iv) Mitochondriale oksidatiewe

kapasiteit is gemeet; (v) Hoë druk vloeistof chromatografie (HPLC) en Western Blot analises is uitgevoer op bloed en hartweefsel vir die bepaling van hoë energie fosfate (HEFe), sowel as proteïenuitdrukking en -fosforilering.

Resultate: Beide gedragsstudies en kortikosteroonvlakke het geen teken van stres in die betrokke groepe getoon nie. Die groep blootgestel aan kreatienaanvulling en oefening se harte het na iskemie funksioneel swakker herstel as harte van die onaktiewe kontrole groep (18.22±6.2% vs 55.9±4.35%; $p<0.01$), asook ‘n groter ikgemiese kontraktuur in vergelyking met die onaktiewe kontrole groep ontwikkel (31.63±4.74 mmHg vs 10.4±4.23 mmHg). Daar was geen verskille in infarktgrootte of mitochondriale suurstofverbruik tussen die verskillende groepe waargeneem nie. HPLC analise het verhoogde fosfokreatien (44.51±14.65 vs 8.19±4.93 nmol/gram nat gewig, $p<0.05$) en adenosientrifosfaat (ATP) bloedvlakke (781.1±58.82 vs 482.1±75.86 nmol/gram nat gewig, $p<0.05$) in kreatien aanvullings vergelyk met die kontrole groepe getoon. Daar was egter geen meetbare veranderings in HEF vlakke in hartweefsel nie. Gepaardgaande hiermee het kreatienaanvulling geen effek gehad op die uitdrukking va die kreatien transporter nie. In vergelyking met onaktiewe kontrole harte was GLUT4, en fosforileerde AMPK en PKB/ Akt beduidend hoër in harte van geoefende rotte met kreatienaangevulling.

Gevolgtrekking: Hierdie data dui daarop dat kreatienaanvulling geen effek op basislyn miokardiale funksie het nie. Kreatienaanvulling het egter die miokardium se weerstand teen iskemiese skade verlaag in harte van rotte blootgestel aan oefening: iskemiese kontraktuur is verhoog en aorta-uitset tydens herperfusie is verlaag. Die presiese meganismes hierby betrokke is egter onbekend en vereis dus verdere studie.

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I love you.

Mom – I miss you every moment. I know you are looking down on me with pride (and relief!) and I wish you were here to share this achievement with me.

I dedicate this to you.

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ABBREVIATIONS

AAR	area at risk
ACC	acetyl-CoA carboxylase
ACS	acetyl CoA synthase
ADP	adenosine diphosphate
AGAT	Glycine Amidinotransferase
alb	albumin
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	one way analysis of variance
AO	aortic output
ATP	adenosine triphosphate
BMI	body mass index
BSA	bovine serum albumin
ºC	degrees celcius
C	cytochrome *c*
c	centi
Ca	calcium
CAD	coronary artery disease
CAT	catalase
C Ex	control exercised
CF	coronary flow
CK	creatine kinase
CO_2	Carbon dioxide
C Sed	control sedentary
CPT	carnitine-palmitoyl transferase
Cr	creatine
CreaT	creatine transporter
Cr Ex	creatine supplemented exercised
Cr Sed	creatine supplemented sedentary
DNA	deoxyribonucleic acid
DP	aortic diastolic pressure
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase

FABP	fatty acid binding protein
FAD	flavin adenine dinucleotide
$FADH_2$	reduced flavin adenine dinucleotide
FAT/ CD36	Fatty acid translocase
FFA	free fatty acids
g	grams
g	gravitational constant
G-6-P	glucose-6-phosphate
GAA	guanidinoacetate
GAMT	Guanidinoacetate methyltransferase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GLUT	glucose transporter
GLUT4	glucose transporter 4
GPx	glutathione peroxidase
GTP	guanidine triphsophate
H	hydrogen
H_2O	water
HDL	high density lipoprotein
HEP	high energy phosphates
HPLC	High Pressure Liquid Chromatography
HR	heart rate
HSP	heat shock protein
IF	infarct size
iNOS	inducible nitric oxide synthase
JNK	c-Jun NH_2-terminal kinase
K	potassium
KOH-KCl	potassium hydroxide potassium chloride
l	litres
LDL	low density lipoprotein
LVEDV	left ventricular end diastolic volumes
LVDP	left ventricular developed pressure
m	milli
M	molar
MAPK	mitogen activated protein kinase
MCD	malonyl-CoA decarboxylase

MI	myocardial infarction
MiCK	mitochondrial CK isoform
min	minutes
MMCK	cytosolic myofibrillar creatine kinase
mmHg	millimeters mercury
MnSOD	manganese SOD
MPTP	mitochondrial permeability transition pore
mRNA	messenger RNA
Na	sodium
NAD^+	Nicotinamide adenine dinucleotide
NADH	reduced Nicotinamide adenine dinucleotide
NCE	Na^+/Ca^{2+} exchanger
NHE	Na^+/H^+ exchanger
NO	nitric oxide
O_2^-	Super oxide free radicals
$OONO^-$	peroxinitrite
p38-MAPK	mitogen activated protein kinase p38
P_i	phosphate
PP_i	diphosphate
PCA	perchloric acid
PCr	phosphocreatine
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PFK	Phosphofructokinase
PKB/ Akt	protein kinase B
RIA	radioactive immunoassay
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RPP	Rate pressure product
RyR	ryanodine receptor
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SERCA	sarcoplasmic reticulum Ca-ATPase
SOD	superoxide dismutase

SP	aortic systolic pressure
SR	sarcoplasmic reticulum
TBS	tris buffered saline
TCA	tricarboxylic acid
TTC	triphenyltetrazolium chloride
U	Ubiquinone
µ	micro
UCP	uncoupling proteins
UDP	uridine diphosphate
UTP	uridine triphosphate
UV	ultra violet
v/v	volume to volume

CHAPTER 1

INTRODUCTION

1.1 ATP: The energy currency of the cell

Each cell needs energy to survive. This energy is primarily in the form of adenosine triphosphate (ATP). Contracting myocytes in the heart require an enormous amount of energy to maintain uninterrupted contractions. ATP is considered the “molecular unit of currency of the cell” [Knowles 1980]. The heart also requires ATP for proper membrane functioning, ion homeostasis and contraction and relaxation [Dzeja et al 2000]. ATP transports chemical energy within cells in the form of phosphate groups which are used for cellular metabolism, and the greater the activity of the heart, the more energy it requires.

ATP is produced as an energy source during breakdown of sugars and fats (glycolysis and β-oxidation) and consumed by many enzymes and a multitude of cellular processes including biosynthetic reactions, and cell division [Campbell et al 2006]. In signal transduction pathways, ATP is used as a substrate by kinases that phosphorylate proteins and lipids, as well as by adenylate cyclase, which uses ATP to produce the second messenger molecule cyclic AMP. Apart from its roles in energy metabolism and signaling, ATP is also incorporated into nucleic acids by polymerases in the processes of DNA replication and transcription [Formosa 2003].

1.1.1 Availability of energy

The ATP to AMP ratio is used by the cell to monitor how much energy is available and controls the metabolic pathways that produce and consume ATP [Hardie and Hawley 2001]. ATP synthase is the enzyme which catalyses the reversable reaction of water and ATP to produce ADP and phosphate, or AMP and diphosphate, as shown below:

$$ATP + H_2O \leftarrow \text{ATP synthase} \rightarrow ADP + P_i$$

$$ATP + H_2O \leftarrow \text{ATP synthase} \rightarrow AMP + PP_i$$

When ATP levels are low the enzyme catalyses the recycling of ATP from its precursors, ADP or AMP, and phosphate groups. ATP can also be produced during oxidative phosphorylation in the mitochondria.

Creatine phosphate occurs in muscle and brain tissue and serves as an energy store. It can "donate" a phosphate group to ADP to reform ATP anaerobically when needed e.g. during exercise. The reversible reaction is catalyzed by creatine kinase (CK).

$$PCr + ADP \underset{(CK)}{\Leftrightarrow} Cr + ATP$$

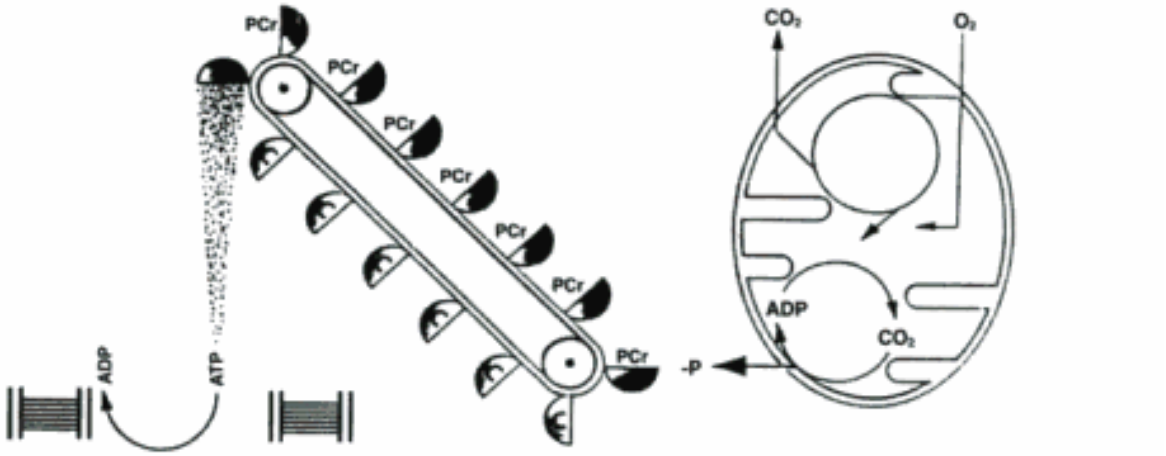
Transfer of energy via this mechanism is called the *phosphocreatine shuttle* [Bessman and Geiger 1981]. This reaction takes place in both the mitochondrion and in the cytosol. This reaction also ensures that the ADP / ATP ratio is controlled – to favour higher ATP and lower ADP concentrations. Thus PCr acts as an energy buffer in the cell [Chung et al 1998].

1.1.2. Energy imbalance

The heart is an aerobic or oxygen consuming organ and therefore relies almost exclusively on the oxidation of substrates for creation of energy. It can only go without oxygen for a short while and still have enough energy to function normally. Thus, in a steady state, determination of the rate of myocardial oxygen consumption provides an accurate measure of its total metabolism. When the supply cannot meet the demand, as occurs when the blood supply is cut off during a myocardial infarction, an energy imbalance ensues because of myocardial ischaemia. Reperfusion is when the blood supply is reinstated, and the energy balance is restored. The hazards and consequences of ischaemia and reperfusion will be described in Chapter 2.

1.2 Limitless energy: the principle of creatine supplementation

The bidirectional reactions highlighted above prompted the use of creatine supplementation that has been predominant in the last decade, particularly in the sports sector. Phosphocreatine is particularly important in tissues that are subjected to fluctuations in energy demand e.g. muscle, brain and nerve tissue. With the high delivery of phosphocreatine to the muscle after supplementation, driving the constant restoration of ATP supply, energy supply is expected to be indefatigable. See Figure 1.1.



<u>Figure 1.1</u>: A simplified schematic of the phosphocreatine shuttle. The more phosphocreatine that is added to the shuttle with supplementation, the greater the store of energy to meet the demands of the cell. (Reproduced from Williams 1999)

CHAPTER 2

LITERATURE REVIEW

2.1 Myocardial metabolism

The energy that the heart requires for the maintenance of normal contraction is supplied by ATP. This high-energy phosphate is primarily produced in the heart by the metabolism of carbohydrates and fatty acids [Lopashuk and Stanley 1997]. Metabolism of these substrates alternates between carbohydrate use as fuel in the fed state, of which glucose and lactate are the major contributors, and fatty acid use as fuel in the fasting state [Most et al 1969, Carlson et al 1972, Drake et al 1980]. This is due to the fact that in the fed state there are more circulating carbohydrates available in the blood, also leading to insulin secretion [Levine and Haft 1970], and in the fasting state there are more free fatty acids (FFA) available. In the latter state, fatty acid oxidation dominates, and glucose oxidation is inhibited [Opie 1991]. The glucose that is taken up by cells is converted to glycogen and stored instead of undergoing glycolysis (Randle et al 1963). Conversely, in the fed-state, when glucose levels in the blood are high, the uptake of fatty acids decreases while glucose uptake and glycolysis increases [Opie 1998].

2.2 Which fuel to use?

2.2.1 Randle's principle of fatty acid and glucose metabolism

The variation in the roles of glucose and fatty acid between the fasting and the fed states forms the basis of the glucose-fatty acid cycle first described by Randle et al in 1962. The basic trigger for the switch in the cycle is the cyclic production and release of free fatty acids (FFA) by the adipose tissue. In the fasting state, adipose tissue is broken down to release FFA which inhibits the metabolism of glucose by the heart. In the fed state the abundance of glucose and insulin inhibits this release of FFA and therefore glucose becomes the major fuel. See Figure 2.

2.3 When the oxygen runs out.

2.3.1 Pasteur effect

Cardiomyocytes can produce energy using two different metabolic pathways. While the oxygen concentration is low, the product of glycolysis, (pyruvate), is turned into lactate and carbon dioxide, and the energy production efficiency is low (2 moles of ATP per mole of glucose). If the myocardial oxygen concentration increases, pyruvate is converted to acetyl CoA that can be used in the Krebs Cycle, which increases the efficiency and ATP yield to 16 moles of ATP per 1 moles of glucose used.

Under low oxygen concentrations (anaerobic conditions), the rate of glucose metabolism is faster, as AMP activated protein kinase (AMPK) is activated (see Chapter 2.4.3), but the amount of ATP produced is less. When exposed to aerobic conditions, the rate of glycolysis slows, because the increase in ATP production acts as an allosteric inhibitor for the pathway, yet more ATP is produced. So, with respect to ATP

production, it is advantageous for cells to utilise the Krebs cycle in the presence of oxygen, as more ATP is produced per glucose molecule [Krebs 1972, Muscari et al 2004, Meisenberg and Simmons 1998].

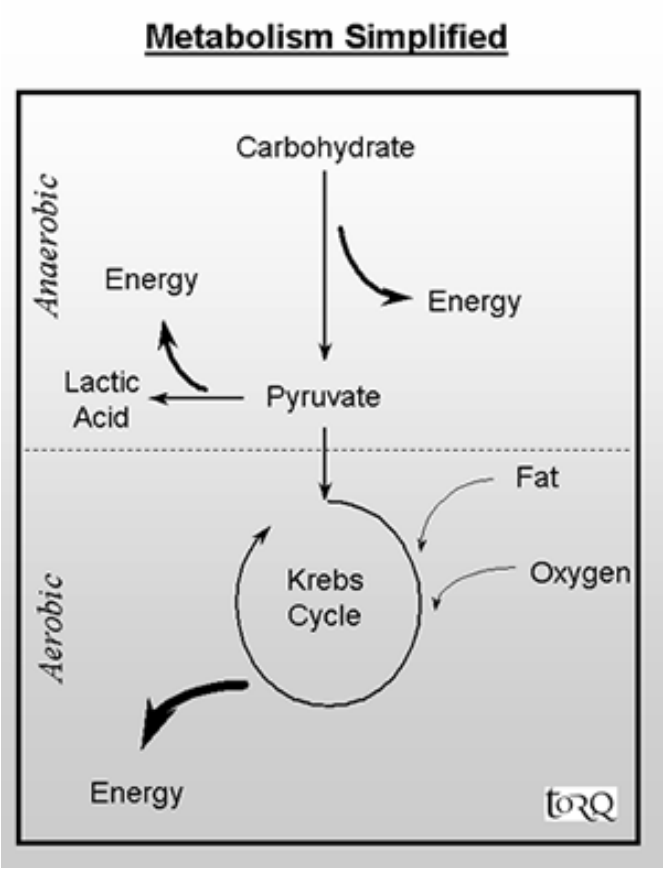


Figure 2.1: Simplified diagram to illustrate the basic metabolism of carbohydrates such as glucose, and fatty acids both aerobically and anaerobically. Carbohydrates are converted to pyruvate via glycolysis with the release of energy (ATP) and carbon dioxide. Pyruvate either enters the Krebs cycle where it is metabolized in the presence of oxygen, or is converted to lactic acid in the absence of oxygen. Diagram from www.google.com.

2.4 Glucose uptake and metabolism

2.4.1 Glucose uptake

Glucose uptake into muscle cells is accomplished through a series of steps from the delivery of blood to the interstitial space to the trans-membrane transport of glucose into the cell [Richter 2001]. This uptake is regulated by a chain of signaling pathways. The cell cannot absorb glucose by simple diffusion. Since the cell membrane is hydrophobic and glucose is hydrophilic, it uses a special carrier protein, the glucose transporter molecule, for this purpose [Lienhard et al 1992]. This carrier requires no energy (ATP) for the transport of glucose since the extracellular glucose concentration is so much greater than the intracellular concentration and the absorption takes place down the concentration gradient (Opie 1991).

2.4.2 Glucose transporters (GLUTs)

The uptake of glucose from the interstitium across the sarcolemma into the myocyte is regulated and performed by the glucose transporters or GLUTs [Lopaschuk and Stanley 1997]. The specific glucose transporters in the heart all belong to the GLUT family and are passive carriers which are energy-independent systems. They can only transport their substrates down a concentration gradient which conserves energy while gaining fuel for the cell. GLUTs are transmembrane proteins containing about 500 amino acid residues and 12 membrane-spanning β-helices [Meuckler 1994].

The glucose transporter that is predominantly expressed in cardiomyocytes is the insulin-sensitive GLUT4 isotype which is also expressed in adipose tissue and skeletal

muscle. GLUT4 is largely confined to an intracellular vesicle storage site in the basal, non-stimulated state [Meuckler 1994, Holman and Kasuga 1997]. It becomes recruited to the cell surface under the influence of insulin [Fischer et al 1997] or other stimuli such as muscle contraction, during exercise [Roy and Marette 1996, Tomàs et al 2001] and hypoxia or anoxia (Sun et al 1994). GLUT4 vesicles respond to insulin in a marked and dramatic way, increasing GLUT4 translocation to the membrane up to nine times that of basal translocation rates [Holloszy 2003]. As soon as blood insulin and glucose levels decrease, the transporter recruitment is reversed and the GLUTs are internalized via endocytosis [Lienhard et al 1992].

The GLUT1-transporter, which is present in most tissues and is also a characteristic feature of fetal tissues (xxi), is also present in cardiomyocytes although it is about 5 times less abundant than GLUT4 [Meuckler 1994]. It is thought to be a specialized “house-keeping” protein that provides the steady basal flow of glucose into cells for homeostasis in their inactive state.

2.4.3 Glucose metabolism

Glucose metabolism comprises two main components, glycolysis and glucose oxidation. (See fig 2)

Glycolysis

Glycolysis ('lysis (or breaking down) of glucose') is the first part of the glucose metabolic pathway and produces ATP from either exogenous glucose or from glycogen stored in the muscle without requiring oxygen [Depré et al 1998]. It is a biochemical process that produces lactate under anaerobic conditions [Opie 1991]. During normal oxidative metabolism, glycolysis yields pyruvate, which is then broken down aerobically in the Krebs cycle (under conditions of adequate mitochondrial capacity). This process is also called aerobic glycolysis. Thus ATP is produced not only during aerobic conditions, but anaerobically too [Opie 1991].

Intracellular glucose is rapidly converted to glucose-6-phosphate by hexokinase, and glycolysis (or more specifically PFK1, see below) then converts this into a compound containing two phosphate groups, fructose-1,6-bisphosphate. After this, each 6-carbon hexose phosphate is converted to two three-carbon triose phosphates, eventually forming pyruvate. In the first stage of glycolysis, two molecules of ATP are used to convert the glucose to two triose phosphate molecules. In the second stage four molecules of ATP are made, independent of oxygen availability, for each glucose 6-phosphate converted to pyruvate. This results in a net production of 2 molecules of ATP per molecule of glucose metabolized [Opie 1991].

Phosphofructokinase 1 (PFK1) is a key enzyme in glycolysis. When its activity increases, fructose-6-phosphate is converted to fructose-1,6-bisphosphate at an increased rate. Since the enzyme which catalyses the reverse reaction (glucose-6-phosphatase) is not present in the heart, this reaction, which uses ATP, is irreversible [Opie 1998]. Thus PFK1 serves as a one directional valve to regulate the rate of

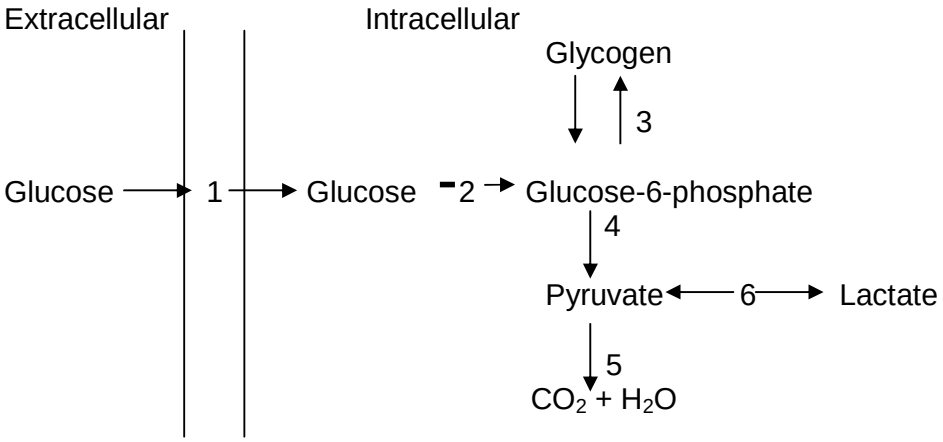
glycolysis. Increased PFK1 activity causes decreased glucose-6-phosphate levels in the cell. PFK1 is allosterically inhibited by ATP and citrate (from the citric acid cycle) and its product, fructose 1,6-bisphosphate. PFK1 is allosterically activated by a high concentration of AMP, but the most potent activator is fructose 2,6-bisphosphate, which is also produced from fructose-6-phosphate by PFK2. Therefore when PFK1 activity is increased the inhibition of hexokinase which is normally caused by glucose-6-phosphate is decreased, and more glucose can be phosphorylated. In contrast, the activity of PFK1 can be inhibited when the oxidation of alternate fuels like fatty acid or lactate produces citrate, and the opposite then occurs. This is therefore a coordinated intracellular control mechanism which regulates the rate of glycolysis. [Opie 1991, Opie 1998]

Anaerobic glycolysis is increased during hypoxia and ischaemia and is controlled by the activity of enzymes, AMP-activated protein kinase (AMPK), PFK and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [Marsin et al, 2000]. The PFK reaction is sensitive to the energy status of the myocardial cells, and is therefore ideally suited for metabolic control. As ATP levels fall, and those of ADP, AMP and Pi rise, the activity of this enzyme is enhanced resulting in increased anaerobic glycolysis and ATP and lactate production [Regen et al 1964]. There is also decreased inhibition of PFK1 by citrate which means glycolysis is further increased.

When glucose is the substrate of glycolysis, the entire glycolytic pathway uses 2 ATP molecules and produces 4 ATP molecules, so the net production is 2 molecules of ATP. When glycogen is the source, 3 ATP molecules are produced [Opie 1991].

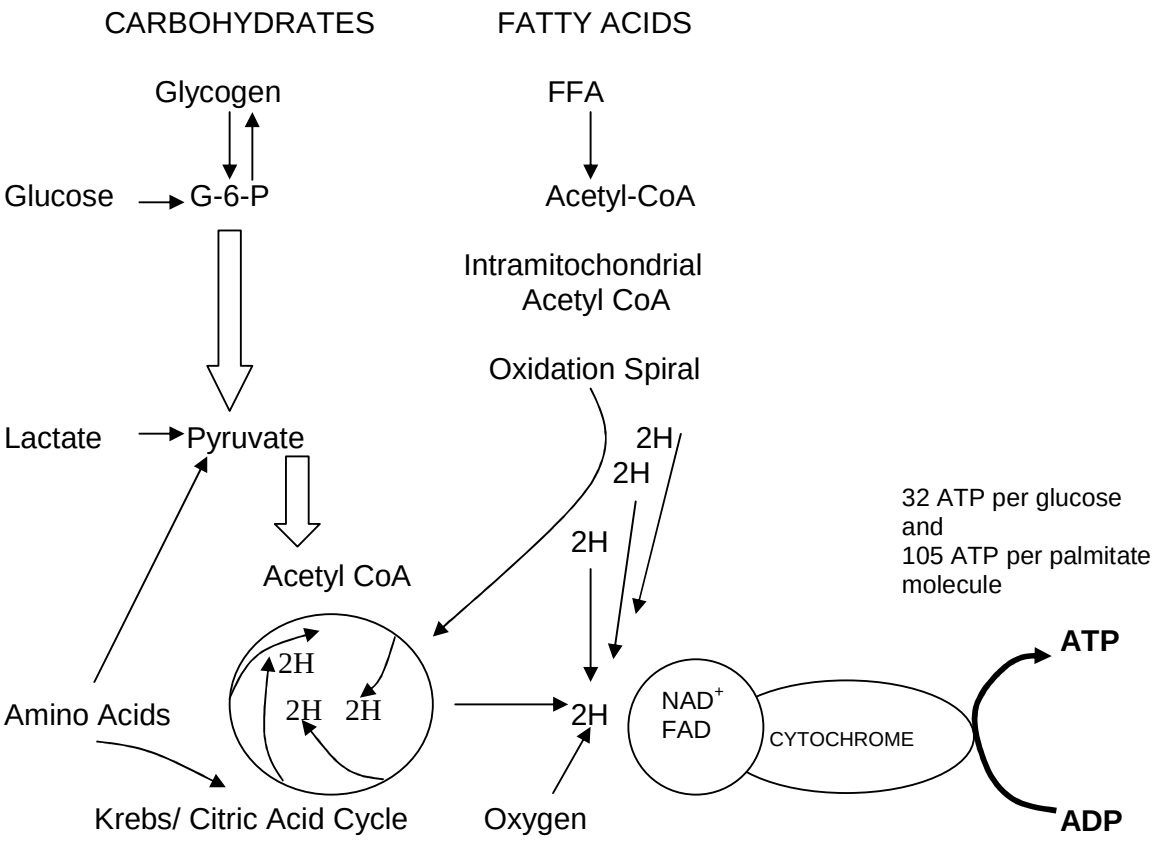
Glucose oxidation

The other component of glucose metabolism is glucose oxidation which involves the pyruvate derived from glycolysis being taken up by the mitochondria and its further metabolism in the citric acid/ Krebs cycle. Glucose is metabolized to pyruvate in the cytosol while glucose oxidation occurs entirely in the mitochondria (see Fig 3). The pyruvate dehydrogenase (PDH) complex is a large complex consisting of proteins spanning the mitochondrial membrane and is a key regulator of glucose entry into the Krebs cycle [Grill and Qvigstad 2000]. Pyruvate is irreversibly converted to acetyl-CoA, NADH and CO_2 by the (PDH) enzyme, which is active when the concentration of its substrates is high and relatively inactive when its substrates are at a low concentration. PDH is inactivated when it is phosphorylated by PDH kinases (PDK) and active when it is dephosphorylated by phosphatases. Pyruvate is also formed from lactate in the healthy human heart [Lopaschuk and Stanley 1997]. PDH links and regulates the flow of energy in cells by determining when pyruvate should be used for oxidative phosphorylation versus "neutralized" to lactic acid to allow continued glycolysis.

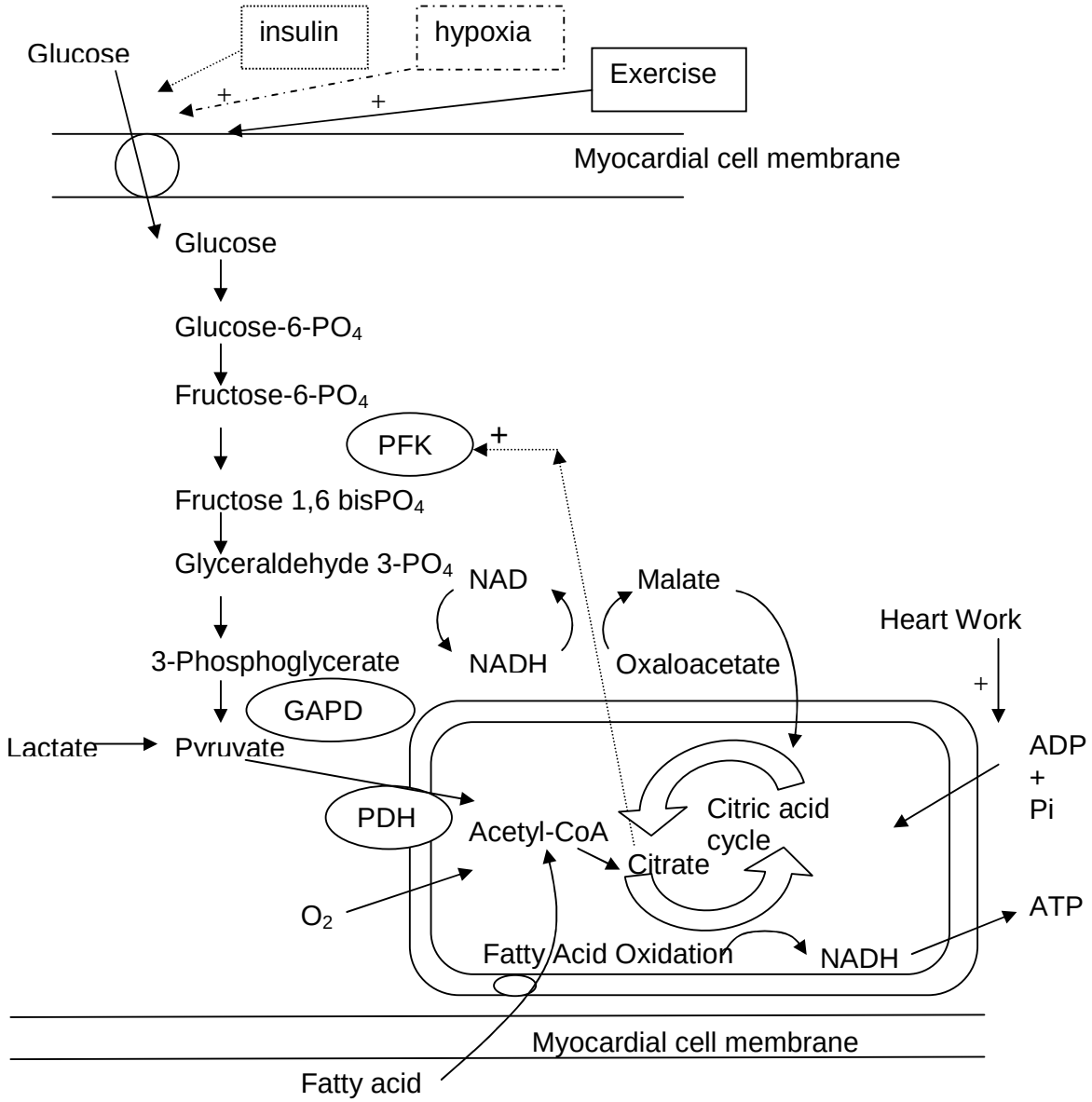


<u>Figure 2.2:</u> Glucose metabolism in muscle

1. **Transmembrane transport of glucose**
2. **Phosphorylation of glucose**
3. **The glycogen cycle**
4. **Glycolysis**
5. **Pyruvate oxidation**
6. **Lactate dehydrogenase reaction**



<u>Figure 2.3:</u> The major fuels of the heart are carbohydrates (glucose and lactate) and non-esterified fatty acids (free fatty acids (FFA)). All fuels are ultimately broken down to acetyl-CoA, which produces hydrogen atoms (H^+) by various dehydrogenase enzymes to produce $NADH_2$ (NADH + H^+), which enters the respiratory chain to produce ATP. Fatty acids also produce $FADH_2$ from the oxidation spiral which enters the cytochrome chain and produces ATP. G-6-P (glucose-6-phosphate). [Adapted from Depré et al 1998, and Opie 2004].



<u>Figure 2.4:</u> Glucose and fatty acid oxidation with the rate limiting steps shown. Glucose transport is regulated by insulin and the energy state of the cell. In the well-oxygenated heart, glucose uptake and glycolysis can be accelerated by heart work and glucose, and partially inhibited by fatty acid oxidation. PFK phosphofructokinase, PDH pyruvate dehydrogenase. [Modified from Opie 1991]

Glycogen storage

During the fed state excess glucose is converted to and stored as glycogen. Glycogen is a form of glucose which can be readily mobilized when needed by being broken down to yield glucose molecules. It is stored in the liver and muscle where it is present in the cytosol in the form of granules.

Although glycogen is not as high in energy yield as fatty acids, it is an important fuel reserve for several reasons. The controlled breakdown of glycogen and release of glucose into circulation increases the amount of glucose that is available between meals. Hence, glycogen serves as a buffer to maintain blood-glucose levels during fasting. Glycogen's role in maintaining blood-glucose levels is especially important because glucose is virtually the only fuel used by the brain, except during prolonged starvation. In the liver, glycogen synthesis and degradation are regulated to maintain systemic blood-glucose levels as required to meet the needs of the organism as a whole. In contrast, in muscle, these processes are regulated to meet the energy needs of the muscle itself.

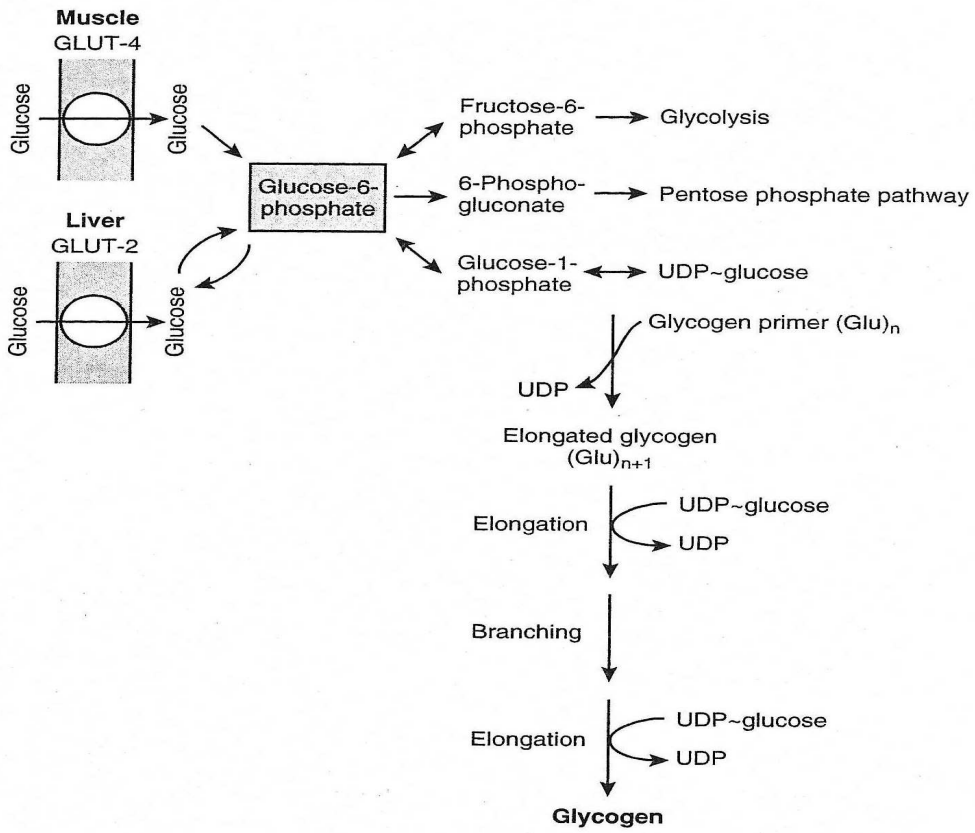
In addition, the glucose from glycogen is readily mobilized and is therefore a good source of energy for sudden, strenuous activity like exercise. Unlike fatty acids, the released glucose can provide energy in the absence of oxygen and can thus supply energy for anaerobic activity [Berg et al 2002].

Glycogen metabolism

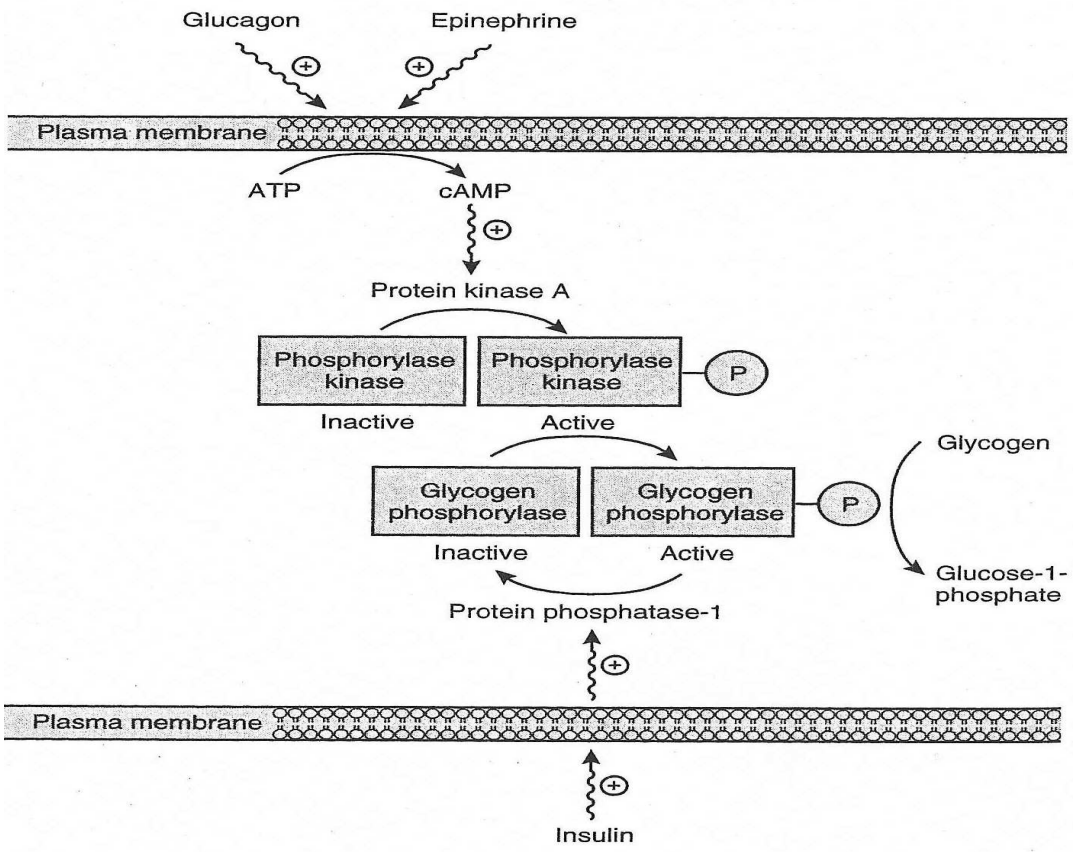
Glycogen synthesis requires an activated form of glucose, uridine diphosphate glucose (UDP-glucose), which is formed by the reaction of UTP (uridine triphosphate) and glucose 1-phosphate. UDP-glucose is added to the nonreducing end of glycogen molecules. As is the case for glycogen degradation, the glycogen molecule must be

remodeled for continued synthesis. Glycogenin initiates glycogen synthesis. It is an enzyme that catalyzes attachment of a glucose molecule to one of its own tyrosine residues thus starting the complex branching process of glycogen synthesis. (Montgomery et al 1990) See Figure 2.6.

Glycogen degradation and synthesis are relatively simple biochemical processes. Glycogen degradation consists of three steps (see figure 7): (1) the release of glucose 1-phosphate from glycogen catalysed by glycogen phosphorylase, (2) the remodeling of the glycogen substrate to permit further degradation, and (3) the conversion of glucose-1-phosphate to glucose-6-phosphate for further metabolism. The glucose-6-phosphate derived from the breakdown of glycogen can either be used as the initial substrate for glycolysis or it can be converted to free glucose for release into the bloodstream. This latter conversion takes place mainly in the liver and to a lesser extent in the intestines and kidneys.



<u>Figure 2.5:</u> Glycogen synthesis from uridine triphosphate (UTP) and glucose 1-phosphate. [Gee 2007] See text for more details.



<u>Figure 2.6:</u> Glycogen degradation via (1) release of glucose 1-phosphate from glycogen catalysed by glycogen phosphorylase, (2) the remodeling of the glycogen substrate to permit further degradation, and (3) the conversion of glucose 1-phosphate to glucose 6-phosphate for further metabolism. See text for more details. [Gee 2007]

2.5 Fatty acid uptake and metabolism

2.5.1 Fatty acid uptake

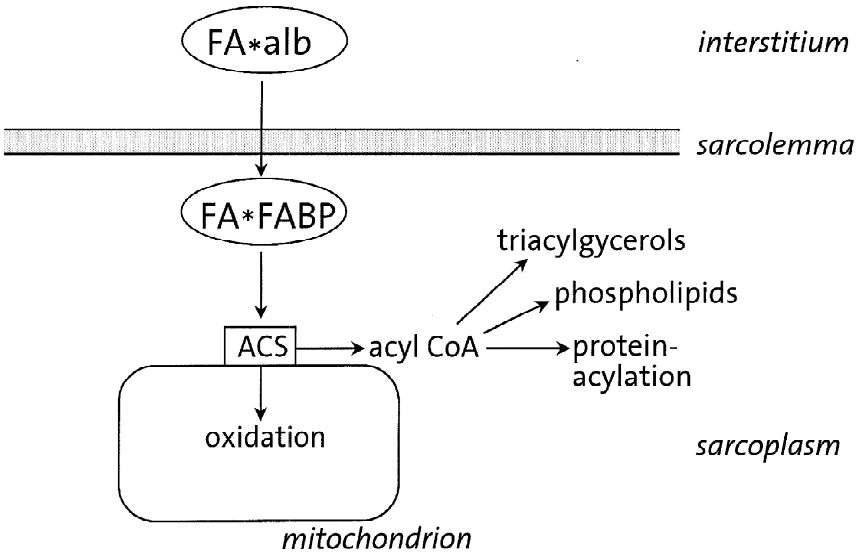
Fatty acids are presented to the sarcolemma of the cardiomyocyte bound to albumin. They can either enter the cell via passive diffusion or with the help of a variety of protein carriers. The albumin is not taken up into the cell with the FFA's and one proposal for a mechanism of cellular uptake is that the FFA-albumin complex binds to a specific high affinity sarcolemmal albumin receptor binding site before the FFA enters the sarcolemma via translocation [Stremmel 1989]. The higher the circulating FFA concentration the greater is the FFA uptake into the myocardium. Eventually feedback systems will limit the uptake i.e. increased tissue acyl-CoA. See Fig 8.

2.5.2 Fatty acid translocase FAT/CD36

Protein-mediated fatty acid uptake seems to be regulated by the translocation of fatty acid translocase/CD36 (FAT/CD36) from intracellular, presumably endosomal, stores to the sarcolemma. This translocation has been shown to be mediated through AMP-activated protein kinase (AMPK) signaling during contraction [Luiken et al 2003]. Insulin is another important hormone which is able to contribute to this process [Luiken et al 2002].

After entry into the cell via either mechanism, fatty acids bind the FABP (fatty acid binding protein) and are converted by acetyl CoA synthase (ACS) into fatty acyl-CoA at the mitochondrial outer membrane or the sarcoplasmic reticulum. In a carnitine mediated process the bulk of these fatty acid derivatives pass through the mitochondrial inner membrane where they are degraded in the β-oxidation pathway and citric acid

cycle. The rest is incorporated into the lipid pool of the cell (e.g. triacylglycerols or phospholipids). [Van der Vusse et al 2000]



<u>Figure 2.7:</u> A simplified diagram showing fatty acid uptake into the cardiomyocyte. FA (fatty acids), alb (albumin), FABP (fatty acid binding protein), ACS (acetyl-CoA synthase). [Gees 2007]

2.5.3 Fatty acid metabolism

Fatty acid degradation is the process by which fatty acids are broken down, resulting in release of energy. It includes three major steps:

- Fatty acid activation and transport into mitochondria,
- β-oxidation
- Electron transport chain

Fatty acids are transported across the outer mitochondrial membrane by carnitine-palmitoyl transferase I (CPT-I), and then couriered across the inner mitochondrial membrane by carnitine (De Palo et al 1981). Once inside the mitochondrial matrix, the enzyme CPT II catalyses the transfer of the acyl group from fatty acyl-carnitine to coenzyme A and produce acetyl-CoA. CPT-I is believed to be the rate limiting step in fatty acid oxidation [Lopaschuk and Stanley 1997].

β-oxidation then converts intramitochondrial long chain acyl-CoA to acetyl-CoA, and the fatty acid oxidation spiral then continuously removes acetyl-CoA from the carboxyl end of the chain, in the TCA (tricarboxylic acid) Cycle. The TCA (Citric Acid or Krebs) cycle is the major energy producing pathway in the body, and starts with the condensation of oxaloacetate to acetyl-CoA by citrate synthase to form citrate.

As acetyl-CoA is oxidized to CO_2, electrons are donated to the oxidation-reduction coenzymes, FAD and NAD^+. Three NADH, 1 $FADH_2$, and 1 GTP are produced in the Krebs Cycle. The NADH and $FADH_2$ generate ATP by donating electrons to O_2 in the process of oxidative phosphorylation. ATP is also produced from GTP (substrate-level phosphorylation). One turn of the cycle generates 12 ATP molecules. [Marks 1990, Martin et al 1983]

Regulation and control of fatty acid oxidation

Malonyl-CoA, which is produced by acetyl-CoA carboxylase (ACC), is a potent inhibitor of CPT-1 and acts at a site distinct from the catalytic site of CPT-1. ACC is a very important determinant of malonyl-CoA levels and fatty acid oxidation rates in the heart [Saddik et al 1993]. A key kinase responsible for the control of ACC activity is AMPK [Sakamoto et al 2000] (see Fig 9). Thus AMPK is an important regulator of fatty acid oxidation in the heart, since it phosphorylates and inactivates ACC, resulting in a decrease in malonyl-CoA production and an increase in fatty acid oxidation rates (Kudo et al 1995). It has been shown that the heart contains an active malonyl-CoA decarboxylase (MCD) that decarboxylates malonyl-CoA back to acetyl-CoA [Sakamoto et al 2000].

Any activated intracellular fatty acid not oxidized can either be stored as triglycerides or transformed to structural lipids and incorporated into the membrane.

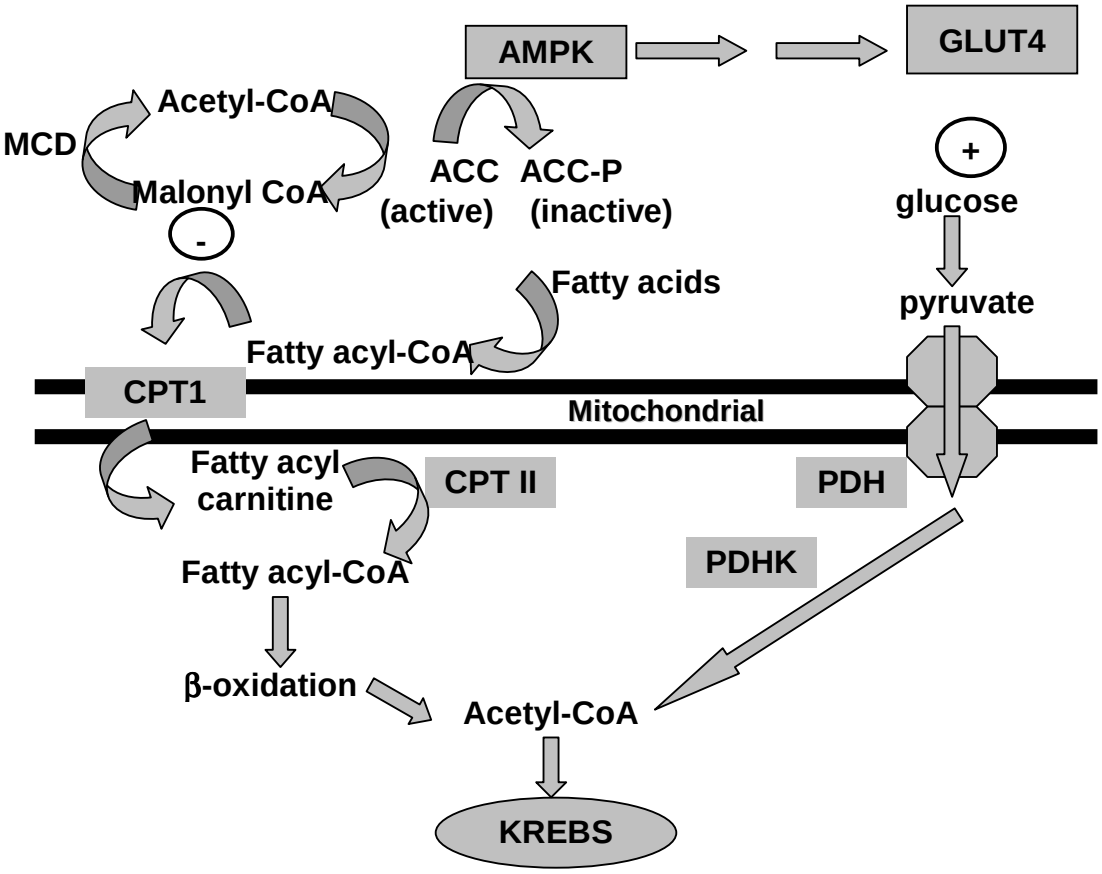


Figure 2.8: **A simplified diagram showing the role of ACC, AMPK, and MCD in the regulation of fatty acid and glucose oxidation in the heart. Fatty acids are converted to fatty acyl-CoA. These fatty acyl-CoA esters are then converted to fatty acyl carnitines and shuttled into the mitochondria via the carnitine translocase system. Once inside the mitochondria, the fatty acyl carnitines are converted back into fatty acyl-CoA esters and enter into the β-oxidation spiral to produce acetyl-CoA. In addition, exogenous glucose is transported into the cell via the cell glucose transporters, and can be converted to pyruvate via glycolysis. Pyruvate enters the mitochondria via the pyruvate carrier and is converted to acetyl-CoA by the pyruvate dehydrogenase complex. Fatty acid-derived or glucose-derived acetyl-CoA enters the Krebs cycle, which produces reduced equivalents that are used by the electron transport chain to produce ATP. (Adapted from Dyck & Lopaschuk, 2002).**

2.6 Mitochondrial energetics

The mitochondrion has been called the “powerhouse” of the cell as it is the place where most of the ATP used by the cell is produced. The Krebs cycle, β oxidation and oxidative phosphorylation are all biological processes which occur in the mitochondria and are essential to life. β oxidation has been briefly discussed above and the Krebs cycle and oxidative phosphorylation will be discussed below.

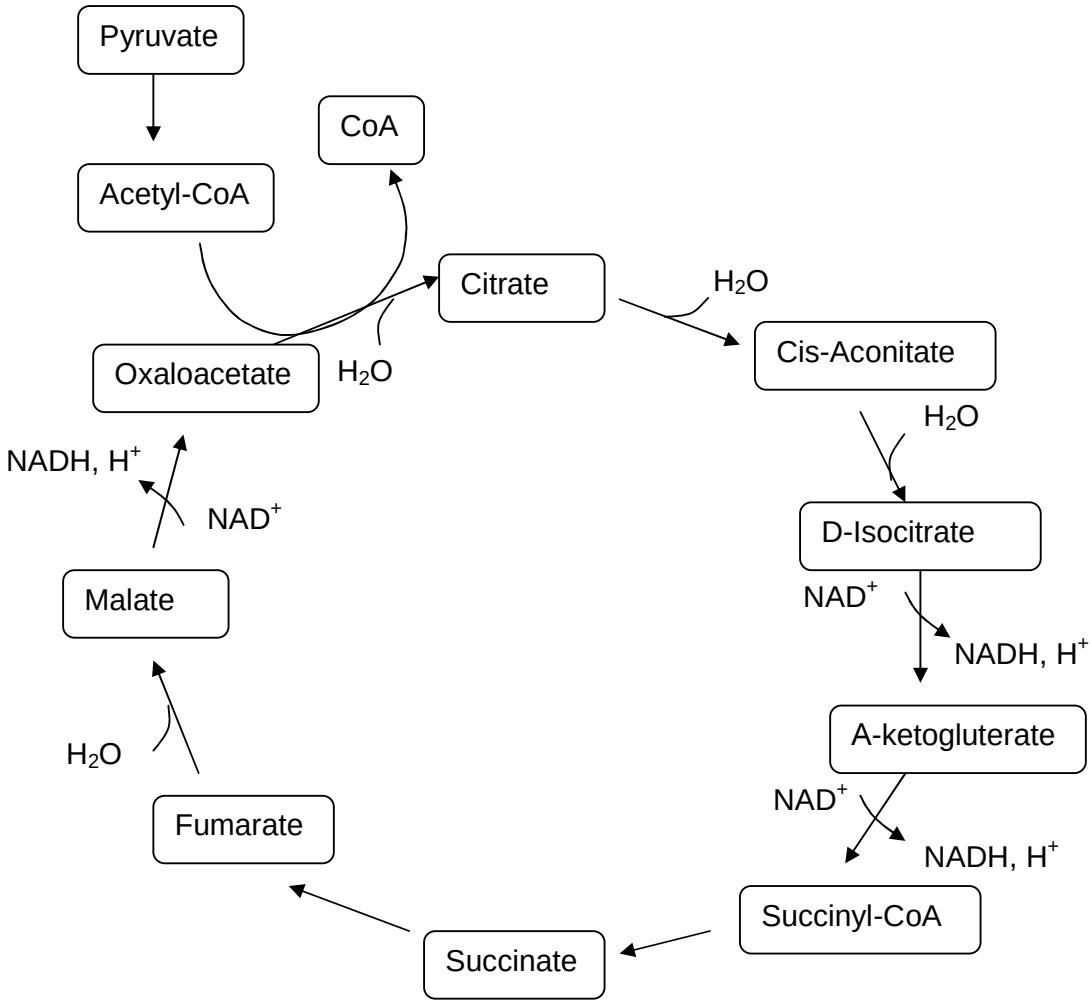
2.6.1 Krebs cycle

The Krebs, Citric Acid or tricarboxylic acid (TCA) cycle is a series of reactions which is a step in the metabolic pathway that uses oxygen in the conversion of fats, carbohydrates and proteins to carbon dioxide (CO_2), water and ATP. Glycolysis and β-oxidation occur before the Krebs cycle and oxidative phosphorylation occurs afterwards.

The cycle begins with acetyl-CoA transferring its acyl group to oxaloacetate to form the 6 carbon compound citrate. This citrate then goes through a series of reactions noted in the figure below, losing 2 carboxyl groups in the form of CO_2, and forming NADH or $FADH_2$ from NAD^+ or FAD^{2+} and electrons. These are energy carriers which then convey the electrons to the electron transport system in oxidative phosphorylation, where ATP is the end product. At the end of the Krebs cycle, oxaloacetate has been reformed and the cycle begins again.

The cycle is regulated by substrate availability and feedback mechanisms from its product NADH. Calcium is used as a regulator. It activates pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase [Denton et al 1975],

which increases the reaction rate of many of the steps in the cycle, and thus increases flow throughout the cycle. Citrate formed in the cycle feeds back and inhibits glycolysis at the level of PFK1.



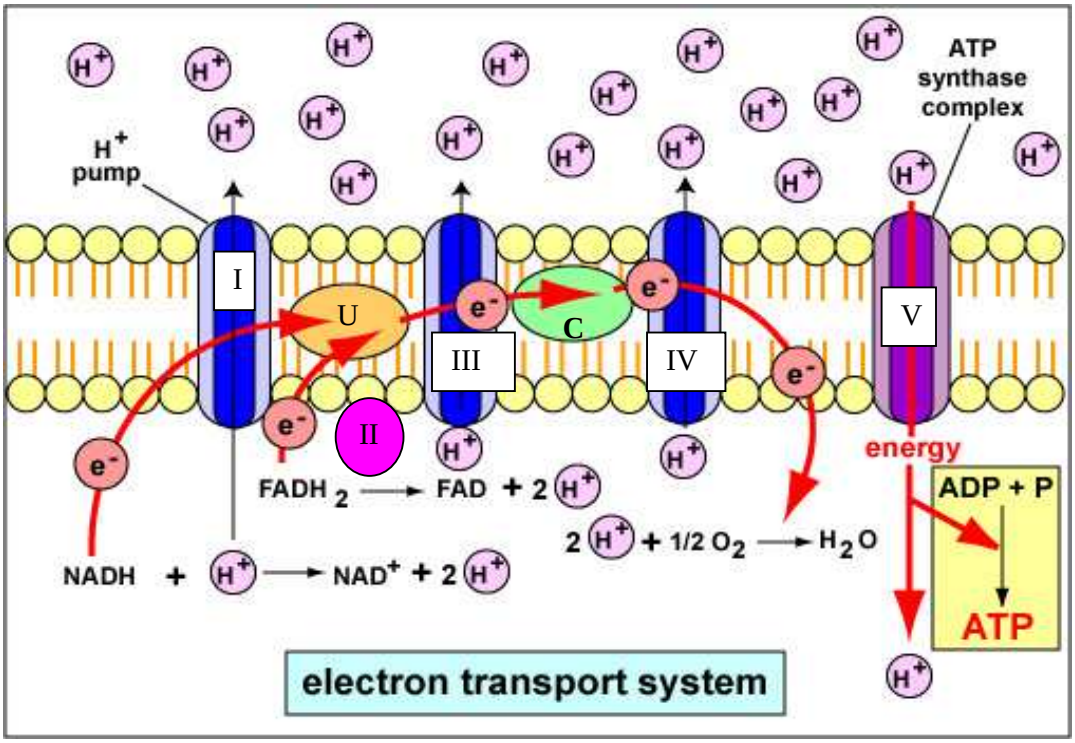
<u>Figure 2.9:</u> Simplified diagram of the Krebs cycle, showing basic substrates, products, and energy carriers. Adapted from Montgomery et al 1990.

2.6.2 Oxidative phosphorylation

Oxidative phosphorylation uses the energy released by the oxidation of nutrients to produce adenosine triphosphate (ATP).

During oxidative phosphorylation, electrons are transferred from electron donors to electron acceptors such as oxygen. These reactions release energy, which is used to form ATP. These reactions occur in protein complexes in the mitochondrial membrane, and the whole process is known as the electron transport chain system.

NAD donates electrons to NADH dehydrogenase (Complex I) which transfers these electrons to Ubiquinone and simultaneously pumps protons out of the mitochondrial matrix into the inner membrane space. Ubiquinone then donates these electrons to Cytochrome *b-c1* (Complex III). After affecting the pumping of a proton across the membrane, the electron leaves III and enters the mobile carrier protein, cytochrome *c* (C). Cytochrome oxidase (complex IV) uses 4 electrons, 4 hydrogens and an oxygen molecule to release 2 water molecules into the matrix and pump 4 protons into the inner membrane. ATP synthase (Complex V) accepts one proton from the intermembrane space and releases a different proton into the matrix space to create the energy it needs to synthesize ATP. It must do this three times to synthesize one ATP molecule from the substrates ADP and Pi (inorganic phosphate). The supply of NADH is the rate limiting step as when there is no electron transport there is no maintenance of a proton gradient to power ATP synthase. Complex II (Succinate dehydrogenase) is not a proton pump. It serves to funnel additional electrons by removing electrons from succinate and transferring them to Ubiquinone. [Pedersen, 1999] See Figure 2.10.

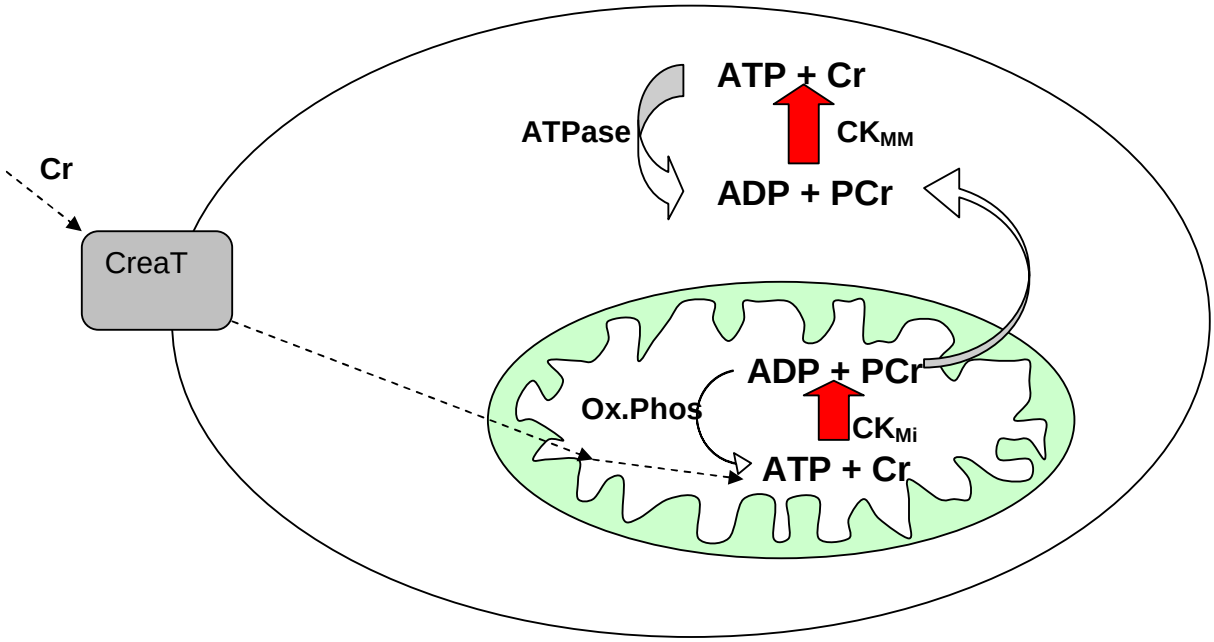


<u>Figure 2.10:</u> Diagram to show the electron transport system. NADH dehydrogenase (Complex I), Ubiquinone (U), Cytochrome *b-c1* (Complex III), cytochrome *c* (C), Cytochrome oxidase (Complex IV), ATP synthase (Complex V), Succinate dehydrogenase (Complex II). Adapted from images from www.google.com).

2.6.3. Phosphocreatine shuttle

ATP is created in the mitochondrion by oxidative phosphorylation, and this ATP is then stored in the form of phosphocreatine (PCr) in the cytosol. In the inner membrane space in the mitochondrion a phosphate group is transferred from ATP to Cr, forming ADP and PCr. This reaction is catalysed by the mitochondrial CK isoform (MiCK). PCr leaves the intermembrane space by diffusion and reaches the cytosol where it is used by cytosolic myofibrillar creatine kinases (MMCK) for the rephosphorylation of cytosolic ADP into creatine and ATP for use by ATPases for energy in cytosolic reactions. Such transfer of

energy has been termed the phosphocreatine shuttle. This also ensures that there is never a build up of ATP in the mitochondria, thus ensuring a gradient in the mitochondria for continuing ATP production [Soboll et al 1997].



<u>Figure 2.11:</u> Schematic representation of the phosphocreatine shuttle model, adapted from Kongas and Van Beek 2007. During oxidative phosphorylation (OxPhos), ADP is converted to ATP when phosphocreatine In the mitochondrial intermembrane space, ATP donates a phosphate group to Cr and produces PCr. This reaction is controlled by mitochondrial creatine kinase (MiCK). ADP is released into the cytosol where myofibrillar creatine kinase (MMCK) produces ATP and Cr from ADP and PCr, and ATPase controls the reverse reaction.

2.7 Myocardial ischaemia and reperfusion injury

The heart is continually using energy and consuming oxygen for contractions [Graham et al 1968], maintenance of cell viability, fatty acid uptake, regulation of coronary blood flow and metabolism [Braunwald 1999] to name a few. The disturbance in the availability of oxygen, or an increase in the demand for oxygen and substrates above the ability to supply it, is termed myocardial ischaemia.

2.7.1 Myocardial ischaemia

Myocardial ischaemia occurs when the heart muscle is deprived of oxygen and this is accompanied by inadequate removal of metabolites because of reduced blood flow or perfusion [Hoffman and Buckberg, 1977]. During ischaemia, there is in imbalance between the energy and oxygen supply to the heart, and the myocardial energy and oxygen demand. Myocardial ischaemia can occur as a result of increased myocardial oxygen and substrates demand, reduced myocardial oxygen and substrates supply, or both.

2.7.2 Causes of ischaemia

Myocardial ischaemia is usually due to coronary artery disease (atherosclerosis of the coronary arteries). The risk of CAD increases with age [Aronow 2006], smoking [Thevenin et al 1986], hypercholesterolaemia (high cholesterol levels) [Gotto 1984], diabetes [Lemp et al 1987], and hypertension [Taylor 1991].

2.7.3 Pathophysiology of ischaemia

In the presence of a coronary artery obstruction, an increase in myocardial oxygen requirements e.g. through exercise, leads to a temporary imbalance in the demand and supply homeostasis. This condition is frequently termed *demand ischaemia* and is responsible for most episodes of chronic stable angina. In other situations, the imbalance is caused by acute reduction of oxygen supply due to e.g. coronary vasospasm or by marked reduction or cessation of coronary flow as a result of platelet aggregates or thrombi. This condition, termed *supply ischaemia*, is responsible for myocardial infarction (MI) and most episodes of unstable angina (UA). In many circumstances, myocardial ischaemia results from both an increase in oxygen demand and a reduction in supply [Zevitz 2006].

2.7.4 Reperfusion injury

Reperfusion is the process where blood flow is restored to the occluded tissue. This procedure, although unavoidable in itself, can bring about a phenomenon known as reperfusion injury. The absence of oxygen and nutrients provided by blood creates a condition in which the restoration of coronary circulation results in inflammation and oxidative damage through the creation of oxidative stress from reactive oxygen species (ROS) rather than restoration of normal function.

2.7.5 Mechanisms of reperfusion injury

The mere principal of reperfusion injury is a contradiction in itself, as the restoration of blood flow to the heart would be expected to be beneficial to the tissue. However, this concept was first described in 1960 by Jennings et al and has since been observed and documented in detail [Peterson et al 1985, Ravingerová et al 1991, Verma et al 2002, Yellon and Hausenloy 2007]. This injury takes place and is described as a series of

paradoxes, which ultimately all contribute to the death of cardiac myocytes that were viable prior to the ischaemic incident [Piper et al 1998].

Oxygen paradox

Reperfusion of the myocardium creates oxidative stress and free radicals which in itself is part of the oxygen paradox and may create more damage than ischaemia alone [Zweier 1988]. Super oxide free radicals (O_2^-) are generated in the reperfused myocardium and are important mediators of reperfusion injury [Zweier 1988]. Oxidative stress also decreases the availability of nitric oxide (NO), as the excess super oxide binds to and quenches the NO to form peroxinitrite ($OONO^-$), and thus stops the beneficial effects of NO i.e. improvement of blood flow, inhibition of neutrophil accumulation and inactivation of free radicals [Zweier and Talukder 2006].

ROS (reactive oxygen species) also activate the Na^+/H^+ exchanger (NHE), which brings more Na^+ into the cell, activating the Na^+/Ca^{2+} exchanger (NCE) to expel more Na^+ out and bring in more Ca^{2+}, producing calcium overload [Sabri et al 1998]. Inhibition of the sodium potassium ATPase by ROS also leads to sodium mediated calcium gain [Hess et al 1981].

pH paradox

Under basal conditions, the Na^+/H^+ exchanger on the cell surface is relatively inactive, the Na^+/K^+ ATPase (Na^+ pump) uses ATP to remove Na^+ and increase intracellular K^+, and the bidirectional Na^+/Ca^{2+} exchanger (NCE) works mainly to pump Ca^{2+} out of the cell. This maintains ion homeostasis in the cellular environment.

During myocardial ischaemia, the Na^+/H^+ exchanger becomes activated in response to intracellular acidosis from an increase in buildup of lactic acid and protons in the cell [Opie 1978] which causes protons to leave the cell down a concentration gradient, through the NHE. The resulting influx of Na^+, occurring as a result of a reduction in ATP and thus a reduction of Na^+/K^+ pump activity, causes the intracellular accumulation of Na^+. Such a rise in the intracellular Na^+ concentration during ischaemia alters the reversal potential of the NCE in that its operation in reverse (Ca^{2+} influx) mode is favoured, thus producing intracellular Ca^{2+} accumulation (contributing to Ca^{2+} overload) during both ischaemia and subsequent reperfusion [Piper et al 2004].

This resulting decrease in pH during ischaemia returns to normal after reperfusion. This return to normal pH does not go hand in hand with a recovery in cell viability and this is what is termed the pH paradox [Lemasters et al 1996].

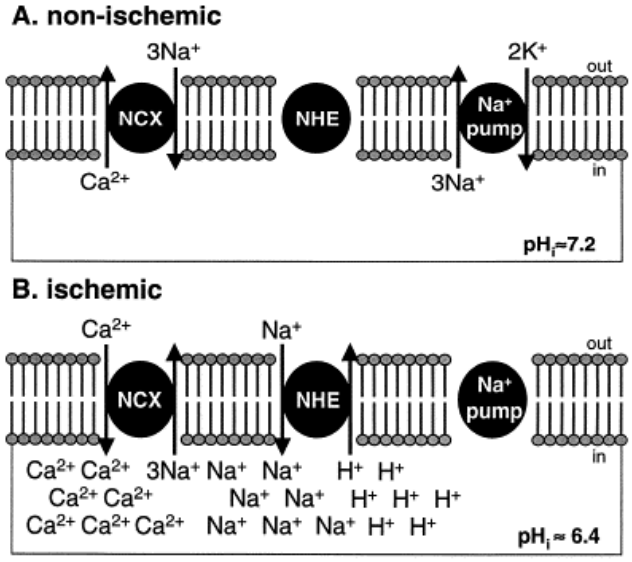
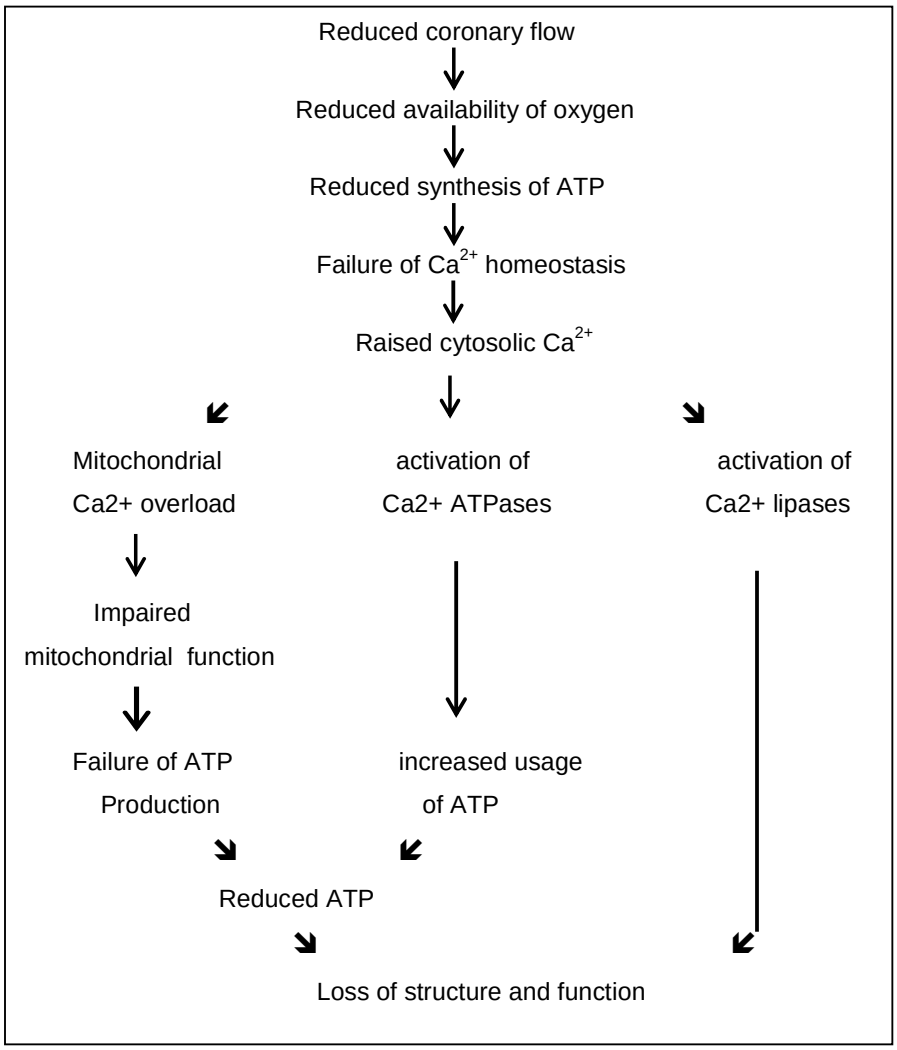


Figure 2.12: (A) The cell under basal conditions, and (B) during ischaemia, showing the pH paradox of ischaemia and reperfusion. Na^+/ Ca^{2+} exchange (NCX in diagram – NCE in text), See text for details. [adapted from Avkiran and Marber 2002]

Calcium paradox.

After cellular depolarization and contraction, calcium is released from the sarcoplasmic reticulum (Ca^{2+} store of the cell) into the cytosol, via a channel called the ryanodine receptor (RyR). During relaxation Ca^{2+} is transported back into the SR by an ATP driven pump called the SR Ca-ATPase (SERCA) [Zucchi et al 1996]. The RyR is opened by increased Ca^{2+} in the cytoplasm, and this results in calcium induced calcium release. The opening of the RyR is also influenced by many intracellular metabolic components like lowering ATP concentrations and lowering intracellular pH (Xu et al 1996) and these occur during ischaemia, thus opening the RyR.

Cytosolic Ca^{2+} increases in large amounts during the first few moments of ischaemia [Lee et al 1987, Kihara et al 1989]. Calcium is then suddenly elevated further in reperfusion and this rapid increase in intracellular calcium overwhelms the normal Ca^{2+} regulatory mechanisms. This increased Ca^{2+} inside the myocyte leads to overload and hypercontracture and mitochondrial permeability transition pore (MPTP) opening (Piper et al 1998). Many ATP consuming enzymes also require Ca^{2+} for activation, and thus Ca^{2+} overload causes a concomitant ATP decrease [Nayler 1981]. Phospholipases and proteases are also activated by Ca^{2+}, leading to a disturbance in the structures of the cell [Suleiman et al 1994, Schwertz and Halverson 1992, Toyo-oka et al 1985].



<u>Figure 2.13:</u> Simplified diagrammatic representation of the sequence of events induced by ischaemia and reperfusion that promote Ca^{2+} overload. See text for details. [Reproduced from Nayler 1981].

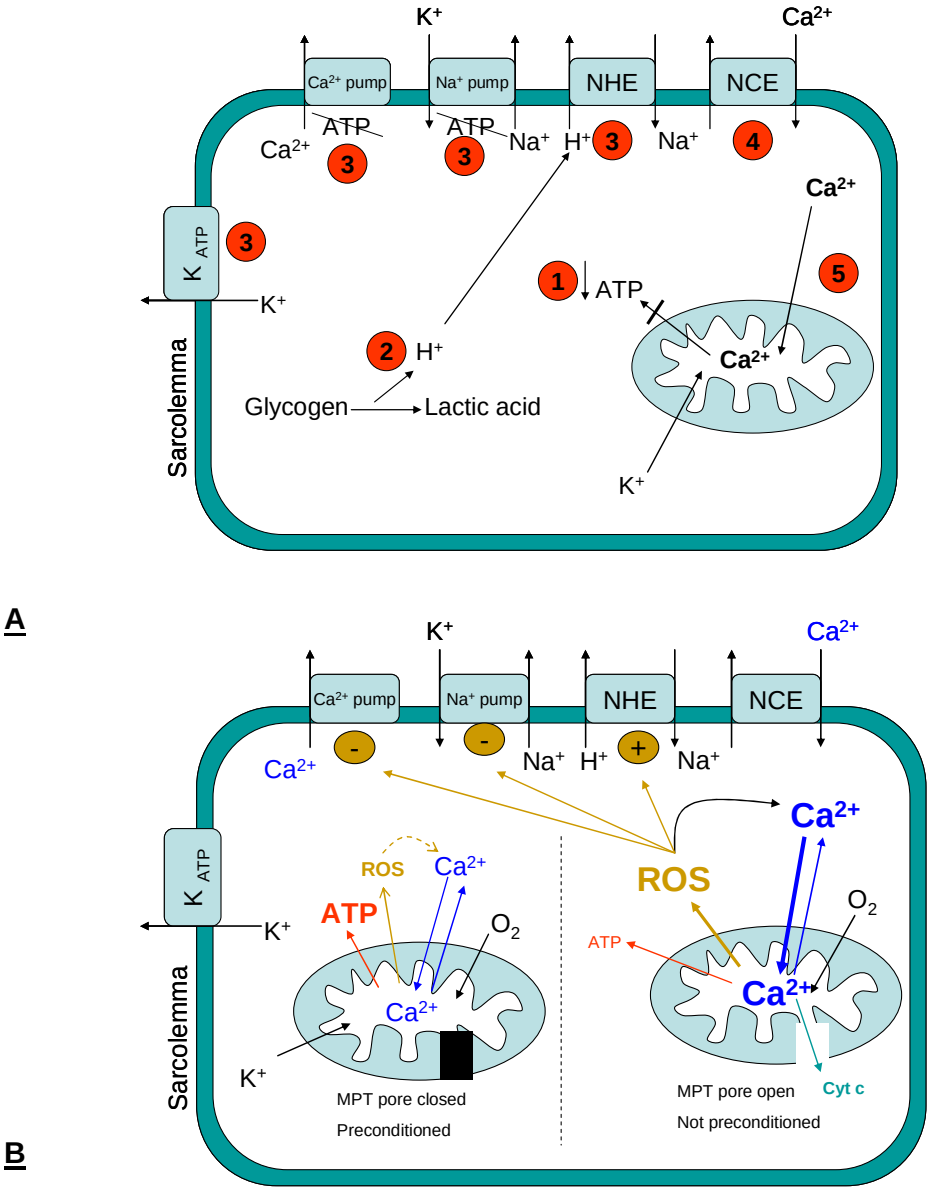


Figure 2.14: Events occurring during A. ischaemia and B. reperfusion. Decreased levels of ATP in ischaemia (1), lead to increased protons (2) in the cell as well as malfunctioning of the ATP-dependent ion exchangers (3) on the cell membrane. With more H^+ ions being released from the cell in exchange for sodium, the sodium is exchanged for calcium and leads to increased intracellular calcium (5). During reperfusion, and the return of oxygen, a surge of reactive oxygen species (ROS) occurs, which further activates the NHE, and deactivates the Na^+ and Ca^+ pumps. The high [Na^+] causes the NCE to work in reverse, further aggravating the Ca overload, and opening of the MPTP. To the left on Figure B shows events in a preconditioned cell. Adapted from Starnes and Taylor 2007.

The damage of reperfusion injury is also partly due to the inflammatory response of damaged tissues. Blood returning to the infarcted area carries with it white blood cells which release a host of inflammatory factors such as interleukins as well as free radicals in response to tissue damage. The restored blood flow reintroduces oxygen to cells that damages cellular proteins, DNA, and the plasma membrane, called the oxygen paradox –as discussed previously. Damage to the cell's membrane may in turn cause the release of more free radicals. Such reactive species may also act indirectly to turn on pro-apoptotic signalling.

Cellular levels of oxygen and ATP decrease during ischaemia and this is associated with increases in cellular oxidant production, calcium overload and increased levels of hydrogen ions. Early in reperfusion, there is an acute increase in both cellular calcium overload and free radical production in cardiac myocytes. Collectively, the increased levels of calcium and oxidants promote activation of pro-apoptotic proteins which contribute to cellular injury, and can lead to cell death. Ischaemia-Reperfusion (IR)-induced cellular injury results in neutrophil activation and the production of ROS that can further contribute to cellular injury. Collectively, these IR-induced disturbances in cellular homeostasis contribute to cellular injury and cell death due to both necrosis and apoptosis.

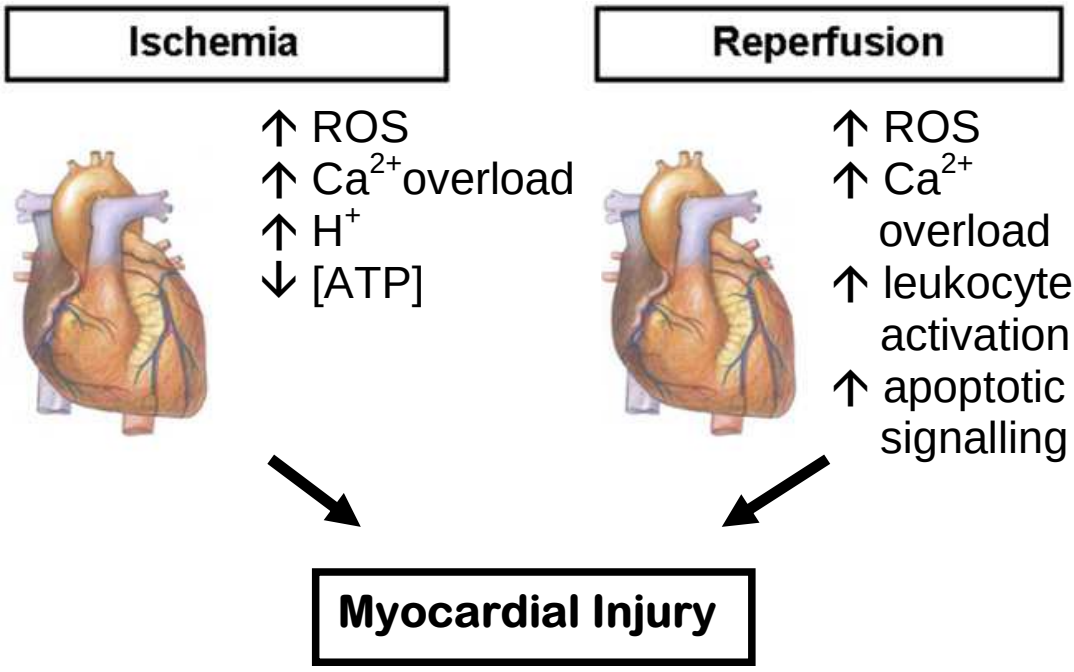


Figure 2.15: **Factors leading to myocardial injury during ischaemia and reperfusion. [Adapted from Powers et al, 2008] See text for more details.**

2.8 Exercise

In 1992, the American Heart Association declared that physical inactivity is an independent risk factor for the development of coronary artery disease [Fletcher et al 1992], highlighting what a large role physical activity plays in procuring health and physiological harmony.

For decades exercise has been described as both a preventative measure and a prophylactic for many diseases and ailments. This is especially relevant in cardiovascular disease prevention and treatment. The beneficial cardiovascular effects of regular exercise were documented as early as 1960 [Raab et al 1960], with many studies since providing solid corroborative evidence to support this [Holloszy 1964, Froelicher et al 1980, Morris et al 1997, Zhang et al 2007].

2.8.1 Beneficial effects of exercise

Exercise from early on in life has been seen to be beneficial for the myocardium [Rockstein et al 1981], and has also been found to prolong life expectancy and quality of life in the elderly [Marom-Klibansky et al 2002]. Exercise also protects against death from coronary artery disease and death from other causes [Rosengren et al 1997]. An increase in physical activity, albeit moderate, can decrease the chances of a myocardial infarct (MI) and may accelerate recovery after a MI [Le Page et al 2009]. Animal and human studies have also shown that exercise results in reduced myocardial ischaemia/ reperfusion injury [Bowles and Starnes 1994, Niederberger et al 1977].

In normal subjects, regular exercise or training results in enhanced body sensitivity to insulin [Koivisto 1979]. This has implications for diabetic and insulin sensitive people, where increased physical activity is beneficial in counteracting high-fat diet-induced insulin resistance [Kraegen 1989] as well as delaying the onset of non insulin-dependent diabetes mellitus (type 2 diabetes) or even preventing the disease.

Other risk factors for coronary heart disease i.e. body weight, body mass index (BMI), cholesterol, LDL cholesterol and triacylglycerols are also decreased with an exercise regime [Ponjee et al 1996], as is the progression of atherosclerosis [Kramsch et al 1981]. Physical training also improves cardiac function as evidenced by increased left ventricular end diastolic volumes (LVEDV), stroke volumes, ejection fractions and interventricular wall thickening during diastole and systole. Eccentric hypertrophy is due to hypertrophic growth of the walls of a hollow organ, especially the heart, in which the overall size and volume are enlarged [Cox et al 1986]. This hypertrophy is associated with an improved left ventricular systolic and diastolic function rather than fibrosis which would be expected to compromise mechanical function [McMullen and Jennings 2007].

Exercise also results in weight loss, and thus reduces blood pressure resulting in reduced hypertension in both men and women [Blumenthal et al 2000, Reger et al 2006]. Haemodynamic changes in response to exercise can also decrease the chance of ischaemic heart disease by reducing platelet aggregation and increased fibrinolytic activity [Watts 1991].

2.8.2 Detrimental effects of exercise

Article titles such as ‘‘Runners who don`t train well can have a marathon of miseries’’ (Foreman 2006) and ‘‘Ironman athletes put hearts at risk of fatal damage, experts warn’’ (Rose 2007), imply that exercise is not necessarily as infallible as it is made out to be. Thompson et al [2007] suggests that exercise is not always beneficial as forceful activity can also acutely and rapidly increase the risk of sudden cardiac death or myocardial infarction in susceptible persons. Exercise is a stress, and although prolonged exposure to moderate episodes of this stress may precondition the heart and protect it, the question of “how much is too much” has arisen [La Gerche et al 2007, George et al 2008]. Heart hypertrophy and associated alterations in the structural properties of the microvasculature have been seen with chronic strenuous exercise [Loud 1984]. Similarly alterations in the structure and function of the sarcoplasmic reticulum with acute strenuous exercise have been observed e.g. depression in the rate of Ca^{2+} uptake, a diminished Ca^{2+} release, and an increase in the intracellular free Ca^{2+} concentration which in turn could activate proteolytic pathways [Byrd 1992]. There is also evidence for a simultaneous activation of the coagulation, fibrinolysis, and complement system as well as for a release of histamine after a short maximal intensity exercise regime [Dufaux et al 1991].

Short-term, high-intensity exercise can lead to significant and prolonged dysfunction of the mitochondrial energy status of peripheral blood leucocytes, and an increased predisposition to apoptosis and raised pro-inflammatory mediators [Tuan et al 2008]. This could in turn lead to coronary artery disease [Diamant et al 2004]. These results support the evidence for the immunosuppressive effects of excessive exhaustive exercise training [Hsu et al 2002].

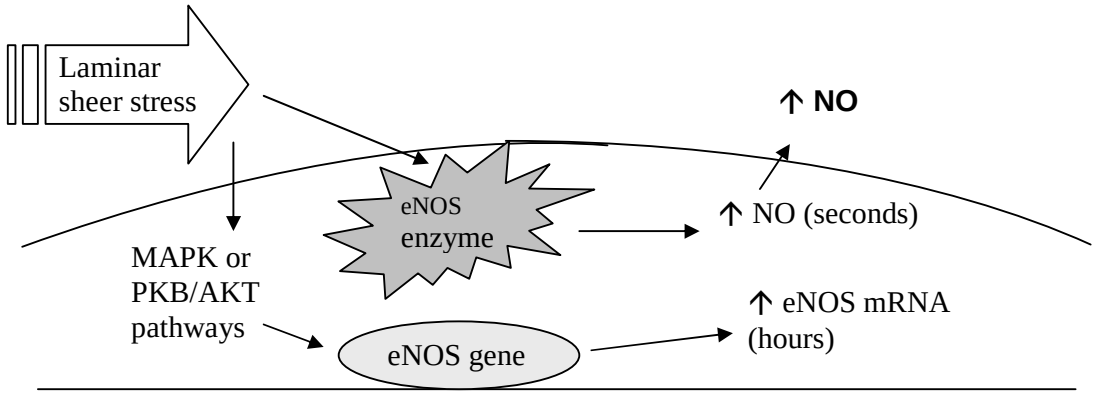
2.8.3 Mechanisms of exercise induced cardiac protection

As expressed in the preceding sections of this literature review, exercise training has been shown to not only protect the heart from ischaemia and reperfusion induced damage, but also has the known benefit of reducing the risk of myocardial infarction. Briefly stated, some of the mechanisms thought to induce this protection are by decreasing many of the causes of ischaemia/ reperfusion injury as described previously in this chapter. To name but a few mechanisms, this exercise training increases coronary circulation [Laaksonen et al 2007], increases heat shock protein expression (HSPs) in the heart [Hamilton et al 2003, Boluyt et al 2006], increases myocardial antioxidant levels [French et al 2008] and improves function of the sarcolemma K_{ATP} channels [Brown et al 2005].

Sheer stress and vascular remodeling

Exercise increases oxygen demand of working skeletal muscles which leads to an increase in cardiac output and blood flow through the vasculature. Shear stress, the stress placed on the vascular wall by the circulating blood, increases during exercise and elevates free radical production in endothelial cells, up-regulates protective antioxidant enzymes and heat-shock proteins and down-regulates pro-apoptotic factors [Marsh and Coombes 2005]. Exercise also activates endothelial- and inducible- nitric oxide synthase (eNOS and iNOS) which leads to a greater nitric oxide (NO) availability [Davis et al 2001, Shen et al 1995]. NO contributes to vessel homeostasis by inhibiting vascular smooth muscle contraction thus inducing blood vessel dilation, platelet aggregation, and leukocyte adhesion to the endothelium. See Figure 2.16.

Long term chronic exercise training can result in angiogenesis and arteriogenesis in the heart [White et al 1998] and skeletal muscle [Gute et al 1996] which results in an increased and improved blood flow capacity to the vasculature and muscle [Brown and Hudlická 1999, Leung et al 2008].



<u>Figure 2.16:</u> Shear stress induced NO production by vascular endothelial cells.

<u>Heat Shock Proteins</u>

Heat shock proteins (HSP) are a class of functionally related proteins whose expression is increased when cells are exposed to stress e.g. increased temperature, ischaemia or exercise. HSPs reduce apoptotic and necrotic cell death by antagonising apoptosis inducing factors e.g. caspases [Ravagnan et al 2001] or enhancing the activity of mitochondrial complexes (complexes I-V) [Summut et al 2001]. HSP70's role in exercise induced cardioprotection has been studied and shown to be effective in protecting hearts against ischaemic injury [Paroo et al 2002, Shin et al 2004].

Antioxidants

Free radicals are atoms, molecules, or ions with unpaired electrons which are usually highly reactive, and are likely to take part in chemical reactions.
Oxygen-centered free radicals are superoxide, hydrogen peroxide and hydroxyl radical. They are derived from molecular oxygen under reducing conditions. Because of their reactivity, these free radicals can participate in unwanted side reactions resulting in cell damage [Dart and Sanders 1988].

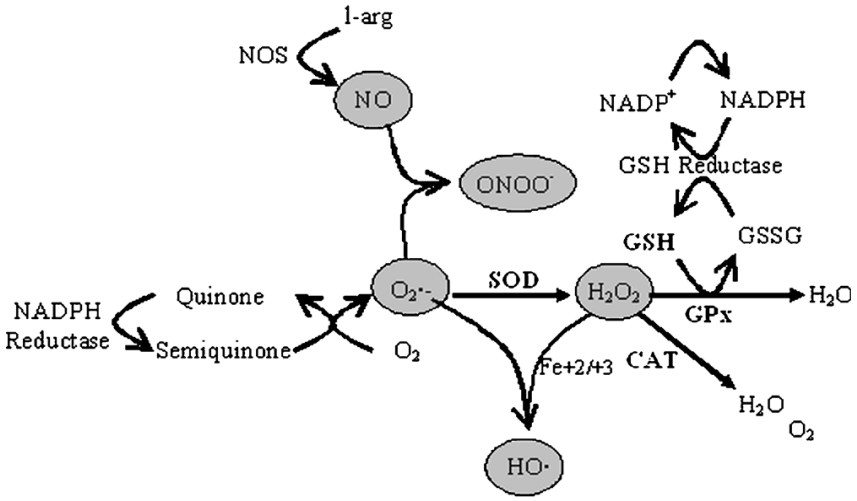
Increased reactive oxygen species (ROS) production by the mitochondria during reperfusion as a by product of the electron transport chain from complexes I and II is at least in part responsible for injury. An increase in antioxidants thus helps scavenge these ROS.

Antioxidants are molecules capable of slowing or preventing the oxidation of other molecules which produces free radicals. Antioxidants stop these reactions by removing free radicals, and inhibit oxidation reactions by being oxidized themselves [Sies 1997].

Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). SOD exists in cytosolic (requires copper and zinc as cofactors) and mitochondrial (requires manganese as the cofactor) isoforms. Important nonenzymatic antioxidants include reduced glutathione and vitamins E and C [Powers and Hamilton 1999].

Although there are reports suggesting that GPx activity increases with exercise [Husain and Somani 2005] there are also reports that suggest the contrary [Demirel et al 2001].

This is also the case with CAT [Demirel et al 2001, Husain and Somani 2005]. MnSOD is however the antioxidant which has been conclusively proven to be increased with exercise [Yamashita et al 1999, Demirel et al 2001, Hamilton et al 2003, Quindry et al 2005]. Despite the association between exercise and increased SOD it has still not been established whether this antioxidant is essential for cardioprotection [Lennon et al 2004].



<u>Figure 2.17:</u> Pathways of major cellular oxidant formation and endogenous antioxidant action. Species noted in gray circles represent some of the reactive oxygen and nitrogen species capable of mediating damage to cellular protein, lipid, and DNA. GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; H_2O, water; NO, nitric oxide; NOS, nitric oxide synthase; O_2, oxygen; O_{2^-}, superoxide; ONOO-, peroxynitrite. From Starnes and Taylor 2007.

K_{ATP} channels

The ATP-sensitive potassium channel (K_{ATP}) is normally inhibited by intracellular ATP and opens during periods of energy depletion [Noma 1983]. K_{ATP} channels are known to exist in the sarcolemmal membrane as well as the mitochondrial membrane of cardiomyocytes and there is evidence both for [Das and Sarkar 2003] and against [Brown et al 2005] the mitochondrial channels' role in cardioprotection. It has been shown to be a mediator of cardioprotection induced by preconditioning either by ischaemia [Budas et al 2004], pharmacological manipulation [du Toit et al 2008, Wan et al 2008] or through exercise [Brown et al 2005].

When the concentration of ATP in the cell falls as during ischaemia, the channel opens and there is thus an outward flux of K^+, consequently hyperpolarising the cell and shortening the cardiac action potential. This in turn causes the voltage dependant Ca^{2+} channels to be inhibited and thus promotes decreased calcium overload of the cell and hypercontractility. Thus this is the suggested mechanism for the sarcolemmal channel's role in protection. [Sandhiya and Dkhar 2009].

Although sarcolemmal K_{ATP} channel activation in the ischaemic myocardium is critically important for cell survival and protection of function, its electrophysiological effects include shortening of the action potential duration and the refractory period. These effects are potentially proarrhythmic and can promote the development of lethal arrhythmias, [Janse and Wit, 1989]. Consequently, the inhibition of sarcolemmal K_{ATP} channels in ischaemic myocardial cells can prevent lethal ventricular arrhythmias and sudden cardiac death [Englert et al 2003, Vajda et al 2007], implicating increased K_{ATP} opening in sudden cardiac death associated with exercise.

The opening of the mitochondrial K_{ATP} channels has also been implicated in improved calcium handling by the cell, reduced mitochondrial matrix swelling and increased oxidative metabolism and the decreased release of ROS by the mitochondria during preconditioning [O'Rourke, 2000, Gross and Peart 2003]. However Brown et al [2005] have shown that the mitochondrial K_{ATP} channels are not an essential mediator in exercised induced cardio protection.

Mitochondria

The mitochondria are the "powerhouse" of the cell, and thus, during exercise, when the energy demand increases substantially, the mitochondria's ATP output needs to increase to meet this increased demand. Besides ATP synthesis, mitochondria also play a significant role in osmotic regulation, pH control, signal transduction and calcium homeostasis. [Cadenas 2004, Brookes et al 2004].

Exercise training has been shown to improve mitochondrial efficiency of oxidative phosphorylation by increasing removal of ROS and decreasing free radical production in skeletal muscle [Servais et al 2003]. Bo et al [2008] showed that exercise training also increases mitochondrial ATP synthetase activity, ADP to oxygen consumption (P/O) ratio, respiratory control ratio (RCI = State 3/state 4 respiration), and MnSOD activity in cardiac muscle. The increased ATP usage is perhaps what increases the ADP/O ratio and drives the production of more ATP in the mitochondria, thus increasing the RCI. Inhibition of the cardiac apoptotic pathways has also been observed in response to exercise training [Quindry et al 2005]. Ascensao et al [2005] showed that endurance training decreased heart mitochondrial susceptibility to MPTP opening.

However not all studies have shown that exercise benefits the mitochondrion. In exhausted guinea pigs, the rate of oxygen consumption in cardiac mitochondria was lower in the exhausted animals than in the controls [Taylor et al 1976]. Leucocyte mitochondria show a lowered energisation status and a higher incidence of apoptosis during high intensity training [Hsu et al 2002].

Pro-survival pathways

Exercise activates the PI-3 kinase – PKB/Akt pathway in the brain, which is known for its role in enhancing neuronal survival [Chen et al 2005]. Exercise training has also been shown to both increase PKB/Akt phosphorylation in the hearts of spontaneously hypertensive rats [Lajoei et al 2004] and normalise the PKB/Akt phosphorylation in the myocardium of Zucker diabetic rats [Lajoei et al 2004b]. Increased PKB/Akt signaling would also be expected to increase Glut4 translocation for increased glucose uptake and usage [Wang et al 1999]. Cardioprotection via the pro-survival pathways is underscored by the findings of Siu et al [2004] who found that exercise training decreased the extent of apoptosis in cardiac and skeletal muscle.

Iemitsu et al [2006] concluded that exercise activated multiple mitogen activated protein kinase (MAPKs: ERK, JNK, and p38) pathways in the heart. P38-MAPK is important in many biological processes including cell growth, differentiation, myocyte hypertrophy, and apoptosis [Wang et al 1998, Bassi et al 2008], but it has been implicated as a mediator of ischaemic injury [da Silva 2004]. P38-MAPK activation has been seen to gradually decline with the development of exercise-induced cardiac hypertrophy after approximately 12 weeks [Iemitsu et al 2006].

AMPK

AMPK is an enzyme that is expressed in most mammalian tissues including cardiac muscle. One of its functions is the regulation of fuel supply and energy-generating pathways in response to the metabolic needs of the organism by regulating the activity of acetyl-coenzyme A carboxylase. AMPK affects levels of malonyl-coenzyme A, a key energy regulator in the cell. AMPK is generally inactive under normal conditions but is activated in response to hormonal signals and stresses such as strenuous exercise, anoxia, and ischaemia which increase the AMP/ATP ratio. Once active, muscle AMPK enhances both the uptake and oxidative metabolism of fatty acids and glucose transport and glycolysis [Arad et al 2007] AMPK enhances glucose uptake via activation of GLUT4 translocation, fatty acid oxidation via acetyl-CoA carboxylase [Hardie and Carling 1997], and glycolysis by inhibiting glycogen synthase [Halse et al 2003]. AMPK is activated during exercise [Chen et al 2000, 2003]. However it has also recently been shown that although AMPK is activated by exercise, the alpha2 isoform of AMPK seems to not be essential for glucose uptake in exercising, AMPK deficient mice [Maarbjerg et al 2009].

2.8.4 Swim training as a model in the rat

For many years swim training has been used in rats as an exercise model to elicit a number of beneficial physiological and metabolic responses. These benefits include enhancement of immunity [Kaufman et al 1994], improved resistance to myocardial ischaemia and reperfusion damage [Margonato et al 2000, Freimann et al 2005, Zhang et al 2007 See Table 1], improved recovery after spinal cord injury [Smith et al 2006] and halting of the deleterious effects of aging [Iemitsu et al 2002, 2006].

Swimming has also been used to elicit stress responses in rats [Salman et al 2000] although this stress is usually more pronounced during an acute swim session than during chronic swim training where animals are habituated to the swimming program [Cox et al 1985]. Raised levels of corticosterone in the rat, as occurs during psychological stress [Scheuer and Mifflin 1998], lead to increased myocardial infarct size [Scheuer and Mifflin 1997].

2.8.5 Other models of exercise training in rats

Other models of exercise training in rats include treadmill (Brown et al 2005, Reger et al 2006, Chicco et al 2007) and wheel running (Chicco et al 2005). These above studies have all also shown cardioprotection with these models of exercise training.

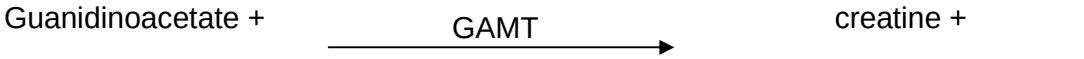
Table 2.1 Table to show length and duration of swimming training, interventions used and outcomes for myocardial research in rats.

	length	duration	intervention	outcome
Zhang et al 2007	10 weeks	3hrs/day 5 days/ week	Insulin stimulated	Improved contraction Increased GLUT4 translocation and eNOS & PKB/Akt, expression
Margonato et al 2000	3 weeks	2 hrs / day 7 days/ week	60 minutes low flow ischaemia	↓ arrythmias ↑RPP recovery ↓diastolic contracture
Freimann et al 2005	7 weeks	90mins/ day 6 days/ week	MI for 4 weeks	Reduced infarct size
Ravi Karan et al 2004	4 weeks	20 mins/day 2% of body mass, weight on tails		↑ Mn-SOD ↓LDL
Ravi Karan et al 2004	4 weeks	20 mins/ day 3%of body mass weight on tails		↑ Mn-SOD ↓LDL
Ravi Karan et al 2004	4 weeks	20 mins/ day 5%of body mass weight on tails		No change in Mn-SOD or LDL

2.9 CREATINE

2.9.1 Creatine biosynthesis

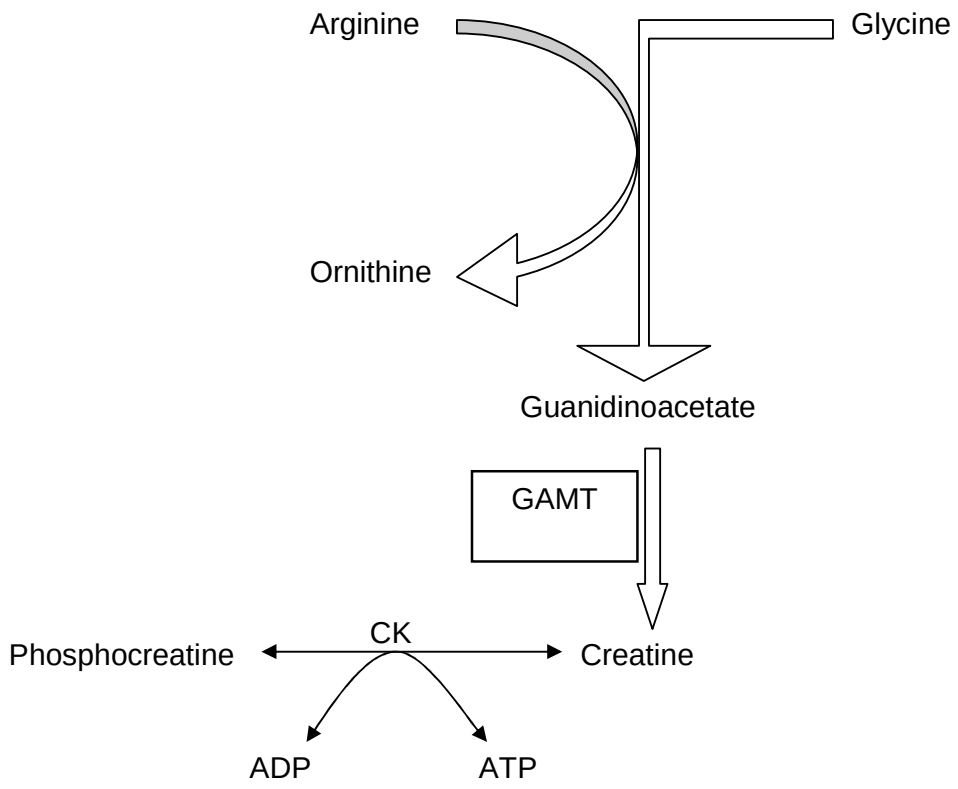
Creatine is a non-essential amino acid derived molecule which is both dietary and synthesized de novo from arginine and glycine. [Walker 1979]



[Borsook and Dubnoff 1941]

This synthesis takes place mostly in the liver and pancreas and to a lesser extent, recent evidence suggests, in the brain and testes too [Moore 2000, Braissant et al 2001]. Glycine Amidinotransferase (AGAT), which is involved in reaction (1) is predominantly expressed in the kidney and pancreas and Guanidinoacetate methyltransferase (GAMT) which is responsible for reaction (2) is predominantly expressed in the liver and pancreas. This implies there is inter organ movement of the metabolites ornithine and guanidinoacetate (GAA) from the kidney to the liver.

The regulation of the process of creatine biosynthesis is thought to be modulated by AGAT (Walker 1979). An increase in serum levels of creatine as a result of supplementation results in a decrease in AGAT enzyme activity, enzyme level, and mRNA expression in rat kidney [McGuire et al 1984], producing less GAA and thus less creatine [Edison et al 2007].



<u>Figure 2.18:</u> Biosynthesis of creatine. GAMT, Guanidinoacetate methyltransferase, ADP, adenosine diphosphate, ATP, adenosine triphosphate, CK, creatine kinase. Adapted from Ensenauer et al 2004.

Creatine is non-enzymatically broken down into creatinine and excreted by the kidneys in the urine [Borsook and Dubnoff 1947]. The rate at which creatine is degraded is 1.6% which equates to 2g per day. This amount needs to be replenished either by endogenous synthesis or by dietary intake [Hoberman et al 1948]. About half of this (±1g per day) is provided by the diet, from sources such as meat and fish and the remainder is synthesized endogenously [Hoogwerf et al 1986].

2.9.2 Creatine absorption

The mechanism of creatine absorption into the body through the gut is not completely clear. Creatine has been seen to be absorbed from the jejunum [Tosco et al 2004] and the ileum [Peral-Rubio et al 2002] of the small intestine into the blood stream, via a CT1 transporter. Creatine is also structurally similar to amino acids arginine and lysine, and may enter via amino acid transporters or peptide transporters in the intestine.

The ingestion of carbohydrate containing solution (e.g. fruit juice) aids in the absorption of creatine from the gut, and may increase total creatine in the muscle up to 60% [Green 1996]. However, while insulin and insulin–stimulating food appears to enhance muscle uptake of creatine, high carbohydrate meals may slow the absorption of creatine from the intestine [McCall and Persky 2007].

2.9.3 Creatine Uptake

Skeletal muscle is the tissue in which most (approximately 95%) of the body's creatine is stored. The remaining 5% is stored in the heart, brain and testes [Snow and Murphy 2001]. Generally, creatine is transported in the blood from areas of production (liver, kidney and pancreas) to tissues requiring it (skeletal and heart muscle, brain and testes). We also know that the brain and testes have been found to produce their own creatine. Creatine is then taken up into cells by a special creatine transporter which is located on the cell membrane, called the CreaT.

2.9.4 Creatine transporter (CreaT)

Over 90% of cellular creatine uptake occurs via the Na^+/Cl^- CreaT, against a large concentration gradient [Loike 1986]. The extracellular creatine content regulates the transport of creatine into cells [Loike 1988]. 2 genes have been isolated that encode for the creatine transporter, CreaT1 (SLC6A8) and CreaT2, of which the latter is predominantly found in the testes. There is a 97% homology between the 2 transcripts; with only small differences in the stop codon [Iyer 1996, Sora 1994]. CreaT1 protein is exclusively found in muscle cells, including the heart, and as this study concentrated on heart muscle we will be referring to it as CreaT for this paper.

CreaT protein consists of 635 amino acids (~70.5kDa) with 12 membrane spanning domains and has been classified as a member of the Na^+- dependent plasma membrane transporter family [Guimbal and Kilimann 1993] to which the transporters of a number of neurotransmitters and osmolytes belong, including transporters for serotonin [Blakely et al 1991] and glycine [Smith et al 1992]. They comprise a family of proteins related in structure and whose transport substrates are biogenic amines (substance produced by a life process, containing an amine group) or zwitterionic compounds (a chemical compound that carries a total net charge of 0, thus electrically neutral but carries formal positive and negative charges on different atoms) [Liu et al 1992, Uhl 1992] .

Immunoblotting has identified two isoforms (55kDa and 70kDa) of the CreaT in rat skeletal and heart muscle, kidney and brain [Wallimann et al 1994], but other isoforms (40, 60 and 115 kDa) have also been seen [Tran et al 2000, McCall and Persky 2007]. The functional significance of these isoforms is not yet resolved, but recent evidence

[Tran et al 2000] suggests that the core CreaT protein is probably a 40kDa protein and that all the other isoforms are produced as a result of different levels of glycosylation of the CreaT, since there are 3 phosphorylation and 5 glycosylation sites on the protein [Sora et al 1994, Nash et al 1994]. This may be important in targeting the CreaT to different cellular locations [McCall and Persky 2007].

CreaT content is reduced in heart failure [Neubauer et al 1999]. This may contribute to the depletion of intra-cellular creatine compounds and thus to the reduced energy reserve in the failing myocardium. This discovery has clinical implications, suggesting that the CreaT is a target for therapeutic studies.

2.9.5 Beneficial effects of creatine

Creatine has been used for years by sportsmen and women as a legal and natural aid to enhance their endurance and power and decrease recovery time. Creatine monohydrate, creatine phosphate and creatine ethyl esters are all forms of creatine that are taken by athletes and body builders and which are metabolized in the gut for absorption and ultimately enhance exercise performance [http://www.creatine-monohydrate.org]. Whichever form of creatine is used, the outcome achieved is the same – building body bulk [Stone et al 1999, Yolek et al 1999], increased muscle power and strength [Stone et al 1999, Dempsey et al 2002], increased endurance [Little et al 2008], increased muscle glycogen accumulation [Op't Eijnde et al 2001, van Loon et al 2004] for increased energy storage and utilization capacity, decreased lactate production [Ceddia and Sweeney 2004] and decreased inflammation and muscle soreness [Santos et al 2004].

Not only does creatine have ergogenic effects but it has also been used as a prophylactic in many muscular and neurological diseases. Since the decrease in cellular creatine in diseased states is a possible reason for muscle weakness and atrophy and disturbances in cellular homeostasis, the normalization of creatine in the cells with supplementation may be a reason for this [Wyss et al 1998]. Studies with creatine supplementation in muscular dystrophies have shown the potential of creatine to alleviate the clinical symptoms of the disease [Felber et al 2000, Kley et al 2007]. Creatine supplementation in heart failure patients also increases the skeletal muscle's performance due to an increase of creatine in the muscle [Gordon et al 1995].

In MELAS disease (Mitochondrial encephalopathy, lactic acidosis) it has been shown that creatine supplementation completely abolished the symptoms after 4 weeks [Barisic et al 2002], and in Parkinson's disease creatine supplementation enhances the benefits of weight training [Hass et al 2007]. There is also data to suggest that creatine supplementation has positive effects on bone function and structure [Antolic et al 2007]. Recent work has eluded to the fact that creatine supplementation may also help improve insulin sensitivity in type 2 diabetes [Op't Eijnde et al 2006]. Interestingly, creatine has been found to increase antioxidants in skin, and can therefore be protective against UV and other environmental damage [Lenz et al 2005].

Therefore it is evident that many positive effects have been documented with the use of creatine as a supplement, in the diseased state. Nevertheless, care should be taken because the effect of creatine loading on skeletal muscle ergogenics can be negated by the intake of caffeine [Vandenberghe et al 1996].

2.9.6 Detrimental effects of creatine

However, not all the evidence provided in the literature is encouraging. There have been reports of adverse effects of creatine supplementation. For example, creatine supplementation has been found to bring about gastrointestinal stress and diarrhea [Ostojic and Ahmetovic 2008]. Short-term, high-dose oral creatine supplementation increases the production and thus excretion of potential cytotoxic compounds, methylamine and formaldehyde but does not have any detrimental effects on kidney permeability [Poortmans et al 2005]. In addition creatine supplementation exacerbates the lung's allergic response in mice [Vieira et al 2007].

There has been a single case study where creatine supplementation was associated with atrial fibrillation, in a 30 year old man who was admitted to the emergency room with atrial fibrillation and rapid heart rate [Kammer 2005]. The medics could not find any reason for his condition, and when his medical history was examined it was revealed that he had been using creatine as a supplement. He was treated with anticoagulants, his heart rate stabilized and was sent home 24 hours later, with no obvious adverse consequences.

2.9.7 Mechanisms of creatine induced cardiac protection

The primary mechanism by which creatine supplementation works is highlighted in Chapter 1.1.1 and 1.1.2 (see Figure 1.1). This increased intracellular creatine potentially acts as a store of phosphate groups to be used during ATP synthesis as energy for the cell.

In a study by Brzezińska and colleagues in 1998 they concluded that dietary Cr did increase cardiac muscle high energy phosphate reserves and its oxidative potential in the rat model after 7 days of supplementation. Creatine supplementation has been shown to increase cardiac creatine reserves only slightly since initial total creatine concentrations are high [Ipsiroglu et al 2001]. They also showed that a minimum of 2 weeks was required to raise levels. However, Boehm et al [2003] showed that there was no difference in creatine transporter levels in cardiac muscle from rats after 6 weeks of creatine supplementation, nor was there an increase in PCr or Cr in the heart tissue, although total creatine levels were raised in the heart and plasma. McClung et al [2003] reported similar results after 3 weeks of supplementation, but although the Cr content of the heart tissue increased, total Cr (TCr) did not.

These conflicting results may be a consequence of rat model, housing, dosage of creatine, duration of study or method of sacrifice and tissue extraction. The manner of feeding may also be a factor e.g. intubation tube/ gavage.

In brain tissue from rats, creatine administration stops the inhibition of the Na^+,K^+ ATPase pump in a model of metabolic disease, where the pump is inhibited and thus

the membrane potential is negatively affected [Ribeiroa et al 2009]. In a study by Zhu et al [2004] creatine supplementation reduced caspase- induced cell death cascades.

GLUT4 expression increases in rat and human skeletal muscle with creatine supplementation [Ju et al 2005, Op't Eijnde 2001b], as does AMPK phosphorylation [Ceddia and Sweeney 2004]. They have also observed decreased lactate production and increased glucose oxidation with creatine supplementation.

Studies have also shown increased glycogen storage in skeletal muscles in humans during creatine supplementation [Robinson et al 1999, Derave et al 2003].
An increase in myosin heavy chain (MHC) type I and II in human skeletal muscle has been shown after 12 weeks of creatine supplementation and resistance training [Willoughby and Rosene 2001], however this has not been investigated in the heart.

A combination of swim training and creatine supplementation for 2 months in rats increased mitochondrial creatine kinase (MB-CK) expression [Golden et al 1994]. Increased MB-CK expression in the myocardium is characteristically associated with hypertrophy. However both pressure overload and coronary artery disease cause increased CK expression [Ingwall et al 1985]. This anomaly was not addressed in Golden et al's study.

In a study by McClung et al 2003, chronic exercise stress in rats induced a significant decrease in cardiac-muscle total RNA. A loss of cardiac RNA results in a decrease in muscle protein which is detrimental for the functioning of the heart. Creatine supplementation, in conjunction with the same exercise stress, corrected this

attenuation and resulted in values of RNA that were comparable to those of control animals.

From the above evidence it would seem that creatine supplementation increases the energy reserves in the heart and would thus leave it better prepared to overcome an ischaemic event. Increased phosphate for regeneration of ATP stores, increased glycogen for energy, as well as increased GLUT4 for glucose uptake and glycolysis during an ischaemic incident would be expected to afford protection against ischaemia and reperfusion injury.

HYPOTHESIS

Based on the knowledge that exercise is of benefit to the heart, both under basal conditions and as a cardioprotective factor for ischaemic injury, and that creatine has been proven to be of advantage to skeletal muscle during contraction and relaxation to increase energy and decrease recovery time, it is our opinion that the combination of these two interventions will be advantageous to the heart.

We therefore hypothesise that creatine alone, and in combination with exercise, will be advantageous to the heart, and provide protection against ischaemia/reperfusion injury.

AIM

We therefore aimed to investigate the effects of dietary creatine supplementation on 1) basal cardiac function,

2) myocardial susceptibility to ischaemia/reperfusion injury and

3) myocardial signaling protein expression and phosphorylation and

4) mitochondrial function

In addition, we investigated the effects of creatine in combination with exercise on these parameters in rats.

CHAPTER 3

METHODS

3.1 ANIMAL MODEL

Male Wistar rats weighing between 200-220g were used in all experiments. The project was approved by the CEAR (Committee of Experimental Animal Research) of the Faculty of Health Sciences, University of Stellenbosch and complied with the guidelines of the South African Medical Research Council for the humane use of laboratory animals. The rats were allowed free access to food (standard rat chow) and water, and maintained in the University of Stellenbosch (US) Central Research Facility at 22°C with a 12 hour day/night cycle.

In this study, swimming was used as exercise. After completion of the 8 week supplementation and swimming programme animals were anaesthetised with an intraperitoneal injection of pentobarbitone sodium at a concentration of 0.12mg/gram body weight. Animals were weighed at the outset of the supplementation and swimming program and again at the time of sacrifice and the weight gain over the 4 weeks was calculated.

3.1.1 Creatine supplementation

Animals were randomly divided into control or creatine supplemented groups. Creatine (EAS Phosphagen Creatine Dietary Supplement) was given daily in jelly cubes (Royal jelly, *Kraft Foods South Africa* Pty Ltd) for multiple reasons. Creatine absorption is

enhanced with carbohydrate ingestion [Green et al 1996] and to ensure minimum psychological stress to the rat and to ensure that they received appropriate dosages. Rats received a dose of 0.07g per kg per day as the supplementation dose for a 70kg human male is 5g per day. Starting doses for the first 5 days was a 4 times stronger loading dose (0.24g per kg per day). The rest of the 8 weeks regime was with the above mentioned maintenance dose. This creatine supplementation program was followed 5 days a week for 8 weeks. Control animals received jelly cubes without creatine. Thus all groups investigated received equal amounts of carbohydrates that are contained in the jelly.

3.1.2 Exercise Program

Each group was randomly divided into exercise trained and sedentary groups. Training comprised swimming at set times of the day, each day. Training duration was started at 5 minutes per day to minimise stress and was increased by 5 minutes increments each day to a maximum of 60 min per day. Rats were exercised for a minimum period of 8 weeks, 5 days per week in order to elicit metabolic changes (Iemitsu et al 2002, Venditti et al 2009) , with a 2 day recovery period in between. Water temperature was kept at 30ºC for the duration of the swim session. Rats were sacrificed 24 hours after the last bout of exercise and the hearts rapidly excised.

The 4 experimental groups investigated were as follows: Control sedentary (C Sed), creatine sedentary (Cr Sed), control exercised (C Ex) and creatine exercised (Cr Ex).

3.1.3 Behavioural Studies

The behaviour of the rats was documented the day before rats were sacrificed for the myocardial studies. Each rat was placed in the open field for 5 minutes (see figure 3.1) to test for differences in anxious-like behaviour and activity.

The open field test is designed to measure behavioural responses such as locomotor activity, hyperactivity, and exploratory behaviour. The open field is also used as a measure of anxiety. Rats tend to avoid brightly illuminated, novel, open spaces. Open field testing is a once off trial test with little or no impact on the animal's subsequent behaviour. The apparatus for the open field test is a square enclosure (1m by 1m) made of black Perspex.

Each rat is placed individually in a corner of the field and its behaviour recorded for 5 minutes. All activity is recorded using a video camera mounted above the open field and scored later by an advanced motion-recognition software package (Noldus Ethovision version 3.1 software) that detects and analyzes the movements of the rat. The video image of the open field arena is partitioned into 36 equal-size squares; 24 border squares and 12 centre zone squares.

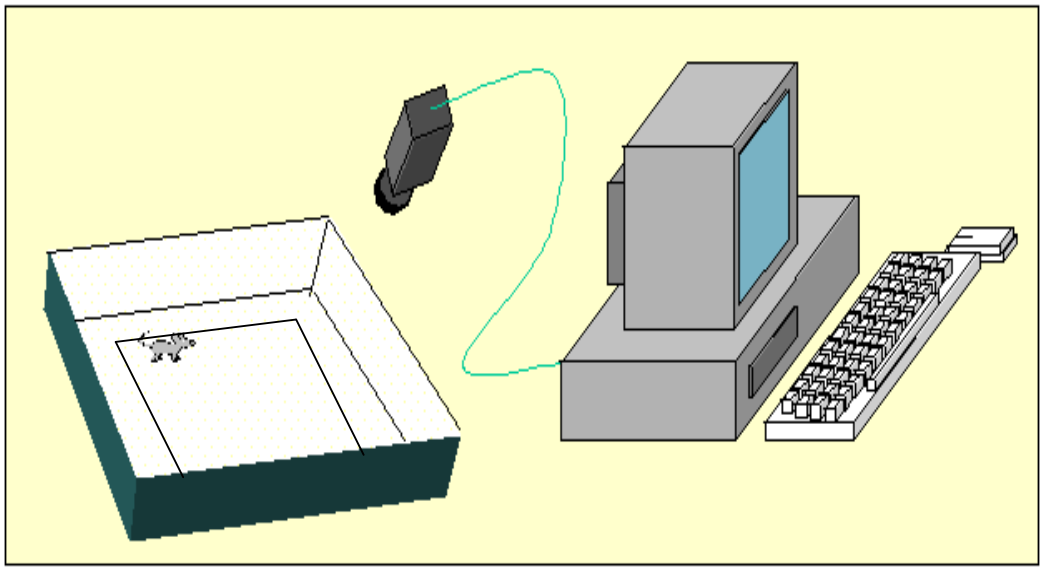


Figure 3.1: Schematic to illustrate the rat in the open field.

Total distance, average speed, and time spent in various parts of the field (e.g. the border areas vs. the open, middle area) were measured and analyzed. Testing was carried out in a temperature, noise and light controlled room. The rats were placed in a cage in the testing room an hour before the test in order for them to acclimatize to the new environment. The open field was cleaned with 70% ethanol after each rat had been tested.

Each rat was tested individually and in a separate test room. Throughout the entire testing-session, the sequence of events was always the same and the test circumstances (handling, room-features, equipment used) were as standardised and controlled as possible. The entire test procedure lasted approx. 20 minutes per animal, and was recorded entirely on videotape to allow analysis at a later time. During the test procedure silence was maintained in the test room. The behavioural tests were performed from 1-1:30pm daily to ensure that normal daily fluctuations in corticosterone, circadian rhythm and activity did not affect the results [Richter 2004]. Rats were sacrificed the following day and blood collected for analysis of corticosterone levels.

3.2 ISOLATED HEART PERFUSIONS

Hearts were excised, arrested in cold (4ºC) Krebs-Henseleit buffer and mounted on a working rat heart perfusion system.

The isolated hearts were perfused with a Krebs-Henseleit buffer equilibrated with 95%O_2 and 5%CO_2 at 37^{o}C (Krebs-Henseleit bicarbonate buffer: 119mM NaCl, 25mM $NaHCO_3$, 4.75mM KCl, 1.185mM KH_2PO_4, 0.6mM $MgSO_4$, 0.6mM $NaSO_4$,1.25mM $CaCl_2.2H_2O$, 10mM glucose) at a preload of 15cm H_2O and at an afterload of 100cm H_2O. Myocardial temperature was closely monitored for the duration of the experiment by placing a temperature probe inside the coronary sinus , Myocardial temperature was kept between 36.5 and 37ºC during Langendorff and work heart perfusions.

3.2.1 Working Heart Perfusions

During Langendorff retrograde perfusion, the heart is perfused through the aorta with a preload of 100 cm H_2O. After cannulation of the aorta the left atrial cannula was inserted into the pulmonary vein for working heart perfusion. In the working heart mode the heart functions with a preload of 15 cm H_2O and an afterload of 100 cm H_2O and buffer enters the heart through the left atrium through the pulmonary vein, and is ejected by the left ventricle via the aorta.

3.2.1.1 Mechanical function recovery after global ischaemia

During working heart perfusion, mechanical function of the hearts was documented (coronary flow (CF), aortic output (AO), heart rate (HR), aortic systolic pressure (SP) and aortic diastolic pressure (DP)).

Global ischaemia was induced by reverting to retrograde perfusion and stopping perfusion of the retrogradely perfused heart for 20min. The temperature was strictly monitored and kept between 36 and 36.5 ºC. Hearts were reperfused and mechanical function was again documented in the working heart mode. The percentage recovery of aortic output was then calculated by expressing the post ischaemic aortic output as a percentage of the pre- ischaemic aortic output. See figure 3.2.

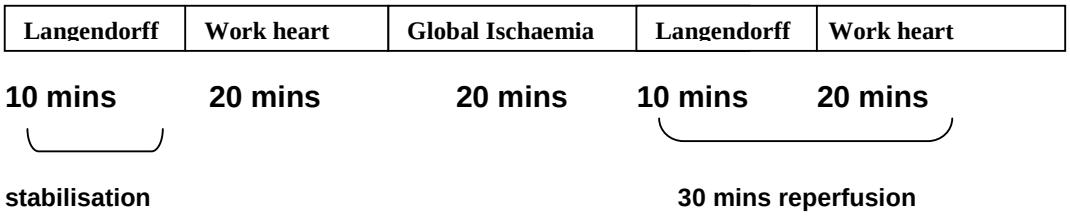


Figure 3.2: Diagram to show the perfusion protocol for the isolated rat heart perfusions used to document functional recovery after 20 minutes of total global ischaemia.

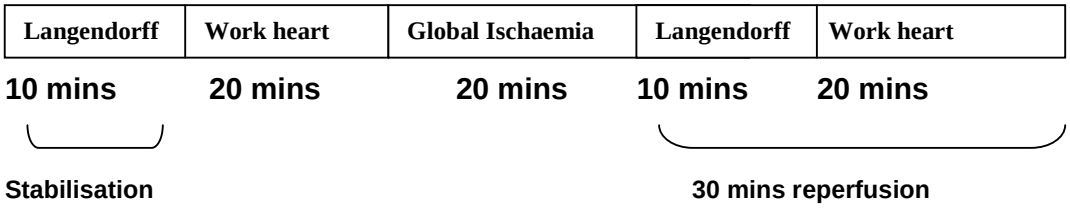
3.2.2 Langendorff Perfusions

A water filled balloon, connected to a pressure transducer (Viggo Spectromed), was inserted into the left ventricle via the left atrium. The balloon was inflated with water to a volume where the end-diastolic pressure of the left ventricle was between 10 and 20mmHg.

Myocardial mechanical function was documented by measuring heart rate (HR), and left ventricular developed pressure (LVDevP) which is the difference between systolic (SP)

and diastolic pressures (DP). By multiplying these 2 parameters (HR x LVDevP), the rate pressure product (RPP) is calculated. These functional parameters as well as Aortic Output (AO) and Coronary flow (CF) were recorded on a computerised GENTRONICS chart recorder v3.00 system throughout the experiment.

During the 20 minutes of global ischaemia, where the temperature was strictly maintained between 36 and 36.5ºC, the increases and ischaemic contracture was documented. This measurement allows us to determine time to the onset of ischaemic contracture and the magnitude (in mmHg) of this contracture. The mechanical function during reperfusion was also calculated, and the percentage recovery calculated as post-ischaemic function divided by pre-ischaemic function. See figure 3.3. At the end of the reperfusion period the heart was dabbed dry and weighed for determination of heart weight to body weight ratio.



<u>Figure 3.3:</u> Diagram to show the perfusion protocol for the isolated rat heart perfusions used to document ischaemic contracture during 20 minutes of global ischaemia.

3.2.3 Infarct size determination

In a separate set of experiments, hearts were subjected to regional ischaemia to determine infarct size. After 30 minutes of stabilisation (10 minutes retrograde perfusion and 20 minutes working heart perfusion) during which mechanical function was documented (AO, CF, RPP), the heart was retrogradely perfused again and the left anterior descending coronary artery was ligated for 35min to induce regional ischaemia before being reperfused for 30 min. The temperature was strictly maintained at 36-36.5ºC during regional ischaemia. See figure 3.4.

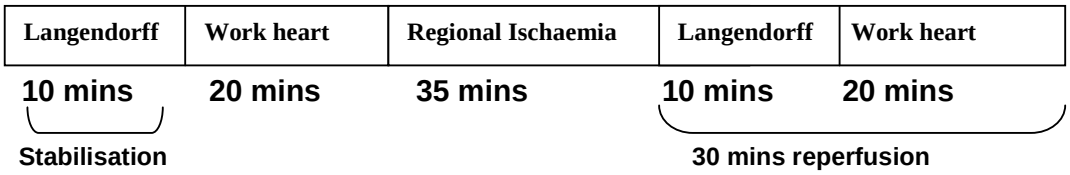


Figure 3.4: Diagram to show the perfusion protocol used for the isolated rat heart perfusions for the analysis of infarct size after 35 minutes of regional ischaemia.

At the end of the experiment the left anterior descending coronary artery was re-ligated and hearts were stained with an Evans Blue suspension (0.5%) for demarcation of the area at risk. Hearts were then frozen at -20ºC overnight.

The hearts were cut into slices of equal thickness (±2mm), and stained by incubation in 1% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH7.4) for 15 minutes and then fixed in 10% formaldehyde to enhance the stained areas. The area at risk, infarcted area and viable tissue were then delineated, drawn and quantified using planimetry on the UTHSCSA ImageTool program and the infarct size (IF) expressed as a percentage of the Area at Risk (AAR). AAR size was the same for all hearts.

3.3 BLOOD AND TISSUE COLLECTION

3.3.1 Blood Collection

3.3.1.1 Corticosterone levels

Rats were sacrificed the day after the behaviour tests were performed. After excision of the heart, blood was taken from the thoracic cavity, placed in BD Vacutainer^{R} serum separation tubes and spun down at 2000 *g* for 10 minutes at 4ºC for separation of the serum. Serum was stored at -80ºC for later analysis. An ImmuChem Corticosterone ^{125}I RIA kit (MP Biomedicals) was used to determine levels of corticosterone in the serum.

3.3.1.2 HEP analysis

After excision of the heart for isolated heart perfusions, blood was taken from the thoracic cavity, placed in eppindorf tubes coated with heparin and spun down at 2000 *g* for 10 minutes at 4ºC for separation of the red blood cells and plasma. This was stored at -80ºC for later HEP analysis.

3.3.2 Tissue collection

3.3.2.1 HEP analysis

Hearts were mounted on the perfusion system and after 2 minutes of perfusion to wash out the blood, hearts were freeze clamped with pre-cooled Wollenberger tongs and immediately plunged into liquid nitrogen and stored for later analysis. Blood was collected from the chest cavity of sacrificed animals and centrifuged in heparin coated eppindorf tubes to separate red blood cells and plasma. These fractions were stored at -80ºC until needed.

3.4 HIGH ENERGY PHOSPHATE ANALYSIS

3.4.1 Extraction of HEPs

For tissue high energy phosphate (HEP) analysis, 100-200 mg of tissue, blood serum or red blood cells were extracted with 1.2ml 6% perchloric acid (PCA). Tissue was pulverised to fine powder in a pre-cooled mortar and pestle, weighed, and then placed in PCA and the exact time documented.

Exactly 2 hours after this, with frequent vortexing every 10 minutes in between, extracts were centrifuged and the supernatant taken for neutralisation. Extracts were neutralised gradually with neutralisation mix (40%saturated KOH-KCl and 0.2MTris-HCl in a 2:3 ratio) and the exact amount of neutralisation mix added to each sample was noted.

Each sample was then filtered through a 0.45µm (Millipore) filter into a clean eppindorf tube, and kept on ice for High Pressure Liquid Chromatography (HPLC) separation and analysis. Samples not separated the same day were stored at -80ºC overnight and then used within 24 hours.

3.4.2 Separation of HEPs

Separation of HEPs (adenosine mono-, AMP, di-, ADP, and tri-phosphate, ATP), creatine (Cr) and creatine phosphate (CrP)) was done using a reversed phase HPLC technique developed by Victor and co-workers (1987). Samples were separated by a LUNA 5µ C18 (2), 250 x 4.6 mm Phenomenex column with on-line UV detection (210 nm) and quantified with appropriate standards (Sigma). The mobile phase (KH_2PO_4

257mM, Tetrabutylammoniumphosphate 1.18mM, HPLC graded methanol 12.5% (v/v)) ran at a flow rate of 2.0 ml/min.

3.4.3 Analysis of HEPs

The concentration for each HEP was determined by dividing its area under the curve by the standard's area under the curve, and multiplied by the total volume and weight of the sample analysed.

3.4.3.1 Determination of HEP ratios

Two of the most important cellular energetic signals are the PCr/Cr ratio and the ATP/AMP ratio. Cellular metabolism is brought about by the use of PCr to keep ATP levels constant, but when PCr levels drop, and ADP and AMP start increasing, then the cellular energy reserve may be in jeopardy, and this has negative implications in muscular dystrophies, atrophies, myopathies and other energy linked diseases (Ye et al 2001).

We determined the PCr/Cr and ATP/ADP as well as the ATP/AMP ratios in all groups, from the values obtained from HPLC.

3.5 MITOCHONDRIA STUDIES

3.5.1 Isolation of Mitochondria from the rat heart

All procedures were performed on ice.

Freshly excised hearts were cut into smaller pieces and placed in a 30ml glass dounce homogeniser and washed 3 to 4 times with ice cold KE isolation buffer (0.18M KCl, 0.01M EDTA, pH adjusted to 7.4 at 4 degrees C, using a 2M Tris solution) to remove all traces of blood. The homogeniser was filled to capacity with ice cold KE isolation buffer and tissue homogenised manually into a slurry which was decanted into Beckman Centrifuge tubes and centrifuged for 10 minutes at 2300 rpm in a JA20 rotor in a Beckman Centrifuge at 4ºC. The supernatant was then transferred to a clean tube and centrifuged for 10 minutes at 12000 rpm in a JA20 rotor in a Beckman Centrifuge at 4ºC. The mitochondrial pellet was then re-suspended in cold isolation buffer and kept on ice until further use. A small sample of the suspension was taken for protein determination.

NB: Prior to resuspension, the homogeniser was cleaned using chromic acid – soaked for 10 minutes and rinsed thoroughly with water between consecutive hearts.

The mitochondrial protein content was determined with the Lowry Protein determination method (Lowry et al, 1951).

3.5.2 Protein content determination

3.5.2.1 Lowry Protein Determination

1ml of 10% TCA was added to 100 µl of mitochondrial suspension in a glass tube and left on ice for at least 30 minutes to precipitate all protein. The sample was then centrifuged at 2000 rpm for 10 minutes at 4°C and the supernatant carefully decanted and discarded. The sides of the tubes were carefully blotted dry. The precipitate was then dissolved in 1N NaOH in a water bath at 70°C. The solution was diluted 1:1 with dH_2O, rendering a 0.5N NaOH solution.

The assay was done in triplicate on 50 µl of sample or standard (3 different BSA solutions of known concentration dissolved in 0.5N NaOH) or 0.5N NaOH (blank), in Lucham tubes.

1 ml of work solution 1 (98% of a 2% Na_2CO_3 solution,1% of 2% Na-K-Tartrate solution and 1% of a 1% $CuSO_4.5H_2O$ solution) was added to the 50 µl of sample or standard or blank, mixed well and allowed to stand for 10 minutes at room temperature. 0.1 ml of work solution 2 (33% Folin-Ciocalteu's phenol reagent) was then added and very rapidly vortexed. After at least 30 minutes, the colour development was read in a spectrophotometer at 750 nm against the blank. The unknown protein concentrations were plotted from the standard curve (Lowry et al, 1951).

3.5.3 Mitochondrial Respiration.

Respiration studies were performed using an Oxytherm respirometer equipped with a Clarke-type electrode and a Peltier temperature control unit (Oxytherm, Hansatech, Norfolk, UK). The oxygraph was calibrated using oxygenated water for the air line and sodium dithionite for the zero air line. It was rinsed out thoroughly and the oxygen content of 650µl of the incubation buffer (250mM Sucrose, 25mM Tris pH 7.4, 8.5mM KH_2PO4) checked until there was a stable baseline. The response of the membrane was checked by switching the stirrer on and off.

3.5.3.1 Glutamate

The mitochondrial suspension (25µl) was added to the incubation buffer in the chamber and the baseline respiration (State 1) measured. The temperature throughout the duration of the experiment was kept at 25ºC.

Glutamate (20µl) was then added to measure O_2 tension and the activity of the whole oxidative phosphorylation chain, starting at complex I (State 2 respiration).
This was followed by the addition of 350µM ADP (28µl of a 10mM solution) to measure state 3 respiration. The mitochondria were allowed to use up all the ADP and when stable, state 4 respiration was measured.

The ADP/O ratio was calculated, which is an indication of the relationship between ATP synthesis and oxygen consumption. The respiratory control index (RCI) was also calculated. The Respiratory Control Index (RCI) is a measure of mitochondrial respiration rate and efficiency of oxygen usage, and is a ratio of State 3 respiration over

State 4 respiration rate. Oligomycin (8µl) was then added to measure oxygen consumption during inhibition of oxidative phosphorylation, in the presence of ADP. See Chapter 3.5.3.1 below for details of the inhibitor used.

Inhibitors of Mitochondrial Respiration

Oligomycin

Oligomycin is an antibiotic which acts by binding and inactivating the F_o subunit of ATP synthase so that it blocks the proton channel thus inhibiting oxidative phosphorylation. Experimentally, oligomycin has no effect on state 4 respiration or electron transport, but it completely prevents state 3 respiration. Refer to Figure 3.5 below. Oligomycin was added to the chamber to a final concentration of 1ug/ml.

3.5.3.2 Succinate

In another experiment using the same mitochondrial preparation, succinate was used as the substrate, and the activity of the oxidative phosphorylation chain from complex II was then determined. ADP was added to measure state 3 respiration, as above, and then state 4 respiration was measured when all the ADP had been utilised. Oligomycin (8µl) was then added, to measure oxygen consumption in the presence of an oxidative phosphorylation inhibitor (see Chapter 3.5.3.1 above). GDP (10mM) was added to measure the involvement of the uncoupling proteins (UCP) (see Chapter 3.5.3.2 below for details of the inhibitor used).

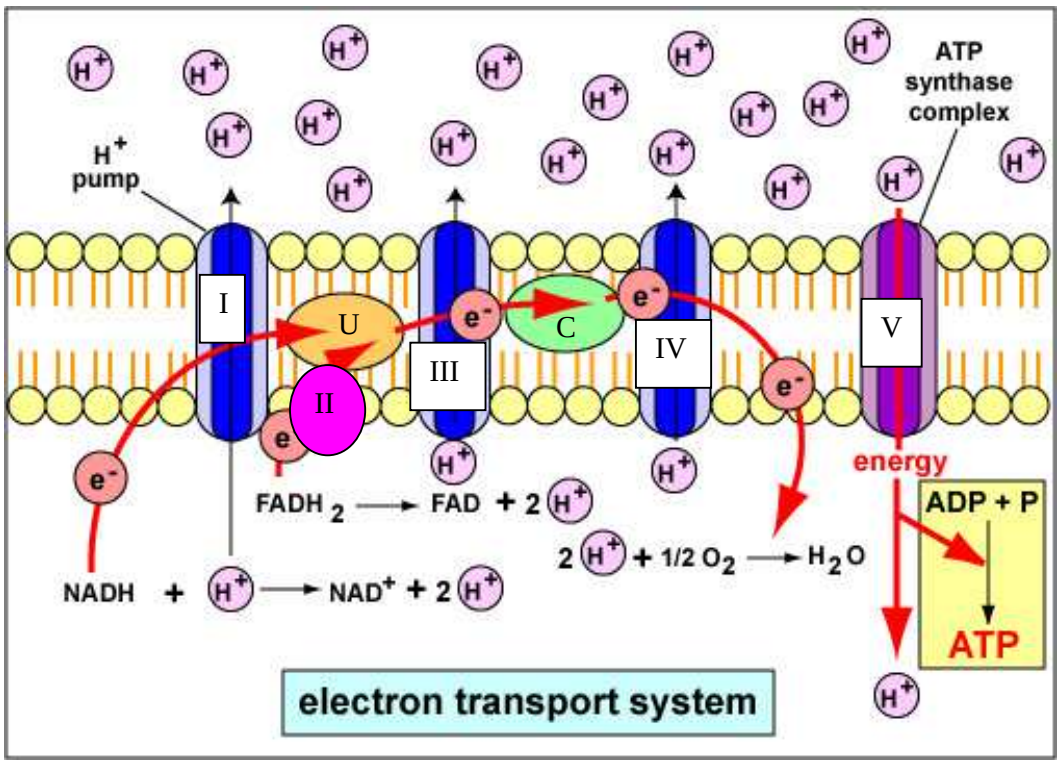


Figure 3.5: **Electron Transport System. NAD donates electrons to NADH dehydrogenase (Complex I) which transfers these electrons to Uniquinone (U) & simultaneously pumps protons out of the mitochondrial matrix into the inner membrane space. U then donates these electrons to Cytochrome *b-c1* (Complex III). After affecting the pumping of a proton across the membrane, the electron leaves III & enters the mobile carrier protein, cytochrome *c* (C). Cytochrome oxidase (coplex IV) uses 4 electrons, 4 hydrogens & an oxygen molecule to release 2 water molecules into the matrix & pump 4 protons into the inner membrane. ATP synthase (Complex V) accepts one proton from the intermembrane space & releases a different proton into the matrix space to create the energy it needs to synthesize ATP. Complex II (Succinate dehydrogenase) is not a proton pump. It serves to funnel additional electrons by removing electrons from succinate and transferring them to U. Adapted from images from www.google.com)**

Inhibitors of Mitochondrial Respiration

GDP

GDP inhibits the uncoupling proteins (UCP's), which uncouple oxidative phosphorylation from ATP generation. An uncoupling protein is a mitochondrial inner membrane protein that can dissipate the proton gradient before it can be used to provide the energy for oxidative phosphorylation [Nedergaard 2005] There are five types known in mammals: UCP1, also known as thermogenin, UCP2, UCP3, SLC25A27, also known as UCP4 and SLC25A14, also known as UCP5. Uncoupling proteins play a role in normal physiology, as in hibernation, because the energy is used to generate heat instead of producing ATP. By inhibiting the UCPs we aimed to see if there is a change in state 4 respiration. If there is, the greater the decrease in state 4 respiration, the more involved the UCPs are, with the percentage change being proportional to the involvement of these UCPs

3.5.3.3 Anoxia / reoxygenation

In yet another experiment on the same mitochondrial suspension, after state 4 respiration was reached, the mitochondria were given an excess of ADP (100nM) and then the oxygen was limited by shutting off the air flow from the chamber. After 20 minutes of anoxia, the air supply was reintroduced by gently bubbling air through a squeeze pipette into the suspension.

We measured the respiration rates during basal mitochondrial respiration, state 3 and state 4 for both succinate and glutamate substrates. We calculated the basal respiratory control index (RCI) (state 3 respiration divided by state 4), and ADP/O ratios as well as the percentage recovery of state 3 after anoxia/ reoxygenation.

3.6 WESTERN BLOT ANALYSIS

3.6.1 Preparation of lysates (protein extraction)

Hearts which had been freeze clamped after 2 minutes of perfusion to wash out the blood, hearts freeze clamped at the end of ischaemia, after 10 minutes and 30 minutes of reperfusion (end of experiment), were used for lysates (see Figure 3.6).

Approximately 0.2g of frozen ventricular tissue was pulverised in a liquid nitrogen pre-cooled mortar and pestle. Pulverised tissue was then added to tubes each containing 900µl of lysis buffer for extraction (20mM Tris-HCL (pH7.4), 1mM EGTA, 25mM NaCl, 1mM Na_3VO_2, 10mM NaF, 1% (vol/vol) Triton X-100, leupeptin (10ug/ml), aprotonin (10ug/ml), 1mM benzamidine, 1mM phenylmethyl-sulphonyl fluoride (PMSF, added immediately before use). The same lysis buffer was used for all lysates and proteins.

The tissue and lysis buffer was then homogenised mechanically with a Polytron PT10 homogeniser for 2 cycles of 5 seconds each on setting 5 and spun down in a microfuge (15min @ 14000 rpm) to remove the particulate matter. Protein content of each sample was determined using the Bradford protein determination method. Samples were diluted with a 3 times Laemmli sample buffer, boiled for 5 min and stored at -20° until Western blot analysis was performed within 2 weeks.

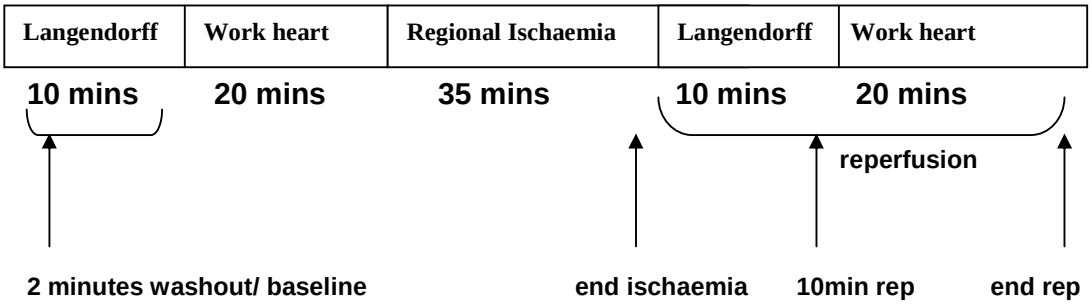


Figure 3.6: Diagram to show the time points during the perfusion experiments when hearts were freeze-clamped for preparation of lysates for Western Blot analysis.

3.6.2 Protein content determination

3.6.2.1 Bradford Protein Determination

The method of Bradford (Bradford, 1976) was used to determine the protein content of samples known to have low concentration of protein. Bradford reagent composition: (0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (v/v) ethanol, and 8.5% (v/v) phosphoric acid.

Standard Curve

Protein standards (bovine serum albumin, BSA in dH_2O) containing 1 to 20 μg protein was pipetted into 12 x 100 mm test tubes. The volume in the test tube was adjusted to 0.1 ml with Millipore H_2O. 0.9 millilitres of Bradford reagent (described above) was added to the test tube and the contents mixed by vortexing. The absorbance was

measured at 595 nm after 15 minutes and before 30 minutes had passed. This was done in 1ml cuvettes against a blank prepared from 0.1ml of Millipore H_2O and 0.9 ml of Bradford reagent. The standard curve obtained in this manner was used to determine the protein content of the unknown samples.

Protein assay

The protein preparation was diluted 1 to 10 with dH_2O in order to dilute all detergents present (e.g. Triton-X) that may interfere with the assay. A suitable aliquot of this dilution (e.g. 5 µl) was adjusted to 0.1ml with dH_2O before the addition of 0.9ml Bradford reagent. The absorbance was measured in duplicate at 595 nM not less than 15 minutes but not more than 30 minutes after the addition of the Bradford reagent. The amount of the protein was determined using the standard curve as described above.

The standard curve generated with Bradford reagent saturates at 20 µg of protein/ sample. Samples were therefore always diluted to fall on the linear portion of the standard curve.

3.6.3 Protein Separation

3.6.3.1 General

After boiling each sample for 5min, equal amounts (20-100μg as indicated) of sample protein from the various fractions were separated on a SDS-polyacrylamide gel with a 4% stacking gel using the standard Bio-Rad Mini-PROTEAN III system (Biorad, Life Science group, US).

Electrophoretically separated proteins were transferred to a PVDF (polyvinylidene fluoride) membrane by electro-blotting. After staining the membranes with Ponceau-S red (reversible staining) for visualisation and verification of transferral of the protein bands, membranes were scanned with laser scanning and saved as a record of equal loading. Membranes were then washed with Tris-buffered Saline (TBS) containing 0.1%Tween-20 (TBST). Non-specific binding sites on the membranes were blocked with 5% fat-free milk powder in TBST for 2 hours at room temperature.

Following the blocking procedure membranes were washed thoroughly with TBST and incubated with the appropriate primary antibody for 5-16 hours at 4°C. After washing the membranes with TBST, the immobilised primary antibody was conjugated with a diluted (1:4000) horseradish peroxidase-labelled secondary antibody for 1 hour at room temperature. The membranes were again washed thoroughly with TBST. Bands were visualised with electro-chemiluminescence (ECL^{TM}) detection agents used according to the manufacturers instructions and quickly exposed to high performance chemiluminescence film (Hyperfilm ECL) to detect the light emission. Bands were quantified by laser scanning densitometry and analysed with suitable software (UN-SCAN-IT, Silkscience, US).

3.6.3.2 Creatine Transporter (CreaT)

Western blotting was performed as stated above with minor adjustments for specificity. Fractions (50-100ug protein) were separated on a 12% SDS-polyacrylamide gel (4.4ml Millipore H_2O, 2.5ml 2.5M Tris-HCl, pH 8.8, 100µl 10% SDS, 3ml Acrylamide, 50µl APS (0.1g/ml) and 20µl TEMED).

Membranes were incubated with the creatine transporter (CT1, CRT, CRTR) primary antibody (US biological and Alpha Diagnostic International) (1:500 dilution in TBS 0.1% Tween) overnight at 4ºC. Membranes were exposed to ECL hyperfilm for an hour of exposure.

3.6.3.3 Glucose transporter 4 (GLUT4)

Western blotting was also performed as stated above with minor adjustments for specificity to quantify GLUT4. Fractions (50µg protein) were separated on a 10% SDS-polyacrylamide gel (4.9ml Millipore H_2O, 2.5ml 1.5M Tris-HCl, pH 8.8, 100µl 10% SDS, 2.5ml acrylamide, 50µl APS (0.1g/ml) and 20µl TEMED).

Membranes were incubated with the GLUT4 (H-61): *sc-7938* primary antibody (Santa Cruz Biotechnology, Inc) (1:1000 dilution in TBS 0.02% Tween) for 5 – 16 hours at 4°C. From this point onwards the membranes were washed with TBS 0.02% Tween instead of TBS 0.1%Tween.

3.6.3.4 Protein kinase B (PKB/Akt)

Western blotting was performed as stated above with minor adjustments for specificity. Fractions (20μg protein) were separated on a 12% SDS-polyacrylamide gel. Membranes were incubated with the total-Akt or the phospho-Akt (Serine 473) primary antibody (Cell Signaling Technology™) for 5 – 16 hours at 4°C.

3.6.3.5 ERK p42/44

Western blotting was performed as stated above with minor adjustments for specificity. Fractions (20μg protein) were separated on a 12% SDS-polyacrylamide gel membranes were incubated with the total-ERK p42/44 MAP Kinase or the phospho-ERK p42/44 MAP Kinase (Threonine 202 / Tyrosine 204) primary antibody (Cell Signaling Technology™) for 5 – 16 hours at 4°C.

3.6.3.6 P38 MAPK

Western blotting was performed as stated above with minor adjustments for specificity. Fractions (20μg protein) were separated on a 12% SDS-polyacrylamide gel membranes were incubated with the total-p38 MAP Kinase or the phospho-p38 MAP Kinase (Threonine 180 / Tyrosine 182) primary antibody (Cell Signaling Technology™) for 5 – 16 hours at 4°C.

3.6.3.7 AMP-activated protein kinase (AMPK)

Western blotting was performed as stated above with minor adjustments for specificity. Protein fractions (30μg protein) were separated on a 10% SDS-polyacrylamide gel. Membranes were incubated with the total or phospho-AMPK (Thr172) primary antibody (Cell Signaling Technology™) (1:1000 dilution in TBS 0.1% Tween, 5% BSA and 5% blocking agent) for 5 – 16 hours at 4°C. 5% blocking agent was also added to the secondary, horse-radish peroxidase conjugated antibody.

3.7 STATISTICAL ANALYSES

In all instances, significance of observed effects was determined using Microsoft GraphPad Prism. The one way analysis of variance (ANOVA) with Bonferroni corrections for multiple comparisons or the paired Students t-test were used for comparisons between 2 groups, and the one or two way ANOVA, as indicated, for comparison of 4 groups. All values are expressed as mean ± standard error of the mean (SEM). A p-value smaller than 0.05 was considered significant.

3.8 MATERIALS

Materials purchased from Sigma Aldrich (Johannesberg, South Africa)

HEPES hemisodium salt technical grade minimum 99.5% titration, pyruvate, mercaptoethanol, N,N,N',N'-tetramethylethylenediamine (TEMED), ponceau S, phenylmethylsulphonyl fluoride (PMSF), Na-p-nitrophenylphosphate (p-NPP), triton-X-100, benzamidine.

Materials purchased from Fluka (Sigma Aldrich, St Louis, United States)

Acrylamide.

Materials purchased from Merck NT laboratory supplies (Pty). LTD [Darmstadt, Germany]

Sodium dodecyl sulphate (SDS), ammonium peroxodisulphate (APS), tris(hydroxylmethyl) aminomethane, all other laboratory salts.

Materials purchased from Millipore, UK .(Microsep Pty (ltd) Johannesberg, SA)

Millipore immobilon-p polyvinylidene fluoride (PVDF) microporous membrane.

Materials purchased from AEC-Amersham Biosciences, UK Ltd (Johannesburg, SA)

ECL Western blotting detection reagents, anti-rabbit Ig, horseradish peroxidase linked whole secondary antibody.

Materials purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany)

GLUT4 (H-61): sc-7938 rabbit polyclonal antibody.

Materials purchased from Cell Signaling technology (Laboratory Specialist Services (PTY) Ltd , Cape Town, SA)

Total and phospho-PKB/Akt (Ser473) antibody, total AMPK and phospho-AMPK-α (Thr172) antibodies.

CHAPTER 4

RESULTS

4.1 ANIMALS

4.1.1 Body weights

Because it is known that creatine supplementation increases body weight in humans, and exercise also has effects on body weight, decreasing fat content and increasing muscle mass we followed the body weights of the animals in the study closely. The rats were weighed at the beginning of the project after they had been randomly divided into groups, before either swim training or creatine supplementation began.

Animals were weighed again after the 8 week program of swimming and creatine supplementation, before sacrifice. The control, vehicle supplemented, sedentary group will be referred to as C Sed, the creatine supplemented sedentary group as Cr Sed, the control, vehicle supplemented, exercised group as C Ex and the creatine supplemented exercised group as Cr Ex.

4.1.1.1 Body weight of rats at the end of the 8 week protocol

C Sed rats and Cr Sed rats had similar body weights at the end of the training and supplementation protocol (396 ± 6.0 grams (g) vs. 390.6 ± 6.0 g). There were no significant differences in weight between these groups or the C Ex (380.7 ± 5.3 g) and Cr Ex rats (379.3 ± 6.2 g). See Figure 4.1.

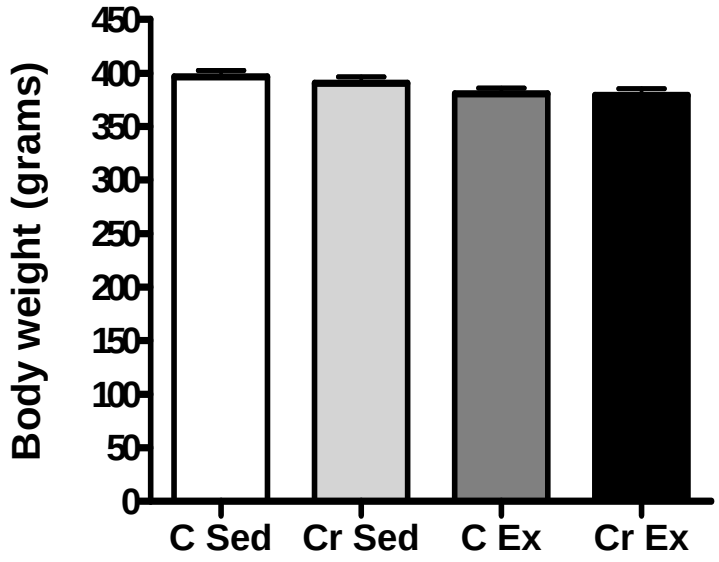


Figure 4.1 **Body weights of Control Sedentary (C Sed), Creatine Sedentary (Cr Sed), Control Exercised (C Ex) and Creatine Exercised (Cr Ex) rats after 8 weeks of creatine supplementation and/or swim training. n=30 per group.**

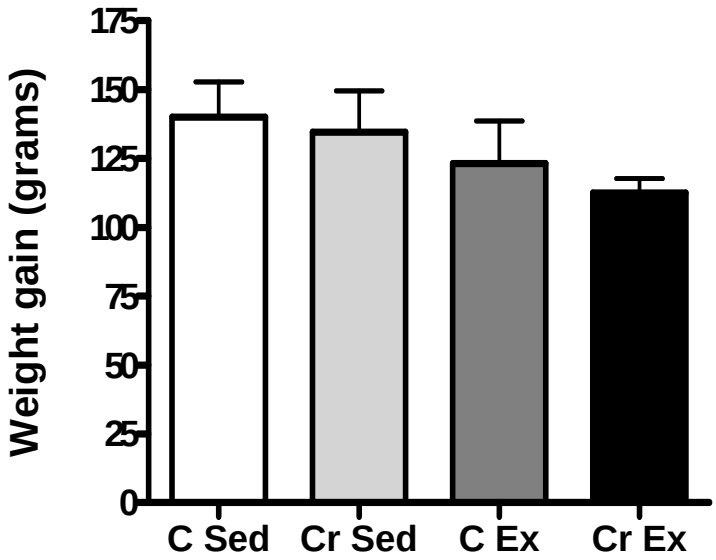


Figure 4.2: **Average body weight gain of rats after 8 weeks of creatine supplementation and/ or swim training. n = 20 per group.**

4.1.1.2 Weight gain

The weight gain, the difference in weight between rats at the beginning and at the end of the swimming and/ or creatine supplementation program, was not different between groups. [C Sed (140.1 ± 12.7 g), Cr Sed (134.6 ± 14.9 g), C Ex (123.2 ± 15.4 g) and Cr Ex (112.5 ± 5.35 g)]. See Figure 4.2.

4.1.1.3 Heart weight / body weight ratio

The ratio of heart weight to body weight is a measure of hypertrophy of the heart [Dadgar *et al* 1979]. Exercise is known to induce either eccentric or concentric cardiac hypertrophy [Mihl *et al* 2008] depending on the type of exercise (see figure 4.3). Hypertrophy has been implicated in heart failure [Hilfiker-Kleiner 2006], and although exercise induced hypertrophy is more often physiological than pathological in nature (eccentric hypertrophy – see Figure 4.3), recent evidence [Rawlins et al 2009, Hart 2003] indicates that there is a fine line between these two forms of hypertrophy and a possible overlap between the two exists. For this reason we weighed the hearts at the end of the perfusion protocol, after ensuring that all excess fluid had been removed, and calculated the heart weight to body weight ratios. These ratios showed no significant differences between C Sed (0.003 ± 0.0001), Cr Sed (0.003 ± 0.0001), C Ex (0.003 ± 0.0001) and Cr Ex groups (0.003 ± 0.0008). See Figure 4.4.

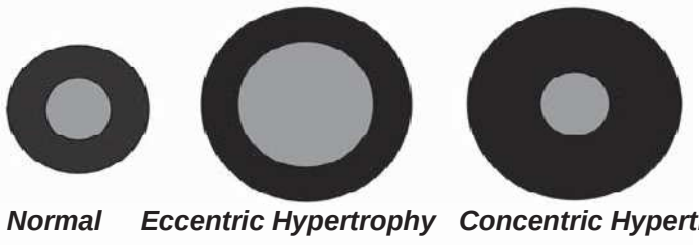


Figure 4.3: Illustration of eccentric and concentric hypertrophy.

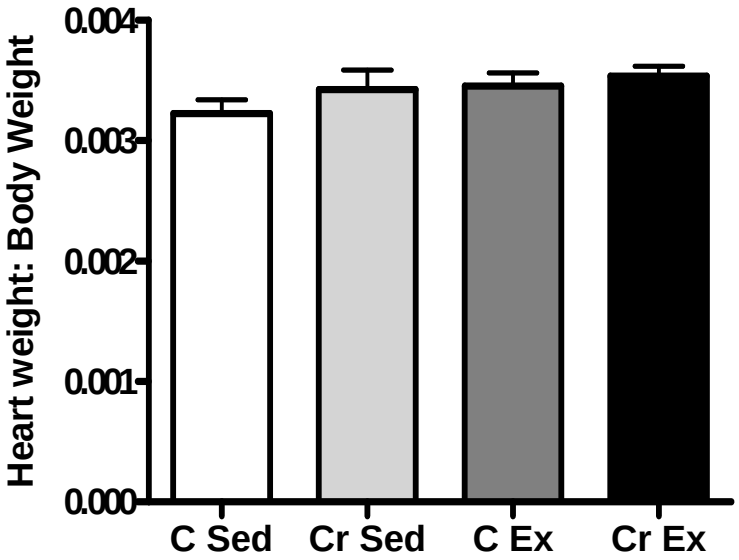


Figure 4.4: Heart weight: body weight ratio of rats from the 4 experimental groups after 8 weeks of creatine supplementation and/ or swim training. n = 20 per group.

4.1.2 Behavioural studies

Swimming has been used in animal studies not only as a model for exercise to induce cardio-protection [Zhang et al 2007] but also as a model for stress in neurological studies [Salman et al 2000, Hall et al 2001]. Although stress studies utilize acute swim training to induce this stress, we used chronic swim training in our study. We performed behavioural studies to determine whether chronic swim training as used in this study induced stress in our animals.

Consequently behavioural tests were performed on the rats to determine whether the training protocol elicited a stress response. Parameters measured included time in the inner and outer zones, frequency of crossing into the inner and outer zones, and distance covered using the open field test as described in Chapter 3.1.3.

To analyze exploratory and locomotor activities, animals were placed in the left rear quadrant of an open field. The number of line crossings and the total distance covered by the rat were measured over 5 minutes. These are classical measures of locomotor and exploratory activities. The more time the rat spends in the inner zone of the open field, and the more exploratory the rat is, the less stressed it is perceived to be.

4.1.2.1 Distance covered

There were no differences in the average distance covered between the 4 groups. C Sed rats moved an average of 1657±144.6cm in 5 minutes compared to Cr Sed rats (1780 ± 119.4cm), C Ex (1810 ± 141.4cm) and Cr Ex (1894 ± 132.8cm) rats. See Figure 4.5.

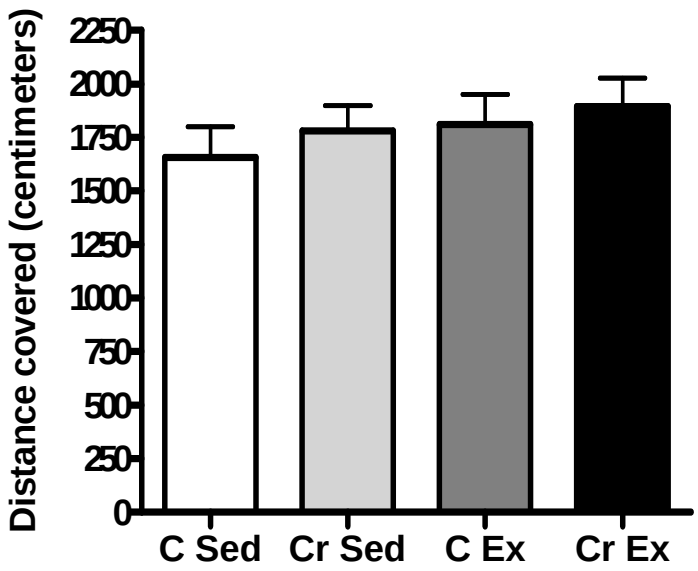


Figure 4.5: Average distance covered by each of the four groups of rats in the open field. n=10 per group.

4.1.2.2 Frequency of movement between inner and outer zones

No differences were found in the frequency of movement from the inner zone into the outer zone and vice versa. Values are given as number of crossings from the inner to the outer zone during a 5 minute period, or from the outer to the inner zone during a 5 minute period.

C Sed rats moved from the inner into the outer zone 9.5 ± 1.3 times, Cr Sed rats crossed 9.7 ± 1.3 times, C Ex rats crossed 13.5 ± 2 times and Cr Ex rats crossed 10.67 ± 2.2 times during the 5 minutes. See Figure 4.6.

C Sed rats moved from the outer to the inner zone 8.7 ± 1.3 times, Cr Sed rats crossed 9.1 ± 1.4 times, C Ex rats crossed 12.4 ± 1.9 times and Cr Ex rats crossed 9.9 ± 2.0 times during the 5 minutes. See Figure 4.7.

4.1.2.3 Time spent in inner and outer zones

As with frequency of movement and distance traveled, there were no significant differences found in the time spent in the two zones between groups. C Sed rats spent 27.44 ± 3.5 sec in the inner zone and 272 ± 3.4 sec in the outer zone. Cr Sed rats spent 26.58 ± 2.6 sec in the inner and 273.4 ± 2.6 seconds in the outer zone. C Ex and Cr Ex rats spent 42.0 ± 9.0 sec versus 257.8 ± 9.1 sec and 26.6 ± 8.1 sec versus 266.6 ± 10 sec in the inner and outer zones respectively. See Figures 4.8 and 4.9.

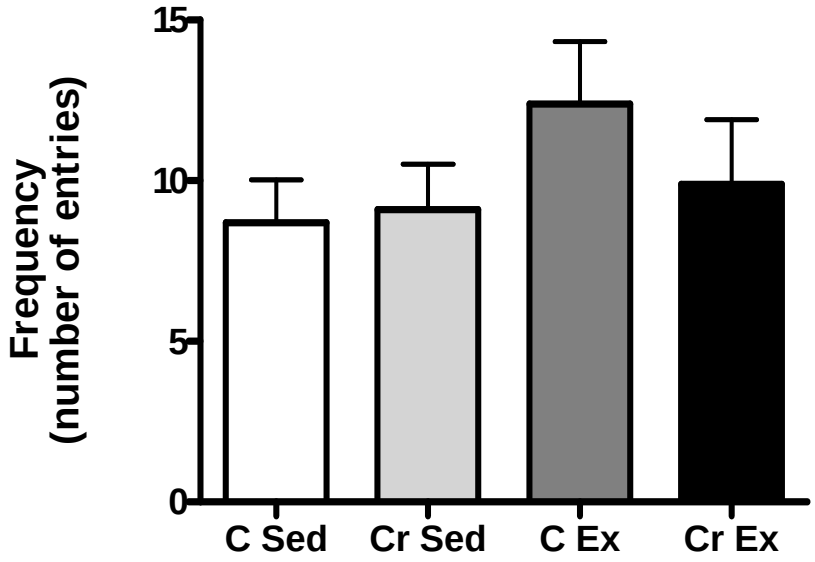


Figure 4.6: **Frequency of movement into the outer zone by rats from each experimental group. n = 10 per group**

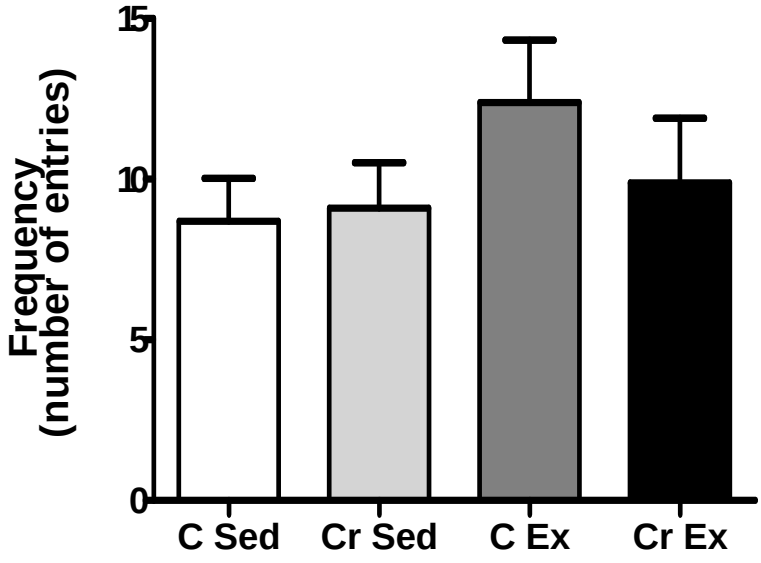


Figure 4.7: **Frequency of movement into the inner zone by rats from each experimental group. n = 10 per group**

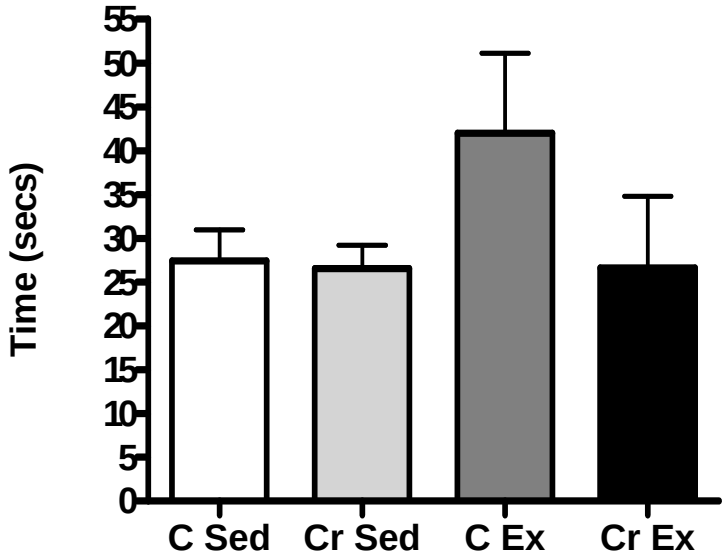


Figure 4.8: **Time spent by the rats from each experimental group in the inner zone of the open field. n = 10 per group**

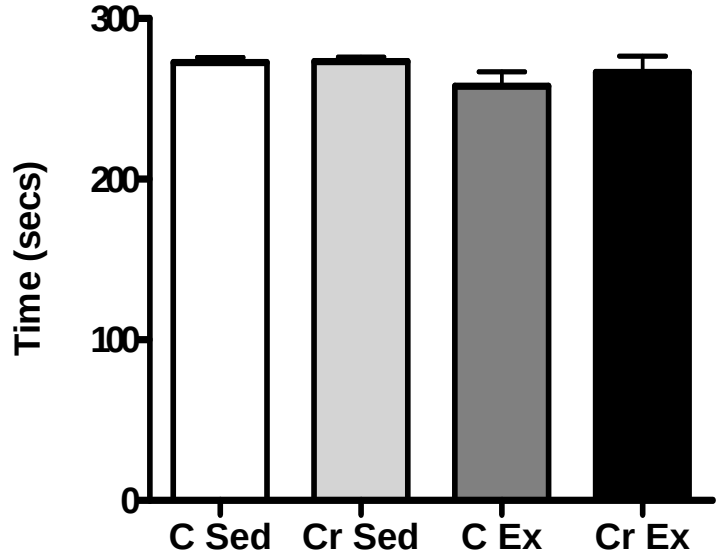


Figure 4.9: **Time spent by the rats from each experimental group in the outer zone of the open field. n = 10 per group**

4.1.3 Corticosterone levels

Stress increases the levels of circulating corticosterone in the rat (the equivalent of cortisone in humans). Elevated levels of this hormone are indicative of stress in these animals.

The serum corticosterone levels were measured in each rat, to determine whether the swim training or creatine supplementation had any effects on stress levels in the animals. C Sed (133.3 ± 37.1 ng/ml), Cr Sed (192.4 ± 49.5 ng/ml), C Ex (130.4 ± 30.1 ng/ml) and Cr Ex (137.8 ± 22.7 ng/ml) rats had similar stress hormone levels. See Figure 4.10.

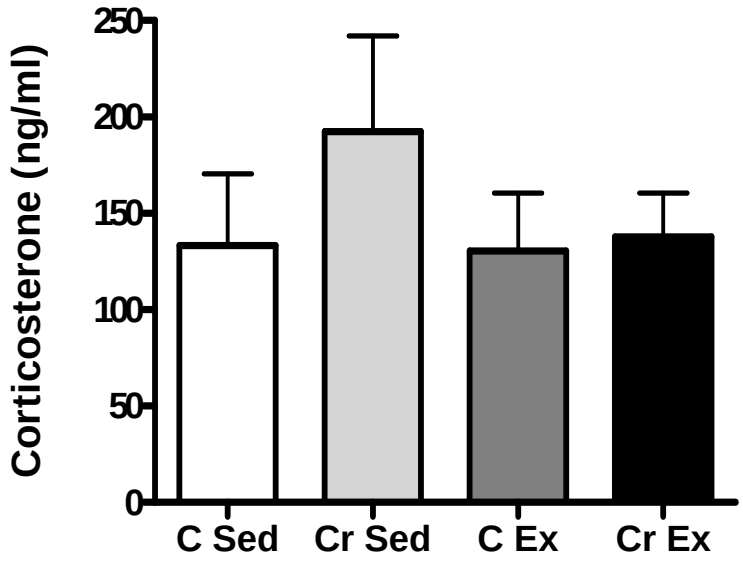


Figure 4.10: Serum corticosterone levels of experimental and control groups. n = 10 per group

Summary of key findings

These data suggest that neither creatine supplementation nor exercise training had any effect on body weight of the rats. Similarly training and creatine supplementation did not alter heart weight to body weight ratios which suggests that cardiac hypertrophy did not occur.

Behavioural studies and blood corticosterone levels in these animals suggest that neither the swim training nor creatine supplementation elicited a stress response in these rats.

4.2 HEART FUNCTION

The effects of creatine supplementation on skeletal muscle have been extensively researched and its effects described (see Chapter 2). However, the effect of creatine supplementation on heart muscle function and metabolism is under-investigated and poorly understood. We used the isolated perfused rat heart model to determine cardiac function and susceptibility to ischaemia and reperfusion injury after creatine supplementation of sedentary animals and animals performing an exercise regime.

Hearts were excised, placed on the Langendorff perfusion apparatus and perfused retrogradely for 10 minutes. They were then perfused in working heart mode for 20 minutes. At the end of this stabilization period, the Aortic Output (AO), Coronary flow (CF), cardiac output (CO = CF + AO), aortic systolic pressure (aortic SP), aortic diastolic pressure (aortic DP), and heart rate (HR) were all measured in work mode. Left ventricular developed pressure (LVDevP = SP-DP) and Rate Pressure Product (RPP = LVDevP x HR) were all documented in Langendorff mode.

4.2.1 Baseline function of hearts

As indicated in Table 1, none of the baseline functional parameters differed between the four experimental groups investigated.

Table 4.1: **Table showing all baseline functional data for control and creatine supplemented, sedentary or exercised groups. (n=15)**

	Work Heart						Langendorff	
	AO (ml/min)	**CF (ml/min)**	**CO (ml/min)**	**aorticDP (mmHg)**	**aorticSP (mmHg)**	**HR (bpm)**	**LVDevP (mmHg)**	**RPP**
Control Sedentary	40.27 ± 2.13	17.2 ± 1.05	57.47 ± 2.7	33.92 ± 2.64	65.69 ± 3.45	263 ± 15.58	43.4 ± 5.19	12305 ± 1311
Creatine Sedentary	44.00 ± 1.97	17.33 ± 0.58	61.33 ± 2.04	35.17 ± 2.23	69.42 ± 2.56	280 ±10.69	48.2 ± 10.82	14064 ± 3012
Control Exercised	36.46 ± 3.50	21.54 ± 1.93	58.00 ± 4.40	39.83 ± 1.47	75.33 ± 1.95	270 ±12.12	46.3 ± 6.01	13587 ± 1736
Creatine Exercised	39.86 ± 1.76	17.21 ± 0.93	57.07 ± 2.1	38.83 ± 1.13	75.67 ± 1.73	273 ± 9.24	49.3 ± 8.23	14767 ± 2759

4.2.2 Ischaemia/ reperfusion

The hearts were subjected to total global ischaemia, as described in Chapter 3, for 20 min and reperfused for 30 min. The function of the heart was measured before ischaemia (See Table 1), and after 30 minutes of reperfusion.

4.2.2.1 Functional recovery after global ischaemia

The reperfusion aortic output was measured and expressed as a percentage of the pre-ischaemic value. This was calculated with the following formula: post-ischaemic aortic output/ pre-ischaemic aortic output multiplied by one hundred. CO recovery, and cardiac work recovery (CW= CO x SP) was also calculated.

When Aortic output recovery was analysed with a 2 way ANOVA, exercise was seen to show a significant decrease in recovery ($F = 20.76$, $p < 0.001$). Creatine also had a significant adverse effect ($F = 4.84$, $p = 0.03$). C Sed rats aortic output recovery was 55.5 ± 4.5% in comparison with the hearts from Cr Sed rats which had significantly reduced AO recoveries of 33.5 ± 4.5% ($p<0.01$). C Ex rats had AO recoveries of 26.1 ± 5.9% which was significantly decreased from that of C Sed ($p<0.05$). Creatine and exercise proved an unfavorable combination as their AO recovery was 18.2 ± 6.2% which was significantly lower than hearts from C Sed rats ($p<0.001$). See Figure 4.11.

In Langendorff mode with the balloon inserted into the left ventricle RPP recovery was calculated, as a percentage of post ischaemic RPP divided by pre ischaemic RPP. Systolic pressure (SP) recovery was also calculated.

RPP recovery showed significance with a one way ANOVA (F=3.36, p=0.03). C Sed rats RPP recovery was 88.1± 7.3 %, in Cr Sed rats it was 72.9 ± 7.9%, and in C Ex rats 58.4 ± 10.7. Cr Ex rat hearts had RPP recoveries that were significantly decreased below C Sed to 49.7 ± 11.1% ($p < 0.05$). See Figure 4.12. This was also seen in CO recoveries, with the Cr Ex hearts (39.9 ± 4.3%) recovering worse than the C Sed hearts (58.9 ± 4.1%) ($p < 0.05$), but Cr Sed (47.1 ± 4.6 %) and C Ex (49.4 ± 5.2%) being no different from C Sed recoveries

Table 4.2: **Table showing recoveries of SP, CO and CW from all groups of rats. n = 12 * = p<0.05 vs C Sed.**

	SP recoveries (%)	LVDevP recoveries (%)	CO recovery (%)	CW recovery (%)
Control Sedentary	66.8 ± 10.7	92.5 ± 8.5	58.9 ± 4.1	41.3 ± 7.7
Creatine Sedentary	69.8 ± 9.7	90.1 ± 9.3	47.1 ± 4.6	34.9 ± 5.7
Control Exercised	73.5 ± 6.9	70.8 ± 11.6	49.4 ± 5.2	40.0 ± 4.6
Creatine Exercised	77.9 ± 2.8	65.3 ± 12.1	39.9 ± 4.3 *	33.25 ± 4.5

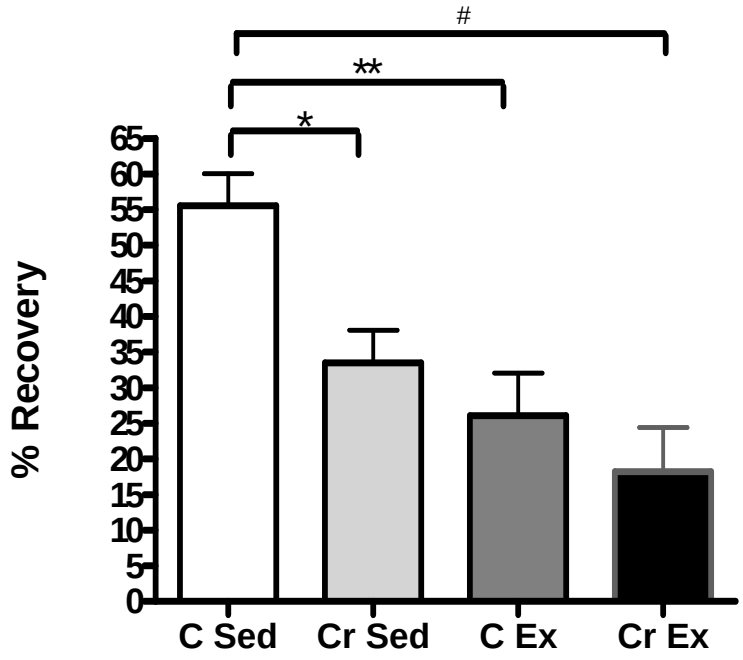


Figure 4.11: **Aortic output recovery of hearts from different groups perfused in the working heart mode. Differences shown are 2 way ANOVA Bonferroni post hoc differences. n = 15 per group. * p < 0.01, ** p < 0.05, # p < 0.001.**

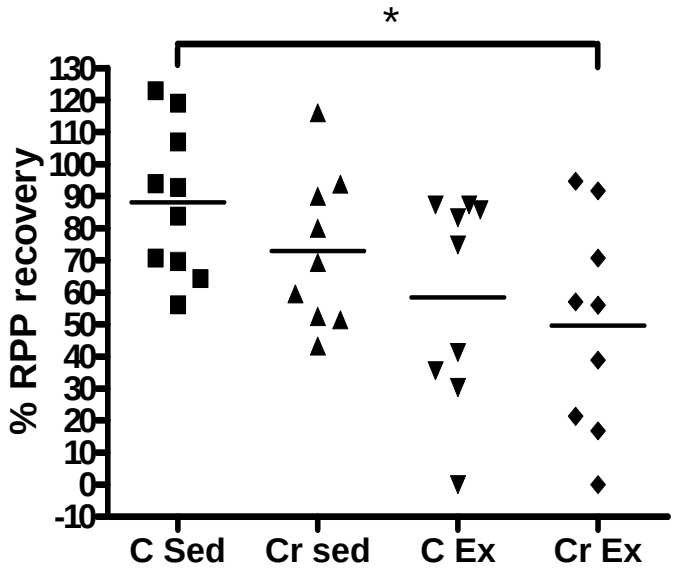


Figure 4.12: **Rate pressure product (RPP) recoveries in Langendorff mode. Differences shown are one way ANOVA Bonferroni post hoc differences. n = 15 per group. * p < 0.05.**

4.2.2.2 Ischaemic contracture during global ischaemia

In another set of experiments a balloon was inserted into the left ventricle through the left atrium and the heart was retrogradely perfused (Langendorff mode). This balloon was connected to a pressure transducer, and the left ventricular pressure during normoxia and ischaemia measured.

Hearts from the Cr Ex group showed significantly higher peak ischaemic contracture development (31.6 ± 4.7 mmHg) compared to the C Sed group(10.4 ± 4.2 mmHg) ($p < 0.05$). Cr Sed (22 ± 6.7 mmHg) and C Ex hearts (23.8 ± 5.8 mmHg) did not differ significantly from C Sed hearts. See Figure 4.13.

The time taken from the onset of ischaemia, for the pressure in the left ventricle to start rising, was measured and referred to as the time to onset of ischaemic contracture.

There was no difference in the time to onset of contracture with C Sed (17.9 ± 0.9 min), Cr Sed (17.7 ± 0.8 min), C Ex (17.1 ± 0.8 min) and Cr Ex (15.6 ± 0.7 min) taking the same time to go into contracture. See Figure 4.14.

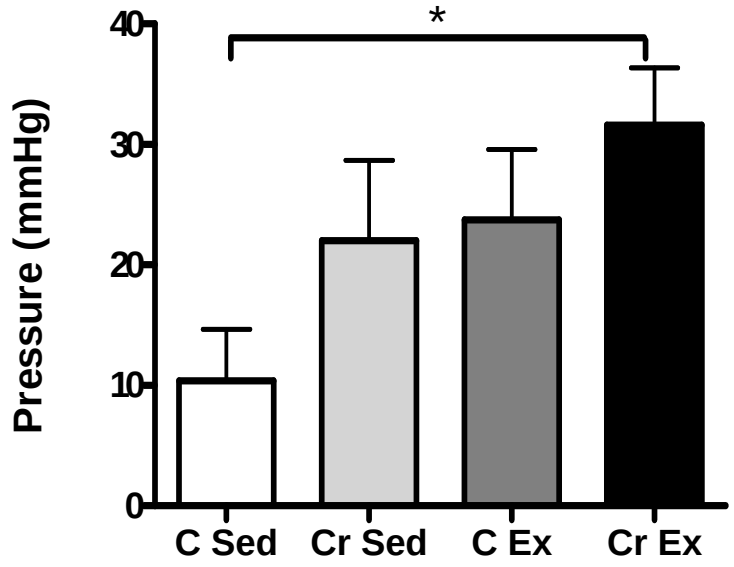


Figure 4.13: Peak pressure development during ischaemic contracture in hearts from C Sed, Cr Sed, C Ex and Cr Ex groups. Differences shown are one way ANOVA Bonferroni post hoc differences. * $p < 0.05$ n = 8 per group.

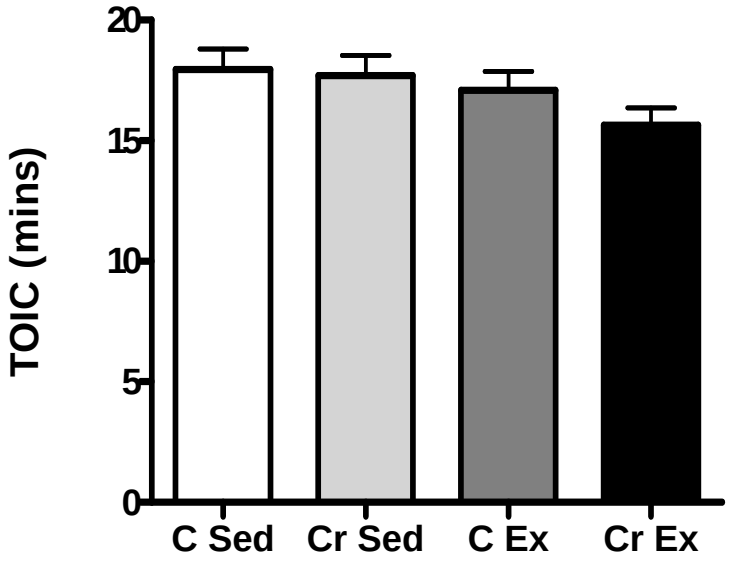


Figure 4.14: Time to onset of ischaemic contracture (TOIC) in hearts from C Sed, Cr Sed, C Ex and Cr Ex groups. n = 8 per group.

A

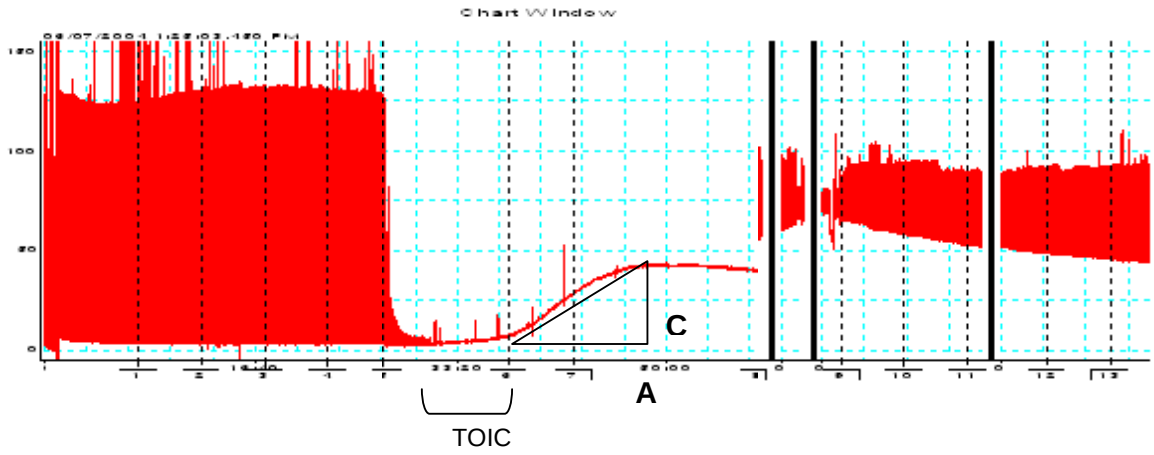


Figure 4.15: Graph depicting the development of ischaemic contracture in isolated balloon- perfused hearts [Lopez et al 2007]. Time to onset of ischaemic contracture (TOIC) is the time taken from the start of ischaemia until a rise in pressure of 4mmHg. A = time to peak pressure, C = Peak developed pressure.

4.2.2.3 Damage after regional ischaemia

Infarct size

After a 30 minutes equilibration perfusion, the left anterior descending coronary artery was ligated to induce 30 minutes of regional ischaemia. The area was then reperfused by untying the ligation. After 30 minutes reperfusion the ligation was tied again and the heart stained for infarct size determination as described in Chapter 3.2.3. The infarct size was expressed as percentage of the area at risk which did not differ between groups and was 40 ± 10%.

C Sed hearts had an infarct size of 25.1 ± 3.5% which was no different to the infarcts in Cr Sed animals (29.9 ± 3.0%), C Ex animals (26.3 ± 4.8%) or Cr Ex animals (30.8 ± 4.3%). See Figure 4.16.

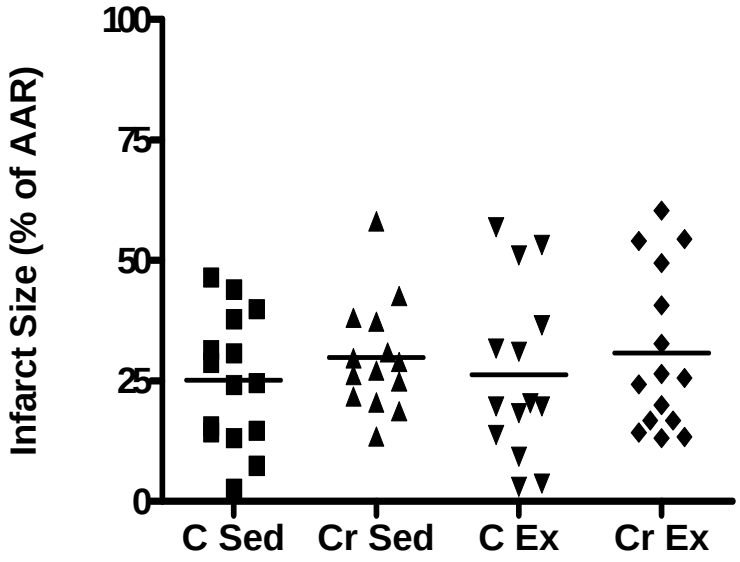


Figure 4.16: Infarct size as percentage of the area at risk (AAR) in C Sed, Cr Sed, C Ex and Cr Ex rats. n = 15 per group.

Summary of key findings

Our infarct size and reperfusion function data suggest that dietary creatine supplementation did not protect hearts against ischaemia/reperfusion injury. Neither functional recovery nor infarct size was improved by creatine supplementationin in our rat model. Creatine supplementation appeared to be detrimental to the ischaemic heart as hearts from both control and exercised animals had poorer functional recoveries after ischaemia and more severe ischaemic contracture than their controls.

4.3 HIGH ENERGY PHOSPHATES

Blood and heart tissue were analyzed to determine whether the high energy phosphates (AMP, ADP, ATP, CrP) and creatine levels were altered by exercise training or creatine supplementation.

Hearts were freeze clamped after 2 minutes of perfusion to wash out the blood, and blood collected from the thoracic cavity and separated by centrifugation into red blood cells and plasma. Tissue and blood samples were then extracted as described in Chapter 3.4 and analyzed by reverse phase HPLC.

4.3.1 Blood

4.3.1.1 Red blood cells

To elucidate what the distribution of HEPs were within the blood, red blood cells (RBCs) and plasma were separated and then analysed.

RBC ATP content

The ATP concentration in the red blood cells of Cr Sed group (781.1 ± 58.82 nmol/gram wet weight (gww)) was higher than C Sed rats (320.3 ± 76.9 nmol/gww, $p<0.05$). The ATP concentration in the red blood cells of the C Ex (405.1 ± 64.7 nmol/gww) and Cr Ex group (446.0 ± 51.8 nmol/gww) were 25 and 40% higher respectively than C Sed levels. However they were both significantly lower than the Cr Sed group ($p<0.01$). See Figure 4.17.

RBC creatine content

As with the ATP concentration, the creatine content of the red blood cells increased in the Cr Sed group vs C Sed (74.9 ± 3.1 vs 94.1 ± 5.5 nmol/gww, $p<0.05$) but no increase was seen between the C Ex or Cr Ex groups (55.7 ± 3.6 vs 53.9 ± 2.84nmol/gww). However these values were significantly less than the Cr Sed group ($p<0.01$). See Figure 4.18.

RBC phosphocreatine content

No differences were seen between the phosphocreatine concentrations in the red blood cells in the 4 groups. C Sed levels were 39.8 ± 20.4 nmol/gww compared to Cr Sed levels of 44.9 ± 16.9 nmol/gww, C Ex levels of 37.3 ± 9.6 nmol/gww and Cr Ex levels of 36.7 ± 4.5 nmol/gww. See Figure 4.19.

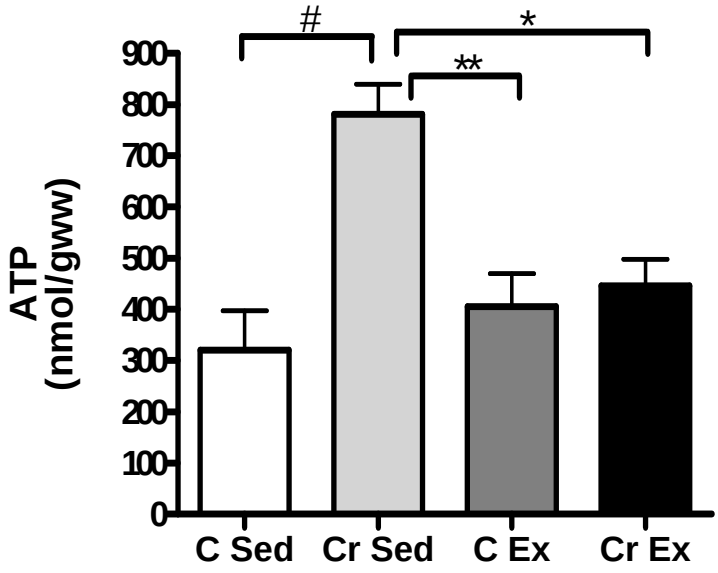


Figure 4.17: ATP concentration in the red blood cells (RBCs) from C Sed, Cr Sed, C Ex and Cr Ex rats. Differences shown are one way ANOVA with Bonferroni post hoc test. n = 10 per group. * p < 0.05; ** p < 0.01, # **p < 0.001**

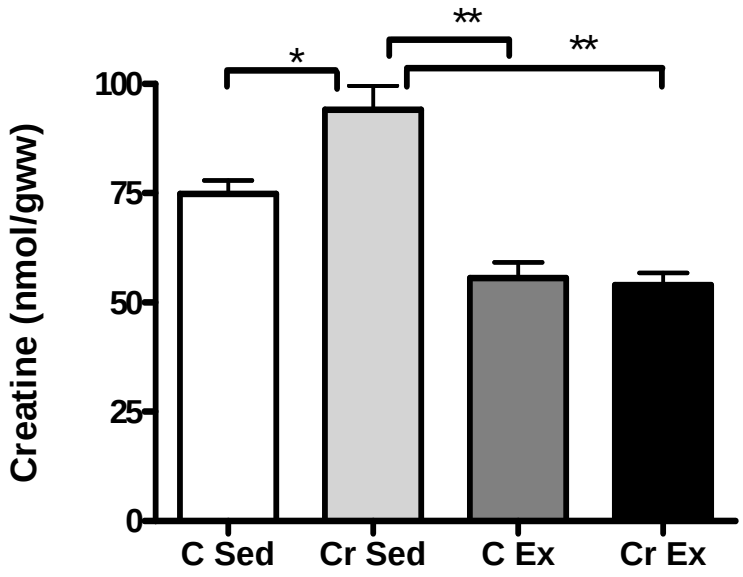


Figure 4.18: **Creatine concentration in the red blood cells (RBC's) in blood from the experimental groups of rats. Differences shown are one way ANOVA with Bonferroni post hoc test. n = 10 per group. * p < 0.05; ** p < 0.001**

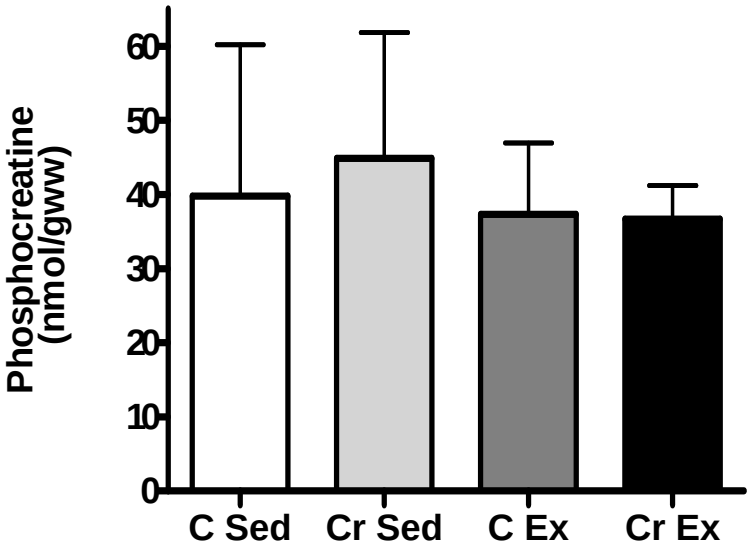


Figure 4.19: **Phosphocreatine concentration in the red blood cells (RBCs) from C Sed, Cr Sed, C Ex and Cr Ex rats. n = 10 per group. * p < 0.05; ** p < 0.001**

4.3.1.2 Blood plasma

Plasma creatine content

No differences were found in the levels of creatine in the blood plasma in the respective groups. Plasma creatine levels in C Sed (93.74 ± 11.05 nmol/gww), Cr Sed (92.93 ± 13.41 nmol/gww), C Ex (58.63 ± 9.28 nmol/gww) and Cr Ex (79.17 ± 9.05 nmol/gww) groups were not significantly different from each other. See Figure 4.20.

Plasma phosphocreatine content

Phosphocreatine levels in the blood plasma of C Sed rats were 8.2 ± 4.4 nmol/gww. These were significantly lower than phosphocreatine levels in Cr Sed rats, which were 49.5 ± 15.4 nmol/gww. However phosphocreatine levels were no different between C Ex (28.1 ± 5.1 nmol/gww), Cr Ex (36.2 ± 8.5 nmol/gww) and C Sed rats. See Figure 4.21.

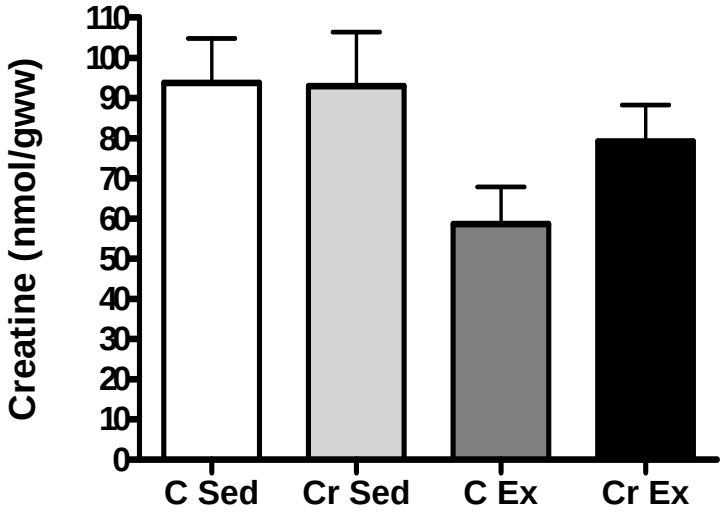


Figure 4.20: **Creatine concentration in the blood plasma from C Sed, Cr Sed, C Ex and Cr Ex rats. n = 10 per group**

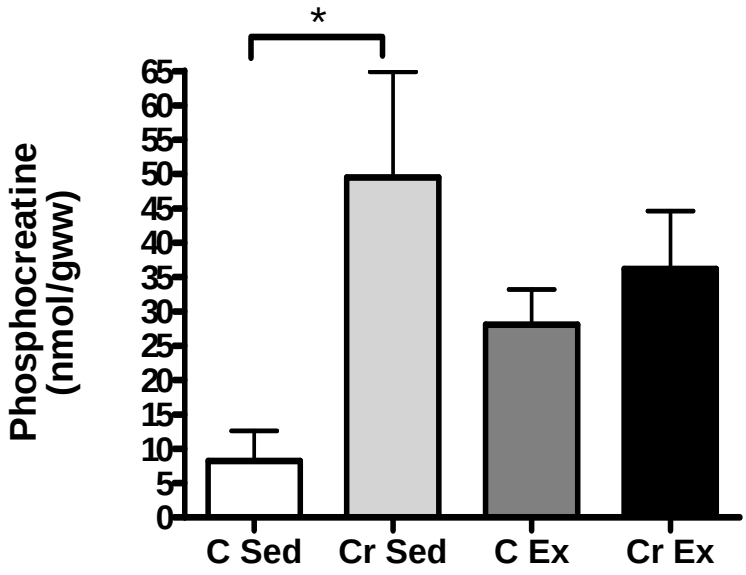


Figure 4.21: **Phosphocreatine concentration in the blood plasma from C Sed, Cr Sed, C Ex and Cr Ex rats. . Differences shown are one way ANOVA with Bonferroni post hoc test. p = 0.03. n = 10, per group. * p < 0.05**

4.3.2 Heart tissue

The ATP, phosphocreatine, creatine and total creatine content of the heart tissue did not differ between any of the groups investigated. (ATP concentrations were: C Sed 4308 ± 205.5 nmol/gww; Cr Sed 4928 ± 103.7 nmol/gww; C Ex 4541 ± 257.9 nmol/gww; Cr Ex 4628 ± 140.5 nmol/gww) (Creatine concentrations were: C Sed 1477 ± 130.7 nmol/gww; Cr Sed 1554 ± 37.6 nmol/gww; C Ex 1513 ± 193.3 nmol/gww; Cr Ex 1510 ± 90.4 nmol/gww) (Phosphocreatine concentrations were: C Sed 4531 ± 342.8 nmol/gww; Cr Sed 5343 ± 159.9 nmol/gww; C Ex 4971 ± 380.4 nmol/gww; Cr Ex 4906 ± 217.2 nmol/gww). (Total creatine: C Sed 6009± 405.6 nmol/gww, Cr Sed 6897 ± 138.4 nmol/gww, C Ex 6485 ± 539.3 nmol/gww and Cr Ex 6318 ± 244.8 nmol/gww) See Figures 4.22, 4.23 and 4.24.

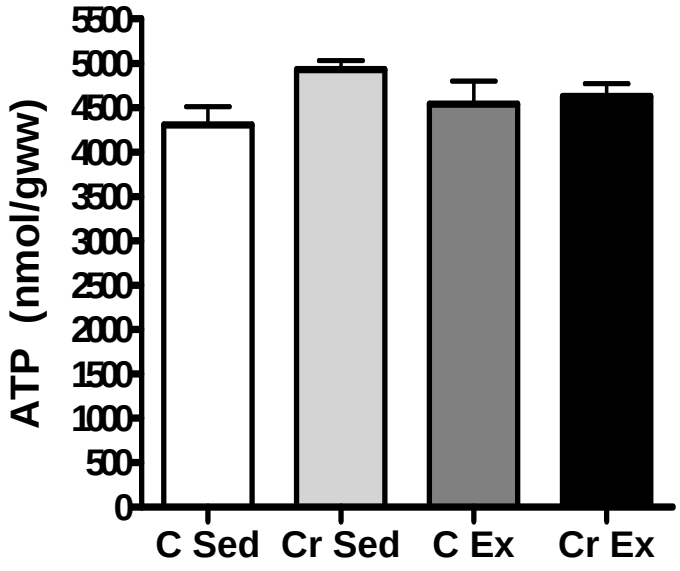


Figure 4.22: ATP concentration in the heart tissue from C Sed, Cr Sed, C Ex and Cr Ex rats. n = 5 per group.

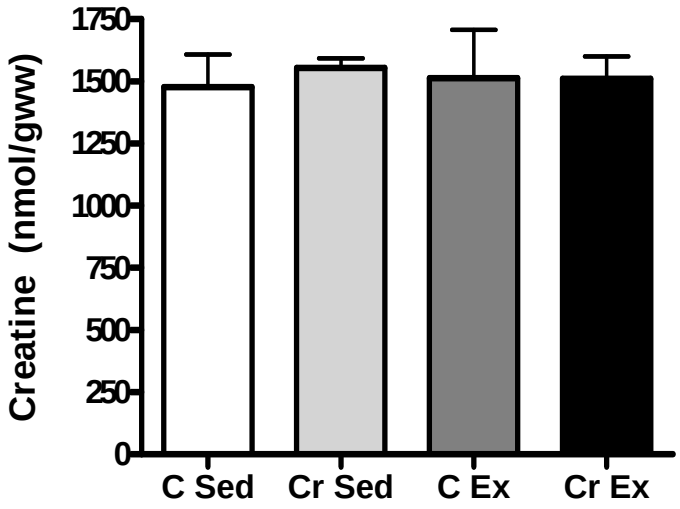


Figure 4.23: Creatine concentration in the heart tissue from C Sed, Cr Sed, C Ex and Cr Ex rats. n = 5 per group.

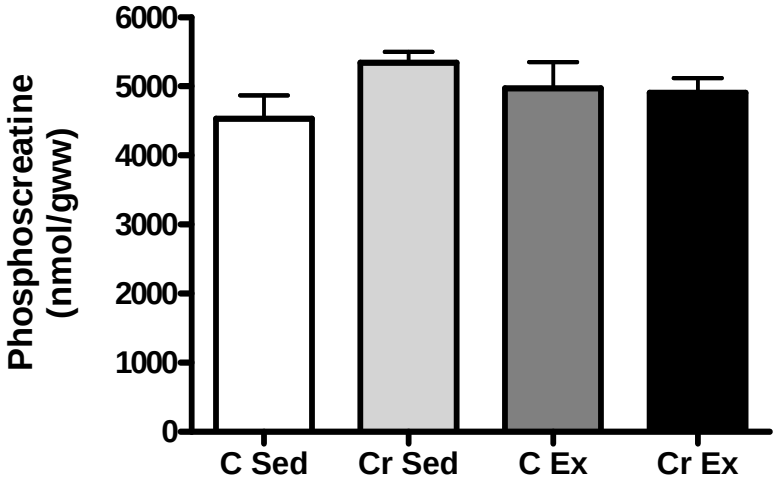


Figure 4.24: Phosphocreatine concentration in the heart tissue from C Sed, Cr Sed, C Ex and Cr Ex rats. n = 5 per group.

4.3.3 HEP ratios in heart tissue

The high energy phosphate ratios, particularly the PCr/ATP ratio, indicate how much energy reserve the heart has, and is an indication of the efficiency of the heart to replenish energy stores. Decreases in PCr/ATP ratios are often an indication of heart failure [Neubauer et al 1995].

4.3.3.1 PCr/ATP

The PCr/ATP ratios for all groups were similar to each other, with the ratios being 1.054 ±0.07 for C Sed hearts, 1.085 ± 0.03 for Cr Sed hearts, 1.094 ± 0.05 for C Ex hearts and 1.037 ± 0.03 for Cr Ex hearts.

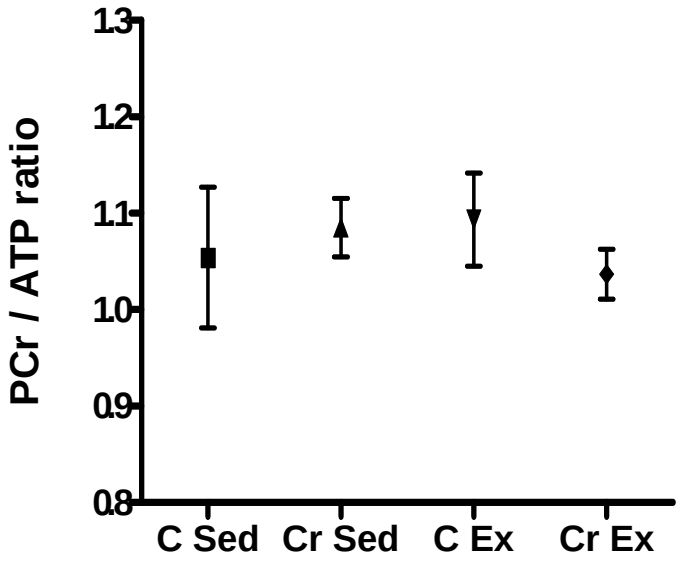


Figure 4.25: PCr/ATP ratios in the heart tissue from C Sed, Cr Sed, C Ex and Cr Ex rats. n = 5 per group.

4.3.3.2 ATP/AMP

The ATP/AMP ratios showed no significant differences between groups. C Sed (14.1 ± 1.1), Cr Sed (16.9 ± 1.5), C Ex (14.0 ± 2.7) and Cr Ex (15.9 ± 4.8) all showed similar ratios. See Figure 4.26.

4.3.3.3 ATP/ADP

Similarly, ATP/ADP ratios were no different between groups, C Sed (3.7±0.26) Cr Sed (4.4±0.22) C Ex (4.3±0.15) and Cr Ex (4.1±0.16) hearts ATP/ADP ratios showed no differences. See Figure 4.27.

4.3.3.3 PCr/Cr

The PCr/Cr ratios were similar for the C Sed (3.1 ± 0.3), Cr Sed (3.5 ± 0.2), C Ex (3.4 ± 0.3) and Cr Ex (3.2 ± 0.2) hearts. They were all within the same range of values. See Figure 4.28.

4.3.3.4 PCr/TCr

The PCr/TCr ratios were no different between groups, C Sed (0.75 ±0.02), Cr Sed (0.77 ± 0.01), C Ex (0.77 ± 0.02) and Cr Ex (0.76 ± 0.01). See Figure 4.29.

Thus there were no differences in the HEP ratios between any of the groups investigated.

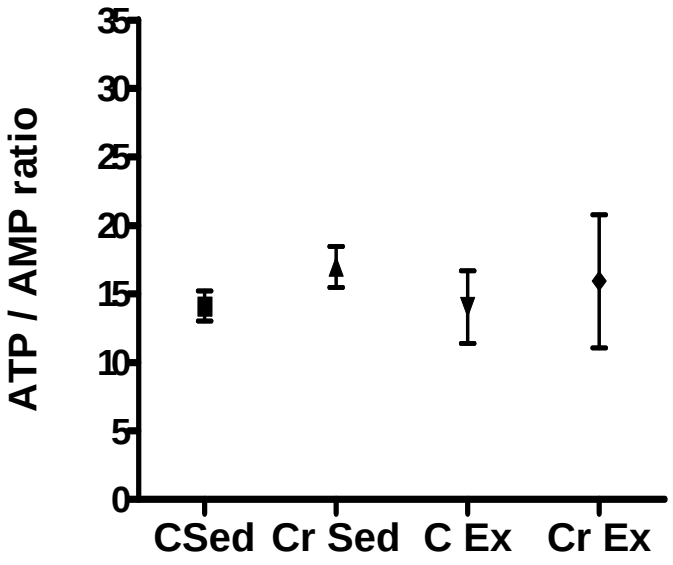


Figure 4.26: ATP/AMP ratios in the heart tissue from C Sed, Cr Sed, C Ex and Cr Ex rats. n = 5 per group.

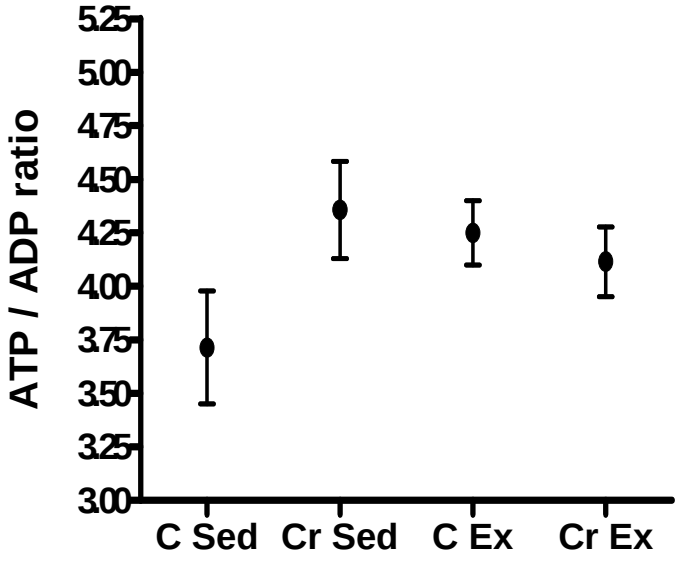


Figure 4.27: ATP/ADP ratios in the heart tissue from C Sed, Cr Sed, C Ex and Cr Ex rats. n = 5 per group.

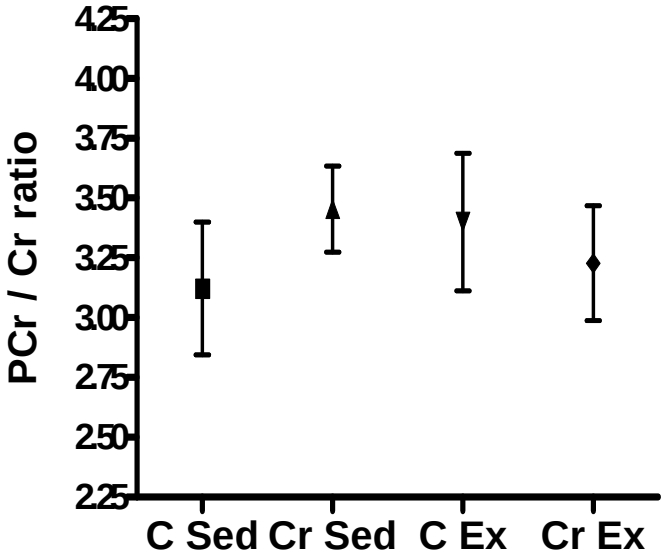


Figure 4.28: **PCr/Cr ratios in the heart tissue from C Sed, Cr Sed, C Ex and Cr Ex rats. n = 5 per group.**

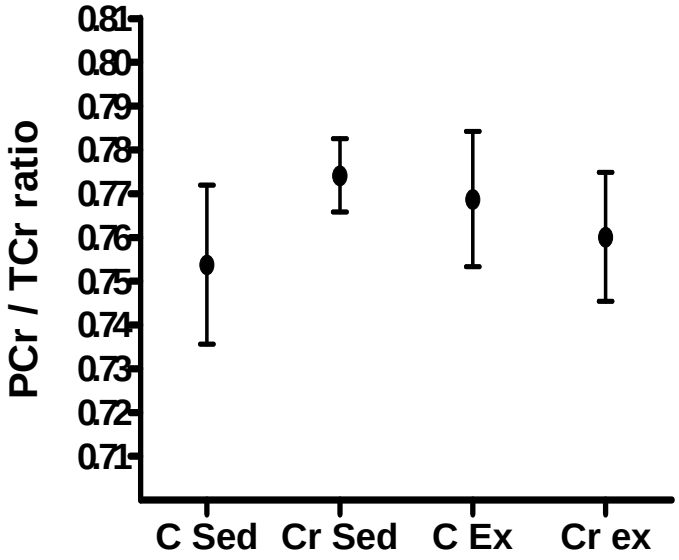


Figure 4.29: **PCr/TCr ratios in the heart tissue from C Sed, Cr Sed, C Ex and Cr Ex rats. n = 5 per group.**

Summary of key findings

Creatine supplementation elevated ATP and creatine levels in the RBCs of sedentary animals but not exercised animals. Creatine supplementation also resulted in high plasma PCr levels. An effect that was also lost with exercise.

In heart tissue neither creatine supplementation nor exercise significantly affected the myocardial levels of HEP or their ratios to one another.

4.4 MITOCHONDRIAL FUNCTION

Mitochondria are the energy centre of the cell. We therefore tested their basal function and function after being given different substrates or after being exposed to anoxia.

Mitochondria were isolated from hearts from the different groups of animals, and suspended in incubation buffer. Their baseline respiration was measured, as well as their respiration when given glutamate as a substrate. They were subsequently given ADP and their state 3 and 4 respiration measured.

Different aliquots from the same mitochondrial sample were subjected to anoxia and re-oxygenation and their function was subsequently measured. In addition, other aliquots were given succinate, GDP and oligomycin and their state 3 and 4 respirations monitored. This was to determine the effect of these inhibitory compounds on respiration rate and the efficiency of complex I and II of the mitochondrial respiratory chain as well as possible leaking of hydrogen back into the mitochondria as described in Chapter 3.5.2.

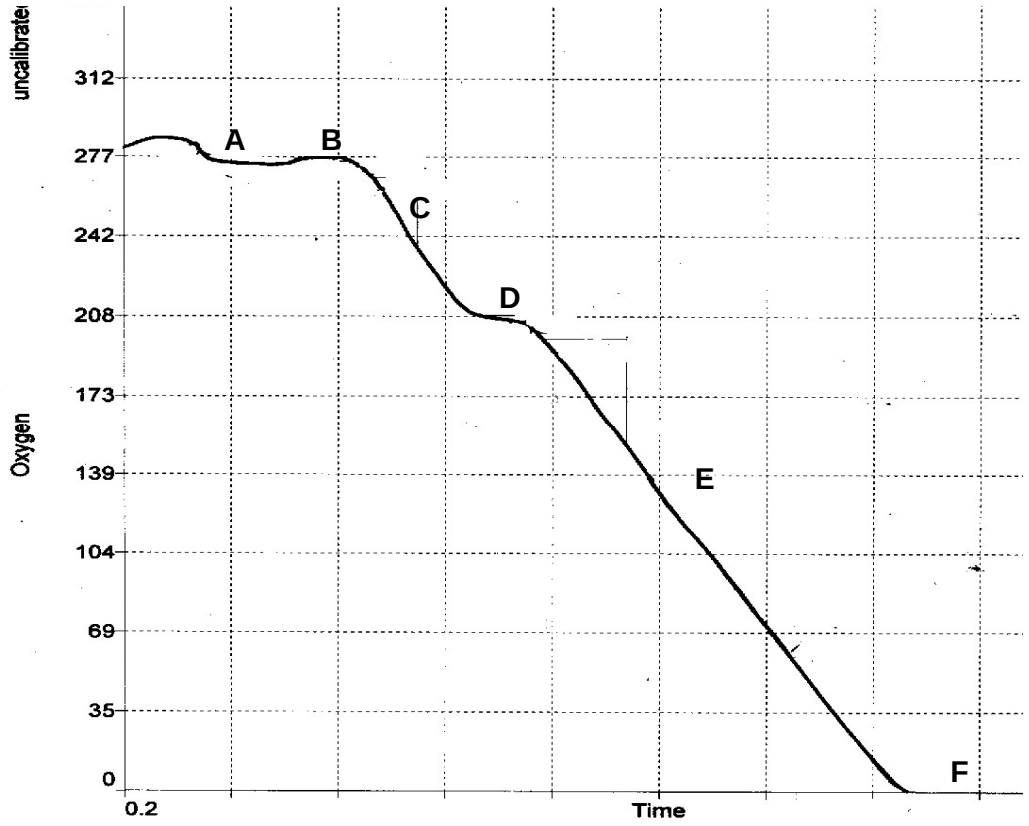


Figure 4.30: **Representation of an oxygen consumption curve. A: state 1 respiration, mitochondrial basal respiration, B: state 2 respiration, in the presence of substrate. C: state 3 respiration, and D: state 4 respiration. E: state 3 in the presence of excess ADP (100nM) to induce anoxia (F).**

4.4.1 Respiration states

Once a substrate (i.e. glutamate or succinate) and ADP is added to the mitochondrial suspension, oxygen is used by these mitochondria to produce ATP, and state 3 respiration is defined as ADP-stimulated respiration of a given substrate. State 4 respiration is oxygen consumption in the absence of ADP or any metabolic poisons or inhibitors [Caprette 2005].

4.4.1.1 State 1 respiration

The basal amount of oxygen consumed by the mitochondria (state 1) showed no significant differences between the groups studied. C Sed rat heart mitochondria had a basal oxygen consumption of 9.3 ± 2.3 natoms O_2/mg protein/min while Cr Sed mitochondria used 9.3 ± 2.2 natoms O_2/mg protein/min. C Ex (10.2 ± 2.4 natoms O_2/mg protein/min) and Cr Ex (8.9 ± 1.6 natoms O_2/mg protein/min) were no different from control values. See figure 4.31.

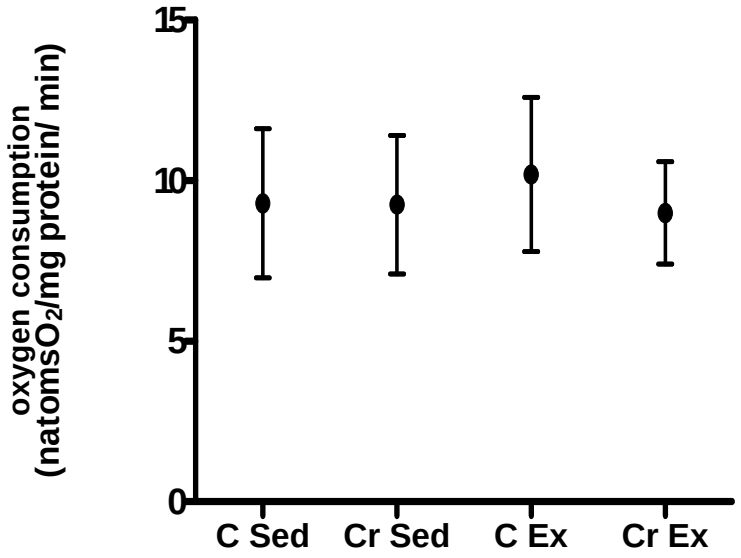


Figure 4.31: Baseline oxygen consumption of mitochondria isolated from C Sed, Cr Sed, C Ex and Cr Ex rats. n = 10 per group.

4.4.1.2 State 2 respiration

State 2 respiration is the respiration of the mitochondria when a substrate (either carbohydrate or fatty acid, in this case glutamate or succinate) is added.

Glutamate as substrate

C Sed rat heart mitochondrial oxygen consumption during state 2 respiration with glutamate was 5.1 ± 1.4 natoms/mg/min, Cr Sed oxygen consumption was 5.0 ± 1.6 natoms O_2/mg protein/min, while C Ex oxygen consumption was 6.1 ± 1.2 natoms O_2/mg protein/min and Cr Ex oxygen consumption was 5.7 ± 1.8 natoms O_2/mg protein/min. See Table 4.3.

Succinate as substrate

C Sed heart mitochondrial oxygen consumption during state 2 respiration with succinate was 81.7 ± 6.6 natoms O_2/mg protein/min, Cr Sed mitochondrial oxygen consumption was 67.3 ± 4.0 natoms O_2/mg protein/min, while C Ex mitochondrial oxygen consumption was 81.9 ± 8.4 natoms O_2/mg protein/min and Cr Ex mitochondrial oxygen consumption was 78.9 ± 5.0 natoms O_2/mg protein/min. See Table 4.3.

4.4.1.3 State 3 respiration

State 3 respiration is ADP-stimulated respiration and is the actively respiring state of the mitochondria, with the use of oxygen and substrate (in our study, glutamate or succinate), and the concurrent production on ATP.

Glutamate as substrate

The state 3 respiration rates were as indicated below in Table 4.3, with no significant differences were seen between any of the groups. Mitochondrial oxygen consumptions were as follows: C Sed: 73.3 ± 5.4 natoms O_2/mg protein/min, Cr Sed: 77.5 ± 4.8 natoms O_2/mg protein/min, C Ex: 91.6 ± 5.4 natoms O_2/mg protein/min and Cr Ex: 78.9 ± 4.3 natoms O_2/mg protein/min.

Succinate as substrate

State 3 respiration rates were as indicated in Table 4.3 and no significant differences were seen between any of the groups: C Sed: 104.7 ± 9.0 natoms O_2/mg protein/min, Cr Sed: 87.1 ± 7.2 natoms O_2/mg protein/min, C Ex: 111.9 ± 8.4 natoms O_2/mg protein/min and Cr Ex: 106.6 ± 7.9 natoms O_2/mg protein/min.

4.4.1.4 State 4 respiration

State 4 is reached when ADP is depleted and no more ATP can be produced, so that a state of uncoupled respiration ensues. The oxygen that is used is uncoupled from ATP production.

Glutamate as substrate

State 4 respiration was also similar for all groups investigated. Mitochondrial oxygen consumption was as follows: C Sed: 8.7 ± 1.0 natoms O_2/mg protein/min, Cr Sed: 9.8 ±

0.8 natoms O_2/mg protein/min, C Ex 10.6 ± 1.4 natoms O_2/mg protein/min and Cr Ex: 8.5 ± 1.4 natoms O_2/mg protein/min.

Succinate as substrate

The state 4 respirations of succinate stimulated mitochondria were not measured.

Table 4.3 Table to show the oxygen consumption rate (nmolO_2/mg protein/min) with glutamate and succinate in State 2, 3 and 4 in the 4 experimental groups.

	State 1		State 2	State 3	State 4
Control Sedentary	9.3 ± 2.3	Glutamate	5.1± 1.4	73.3 ± 5.4	8.7± 1.0
		Succinate	81.8 ± 6.6	104.7 ± 9.0	
Creatine Sedentary	9.3 ± 2.2	Glutamate	5.0 ± 1.6	77.5 ± 4.8	9.8 ± 0.8
		Succinate	67.3 ± 4.0	87.1 ± 7.2	
Control Exercise	10.2 ± 2.4	Glutamate	6.1 ± 1.2	91.6 ± 5.4	10.6 ± 1.4
		Succinate	81.9 ± 8.4	111.9 ± 8.4	
Creatine Exercise	8.9 ± 1.6	Glutamate	5.7 ± 1.8	78.9 ± 4.3	8.5 ± 1.4
		Succinate	78.9 ± 5.0	106.6 ± 7.9	

4.4.2 Respiratory control index (RCI)

The Respiratory control index (RCI) is a measure of mitochondrial respiration rate and efficiency of oxygen usage, and is a ratio of the state 3 respiration rate over the state 4 respiration rate. Glutamate was added to the mitochondria and this was followed by 350uM of ADP. The state 3 and state 4 respiration rates were measured and then RCI calculated.

C Sed mitochondrial RCI of 8.49 ±0.6 was not increased by creatine supplementation (8.8 ± 0.9). Exercise increased the RCI (C Ex: 11.7 ± 1.7), but the combination of exercise and creatine had no effect (9.5 ± 0.7). See Figure 4.32.

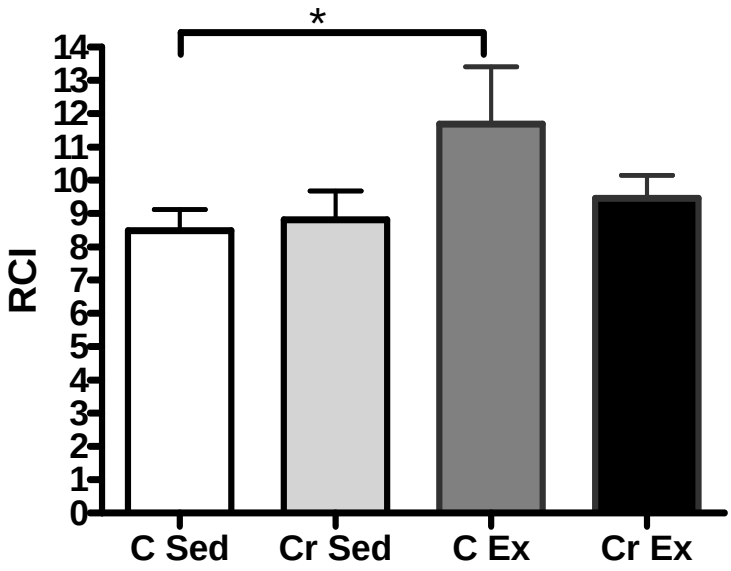


Figure 4.32: Respiratory control index (RCI = state 3 / state 4 respiration) of mitochondria during glutamate respiration. n = 10 per group. * p<0.05 (unpaired Students t-test)

4.4.2.5 Inhibitors of respiration

Oligomycin

Oligomycin inactivates ATP synthase so that it blocks the proton channel thus inhibiting oxidative phosphorylation. Experimentally, oligomycin has no effect on state 4 respiration or electron transport, but it completely prevents state 3 respiration. Thus by adding oligomycin basal proton leak can be measured, as any oxygen used is a result of protons entering the mitochondria independently of complex IV.

C Sed mitochondria had a 5.4 ± 23.2 % decrease in respiration, while Cr Sed had an increase in respiration of 5.6 ± 15.2 %. C Ex had a 9.3 ± 12.9% increase in respiration whereas Cr Ex had a 0.3 ± 5.8% decrease. Neither of these changes was significantly different from each other. See Figure 4.33.

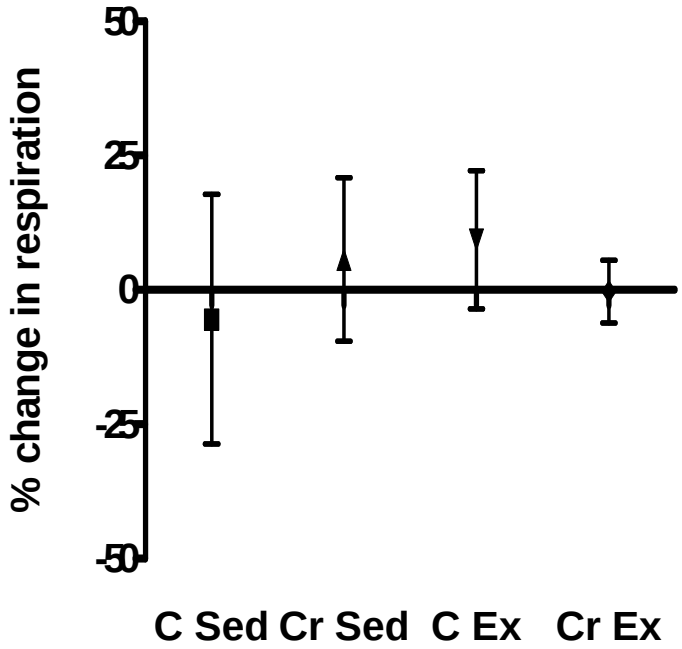


Figure 4.33: Percentage change in state 4 respiration with the addition of oligomycin in glutamate fueled mitochondria as a measure of basal proton leak. n=10.

GDP

GDP inhibits the uncoupling proteins (UCP's)\. By inhibiting the UCPs we aimed to establish whether there is a change in state 3 respiration. If there is, the greater the decrease in state 3 respiration, the more involved the UCPs are, with the percentage change being proportional to the degree of involvement of these UCPs.

C Sed mitochondria had a 10.3 ± 1.8 % decrease in respiration, while Cr Sed had a decrease in respiration of 15.5 ± 1.7 %. C Ex had a 14.9 ± 1.5% decrease in respiration whereas Cr Ex had a 16.6 ± 1.8% decrease. These changes are however not significantly different. See Figure 4.34.

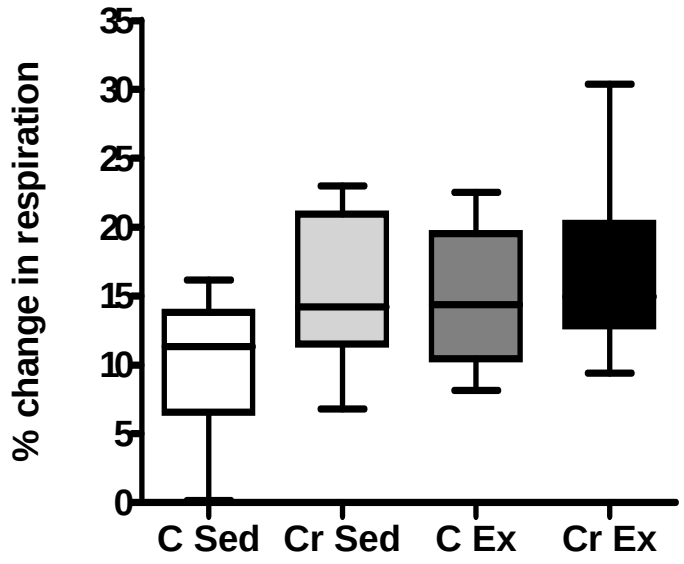


Figure 4.34: Percentage decrease in state 3 respiration in response to oligomycin inhibited respiration with addition of GDP in succinate fueled mitochondria as a measure of UCP involvement. n=10.

4.4.3 ADP/O ratio

This ratio illustrates the relationship between ATP synthesis and oxygen consumption.

Glutamate as substrate

ADP/O ratios were as follows: C Sed: 2.89 ± 0.09, Cr Sed: 2.85 ± 0.12, C Ex: 2.77 ± 0.18 and Cr Ex: 3.03 ± 0.11. These respiration rates were no different from each other. See Figure 4.35.

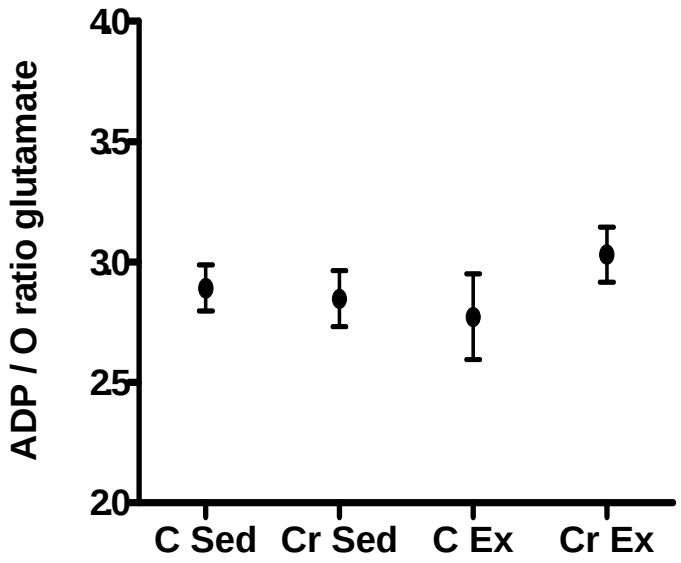


Figure 4.35: ADP/ O ratio of mitochondria from the 4 experimental groups during glutamate oxidation. n = 10 per group.

Succinate as substrate

The ADP/ O ratios for succinate were not determined.

4.4.4 Recovery after anoxia / reoxygenation

Mitochondria were given 100mM ADP and allowed to use up all the oxygen in the chamber. They were then sealed and left anoxic for 20 minutes. The mitochondria were subsequently reoxygenated by bubbling air through the chamber with a squeeze pipette.

After being subjected to anoxia and reoxygenation, mitochondria from C Sed rats recovered 20.95 ± 4.97 % of their initial state 3 respiration as compared to Cr Sed mitochondria which recovered 30.98 ± 8.7 %. C Ex mitochondria recovered 20 ± 3.9 % and Cr Ex mitochondria 24.81 ± 6.6 %. There were no significant differences in post-anoxic oxygen consumption. See Figure 4.36.

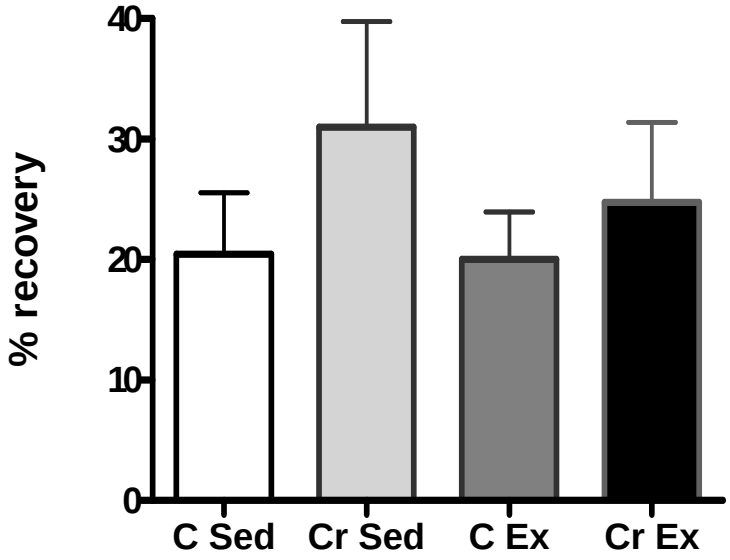


Figure 4.36: Percentage recovery of mitochondrial state 3 respiration after anoxia and re-oxygenation. n = 10 per group.

Summary of key findings

The RCI of mitochondria from exercised animals was significantly elevated when compared to control sedentary animals. This increase in RCI was however lost with creatine supplementation.

4.5 SIGNALLING PATHWAYS IN THE HEART

(i) To obtain myocardial tissue samples for baseline (pre-ischaemic) protein expression and activation determinations, heart were perfused for two minutes after excision from the animal and freeze clamped for later Western blot analysis

(ii) Separate hearts were subjected to 20 minutes of global ischaemia, and freeze clamped without reperfusion.

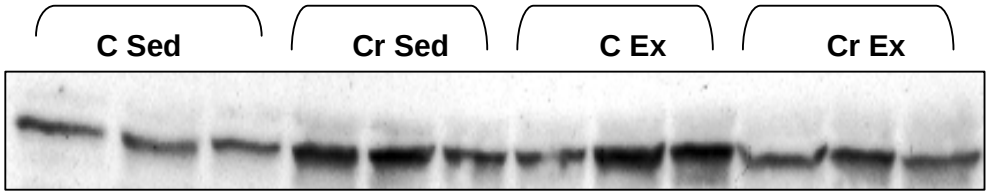
(iii) A third series of hearts were perfused, subjected to 20 minutes of global ischaemia and after 10 minutes of retrograde reperfusion, hearts were freeze clamped and the protein levels measured.

(iv) In a last series of experiments, hearts were perfused for half an hour, made globally ischaemic for 20 minutes and then reperfused for 30 minutes, and freeze-clamped at the end of 30 minutes of reperfusion.

See Chapter 3.6 for more details.

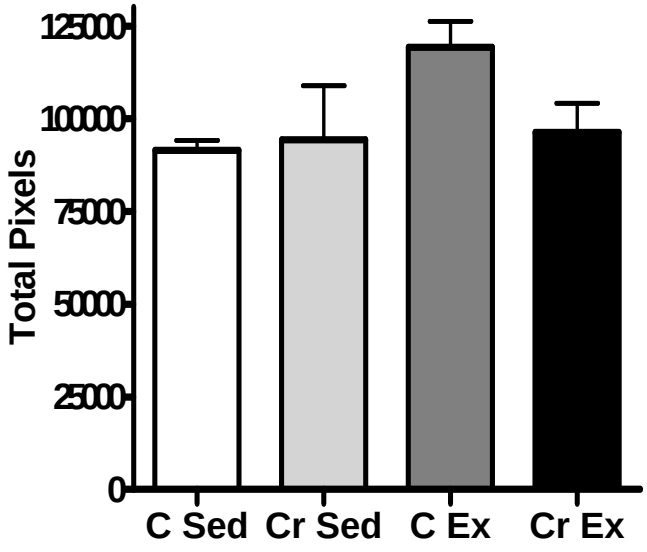
4.5.1 Myocardial creatine transporter

Creatine is taken up into the cell through the creatine transporter (CreaT). To determine whether the CreaT expression was upregulated with creatine supplementation and/or exercise, Western Blot analysis was carried out on samples from hearts perfused for 2 minutes before being freeze-clamped in precooled Wollenberger tongs, for baseline values. No significant up- or down- regulation was found with creatine supplementation or with exercise at baseline. In C Sed hearts CreaT levels were 91475 ± 2760 total pixels, while Cr Sed levels were 94351 ± 14590 total pixels. C Ex levels (119298 ± 6912 total pixels) and Cr Ex levels (96409 ± 7714 total pixels) were not significantly different. See Figure 4.37.



A

Figure 4.37: A: Representative Western blot to show the levels of CreaT expression in hearts from C Sed, Cr Sed, C Ex and Cr Ex rats.



B

Figure 4.37: B: Graph to show the levels of creatine transporter in the heart tissue of rats C Sed, Cr Sed, C Ex and Cr Ex groups. n = 9

4.5.2 Myocardial GLUT4

Glucose Transporter 4 (GLUT4) is the insulin stimulated glucose transporter, and can be up-regulated with exercise. Baseline GLUT4 was measured. GLUT4 in Cr Sed hearts (39890 ± 10548 total pixels) was not significantly increased from C Sed hearts (17481 ± 3815 total pixels), but expression was significantly higher in C Ex (80609 ± 12213 total pixels) but not in Cr Ex hearts (46318 ± 11899 total pixels). See Figure 4.38.

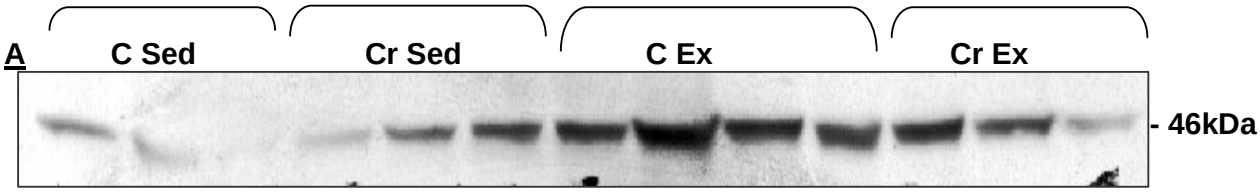


Figure 4.38: A: Representative Western blot of GLUT4 expression from C Sed, Cr Sed, C Ex and Cr Ex rats.

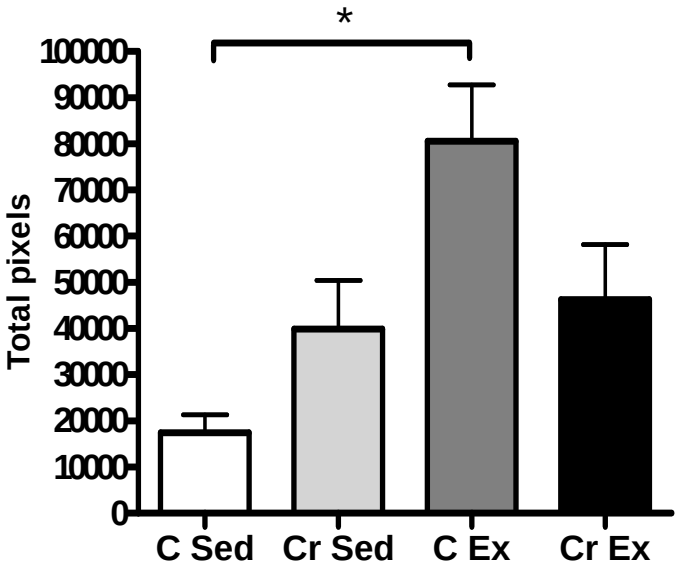


Figure 4.38: B: Graph to show expression of GLUT4 in hearts n = 3, * = p < 0.05, # = p < 0.01.

4.5.3 Myocardial AMPK

AMP-activated protein kinase is phosphorylated and activated during both exercise and stress e.g. ischaemia [Kudo et al 1995] and has a key role in many biological processes which include lipid and glucose metabolism, muscle contraction and energy homeostasis. To ascertain the total levels of the protein and whether it was phosphorylated in hearts from exercised and/or creatine supplemented rats, Western Blot analysis was performed on heart tissue.

4.5.3.1 Total AMPK expression

End of 20 minutes global ischaemia

In the heart, AMPK activity increases during ischaemia and functions to sustain ATP, cardiac function and myocardial viability. AMPK has a fundamental role in glucose metabolism and fatty acid oxidation and therefore increased levels during ischaemia can decrease ischaemic damage [Russel RR 3rd 2004].

Total AMPK values did not differ between the experimental groups. C Sed (178809 ± 3461 total pixels), Cr Sed (149505 ± 16464 total pixels), C Ex (125099 ± 28648 total pixels) and Cr Ex (145364 ± 15931 total pixels) total AMPK levels were all similar (n=3). See Figure 4.39.

10 minutes reperfusion

All total AMPK values for 10 minutes reperfusion were similar with C Sed (20440 ± 2394 total pixels), Cr Sed (23451 ± 1651 total pixels), C Ex (19344 ± 978.5 total pixels) and Cr Ex (18892 ± 478.9 total pixels) not being significantly different from each other. See Figure 4.39.

End of 30 minutes reperfusion

End of reperfusion levels of total APMK were also similar in all the experimental groups. They were: C Sed (217943 ± 7848 total pixels), Cr Sed (191743 ± 12241 total pixels), C Ex (197891 ± 8229 total pixels) and Cr Ex (231106 ± 18124 total pixels) and were all statistically similar. See Figure 4.39.

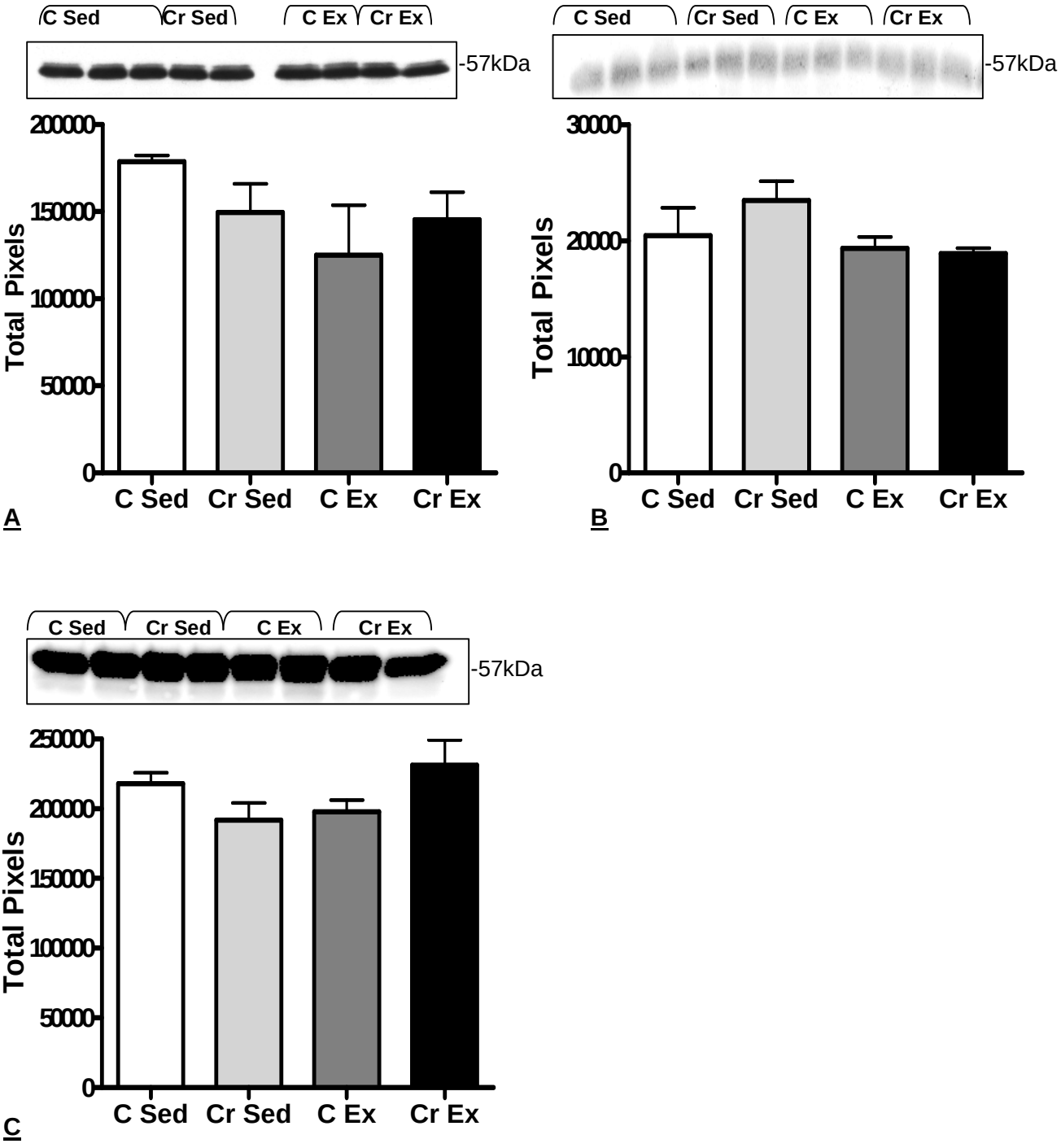


Figure 4.39: Representative Western blot and graphs of total APMK at A: the end of 20 minutes global ischaemia, B: 10 minutes reperfusion and C: at the end of 30 minutes reperfusion in all 4 experimental groups. n=3-6.

4.5.3.2 Phosphorylated AMPK

Baseline

AMPK phosphorylation was significantly higher in Cr Sed (56649 ± 8928 total pixels), C Ex (67242 ± 4346 total pixels) and Cr Ex (39054 ± 7268 total pixels) hearts compared to the values for C Sed (20466 ±4571 total pixels) ($p < 0.05$). See figure 4.40.

End of 20 minutes global ischaemia

There were no differences in AMPK phosphorylation at the end of ischaemia when comparing C Sed (39054 ± 7068 total pixels), Cr Sed (27177 ± 10508 total pixels), C Ex (16876 ± 744.9 total pixels) and Cr Ex (21926 ± 677.7 total pixels) hearts. See Figure 4.40

10 minutes reperfusion

Phosphorylated AMPK levels in C Sed hearts were 5982 ± 695 total pixels, Cr Sed levels were 6677 ± 237 total pixels and C Ex 7325 ± 941 total pixels. Phosphorylated AMPK levels in Cr Ex hearts (18399 ± 2130 total pixels) were significantly higher than all other groups ($p < 0.05$). See Figure 4.40

End of 30 minutes reperfusion

There were no differences in the levels of phosphorylated AMPK between groups at the end of reperfusion. C Sed hearts (35698 ± 10862 total pixels), Cr Sed hearts (79255 ± 15155 total pixels), C Ex hearts (25812 ± 384 total pixels) and Cr Ex hearts (45655 ± 12511 total pixels) all had similar levels of AMPK phosphorylation. See Figure 4.40.

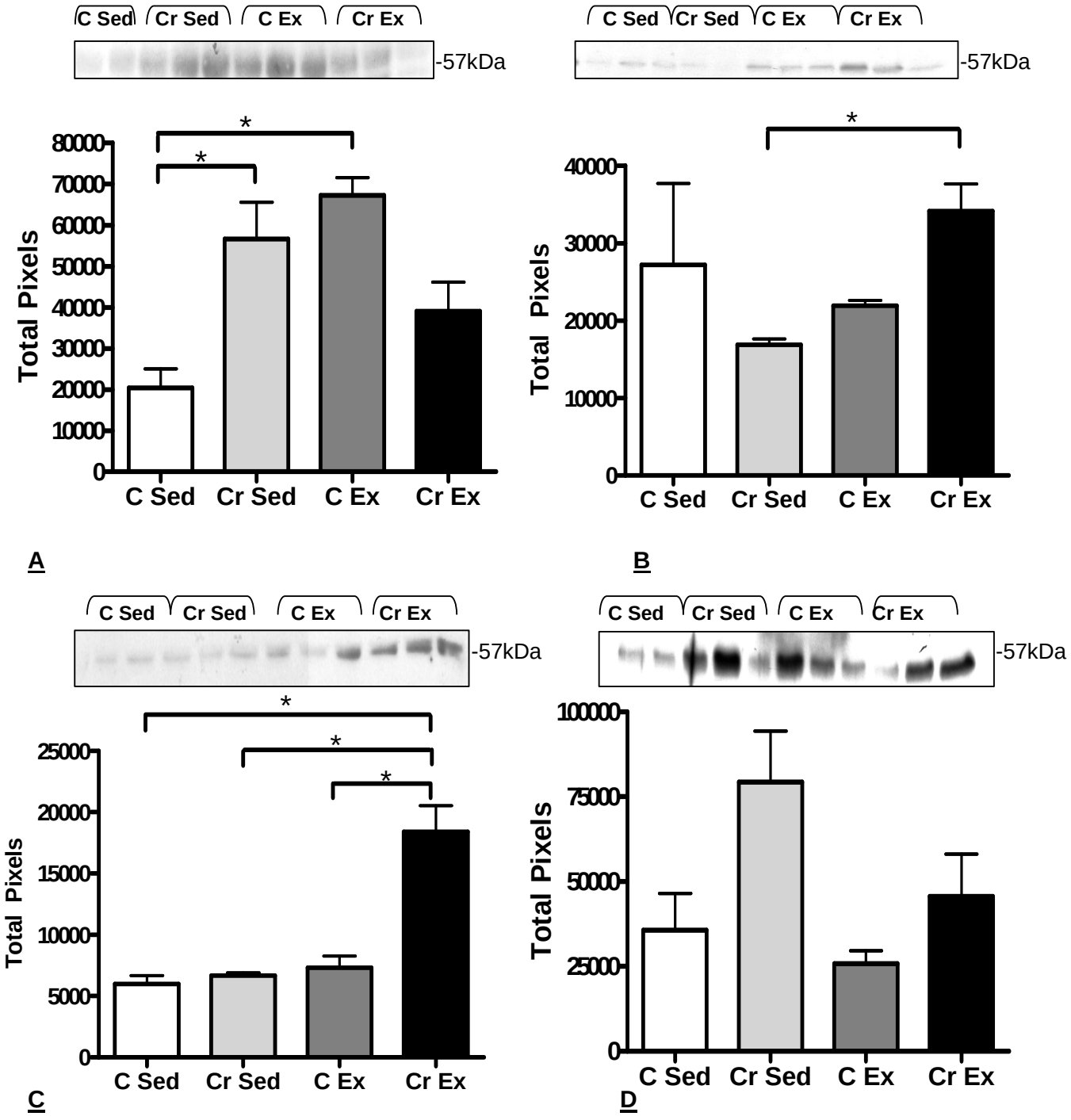


Figure 4.40: **Representative Western blot and graphs of AMPK phosphorylation in the experimental groups during A: baseline, B: end of 20 mins global ischaemia, C: 10 mins reperfusion and D: end of 30 minutes reperfusion time points. n = 3-5, * = p<0.05,**

4.5.4 Myocardial PKB

Phosphokinase B (PKB/ Akt) is involved in glucose metabolism, and it is also a pro-survival kinase with key functions in cell survival. It is also seen to be phosphorylated with exercise leading to increased insulin sensitivity [Jessen 2002]. However basal PKB/ Akt phosphorylation has been seen to be decreased with creatine supplementation [Deldicque 2008]. For these reasons we determined the total and phosphorylated PKB/Akt levels under baseline conditions.

4.5.4.1 Total PKB/ Akt expression

Baseline

In C Sed hearts total PKB/ Akt (57734 ± 9287 total pixels) was no different than Cr Sed (68358 ± 8976 total pixels), C Ex (62994 ± 7116 total pixels) or Cr Ex (49717 ± 5470 total pixels) hearts. See figure 4.41.

End of 20 minutes global ischaemia

Total PKB/Akt in C Sed hearts after 20 minutes of global ischaemia was 58212 ± 3614 total pixels. Cr Sed hearts were no different with 55543 ± 8482 total pixels of total PKB/Akt. C Ex hearts had total PKB/Akt of 45070 ± 14158 total pixels and Cr Ex hearts had 42985 ± 11241 total pixels. See Figure 4.41.

10 minutes reperfusion

Total PKB/ Akt expression was similar in all of the experimental groups at 10 minutes of reperfusion. (C Sed: 60766 ± 2348 total pixels; Cr Sed 62441 ± 1058 total pixels; C Ex 64060 ± 1742 total pixels and Cr Ex 68786 ± 1440 total pixels). See Figure 4.41.

End of 30 minutes reperfusion

PKB/ Akt levels for C Sed hearts were 20407 ± 2425 total pixels and did not differ from levels in Cr Sed hearts (15448 ± 159 total pixels). C Ex hearts (15321 ± 176 total pixels) and Cr Ex (18503 ± 2857 total pixels) total PKB/ Akt were also no different. See Figure 4. 41.

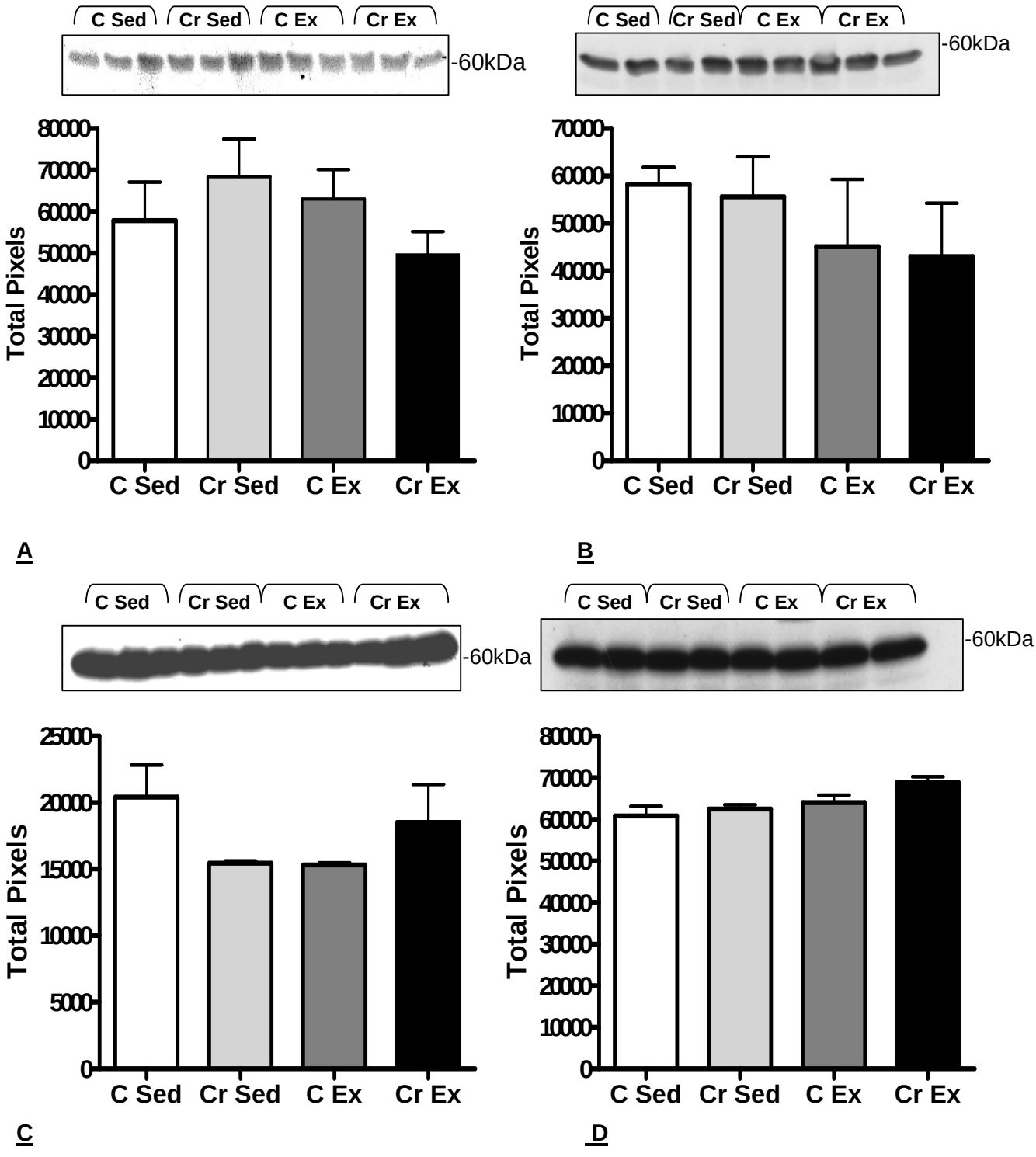


Figure 4.41: Representative blots and graphs of total PKB/Akt in the experimental groups during A: baseline, B: end of 20 mins global ischaemia, C: 10 mins reperfusion and D: end of 30 mins reperfusion time points. n = 3-5

4.5.4.2 Phosphorylated PKB/ Akt

The activation of prosurvival kinases, such as PKB/Akt and ERK42/44 (which are termed the reperfusion injury salvage kinase [RISK] pathway kinases), at the time of reperfusion, has been demonstrated to confer powerful cardioprotection against myocardial ischaemia-reperfusion injury [Zhu *et al* 2006]. However other data has also shown that this is not always the case, as Schwartz and Lagranha [2006] have shown that activation of PKB/Akt is not accompanied by concurrent cardioprotection.

Baseline

PKB/Akt phosphorylation in C Sed hearts was 49609 ± 25665 total pixels in comparison with phosphorylated PKB/Akt in Cr Sed hearts (167900 ± 16220 total pixels). PKB/Akt was phosphorylated significantly more in C Ex hearts (266527 ± 19982 total pixels) and Cr Ex hearts (234546 ± 31600 total pixels)than C Sed hearts ($p<0.01$). See Figure 4.42.

End of 20 minutes global ischaemia

End of ischaemia values for phosphorylated PKB/ Akt were low, with C Sed (3223 ± 98 total pixels), Cr Sed (3200 ± 82 total pixels), C Ex (3911 ± 336 total pixels) and Cr Ex (3225 ± 803 total pixels) being no different from each other. See Figure 4.42.

10 Minutes reperfusion

After 10 minutes of reperfusion the phosphorylated PKB/Akt levels increased. Phosphorylation in C Sed hearts increased to 18320 ± 3530 total pixels, Cr Sed to 21880 ± 1761 total pixels and C Ex to 42041 ± 11997 total pixels, all statistically similar. However PKB/Akt phosphorylation in Cr Ex hearts (58274 ± 5444 total pixels) was significantly higher than both C Sed and Cr Sed hearts ($p < 0.05$). See Figure 4.42.

End of 30 minutes reperfusion

By the end of 30 minutes reperfusion the differences seen at 10 minutes reperfusion were gone. C Sed (17561 ± 3005 total pixels), Cr Sed (20858 ± 1022 total pixels), C Ex (20345 ± 1820 total pixels) and Cr Ex (27602 ± 3127 total pixels) levels were all decreased and no different from each other. See Figure 4.42.

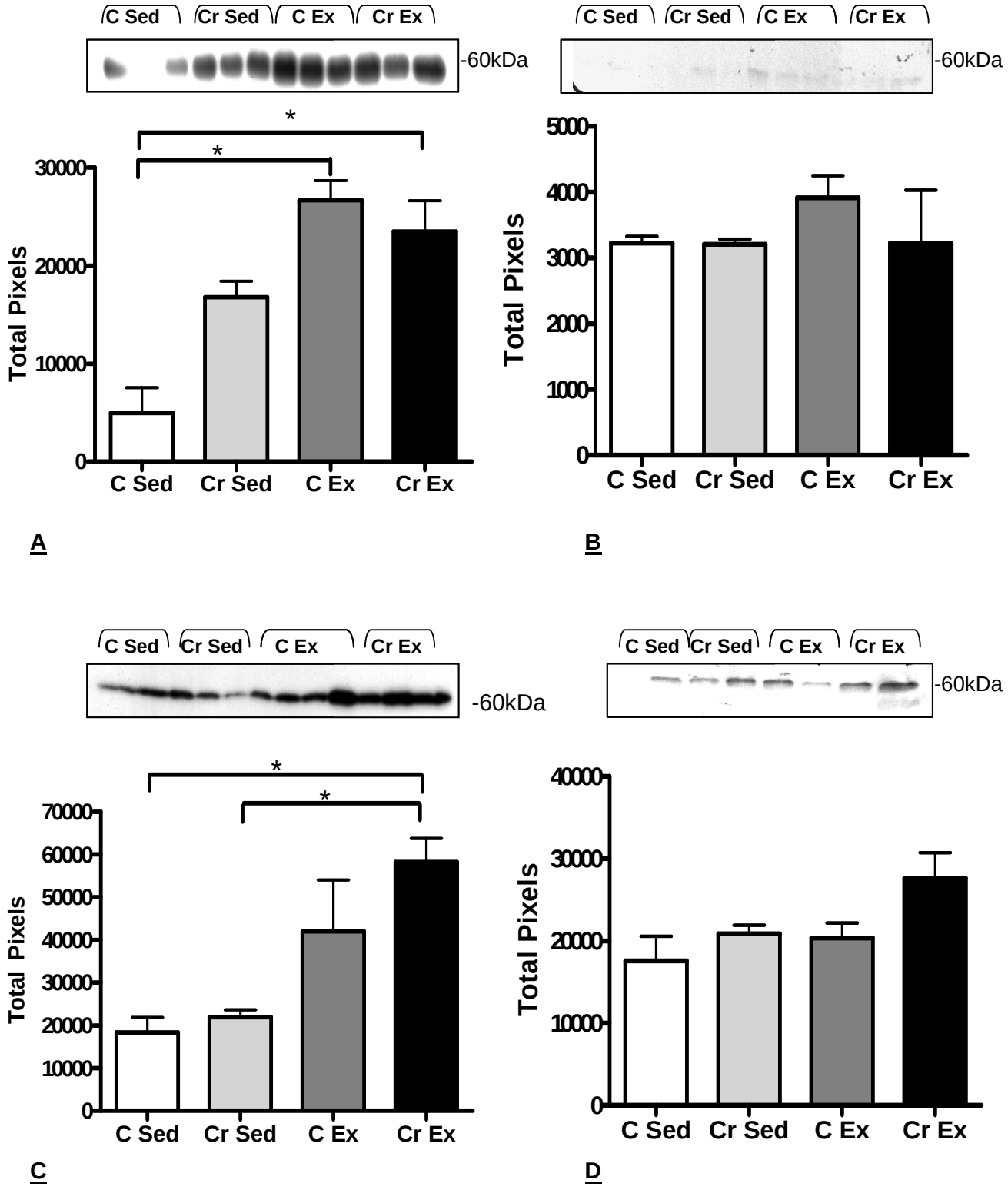


Figure 4.42: Representative Western blots and graphs of PKB/Akt phosphorylation pattern in the experimental groups during A: baseline, B: end of 20 mins global ischaemia, C: 10 mins reperfusion and D: end of 30 mins reperfusion time points. n = 3-5

4.5.5 Myocardial ERK 42/44

ERK42 and ERK44 can be phosphorylated and thus activated by extracellular stresses thus activating cellular survival pathways leading to cell survival [Bogoyevitch 2000]. Therefore we determined the phosphorylation of these survival kinases in the hearts from the four experimental groups.

4.5.5.1 Total ERK 42/44 expression

End of 20 minutes global ischaemia

There were no significant differences between total ERK 42 values. Values were C Sed: 34420 ± 1678, Cr Sed: 30107 ± 618, C Ex: 20810 ± 3507 and Cr Sed: 19743 ± 5195.
The total ERK 44 content of C Sed hearts was 69192 ± 3523 total pixels. Cr Sed (62953 ± 1318 total pixels) and C Ex (46083 ± 5887 total pixels) hearts did not differ from C Sed hearts, but Cr Ex hearts ERK 44 was significantly lower (40264 ± 8254 total pixels) than C Sed heart levels. See Figure 4.43

10 minutes reperfusion

At 10 minutes reperfusion the total ERK42/44 did not differ between groups, with levels of C Sed (ERK42: 7659 ± 1495, ERK44: 40056 ± 6535 total pixels), Cr Sed, (ERK42: 10264 ± 100, ERK44: 32603 ± 1234 total pixels), C Ex (ERK42: 11045 ± 1259, ERK44: 33571 ± 1271 total pixels) and Cr Ex (ERK42: 14994 ± 1888, ERK44: 41337 ± 4095 total pixels) hearts being similar. See Figure 4.43

End of 30 minutes reperfusion

Similarly, total ERK 42/44 levels at the end of reperfusion did not differ between the groups. C Sed (ERK42: 35818 ± 1068, ERK44: 20114 ± 5468 total pixels), Cr Sed (ERK42 39292 ± 548, ERK44: 37249 ± 2595 total pixels), C Ex (ERK42: 39264 ± 1578, ERK44: 37361 ± 287 total pixels) and Cr Ex (ERK42: 43829 ± 905, ERK44: 35902 ± 1769 total pixels) values were all similar. See Figure 4.43.

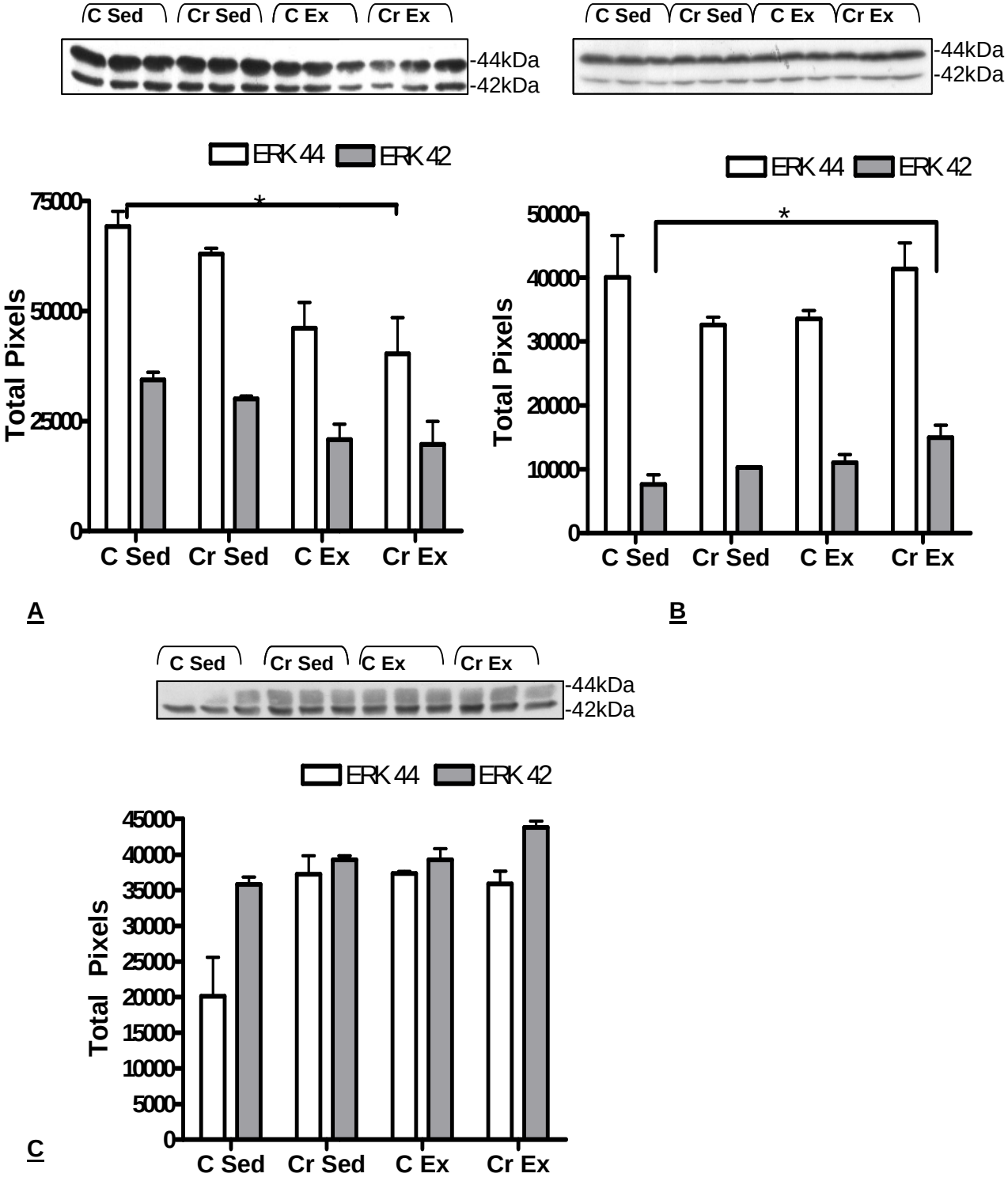


Figure 4.43: Representative Western blots and graphs of total ERK 42/44 in the experimental groups at A: end of 20 mins global ischaemia, B: 10 mins reperfusion and C: end of 30 mins reperfusion time points. n = 3-5

4.5.5.2 Phosphorylated ERK 42/44

Baseline

There were no differences in ERK 44 phosphorylation between C Sed (5393 ± 2049 total pixels), Cr Sed (5603 ± 551 total pixels), C Ex (6629 ± 530 total pixels) or Cr Ex (6184 ± 597 total pixels) hearts. Neither were there any differences in ERK 42 phosphorylation in these hearts at baseline [C Sed: 30986 ± 8329, Cr Sed: 29305 ± 2779, C Ex: 31161 ± 3141 and Cr Ex: 29179 ± 1367]. See Figure 4.44.

End of 20 minutes global ischaemia

ERK phosphorylation was no different between the 2 isoforms nor the experimental groups during ischaemia, with C Sed (ERK44: 18504 ± 5602, ERK42: 23854 ± 3850 total pixels), Cr Sed (ERK44: 24700 ± 3215, ERK42: 29765 ± 4742 total pixels), C Ex (ERK44: 23891 ± 4775, ERK42: 26008 ± 6228 total pixels) and Cr Ex (ERK44: 22337 ± 3874, ERK42: 19307 ± 4911 total pixels) values being similar. See figure 4.44.

10 Minutes reperfusion

At 10 minutes of reperfusion there were also no differences in the phosphorylation pattern of ERK42/44 in any of the groups. C Sed ERK 42 was 35394 ± 2672 total pixels, Cr Sed was 36222 ± 4850 total pixel, C Ex was 33498 ± 8285 total pixels and Cr Ex was 39694 ± 8699 total pixels, while C Sed ERK 44 was 44506 ± 6499 total pixels, Cr Sed was 31712 ± 3470 total pixels, C Ex was 37304 ± 10586 total pixels and Cr Ex was 36831 ± 8804 total pixels. See Figure 4.44.

End of 30 minutes reperfusion

As with the rest of the time points, C Sed (ERK44: 9450 ± 1096, ERK42: 21236 ± 6060 total pixels), Cr Sed (ERK44: 9641 ± 2518, ERK42: 12505 ± 3187 total pixels), C Ex (ERK44:7628 ± 550, ERK42: 22765 ± 4796 total pixels) and Cr Ex (ERK44: 13016 ± 1621, ERK42: 35606 ± 9671 total pixels) phosphorylated ERK 42/44 was no different in any of the groups. See Figure 4.44.

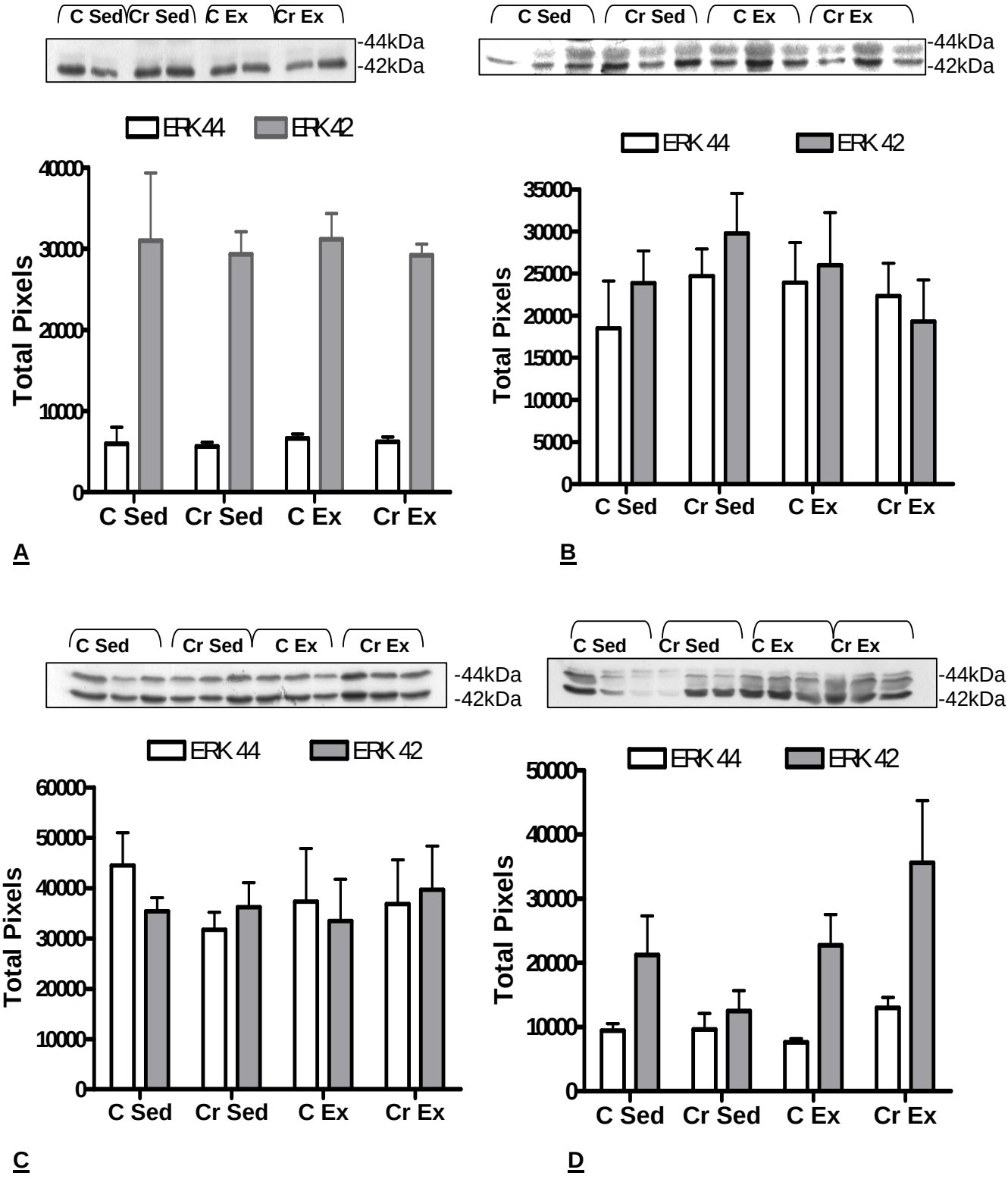


Figure 4.44: **Representative Western blots and graphs of ERK 42/44 phosphorylation pattern in the experimental groups during A: baseline, B: end of 20 mins global ischaemia, C: 10 mins reperfusion and D: end of 30 mins reperfusion time points. n = 3-5**

4.5.6 P38 MAPK

P38 MAPK is strongly activated by environmental stresses and inflammatory cytokines and is therefore also known as stress-activated protein kinase. [Sugden and Clark 1998]. Increased p38 MAPK activation has been implicated in ischaemia and reperfusion injury. For this reason we determined its levels of expression and phosphorylation in the experimental hearts [Clark et al 2007].

4.5.6.1 Total p38 MAPK expression

Baseline

Total p38 MAPK expression was 97066 ± 8934 total pixels in C Sed hearts, 94687 ±7313 total pixels in Cr Sed hearts, 79599 ± 7762 total pixels in C Ex hearts, and 98893 ±1943 total pixels in Cr Ex hearts. These values were all comparable. See Figure 4.45.

End of 20 minutes global ischaemia

Total p38 MAPK levels in exercised hearts (C Ex: 46415 ± 4658 total pixels) were significantly increased compared to C Sed hearts (30715 ± 2409 total pixels). However both Cr Sed (38402 ± 1433 total pixels) and Cr Ex (38779 ± 2402 total pixels) heart total p38 MAPK levels were not significantly different from either C Sed or C Ex values. See Figure 4.45.

10 Minutes reperfusion

There were no differences in the levels of total p38 MAPK, between any of thel groups (C Sed 48874 ± 3637 total pixels; Cr Sed 47445 ± 3337 total pixels; C Ex 40437 ± 5039 total pixels and Cr Ex 39972 ± 1986 total pixels). See Figure 4.45.

End of 30 minutes reperfusion

The levels of total p38 MAPK expression in hearts were similar in all groups at the end of 30 minutes reperfusion. These levels were C Sed (66638 ± 2796 total pixels), Cr Sed (62632 ± 3096 total pixels), C Ex (71182 ± 3169 total pixels) and Cr Ex (69827 ± 1079 total pixels). See Figure 4.45.

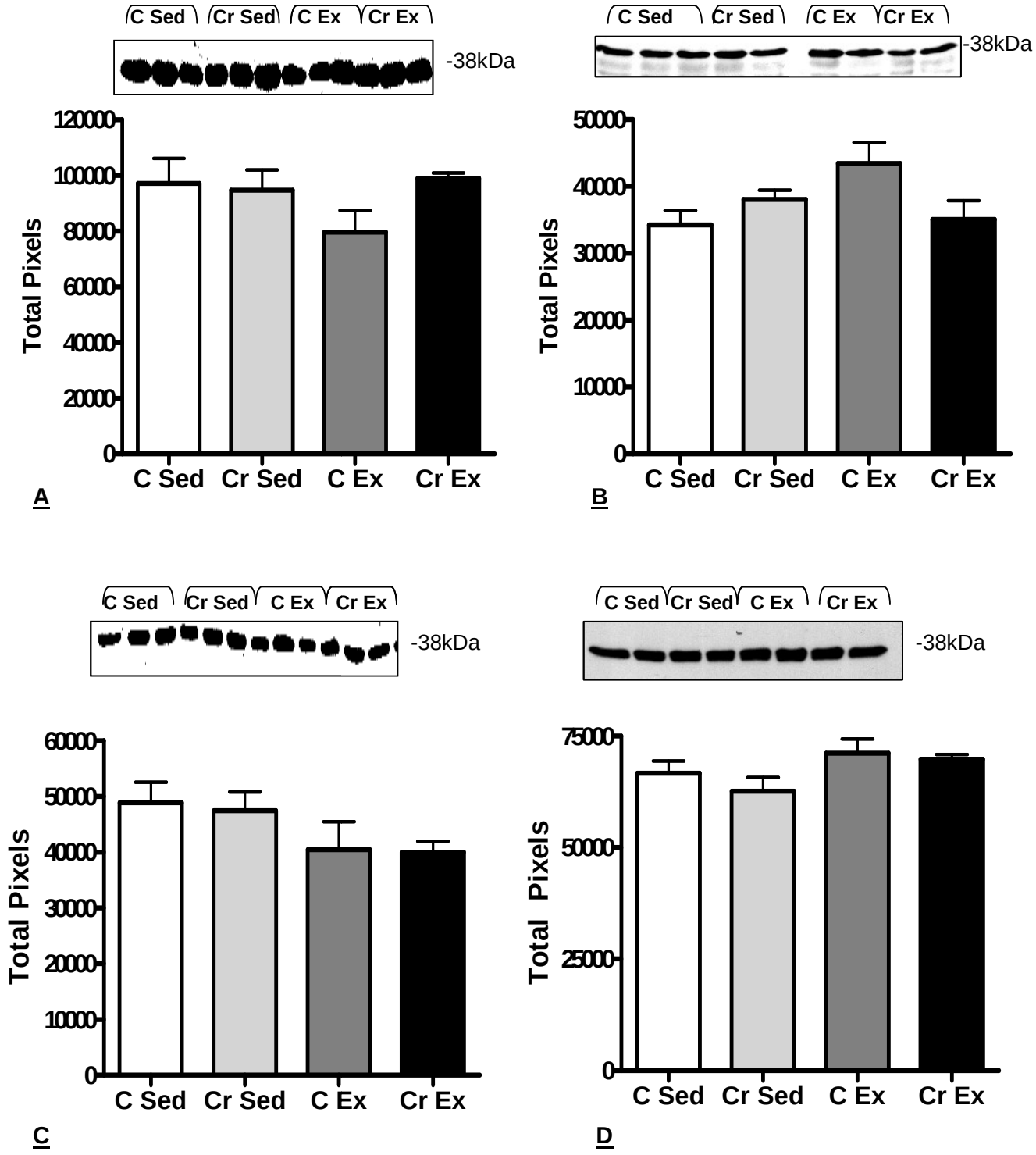


Figure 4.45: Representative Western blots and graphs of total P38 MAPK in the experimental groups at A: baseline, B: end of 20 mins global ischaemia, C: 10 mins reperfusion and D: end of 30 mins reperfusion time points. n = 3-5

4.5.6.2 Phosphorylated p38 MAPK

Baseline

Phosphorylated p38 MAPK levels in hearts were similar in C Sed (43937 ± 21099 total pixels), Cr Sed (39004 ± 8114 total pixels) and C Ex (41694 ± 9327 total pixels) groups while p38 MAPK phosphorylation levels in Cr Ex hearts (8544 ± 5529 total pixels) were significantly lower than in both Cr Sed and C Ex hearts ($p<0.05$, Students t-test). See figure 4.46.

End of 20 minutes global ischaemia

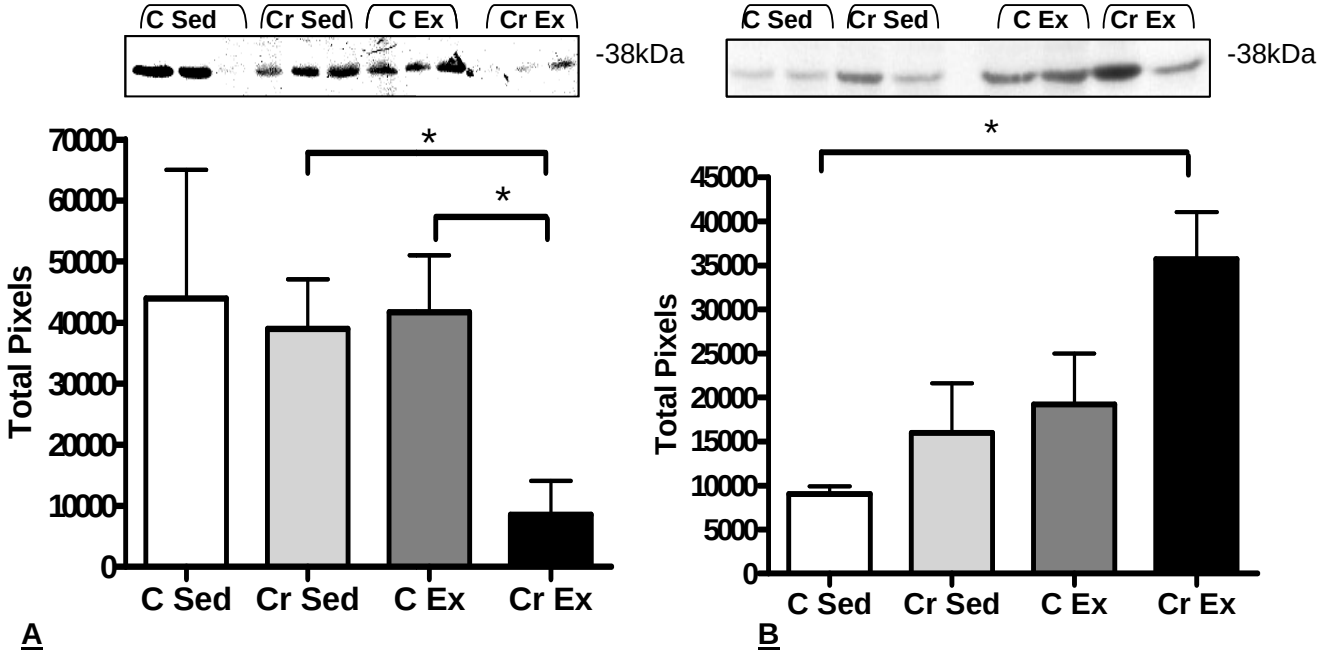
After 20 minutes of global ischaemia C Sed hearts (9039 ± 884 total pixels) had substantially less phosphorylated P38 MAPK than Cr Ex hearts (35712 ± 5314 total pixels, $p<0.05$). C Sed (15978 ± 5642 total pixels) and C Ex (19222 ± 5789 total pixels) hearts levels of phosphorylated p38 MAPK were no different to those of C Sed hearts. See Figure 4.46.

10 minutes reperfusion

By 10 minutes reperfusion levels of phosphorylated P38 MAPK had equalized and no significant differences existed between groups. Levels in C Sed hearts were 75749 ± 20862 total pixels, levels in Cr Sed hearts were 42755 ± 1590 total pixels, C Ex heart levels were 46758 ± 4909 total pixels and Cr Ex heart levels were 40979 ± 5755 total pixels. See Figure 4.46.

End of 30 minutes reperfusion

As was the case after 10 minutes of reperfusion, by the end of reperfusion levels of phosphorylated p38 MAPK were not significantly different between groups, with levels being as follows: C Sed 29402 ± 19163 total pixels; Cr Sed 17926 ± 4924 total pixels; C Ex 40001± 6605 total pixels and Cr Ex 50523 ± 9262 total pixels. See Figure 4.46.



(* p<0.05, Students t-test)

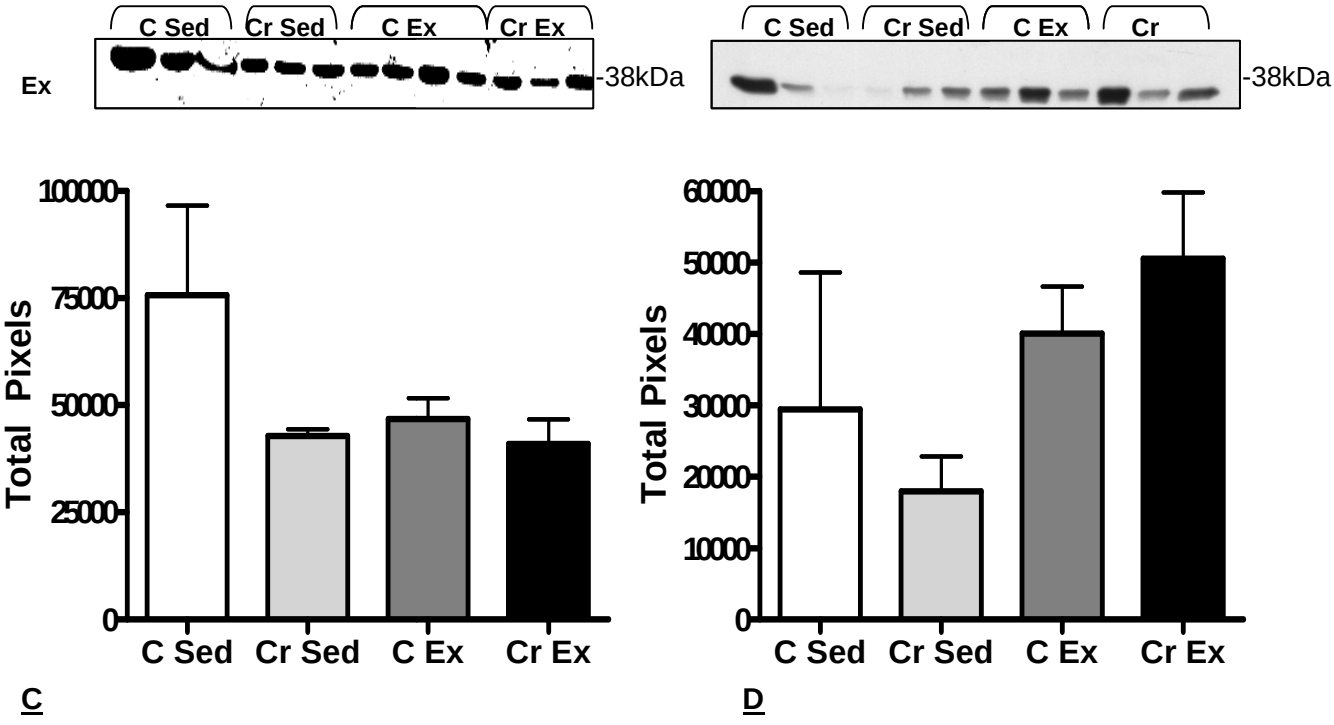


Figure 4.46: Representative Western blots and graphs of P38 MAPK phosphorylation pattern in the experimental groups during A: baseline, B: end of 20 mins global ischaemia, C: 10 mins reperfusion and D: end of 30 mins reperfusion time points. n = 3-5

Summary of key findings

CreaT was not upregulated by creatine supplementation.

Either creatine supplementation or exercise alone increased basal phosphorylated AMPK, but the combination of these interventions had no effect on basal AMPK phosphorylation. During ischaemia phosphorylated AMPK in the Cr Ex group was increased compared to the Cr Sed group and after 10 minutes of reperfusion the Cr Ex group had higher AMPK phosphorylation than any of the other groups.

Exercise increased basal PKB/Akt phosphorylation, but only Cr Ex had elevated phosphorylated PKB/ Akt levels after 10 minutes of reperfusion.

Total ERK 44 expression decreased in the Cr Ex group after 20 minutes of global ischaemia, and total ERK 42 expression increased in the Cr Ex group after 10 minutes of reperfusion.

The combination of creatine supplementation and exercise decreased basal p38 phosphorylation and increased its phosphorylation at the end of 20 minutes of global ischaemia.

CHAPTER 5

DISCUSSION

5.1 ANIMALS

5.1.1 Body weights

5.1.1.1 Body weight gain

According to Francaux and Poortmans [1999] total body water, including intracellular water is increased with creatine supplementation, which leads to increased body weight in users. Corroborating this, the study of Kutz and Gunter [2003] reported that a 4 week creatine supplementation and exercise regime increased total body weight and total body water in human subjects. Similarly Brilla et al [2003] showed supplementation with Mg-Cr increased total body water and intracellular body water after 2 weeks. Taes et al [2004], however, found no changes in body weights in sedentary patients during a 4 week period of creatine supplementation. These findings are supported by Young and Young [2007] who also found that creatine supplementation for 5 weeks had no affect on body mass in rats where the gastrocnemius and plantaris skeletal muscles were ablated to induce muscle hypertrophy. It is possible that the increase in body weight with creatine supplementation is reliant on exercise and because of the sedentary nature of these studies this increase in body weight was not seen.

No change in body weights or accelerated weight gain was evident in our animals with either exercise training or creatine supplementation. This is consistent with results documented by Diffee et al [2003] who failed to see increased body weight with 11

weeks of treadmill running in rats. McClung et al [2003] showed 3 weeks of swimming and creatine supplementation did not change body weights, and Silva et al [2007] found that 21 days of creatine supplementation and swimming in humans had no effect on body composition or body weight. Horn et al [1998] actually found that creatine supplementation of rats with a high dose of creatine (5 & 7% of body weights), for 40 days resulted in a decrease in body weight. Supplementation with creatine equivalent to 1% of body mass, which is similar to the dose we used, had no effect on body weight in their study. We propose that the exercise model we used did not elicit changes in body weight with creatine supplementation because it was not high resistance exercise, which would be expected to elicit an increase in body weight.

5.1.1.2 Heart weight: body weight ratio

Heart weight to body weight (HW: BW) ratios were no different in any of our experimental groups. McClung et al [2003] also found that neither body weights nor HW: BW ratios were changed in either their exercise or creatine supplemented groups. Their exercise regime was 30 minutes of swimming per day, 5 days per week, for 3 weeks, with a weight equivalent to 2% of the rat's body weight attached to the tails and 15.6mg (less than 0.1% of body weight) creatine fed to the rats via oral gavage half an hour before exercise. As was seen in our study the combination group of both interventions failed to affect HW: BW ratios. Similar results have been noted in humans, where creatine supplementation and exercise for a month did not change cardiac structure or function as measured echocardiographically [Murphy et al 2005]. However, Fenning et al [2003] showed increased HW: BW ratios after 6 and 12 weeks of treadmill exercise training (5 days per week, 30 mins per day).

Treadmill endurance exercise training (11 weeks, 5 days per week) has been shown to increase myosin heavy and light chain RNA expression in cardiac muscle, leading to an increase in protein synthesis and physiological hypertrophy [Diffee et al 2003]. McClung et al [2003] however found decreased total cardiac RNA, and decreased MHCα with endurance exercise (21 days of swimming) in the rat model. It was postulated that their training was not intense enough to elicit cardiac changes and hypertrophy. Although our training protocol was double the time course of theirs, it is possible that our swimming protocol was not intense enough to induce cardiac hypertrophy. Kaplan et al [1994] saw no change in MHCα in the heart after 4 weeks of a 3 hour swim training protocol for 5 days a week. McMullen et al [2003] increased this program to 7 days per week, and indeed saw exercise induced hypertrophy in hearts, with increased HW: BW ratios, but no change in MHCα levels. Therefore we propose that the changes in ischaemic tolerance that we saw in our study were unrelated to exercise induced cardiac hypertrophy, as the 1 hour swim for 5 days per week, over an 8 week time period in our study was not intense enough to induce heart weight changes in the hearts of our animals.

However, these lack of changes in body and heart weights were not too disconcerting in this study, as we followed the protocol which is most similar to that of athletes, and we also did not differ too greatly from many studies described here, both in protocol and in results.

5.1.2 Behaviour

Contrary to the effects of increased corticosterone and depressive behaviour of acute forced swim stress on rats [Racca et al 2005, Armario et al 1995, Porsolt et al 1978], the long term swim training in our study elicited no stress response as reflected by either behaviour or corticosterone levels at rest in our rats.

The open field test is a measure of the amount of stressful behaviour displayed by the rats [Walsh and Cummins 1976]. Although we found no significant differences in behaviour between the groups, the control exercised group tended to move into the inner and outer zone and remain in the inner zone longer than control sedentary group. Although these differences were not significant, it may indicate that they may have been less stressed than the other groups.

Contarteze et al [2008] have shown that only acute swimming elicits elevations in the stress hormones ACTH and corticosterone in rats, and Cox et al [1985] showed that training and familiarization with swimming decreases levels of corticosterone during swimming in rats. The forced swim test is used as a model of stress and depression in neurological studies [Armario et al 1995] where rats are subjected to a once off swim for 15 minutes. It has been shown that during these tests, Wistar rats display more passive behaviour compared to other rat strains [Armario et al 1995]. In our model, rats were acclimatized to the swim training, starting with a swim duration of 5 minutes per day and increasing the duration incrementally daily. Acute exposure to swimming rather than trained or chronic swimming has been shown to increase stress hormone levels in rats [Avital et al 2001]. Therefore the effects that were elicited by ischaemia/reperfusion in our model can not be attributed to elevated levels of stress hormones because of the exercise regime.

5.1.3 Choice of exercise program

Body builders, triathlon competitors, sprinters and other athletes are all advised to train 5 days per week and rest for 2 [http://www.thetriathloncoach.com/End-of-season-break-do-athletes-need-it.php]. Exercise for 6 days per week with too little time for recovery is not associated with any improvement in training parameters (i.e. increased performance

and decreased recovery) [Lehmann et al 1991]. Decreased infarct size has been shown in rats exercised 5 days per week, by both swimming and treadmill running [Thorp et al 2007, Zhang et al 2007], but no beneficial effect was seen with a daily exercise program (every day of the week) over 20 weeks [Brown et al 2003], which may be due to the absence of a recovery time. For this reason, our swim and creatine supplementation protocol of 5 days per week was in our opinion sufficient, and representative of the training regimes used by most athletes. Although it has been shown to not necessarily bring about physiological cardiac hypertrophy, or “athlete’s heart”, this approach has previously been shown to induce cardioprotection [Brown et al 2003, Reger et al 2006, Zhang et al 2007, Chicco et al 2007].

5.2 HEART FUNCTION

5.2.1 Baseline Function

No differences in baseline heart function (AO, HR, CF, aortic SP, and aortic DP) were found in our study with either creatine supplementation or exercise or a combination of the two interventions. Zhang [2007b] showed that resting heart rate and systolic blood pressures were not affected by exercise training in healthy individuals.

Although it has been shown that exercise training decreases resting heart rate in young and old healthy subjects after 12 weeks of running [Carter et al 2003], Rakpongsiri and Sawangkoon [2008] also recently showed that supplementation with creatine and an exercise regime did not alter resting heart rate. In a study done on obese individuals, exercise training decreased resting heart rate, and decreased systolic and diastolic blood pressures [King et al 2009]. In Type II diabetic patients, exercise training for a year lowered body weight along with insulin, HbA1c's and cholesterol levels and increased HDL but did not have an effect on systolic blood pressure or heart rate at rest [Loimaala et al 2007].

Murphy et al [2005] presented work which showed that creatine supplementation and exercise in humans had no detectable effects on cardiac structure or function as determined by echocardiography. Resting LVDP, heart rate and coronary flow were not influenced by creatine supplementation in rats [Horn et al 1998]. Our findings are consistent with both of these studies of no alterations in basal heart function. Lennon et al [2004] also found unchanged baseline cardiac function (HR, CF, RPP or cardiac work) in hearts from moderate and high intensity exercise trained rats.

5.2.2 Myocardial susceptibility to ischaemia/ reperfusion injury

Global ischaemia is clinically similar to myocardial injury following cardiac arrest, and regional ischaemia with coronary artery ligation similar to that of coronary bypass surgery [Song et al 2009]. In order to obtain a clinically relevant answer to the question what is the effect of creatine supplementation and exercise on the heart, it was necessaru to investigate both these types of ischaemia and their effects on function and infarct size in these hearts.

5.2.2.1 Effect of creatine and swim training on infarct size

Neither exercise training nor creatine supplementation had an effect on infarct size after regional ischaemia in our study. The AAR for all groups averaged 40% of the left ventricle. The functional recovery after CAL and 25 minutes of regional ischaemia was also no different between groups, although creatine supplemented in combination with exercise decreased AO recoveries below those of the control sedentary group.

Reduction in infarct size with exercise was reported as far back as the 1970's [McElroy et al 1978]. Infarct size was reduced in exercised rats after 48 hours *in vivo* coronary artery occlusion which may be partly related to increased myocardial vascularity which was observed in this study [McElroy et al 1978]. Melling et al [2009] found decreased infarct size in the heart after 24 hours of acute exercise (60mins treadmill running) with an increase in HSP70, possibly providing the protection. Brown et al [2003] showed decreased cardiac infarct sizes with treadmill run training in rats trained for an hour a day for 20 weeks. Exercise training also induced a reduction in infarct size *in vivo* in rats subjected to an 8 week swimming regime (3 hours per day, 5 days per week) [Zhang et al 2007].

De Waard and Duncker [2009] however found that although exercise in mice using voluntary wheel running for 8 weeks reduced post-MI mortality and reduced LV dysfunction it did not reduce infarct size. In these studies the thickness and area of infarct worsened in the exercise trained group.

Infarct size was reduced in the brain after 3 weeks of creatine supplementation in an induced stroke model in mice [Prass et al 2007]. This was independent of levels of Cr, PCr or ATP which were found to be unaltered in the brain tissue. The same group found that life-long creatine administration failed to protect adult mice against having a stroke, suggesting that adaptive mechanisms could compromise the beneficial roles of creatine. Data from Rawson et al [2007] implied that oral creatine supplementation does not reduce skeletal muscle damage or improve functional recovery after hypoxic resistance exercise.

These data illustrate that information on CAL, infarct size and the effect of creatine supplementation and exercise on these parameters are contradictory, and while our data on infarct size is contrary to many previous observations [Prass et al 2007, Zhang et al 2007, McElroy et al 1978, Brown et al 2003], it corroborates others [de Waard and Duncker 2009, Rawson et al 2007]. It has been suggested in previous work that 30 minutes of reperfusion is not substantial enough for calculation of infarct size [Birnbaum et al 1997]. However, previous work done in our laboratory has shown that 30 minutes reperfusion is sufficient to measure differences in infarct size, as no differences were seen between 30 minutes and 2 hours reperfusion time [Marais et al 2005, Fan et al 2009].

5.2.2.2 Post ischaemic cardiac function

Effect of exercise on post ischaemic cardiac function

Aortic output recoveries were decreased in our control (vehicle treated) and creatine supplemented exercised groups.

Zhang et al [2007] found that LVSP improved in rats subjected to regional ischaemia *in vivo*, after 8 weeks of free loading swim training (3 hours per day, 5 days per week). Demirel et al [2001] found an improved myocardial LVDP and RPP recovery after 5 days of treadmill exercise training for 20 minutes per day. This was associated with an increased HSP72 expression and antioxidant enzyme activity, showing beneficial effects of short term exercise. Lennon et al [2004] found that moderate (55% VO_2max) and high intensity treadmill training provided protection against 20 minutes of global ischaemia as reflected by enhanced recovery of CO and cardiac work, while RPP recovery, heart rate and coronary flow were no different from controls.

Burelle et al [2004] also found that treadmill training for 10 weeks (4 days per week) protected isolated hearts against reperfusion injury when using CO as the end point, however they used both palmitate and glucose in the perfusion buffer, which may have affected the outcome. They found the hearts from exercise trained animals had higher glucose and palmitate oxidation rates before and after ischaemia and lower glycolysis rates at these times.

Cardioprotection against ischaemia/reperfusion damage was seen in hearts from exercised rats in males but not females [Thorp et al 2007]. The female's hearts displayed better recovery of LVDP than the male's hearts, but not better than their

control, post-ischaemic values. It was postulated that the female heart was possibly already maximally protected by estrogen and could therefore not be further protected by exercise training. Starnes et al [2005] found low intensity training (55-60% VO_2max) did not improve cardiac recovery of heart work after 20 minutes global ischaemia and reperfusion. Brown et al [2003] found no LVDP or CF differences under baseline conditions, and although LVDP was greater immediately after ischaemia in trained hearts, LVDP had decreased to values comparable to those of control hearts by the end of reperfusion. One study by Mancardi et al [2008] has shown that stressful forced exercise using treadmill training is detrimental to the ischaemic heart, increasing infarct size and decreasing LVDP recoveries in the heart.

Many of the studies that have documented cardioprotection with exercise training have used different end points to assess reperfusion myocardial viability (e.g. CF, active tension, LVDP recovery, cardiac output and cardiac work recovery, infarct size) [Le Page et al 2009, Zhang et al 2007, Reger et al 2006, Brown et al 2005], all of which were unchanged in our study. This is possibly because these groups looked at the effects of regional [Zhang et al 2007, Brown et al 2005] or low flow ischaemia [Le Page et al 2009, Reger et al 2006] on these parameters, while our study was performed using total global ischaemia. The exercise models used were also different, Zhang et al [2007] used swim training similar to our model and Brown et al [2005], Reger et al [2006] and Le Page et al [2009] used treadmill training. These differences in model could also lead to differences seen in our results.

Bowles and Starnes [1994] and Lennon et al [2004] looked at cardiac output and cardiac work recovery and found that it was increased, however considering that cardiac output is a function of both aortic output and coronary flow, this increased CO

may have been due to an increase in CF without an increase in AO. These latter values were not shown. Our CF values remained unchanged, while both AO and CO recoveries were decreased. Cardiac work, which is a function of cardiac output and systolic pressure, was also unchanged between groups in our study.

Effect of creatine supplementation on post ischaemic cardiac function

Aortic output, cardiac output and rate pressure product recoveries were decreased in the hearts of the creatine supplemented exercised group. Creatine supplementation alone also decreased AO recoveries in hearts from sedentary rats. Myocardial function (pressure and stroke work) was preserved by creatine infusion in a model of coronary artery bypass grafting. Creatine infusion for 10 minutes during CAL and 10 minutes of reperfusion increased myocardial cellular ATP levels during ischaemia and reperfusion in the treated animals [Woo et al 2005]. Creatine supplementation in cardioplegic solution during heart surgery also resulted in better post surgery left ventricular work [Thelin et al 1987].

Interestingly, in 1992, Thorelius showed that creatine phosphate in a cardioplegic solution led to better stroke work after aortic valve surgery, even though no increases in myocardial ATP or PCr levels were observed. Creatine supplementation (1% body weight in powdered rat chow) for 21 days did not provide cardioprotection during global ischaemia (which was induced until ATP was completely depleted in the heart - ± 13 minutes) in rats in a study by Osbakken et al [1992]. Here the Langendorff perfusion apparatus was used and mechanical functional measured was HR multiplied by systolic pressure. The time taken to restore function to normal after ischemia was similar in untreated and creatine supplemented hearts. This was similar to our study, in that an

extended period of creatine supplementation did not provide protection to the heart for mechanical functional recovery.

No other studies have looked at AO recovery after 20 minutes global ischaemia. Neither have the effect of creatine supplementation alone or in combination with exercise been investigated. Our study is, to our knowledge the first to demonstrate decreased recovery of function after ischaemia with creatine supplementation.

Pulido et al [1998] found that creatine pretreatment of muscular dystrophic (mdx) skeletal muscle cells improved the calcium handling of these cells in which the baseline calcium concentration is abnormally high. They proposed that creatine supplementation improved calcium handling due to improved function of either the sarcolemmal or SR Ca^{2+}ATPase pumps. This data is refuted by their observations showing that creatine pretreatment of mdx myotubes did not affect the stress-induced increase in Ca^{2+} in the cell. These observations rule out the proposed role for altered cellular Ca^{2+} handling in creatine induced improved muscle function.

In our study we found an increased peak ischaemic contracture in the creatine supplemented exercised group. We propose that the creatine supplementation, when used in combination with exercise, impairs the ability of the cardiomyocyte to manage the high calcium levels in the cell during ischaemia. This may be due to a creatine supplementation induced increase in intracellular sodium in the heart. The CreaT is part of the family of sodium: neurotransmitter symporters which derive energy from the co-transport of Na^+ and Cl^-, in order to transport molecules into the cell against their concentration gradient [http://en.wikipedia.org/wiki, Nash et al 1994]. Thus with increased creatine uptake, there may be a concurrent increase in the Na^+ concentration

in the cell. This increased intracellular Na^{+} could compromise the ability of the cardiomyocyte to export Ca^{2+} via the Na^{+}/ Ca^{2+} antiport system which may be crucial during ischaemia and early reperfusion. This elevated intracellular Na^{+} accompanying creatine uptake may contribute to calcium overload and hypercontracture.

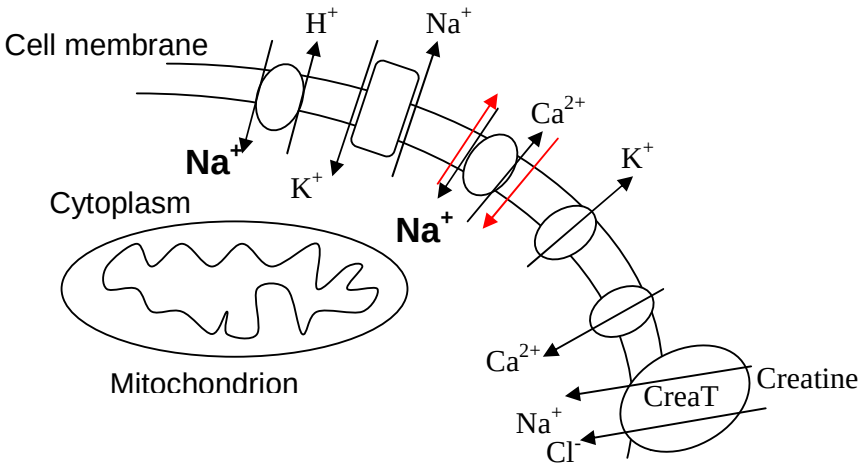


Figure 5.1: Diagram to show ion channels and transporters in the cell membrane and proposed mechanism for creatine induced exacerbation of calcium overload, hyper-contracture and decreased post-ischaemic function.

5.3 HIGH ENERGY PHOSPHATES

Blood was analysed for HEP concentrations to ensure that creatine was indeed being absorbed from the gut into the blood steam for transport to the muscles, and also to determine what the effect was on adenosine phosphate and creatine phosphate profiles. For interest's sake, the blood was separated into plasma and red blood cells to elucidate in which fraction these HEPs were.

5.3.1 Blood

In our study Cr content increased by approximately 20% in RBCs of creatine supplemented sedentary rats, and PCr in the plasma increased 5 fold in creatine supplemented sedentary rats. Although the exercised groups both had between 2 and 3 fold increases in PCr content in the blood plasma, these increases were not significantly more than the control sedentary group. McMillen et al [2001] found that creatine supplementation resulted in a tenfold increase in blood creatine content. Thus, although not as pronounced as in McMillen et al's study, our results are consistent with the observations of increased Cr and PCr levels in blood with creatine supplementation.

ATP in the RBC's was significantly increased in creatine supplemented sedentary rats. In the exercised groups RBC ATP was 33% higher than the control sedentary group but this increase was not significant. Brodthagen et al [1985] compared whole blood of control sedentary individuals with that of elite long distance runners and found that ATP concentrations in the blood were no different at rest or post-exercise. Harmer et al [2000] and Hellsten et al [2004] demonstrated that resting muscle ATP levels were actually decreased by exercise training, however to our knowledge this is the first study

showing that resting ATP levels in the blood are unaffected by creatine supplementation.

A possible explanation for not observing a significant increase in blood Cr, PCr or ATP in the exercised groups is that the increase in energy expenditure associated with exercise may result in a decrease in the high energy phosphates because they are used for muscle contraction during the exercise. During Cr supplementation, ATP donates a phosphate group to Cr, thus forming PCr, so that there is effectively less ATP and Cr and more PCr in the blood, which is also consistent with our findings.

5.3.2 Heart Tissue

In our study, 2 months of creatine supplementation had no effect on high energy phosphate or creatine levels in the heart tissue. Isolated hearts perfused with a cardioplegic solution with an increased creatine content for 20 minutes have been shown to have increased tissue creatine [Thelin et al 1987]. A short term creatine supplementation program of 7 days also showed increased myocardial levels of phosphocreatine and ATP in the rat [Brzezińska et al 1998].

However Osbakken et al [1992] used nuclear magnetic resonance NMR spectroscopy and showed that 21 days of creatine supplementation did not increase the half life of ATP in the heart during ischaemia i.e. ATP stores were depleted during ischaemia within the same time as control hearts that did not receive creatine supplementation suggesting that there were not increased ATP stores in the creatine supplemented hearts. Horn et al [1998] also found unchanged high energy phosphate levels in hearts from rats supplemented with creatine for 40 days . McMillen [2001] demonstrated tissue

specific uptake of creatine in skeletal muscle after 2 weeks of supplementation. They found increases in creatine stores with creatine supplementation in the gastrocnemius but not the soleus muscle. These data suggest creatine uptake may be fibre specific, and may also rely on training and supplementation model.

5.3.3 HEP ratios

The preservation of normal intracellular levels of high-energy phosphates is essential for myocardial function and protection. High energy phosphates include ATP, ADP, AMP and PCr, and these are intermediates in energy transfer in the cell. The ratios between the HEPs are used by the cell as a measure of intracellular energy levels, and determine whether it is necessary to start producing more ATP via glycolysis and the electron transport chain.

In a model of congestive heart failure PCr and total creatine levels were reduced while in severe heart failure ATP was also reduced [Zhang et al 1996]. PCr/ATP ratios are low in infracted hearts and in CHF [Murakami et al 1999]. PCr/ATP ratios are closely correlated with the severity of heart failure and are a prognostic indicator of mortality in patients [Ten Hove and Neubauer 2007]. PCr/ATP ratios for normal human hearts range between 1.2-2.4 [Ten Hove and Neubauer 2007], We measured PCr/ATP ratios of between 1.04 and 1.09 for all our groups. These are perhaps lower than expected not because of heart failure, but because of species differences between humans and the rat model.

Although the cardiac PCr/ATP ratio is decreased in several pathological conditions, such as ischemia and heart failure, it is increased in the heart muscle of GLUT4 null

mice [Weiss et al 2002]. There is currently no information available on the effect of creatine supplementation on heart PCr/ATP ratio. For this reason we looked at studies done on skeletal muscle. Creatine supplementation resulted in elevated PCr/ATP ratios (4.2 ±0.7 vs 3.3 ± 0.3 in control athletes) in the calf muscle of athletes [Zange et al 2002] while run trained rats had decreased PCr and PCr/ATP ratios (1.70 ± 0.24 vs 1.32 ± 0.06) with increased cardiac creatine, AMP and ADP concentrations [Fenning et al 2003]. We found no change in PCr/ATP ratio suggesting no apparent influence of exercise or creatine supplementation on energetic status of the hearts.

PCr/TCr (total creatine) ratio for skeletal muscles ranges between 0.6 and 0.8 depending on muscle type. We observed a ratio of 0.7 in heart muscle for all our study groups, with no changes in this ratio suggesting that there is no apparent influence of either exercise or creatine supplementation on energetic status in the hearts.

5.4 MITOCHONDRIAL FUNCTION

5.4.1 Respiration states and RCI

5.4.1.1 Effect of exercise on mitochondrial respiration

Isolated mitochondria showed no significant difference in state 3 or 4 respiration with either glutamate or succinate as substrate in the four groups in our study. Using glutamate and succinate as substrates therefore demonstrated that the different interventions did not change the activity of either complex I or complex II of the mitochondrial respiratory chain as glutamate in the presence of malate measures only the activity of complex I while succinate measures both complex I and II activities.

Basal RCI of mitochondria of control exercised hearts was higher than that of control sedentary hearts, which suggests a heightened mitochondrial respiratory potential with exercise. This increased respiratory potential was however attenuated with creatine supplementation.

Our findings are similar to work published by Starnes et al [2009] who showed that state 3 respiration, state 4 respiration and ADP/O ratios were no different between exercise trained and control rat myocardial mitochondria with either succinate or glutamate as substrates.

Exercise training results in changes in mitochondrial proteins which may contribute to a cardioprotective phenotype [Kavazis et al 2009]. Farrar et al [1981] reported that exercise training decreased the decline in state 3 respiration in skeletal muscle mitochondria of aged animals.

In a study by Ascensao et al [2006], state 3 respiration in heart mitochondria was improved by 14 weeks of treadmill exercise training, both before and after 1 minute of anoxia. There was however no pre-anoxic differences in RCI or ADP/O ratio. However the attenuation of RCI by anoxia and re-oxygenation was not as pronounced in the exercised group. In streptozitosin induced Type 1 diabetic rats, a 10 week exercise training program normalized state 3 respiration rates in heart mitochondria [Mokhtar et al 1993] and Ferström et al [2004] reported increased state 3 respirations in skeletal muscle mitochondria after 6 weeks of endurance training.

An 8 week period of training, 3 times per week, did not elicit changes in oxygen consumption in skeletal muscle mitochondria from subjects exposed to a continuous training regime. However, in the same study interval training did increase the mitochondrial oxygen consumption. Interval training consisted of 5 minute episodes of 90% maximal power output exercise interspersed with low power output exercise, and continuous training consisted of 20-35 minutes of uninterrupted exercise at the same mean power output as the interval training. Thus, even though the same total amount of work was performed during the two different training protocols, the higher intensity short burst exercise seemed to elicit changes whereas the constant moderate intensity exercise did not [Daussin et al 2008].

Fregosi et al [1987] found that exercise training did not affect state 3 respiration rate, RCI or ADP/O ratios in the rat plantaris or the diaphragm muscle mitochondria from rats exercise trained for 8 weeks. No differences were seen either in state 3 respiration or ADP/O ratios in exercised obese rats subjected to treadmill running daily for 7 weeks [Wardlaw et al 1986]. In similar studies endurance training on a treadmill for 5 days a week for 16 weeks did not alleviate the loss of myocardial mitochondrial ATP production

seen in response to calcium overload. [Starnes et al 2007b]. These studies support the data that we collected.

It would thus seem that the effect of exercise on myocardial mitochondria function is limited and diverse, and that our observations do not differ from several previous studies. Mitochondrial respiration was unaltered by the type and intensity of exercise employed in our study.

5.4.1.2 Effect of creatine supplementation on mitochondrial respiration

Creatine supplementation whether on its own or in combination with exercise had no effect on mitochondrial respiration, RCI or recovery of state 3 respiration after anoxia.
In mice with muscular dystrophy, creatine supplementation normalized skeletal muscle mitochondrial respiration which was decreased by the disease [Passaquin et al 2002]. In another study 16 days of Cr supplementation, reduced state 2 respiration (i.e. in the absence of ADP) but had no effect on state 3 respiration [Walsh et al 2001]. However, there is no other data available regarding the effect of creatine supplementation on its own or in combination with exercise on cardiac mitochondrial respiration. To our knowledge this is the first study showing that creatine supplementation has no effects on basal cardiac mitochondrial respiration or recovery of respiration after anoxia.

5.4.1.3 Effects of inhibitors of mitochondrial respiration

GDP inhibits the uncoupling proteins, which uncouple oxidative phosphorylation from ATP generation. Increased mitochondrial UCPs have been associated with less efficient ATP synthesis [Murray et al 2008], but have also been seen to be down regulated in the

failing heart [Laskowski and Russel [2008]. Murray et al [2004] found increased UCP expression with increased plasma free fatty acid concentrations and have suggested that energy deficiency in heart failure might result from increased mitochondrial UCPs. We thus felt it would be important to determine whether creatine or exercise influenced UCP's in any of the study groups we investigated.

We found no differences in the change in respiration rates between the groups in our study after the addition of GDP. If UCP's were influenced by our interventions we would have expected to see an increase in state 4 respiration in the presence of GDP and succinate. We propose that UCP's were not influenced by exercise training or creatine supplementation in our study.

The inhibition of state 4 respiration with GDP was significantly higher in heart mitochondria from 14 weeks treadmill run trained animals than in heart mitochondria from sedentary animals [Ascensao et al 2006], showing that the UCPs were more activated in trained heart mitochondria. Boss et al [1998] found that 4 weeks of treadmill running (5 days per week for 90 minutes per day) decreased UCP2 and 3 expression in skeletal muscle and the heart. These results were corroborated by Fernström et al [2004] who found down regulation of UCP3 with exercise training, showing less activation of UCPs with long term exercise training. It would appear that neither the length nor intensity of our exercise program brought about changes in the activity of UCPs involved in respiration in our study. Creatine supplementation did not affect the UCPs activity in our study.

Oligomycin (which inactivates complex IV, which produces ATP) was added to mitochondria to measure basal proton leak. If the mitochondria leak H^+ back across the

mitochondrial inner membrane without releasing ATP, then they are not efficient. However, neither exercise training nor creatine supplementation (at the dose and time given), conferred any advantage or cardioprotection on the rat hearts.

One would have anticipated that exercise and/or creatine supplementation would be beneficial to the rats, as the theory behind creatine supplementation is based on the premise that creatine phosphate is the cell's readily available supply of phosphate groups to phoshorylate ADP, as the ADP import export mechanism limits energy availability during exercise. Having a readily available pool of creatine to phosphorylate would have been expected to be of benefit against an acute anoxic stress, but this was not the case in our study.

5.5 SIGNALLING PATHWAYS IN THE HEART

5.5.1 Myocardial Creatine Transporter

There were no differences in myocardial CreaT protein expression in any of the groups we studied.

It has been shown that CreaT is not upregulated with creatine supplementation [Tarnopolsky et al 2003]. It has however also been reported to be downregulated in certain skeletal muscle types after 3-6 months of supplementation [Guerrero-Ontiveros and Wallimann 1998, Loike et al 1988]. Brault et al [2003] also found that 7 weeks of creatine supplementation resulted in no increased Cr, PCr, or total adenonucleotides (ATP+ADP+AMP), and no alterations in CreaT levels in skeletal muscle. After a 6 week creatine supplementation regime no effect was found on CreaT protein expression or PCr, Cr or ATP levels in the heart [Boehm et al 2003]. Consistent with the findings of Boehm et al [2003], we found that myocardial CreaT protein expression and ATP, PCR or Cr levels were unchanged by creatine supplementation.

Our findings are therefore consistent with these data and also provide additional insight about the effect of training on CreaT protein expression. One study examined the effect of exercise and creatine supplementation on intracellular creatine levels and reported increased creatine in the muscle after training [Robinson et al 1999]. However this study did not analyse the CreaT content or the effect of exercise training on it. Neubauer et al [1997] have shown that CreaT is downregulated in the failing human myocardium and in experimental heart failure. Lourdes Guerrero-Ontiveros and Wallimann in [1998] found a downregulation of the CreaT with supplementation in skeletal muscle. This is a possible protective mechanism induced to prevent Cr overload in the cell. This is particularly likely since Wallis et al [2005] found overexpression of CreaT in mouse hearts to be

detrimental, leading to cardiac hypertrophy and LV dysfunction (decreased ejection fraction). They speculated that the high levels of ADP, which they also observed, led to an energy imbalance in the cells.

5.5.2 Myocardial GLUT4

We found a significant increase in basal GLUT4 expression with exercise, but there was no increase in GLUT4 with creatine supplementation alone (creatine sedentary) or in combination with exercise (creatine exercised).

Information about GLUT4 expression or translocation in the heart or skeletal muscle after creatine supplementation is sparse. Treadmill running of diabetic rats for 6 days a week for 10 weeks increased the GLUT4 expression in cardiac muscle [Osborn et al 1997) while glucose transporters (GLUT4) in rat cardiac muscle showed increased translocation to the membrane in response to 10 weeks of swim training (5 days per week, 3 hours per day) [Zhang et al 2007c].

Skeletal muscle GLUT4 translocates to the plasma membrane in response to 2 hours of treadmill exercise [Fushiki et al 1989]. Total GLUT4 translocation but not total tissue GLUT4 protein expression increased in the skeletal muscle of rats after 6 weeks of exercise training in wheelcages [Goodyear et al 1992]. However, Rodnick et al [1990] used the same experimental protocol and found increased GLUT4 expression in the rat skeletal muscle. Langfort et al [2003] also found an increase in GLUT4 expression in human skeletal muscle after 8 days of cycle training.

Thus our data is consistent with other research findings which indicate that GLUT4 expression is increased after exercise training, particularly in the myocardium in our study.

Op't Eijnde et al [2001b] found an attenuated decrease in GLUT4 protein content in skeletal muscle immobilized for 2 weeks in creatine supplemented patients, and a subsequent increase in GLUT4 expression after 3 weeks of rehabilitation resistance exercise and creatine supplementation. However this increase in GLUT4 above control values was no longer evident after 10 weeks of creatine supplementation and resistance exercise. Ceddia and Sweeney (2004] found no increase in GLUT4 with 48 hours of creatine supplementation (0.5mM) in cultured L6 myoblasts.

Rooney et al [2002] have shown that creatine supplementation for 8 weeks increased fasting plasma insulin levels and increased insulin secretion in response to a glucose tolerance test in rats, but did not affect the overall glucose uptake. This was confirmed by Newman et al [2003] in sedentary male subjects with both acute (5 days), and short term (28 days) creatine supplementation, where no effect of creatine supplementation was seen on glucose tolerance. GLUT4 translocation and protein expression was not investigated. However, Gualana et al [2008] studied glucose tolerance in response to creatine supplementation and high intensity exercise run training 3 times per week for 3 months in human subjects. They showed that creatine supplementation and exercise increased glucose tolerance above that observed with exercise alone. Once again, GLUT4 protein expression and translocation was not examined.

To our knowledge ours is the first study to show that simultaneous exercise and creatine supplementation attenuates myocardial GLUT4 protein expression. It is therefore possible that the GLUT4 translocation was increased with both exercise and creatine in our study, as described previously. However GLUT4 expression is down

regulated by their combination. This could be a sensitization response and adaptive mechanism to prevent over stimulation of translocation and excess absorption of glucose and a resulting hypoglycemia.

5.5.3 Myocardial AMPK

5.5.3.1 AMPK phosphorylation at baseline

At baseline, creatine supplemented sedentary and exercised control rats had significantly raised phosphorylated AMPK levels compared with the control sedentary rats. However, creatine supplemented exercised rats had the same levels of phosphorylated AMPK as control sedentary rats.

Musi et al [2005] also found that an acute bout of exercise increased phosphorylation of AMPK in mice hearts. These observations were corroborated by Coven et al [2003], with moderate and high intensity exercise for 10 minutes increasing AMPK phosphorylation. Pold et al [2005] exercised diabetic Zucker rats for 5 days per week for 8 weeks, and found no changes in total AMPK expression in the heart muscle, but found an increased expression of total AMPK in the red gastrocnemius skeletal muscle. However they did not look at phosphorylation or activity of AMPK in the heart. There is little published data available on the effect of long term exercise on the activity or phosphorylation of AMPK in the heart.

Gibala et al [2009] reported that after 4 bouts of acute exercise AMPK phosphorylation was increased in skeletal muscle, and Langfort et al [2003] found increased AMPK content in skeletal muscle after 1 month of training.

Ceddia and Sweeney [2004] found AMPK phosphorylation was increased with Cr supplementation in skeletal muscle cells but contrarily Op't Eijnde et al [2005] showed that neither AMPK phosphorylation nor protein expression was increased by creatine supplementation after 6 weeks of recovery in skeletal muscle previously immobilized for 2 weeks. Ponticos et al [1998] demonstrated that in the presence of PCr in an *in vitro* assay, AMPK activity was inhibited, but the presence of Cr even at supra-physiological doses, had no effect. They also showed that active AMPK phosphorylates and inactivates MM-CK, thus stopping the conversion of PCr and ADP to ATP and Cr. Thus, less phosphorylation of AMPK would translate to more ATP being available for use by the cell. We propose that simultaneous creatine supplementation and exercise training may decrease the AMPK phosphorylation and stop the inhibition of the MM-CK and consequently provide a larger pool of energy for the heart.

5.5.3.2 AMPK during Ischaemia and Reperfusion

We found no differences in total AMPK expression in the heart during ischaemia, but creatine supplemented exercised hearts had higher levels of phosphorylated AMPK than creatine supplemented sedentary hearts. There were no differences between the control sedentary, control exercised and creatine exercised hearts. There was however a significant increase in AMPK phosphorylation in the creatine supplemented exercised group above all the other groups at 10 minutes of reperfusion.

There have been several reports that indicate that the activation of AMPK during ischaemia is a protective mechanism of the heart to preserve and generate ATP and protect the heart from the detrimental effects of ischaemia and reperfusion [Beauloye et al 2001, Baron et al 2005, and Sakamoto et al 2006]. However, one of these studies looked at the effect of AMPK activation on cardiac post-ischaemic functional recovery,

and only Lopaschuk [2008] reports that the activation of AMPK during ischaemia is detrimental. They have also shown that the inhibition of AMPK during reperfusion does not lead to a decrease in cardiac function or an energy supply impairment [Folmes et al 2009].

Our results indicate that increased AMPK phosphorylation during ischaemia and reperfusion was associated with reperfusion injury in the heart, but whether the APMK phosphorylation caused the decreased function, or vice versa, remains to be determined in further studies.

5.5.4 Myocardial PKB/Akt

5.5.4.1 Baseline expression and phosphorylation

In our study 8 weeks of exercise training increased PKB/Akt phosphorylation in the heart muscle, as did a combination of exercise and creatine supplementation. There were no changes in total protein expression of PKB/Akt.

Zhang et al [2007] has shown that long term exercise (3 hours per day, 5 days per week, 8 weeks of swimming) increases PKB/Akt phosphorylation in rat hearts. Similarly, Kemi et al [2008] showed that exercise on a treadmill for 5 days per week for 8 weeks increased phosphorylation of PKB/Akt in the heart. They also showed that this type of exercise induced physiological hypertrophy in cardiomyocytes, increasing cell width and length.

Pathological hypertrophy was also induced by pressure overload after 8 weeks of transverse aortic constriction [Kemi et al 2008]. This increased HW: BW ratios, left ventricle posterior wall and interventricular wall thickness, and down regulated the

Akt/mTOR pathway in these hearts. Thus this implicates PKB/Akt phosphorylation in physiological hypertrophy and may differentiate it from pathological hypertrophy. McMullen et al [2003] also showed that PKB/Akt was phosphorylated after 3 weeks of swim training in rats. When comparing our results to these, it could be speculated that we may have had physiological hypertrophy (cardiac remodeling) without increases in heart weight in our model. This has been shown by Evangelista et al [2003] who showed that an increased heart weight did not necessarily go hand in hand with physiological hypertrophy.

Lajoie et al [2003] documented decreased PKB/Akt phosphorylation after a 13 week swimming program, however the same exercise program increased and thus normalised the levels of PKB/Akt in diabetic rats. Deldicque et al [2007] found that exercise decreased phosphorylation of PKB/Akt in skeletal muscle directly after exercise, but had no effect on baseline levels of phosphorylated protein 72 hours after exercise. They also found that creatine supplementation for 5 days decreased PKB/Akt phosphorylation in the resting state. This differs from our results, in that we found a 50% increase in PKB/Akt phosphorylation with creatine supplementation in sedentary rat hearts – although this was not significantly different when compared to hearts from control sedentary rats. However, the study done by Deldique et al [2007] is the only one published that provides some insight into the effect of creatine and exercise on PKB/Akt.

5.5.4.2 PKB/Akt during ischaemia and reperfusion

Phosphorylation of PKB/Akt was increased after 10 minutes reperfusion in creatine supplemented exercised hearts compared to control and creatine supplemented sedentary hearts. Hearts from control exercised rats had phosphorylated PKB/Akt levels

that were twice as high as hearts from sedentary rats, but did not reach significance. This was possibly due to a small number of hearts analysed. There were no differences in PKB/Akt expression or phosphorylation during ischaemia or at the end of 30 minutes of reperfusion.

Zhang et al [2007] has shown that long term exercise (3 hours per day, 5 days per week, 8 weeks of swimming) increases PKB/Akt phosphorylation in rat hearts subjected to regional ischaemia. This was accompanied by a smaller infarct size in these exercised hearts. On the other hand, Ravingerova et al [2009] has shown that the activation of PKB/Akt is not essential for cardioprotection, because inhibiting its activity still resulted in cardioprotection after ischaemia in pre-conditioned rat hearts. Skyschally et al [2009] has also recently shown that inhibition of the RISK pathway and PKB/Akt with wortmannin did not decrease infarct size in ischaemic preconditioned hearts, suggesting that this kinase did not play a role in cardioprotection.

Taking this conflicting evidence and our own data into consideration, we propose that the phosphorylation of PKB/Akt at 10 minutes of reperfusion in the hearts from creatine supplemented exercised rats was not cardioprotective in this study

5.5.5 Myocardial ERK 42/44

Our study showed that expression of ERK 44 in the creatine supplemented exercised group was significantly decreased at the end of 20 minutes of global ischaemia when compared to control sedentary hearts. Total ERK 42 expression was significantly higher in creatine supplemented exercised hearts compared to control sedentary hearts after 10 minutes of reperfusion. Neither creatine supplementation nor exercise had any effect on the levels of ERK42/44 phosphorylation.

Deldicque et al [2007] found that 5mM creatine supplementation in the culture medium did not affect ERK phosphorylation in cultured skeletal muscle cells, and in 2008 they showed that neither Cr supplementation for 5 days nor short term acute maximal exercise effected ERK phosphorylation in human skeletal muscle cells. McMullen et al [2003] also showed no effect of swim training for 4 weeks on myocardial ERK phosphorylation or expression. Iemetsu et al [2006b] demonstrated that 8 weeks of swim training increased basal ERK 42 and ERK 44 phosphorylation. They did not however look at the effect of this increase on the heart functional parameters. One would expect, with an increase in this pro-survival kinase, that protection would be elicited, however this was not the case in our study, and an increase in total basal ERK 42 in the creatine supplemented exercised group during reperfusion did not protect these hearts against reperfusion injury. However, the decrease in ERK 44 during ischaemia in creatine supplemented, exercised hearts, corresponded with a decreased reperfusion function.

Kovacs et al [2009], du Toit et al [2008] and Yu et al [2008] have all recently showed a correlation between ERK activation during ischaemia and reperfusion, and increased cardioprotection. Taking this into consideration, the decreased ERK 44 levels that we found in the creatine supplemented exercise trained group correlates with decreased cardioprotection. This is supported by the evidence presented above.

5.5.6 Myocardial p38 MAPK

In our study, basal phosphorylated p38 MAPK expression was decreased by a combination of 8 weeks of exercise training and creatine supplementation, and after 20 minutes of global ischaemia, the phosphorylation of p38 MAPK was increased in these hearts compared to control sedentary hearts.

The activation of p38 MAP kinase during ischaemia has been studied in depth by several research groups. Notwithstanding the fact that p38 has been implicated as a trigger and a mediator of the protection elicited by ischaemic preconditioning, there is contradictory evidence concerning its role in cardioprotection. For this reason we documented the p38 expression and phosphorylation profiles in the exercised and creatine supplemented hearts of animals from our study.

Deldicque et al [2007] found that immediately after exercise p38 MAPK increased in skeletal muscle but creatine supplementation for 5 days had no effect on the p38 MAPK phosphorylation. Musi et al [2005] showed increased p38MAPK phosphorylation after a single bout of acute exercise. McMullen 2003 also showed no influence of swim training for 4 weeks on p38 MAPK phosphorylation or expression.

Limited research has been done on the effect of long term exercise training on p38 MAPK levels in the heart and the only study that was done showed that chronic long term (8 weeks) swim training had no effect on basal MAPK levels [Iemitsu et al 2006b]. To our knowledge this is the first study to show that p38 MAPK may be down regulated with simultaneous long term exercise training and creatine supplementation, and increased during ischaemia in hearts from animals of the same group.

P38 MAPK phosphorylation and activation during ischaemia and reperfusion has been associated with myocardial ischaemia/reperfusion injury [Kim et al 2009] and apoptosis [Ma et al 1999]. The combination of creatine supplementation and exercise increased levels of p38 MAPK phosphorylation during ischaemia. This p38 MAPK phosphorylation is associated with a decreased functional recovery of these hearts during reperfusion and increased susceptibility to myocardial ischaemia/reperfusion injury.

CONCLUSIONS

Data from this study suggests that creatine supplementation has no effects on basal cardiac function but does reduce myocardial tolerance to ischaemia/reperfusion in the hearts of sedentary and exercise trained animals. The combination of creatine supplementation and exercise increases ischaemic contracture and decreases aortic output recoveries in the rat hearts. Creatine supplementation alone or in combination with exercise, had no effect on mitochondrial respiration or tolerance to anoxia. Exercise with and without creatine supplementation increased basal PKB/Akt phosphorylation however only the combination of creatine supplementation and exercise increased PKB/Akt phosphorylation after 10 minutes of reperfusion, suggesting that PKB/Akt phosphorylation was not associated with cardioprotection in this study. Although both GLUT4 expression and AMPK phosphorylation increased with exercise training, creatine supplementation attenuated this increase. AMPK phosphorylation in hearts from creatine supplemented exercised rats was much higher after 10 minutes of reperfusion than all other groups, as was p38 MAPK phosphorylation during ischaemia. P38 MAPK phosphorylation was also increased during ischaemia in this group. Basal total ERK44 expression was decreased in hearts from creatine supplemented exercised animals, while ERK42 expression was increased in this group after 10 minutes of reperfusion. This data suggests that increased ERK44, AMPK and PKB/Akt phosphorylation did not offer cardioprotection in this study. The exact mechanisms for the decreased aortic output recoveries in creatine supplemented exercised rat hearts are unknown and need further investigation.

FURTHER STUDIES

The exact mechanism involved in the exercised and creatine supplemented, exercised heart's susceptibility to ischaemia/ reperfusion injury remains elusive. While an increased ischaemic contracture in these hearts has been seen in this study, the precise reason for this is also mysterious.

Since one of the possibilities for contracture is an imbalance in calcium concentrations, it becomes obvious that this is the next step in trying to find the mechanism in this paradox. If raised calcium concentration *is* a factor, then the membrane transporters activity need to be examined.

A possible shortcoming of this study is that baseline values of proteins and signaling molecules were examined at 2 minutes perfusion time. This is the time the hearts were freeze clamped for optimal HEP determination, but it has recently been implied that this is not optimal for baseline signaling molecule assessment [Stenslokken et al 2009].However this study used the balloon model to compare differing lengths of perfusion time on the phosphorylation of stress induced kinases, and we used the working heart model, which in itself is less stressful that the balloon model, and thus has been shown to reduce inadvertent phosphorylation of these kinases. We could also have taken reperfusion samples at more than 30 minutes reperfusion, as 30 minutes may not have been long enough of changes in total protein synthesis to have been brought about. However in our lab it has been shown that for most survival kinases analysed here (Erk, p38, PKB) 30 seconds perfusion is substantial for baseline values and 30 minutes reperfusion is adequate for post-ischaemic values of these proteins [Fan et al 2009].

Our balloon perfusions for determination of ischaemic contracture and heart pressures were performed without the perfusionist having extensive experience on the isolated rat heart perfusion system. This may have contributed to the low LVDevPs (and high DPs) obtained in some experiments. This was a weakness in the study and we are aware of the errors and recognize that the diastolic pressures and LVDevP were not always optimal. We do however believe that as these technical weaknesses were applied to all groups of hearts studied and would therefore have a similar impact on all experimental groups.

Bearing these weaknesses in the study in mind, the data obtained from functional recovery is indeed both surprising and worrying. The fact that exercise alone and in combination decreased the functional recovery of hearts post-ischaemia in this model is disturbing, as it has for decades been thought that exercise training is highly cardioprotective, and sportsmen and - women have for years been and are still using creatine supplementation without being aware of possible risks to their health.

This study draws our attention to the possibility that although creatine supplementation may have beneficial effects on sport performance and post training recovery, there may be long term health risks associated with the use of this supplement. More studies looking at the long-term effects of creatine supplementation are advisable.

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