The Effect of Androgenic Anabolic Steroids on the Susceptibility of the Rat Heart to Ischaemia and Reperfusion Injury

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Thesis presented in partial fulfilment of the requirements for the degree of Master of Physiology at the University of Stellenbosch

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December 2002

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Summary

Background: Athletes use androgenic anabolic steroids (AAS) to enhance their physical performance. The abuse of AAS is however associated with a host of side effects including sudden death due to cardiac arrest. The use of AAS leads to myocardial hypertrophy, which possibly makes the heart more prone to ischaemia/reperfusion injury, since it often develops in the absence of proper vasculature development.

Chronic AAS use also disrupts myocardial β-adrenoreceptor function and possibly cAMP, signalling in the heart. Drugs increasing cAMP and decreasing cGMP levels in the ischaemic myocardium exacerbate myocardial ischaemia/reperfusion injury.

We also know that AAS causes coronary artery disease secondary to the deleterious alteration of lipid profiles by increasing the LDL cholesterol and decreasing the HDL cholesterol levels.

AAS treatment may increase systemic TNFα levels by stimulating lymphocyte TNFα secretion that has been implicated in the depression of myocardial function, myocardial hypertrophy and the worsening of ischaemia/reperfsuion injury.

Aims: To determine whether chronic AAS treatment in trained and untrained rats influences: 1) heart function and susceptibility to ischaemia/reperfusion injury, 2) myocardial cyclic nucleotide levels (cAMP and cGMP) and 3) myocardial TNFα levels.

Material and methods: Male Sprague-Dawley rats (n=100) were divided into 4 groups: sedentary vehicle (placebo) treated group, sedentary AAS treated group, exercise vehicle (placebo) treated group, and exercise AAS treated group. Steroid treated animals received an intramuscular injection of nandrolone laureate (0.375 mg/kg) once a week, for six weeks.

Training consisted of swim sessions 6 days a week for 6 weeks. Swim time was incrementally increased up to a maximum of 50 minutes a day. For biometric parameters heart weight and body weight were documented. Hearts were mounted on a Langendorff perfusion apparatus and left ventricular developed pressure (LVDP), heart rate (HR) and coronary flow (CF) was monitored. The hearts were subjected to a period of 20 minutes of global ischaemia, followed by 30 minutes of reperfusion. Functional parameters was again monitored and documented. For biochemical analysis, blood was collected for the determination of serum lipid levels and myocardial tissue samples were collected before, during and after ischaemia for the determination of myocardial TNFα, cGMP and cAMP levels and p38 activity.

Conclusions: Results obtained would suggest that AAS exacerbate exercise induced myocardial hypertrophy. It also prevents the exercise-induced improvement in cardiac function. AAS use reduces reperfusion function in treated hearts, which may suggest that AAS exacerbates ischaemic and reperfusion injury. Furthermore it was seen that AAS elevates basal (pre-ischaemic) cyclic nucleotide levels and basal (pre-ischaemic) as well as reperfusion TNFα levels. This may also contribute to the exacerbation of ischaemic and reperfusion injury.

Opsomming

Agtergrond: Androgeniese anaboliese steroïede (AAS) word dikwels deur atlete gebruik om sportprestasie te verbeter. Die misbruik van AAS het egter talle newe effekte, insluitende skielike dood wat gewoonlik toegeskryf word aan hartaanvalle. Die gebruik van AAS lei onder andere tot miokardiale hipertrofie wat opsigself, as gevolg van ontoereikende vaskulêre ontwikkeling tydens die ontwikkeling van hipertrofie, die hart nog meer vatbaar vir isgemie/herperfusie skade maak.

Kroniese AAS toediening versteur miokardiale beta-adrenoreseptor funksie en moontlik die tweede boodskapper, sAMP, seintransduksie in die hart. Ons weet ook dat AAS koronêre hartvatsiektes veroorsaak. Laasgenoemde is sekondêr tot die nadelige lipiedprofiel verandering, wat 'n verhoging in LDL-C en 'n verlaging in HDL-C insluit. Middels wat miokardiale sAMP vlakke verhoog en sGMP vlakke in die isgemiese miokardium verlaag, vererger miokardiale isgemie/herperfusie skade.

AAS behandeling kan moontlik ook sistemiese TNFα vlakke verhoog deur limfosiet TNFα sekresie te stimuleer. Die verhoogde TNFα vlakke word verbind aan die onderdrukking van miokardiale funksie, miokardiale hipertrofie en die verergering van isgemie/herperfusie skade.

Doelwitte: Die doelwitte van die studie was om te bepaal of kroniese AAS toediening in geoefende en ongeoefende rotte 1) hartfunksie en die hart se vatbaarheid vir isgemie/herperfusie skade beïnvloed, 2) miokardiale sikliese

nukleotiedvlakke (sAMP en sGMP) beïnvloed en 3) miokardiale TNFα-vlakke beïnvloed.

Materiale en metodes: Manlike Sprague-Dawley rotte (n=100) is gebruik en in 4 groepe verdeel: 'n ongeoefende placebo groep (kontrole); 'n ongeoefende steroïedbehandelde groep; 'n geoefende placebo groep (kontrole) en 'n geoefende steroïedbehandelde groep. Steroïed behandelde diere het 'n intramuskulêre nandroloon lauraat inspuiting (0.375 mg/kg) een keer per week vir ses weke ontvang. Die oefenprogram het bestaan uit ses swemsessies 'n week vir ses weke. Die swemtyd is geleidelik weekliks verhoog tot by 'n maksimum tyd 50 min. Die waterbadtemperatuur is tussen 30 - 32 °C gehandhaaf. Vir biometriese parameters is hartgewig en liggaamsgewig genoteer. Harte is op 'n Langendorff perfusie apparaat gemonteer en linker ventrikulêre ontwikkelde druk (LVOD), koronêre vloei (KV) en harttempo (HT) is genoteer. Die harte is vervolgens blootgestel aan 20 minute van globale isgemie gevolg deur 'n 30 minute herperfusieperiode. LVOD, KV en HT is weer eens noteer. Vir biochemiese doeleindes is bloed voor perfusie versamel om serum lipied vlakke te bepaal. Miokardiale weefsel is versamel voor, tydens en na isgemie vir die bepaling van TNFα, cGMP en AMP vlakke asook p38 aktiwiteit.

Gevolgtrekkings: Na aanleiding van resultate verkry wil dit voorkom asof die gebruik van steroïde oefeningsgeïnduseerde miokardiale hipertrofie vererger. Dit verhoed ook oefeningsgeïnduseerde verbetering in miokardiale funksie. AAS lei tot 'n verlaagde herperfusiefunksie in behandelde harte, wat dalk mag

dui op AAS verergering van isgemie en herperfusie skade. Verder was daar ook waargeneem dat AAS basale (pre-isgemiese) sikliese nukleotiedvlakke en basale TNFα-vlakke sowel as herperfusie TNFα vlakke verhoog. Die verhoging in TNF-α vlakke mag dus moontlik ook bydra tot die verergering van isgemie- en herperfusieskade.

Acknowledgments

I would like to express my sincere gratitude to my promotor, dr. EF du Toit, for his patience and guidance throughout this study.

I would like to thank my project co-leader, dr. J. van Rooyen, for his assistance.

I would like to thank the department at Tygerberg Medical Campus and prof. Amanda Lochner for their support and warm welcoming.

For financial support, I would like to thank the National Research Foundation.

I wish to thank my parents, family and friends for their motivation and trust in my ability to complete this study.

ABBREVIATIONS

Units of measurement

%	percentage
°C	degrees Celsius
µl	microliter
bpm	beats per minute
cm	centimeter
cmH_2O	centimeters water
DP	diastolic pressure
fmol	femtomole
fmol/gww	femtomole per gram wet weight
g	gram
gww	gram wet weight
HR	Heart rate
KDa	kilodalton
kg	kilogram
l/L	liter
LVDP	Left ventricular developed pressure
M	molar
mg	milligram
min	minute
ml/min	milliliter per minute
mm	millimeter
mM	millimolar

mmHg	millimeter mercury
mmol	millimole
mol	mole
pg	picogram
pmol	picomole
pmol/gww	picomole per gram wet weight
Rpm	revolutions per minute
RPP	rate pressure product
SEM	standard error of the mean
SP	systolic pressure
TC	total count
w	weight
w/v	weight per volume
N	normal
nm	nanometre

Chemical compounds

APS	ammonium persulphate
BSA	bovine serum albumin
Ca^{2+}	Calcium
$CaCl_2.H_2O$	calciumchloride 2-hydrate
CO_2	carbon dioxide
DTT	dithiotretiol
EDTA	Ethylenediaminetetra- acetic acid
EGTA	Ethylene-bis(β-Aminoethylether)- N,N,N',N'-tetra-Acetic acid

H_2O	water
H_2SO_4	sulphuric acid
H_3PO_4	ortho-phosphoric acid
K^+	potassium
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
KOH	potassium hydroxide
$MgSO_47H_2O$	magnesium sulphate 7-hydrate
Na_2CO_3	sodium bicarbonate
Na_2HPO_4	di-sodium hydrogen phosphate
NaCl	sodium chloride
$NaHCO_3$	sodium hydrogen carbonate
O_2	oxygen
Panceau-S	3 Hydroxy-4-[2-sulfo-4-(4-sulfo-phenylazo)phenylazo]-2.7-naphtalene disulphonic acid
PBS	phosphate buffered solution
PCA	perchloric acid
PMSF	phenylmethyl sulphonyl fluoride
p-NPP	p-nitrophenylphosphate
PVDF	poly vinylidene fluoride
SDS	sodium dodecyl sulphate
TCA	trichloro acetic acid
Temed	N,N,N',N'- Tetramethyl ethylene diamine ammonium
TMB	3,3', 5,5' tetramethylbenzidine
TRIS-EDTA	tris(hydroxymethyl) aminomethane Ethylenediamintetra-

TRIS-HCL	tris(hydroxymethyl) aminomethane hydrochloride

Other abbreviations

AAS	androgenic anabolic steroids
ADH	antidiuretic hormone
ANF	atrial natriuretic factor
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
cAMP	cyclic 3',5'- adenosine monophosphate
CF	coronary flow
cGMP	cyclic 3',5'- guanosine monophosphate
CHOP	C/EBP homology protein
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular-regulated kinases
ET-1	endothelin-1
Exerplac	exercise placebo (control)
Exerster	exercise steroid treated
FADD	Fas-associated death domain
FFA	free fatty acids
G-protein	guanine nucleotide-binding protein
G_s	Stimulatory G-protein
GTP	guanine triphosphate
HDL	high density lipoprotein
HDL-C	high density lipoprotein cholesterol
HL	hepatic lipase

Hsp25/27	heatshock protein 25/27
HTGL	hepatic triglyceride lipase
IL	interleukin
IL-1	interleukin-1
IL-6	interleukin-6
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
Isch	ischaemia
JAK	janus-associated kinases
JNK	c-Jun NH_2-terminal kinase
K_{Ca}^{2+} channel	calcium dependent potassium channel
LDL	low density lipoproteins
LDL-C	low density lipoprotein cholesterol
MAPK	mitogen activated protein kinase
MAPKAP	mitogen activated protein kinase associated kinase
MEF2C	myocyte enhancer factor-2C
MEK	MAPK/ERK
MEKK	MEK kinase
MHC	myosin heavy chain
MKKK	MAPK kinase kinase
MLC	myosin light chain
MRNA	messenger ribonucleic acid
NF-κB	nuclear factor-kappa B
NO	nitric oxide
NOS	nitric oxide synthase

PDGF	platelet derived growth factor
Perf	perfusion
PHLPL	post heparin lipoprotein lipase
PKA	protein kinase A
PKC	protein kinase C
PKG	cGMP dependent protein kinase
Repf	reperfusion
RNA	ribonucleic acid
ROS	reactive oxygen species
SAPK	stress activated protein kinases
Sedplac	sedentary placebo (control)
Sedster	sedentary steroid treated
SERCA	sarcoplasmatic reticulum Ca^{2+} ATPase
STAT	signal transducers and activators of transcription
TAG	triacylglycerol
TGF-β	transforming growth factor-beta
TNF- β	tumor necrosis factor beta
TNF-RI	TNF receptor I (TNF-R55)
TNF-RII	TNF receptor II (TNF-R75)
TNF-α	tumor necrosis factor alpha
TRADD	TNF receptor associated death domain
TRAF2	TNF receptor associated factor 2

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Chapter 1

Introduction

It is a well-known fact that athletes use androgenic anabolic steroids (AAS) to enhance their performance (Shahidi, 2001). The abuse of AAS has several side effects including so-called sudden death. This phenomenon is reported in several case studies and is generally attributed to cardiac arrest (Alen and Hakkinen, 1985; Mewis *et al.*, 1996; Sullivan *et al.*, 1998).

One of the most important side effects of AAS use is the alteration of lipid profiles. Several studies reported that the use of AAS leads to both an increase in LDL-cholesterol and a reduction of the HDL-cholesterol (Leeds *et al.*, 1986; Weyrich *et al.*, 1991). These detrimental lipid profiles are known to place subjects at an increased risk of cardiovascular diseases (Glazer, 1991).

Several cardiovascular adaptations to exercise are documented. One of the most important relates to heart size. As mentioned earlier, endurance training leads to an increase in heart weight, which is beneficial at first (Frenzel *et al.*, 1988). This may progress to a pathological hypertrophy (De Maria *et al.*, 1978; Steinberger, 2001). Further adaptations include an overall increase in stroke volume (Jensen-Urstad *et al.*, 1998), a decrease in resting heart rate (Wayne *et al.*, 1998), an increased cardiac output at maximum exercise levels (Charlton and Crawford, 1997), a lowering in resting blood pressure (Lewis *et al.*, 1999) and an increase in blood volume (Green *et al.*, 1991).

Androgenic anabolic steroid use leads to myocardial hypertrophy- a condition that on its own makes the heart more vulnerable to ischaemia/reperfusion injury due to, amongst other things, inadequately developed vasculature (Opie, 1995). Resistance training on its own stimulates cardiac hypertrophy independent of AAS use (Sullivan *et al.*, 1998). This is thought to be an adaptational hypertrophy to compensate for an increase in workload (afterload). This compensatory hypertrophy often progresses to decompensatory hypertrophy. This condition exists when work output per unit of cardiac mass decreases (Meerson, 1982). It is thought that AAS pathologically alters this beneficial physiological adaptation to exercise (Sullivan *et al.*, 1998).

There are two main signalling pathways implicated in cardiac hypertrophy: the mitogen activated protein kinase (MAPK) pathway and the janus associated kinase/signal transducers and activators (JAK/STAT) pathway (Ruwhof and Van der Laarse, 2000). Other models might also explain the negative effects of AAS on the heart and shed some light on the possible causes for an increased risk for heart failure. These include the thrombosis, vasospasm and direct injury hypotheses (Melchert and Welder, 1995).

Chronic AAS administration might also disrupt myocardial beta-adrenoreceptor and second messenger function (Norton *et al.*, 2000). The cellular mechanisms for this disruption of function have not been elucidated, but it has been well demonstrated that myocardial ischaemia/reperfusion injury is exacerbated by agents that increase ischaemic cAMP levels

(Wollenberger *et al*, 1969; Depre and Hue, 1994) and is lessened by agents that increase cGMP levels in the ischaemic myocardium (Du Toit *et al.*, 2001).

Mitogen activated protein kinases also play an important role in stress signal transduction from the membrane to the nucleus (Cobb, 1999). Of special interest is the p38 MAPK pathway, since it might play a protective or deleterious role in ischaemia/reperfusion injury, and in cardiac hypertrophy. The protective role of p38 against ischaemia/reperfusion injury involves the phosphorylation of small heat shock proteins (hsp25/27) (Rouse *et al.*, 1994). Regarding the deleterious role of p38, it has been shown that the activation of p38 generally leads to myocardial damage and apoptosis (Ma *et al.*, 1999). The activation of p38-MAPK and c-Jun NH2-terminal pathways has also been implicated in alpha-1 adrenergic stimulation induced hypertrophic growth (Wang *et al.*, 1998).

AAS treatment has also been shown to possibly increase systemic TNF-alpha levels by stimulating lymphocyte secretion of TNF-alpha (Hughes *et al.*, 1995). Tumour Necrosis Factor alpha is a pro-inflammatory cytokine associated with ischaemia and reperfusion injury and cardiac hypertrophy. Locally produced TNF-alpha can induce post-ischaemic dysfunction by causing contractile dysfunction and myocyte apoptosis (Meldrum *et al.*, 1998). It would seem that TNF-alpha depresses cardiac function by interfering with the calcium-induced calcium release by the sarcoplasmatic reticulum (Krown *et al.*, 1995). It is also possible that TNF-alpha is a mediator of myocardial

cell growth when the heart is exposed to chronic low TNF-alpha concentrations (Sack *et al.*, 2000).

The aim of this study was to determine whether chronic AAS treatment in trained and untrained animals had an effect on basal heart function and if it increased the susceptibility of the heart to ischaemic/reperfusion injury. A further aim was to determine whether chronic AAS use influenced the myocardial cyclic nucleotide levels, myocardial p38 activity and myocardial TNF-alpha levels. Changes in the levels of these cytosolic proteins may explain some of the putative negative effects of AAS on the normoxic and ischaemic heart.

Chapter 2

Literature review

2.1 Androgenic Anabolic Steroids

Androgenic anabolic steroids (AAS) are almost identical to the male sex hormones. The anabolic properties of these drugs accelerate growth by promoting the development of muscle mass and increasing the rate of bone maturation. In theory, the administration of AAS will increase fat free mass and strength, which makes it an attractive supplement for athletes. It has been postulated that anabolic steroids facilitate recovery from exhaustive training, allowing the athletes to train harder on subsequent days. The potential for anabolic steroid abuse among athletes is very high, and is currently a major problem in sport. Some athletes are reported to be taking five to ten times the recommended daily maximum dosage (Willmore and Costill, 1994). Studies on humans and steroid abuse are relatively limited because of the stigma attached to its use and it is therefore relied on a few reported case studies for most of the information.

2.1.1 Case studies of anabolic steroid abuse

Sullivan and colleagues (1998) reported that a 22-year old man was hospitalised complaining of generalized weakness and a "funny feeling" in his chest. The patient had been in his usual state of health until 2 weeks earlier when he started experiencing headaches and palpitations while exercising. His symptoms gradually worsened and became progressively associated with generalized weakness, shortness of breath, anxiety and diaphoreses. He denied any drug abuse (including alcohol and tobacco). He considered

himself a competitive body builder, lifting weights 5 times a week for several years. His physical examination revealed no inconsistencies. The chest radiograph was unremarkable and his lungs clear. However, the electrocardiogram demonstrated artrial fibrillation with rapid ventricular response at approximately 175 beats per minute, a right axis deviation, and inverted T-waves. He eventually confessed to using 200 mg of testosterone cypionate, 200 mg of extrabolin decanoate and 120 mg of stanozolol two days a week, for the previous five weeks. These are supra-physiological dosages. An echocardiogram showed an area of significant septal hypokinesis, posterior and septal wall thickness at the upper limit of normal, preservation of left ventricular function, moderate left atrial hypertrophy and mild mitral and tricuspid regurgitation.

In another case a 28-year-old man was admitted to hospital with ventricular tachycardia (Mewis *et al.*, 1996). Coronary angiography revealed severe underlying coronary heart disease. The patient again had no coronary risk factors such as a history of smoking, hypertension, diabetes, or a positive family history for coronary heart disease. However, six months prior to his admission to hospital, he had discontinued stanazolol use, which had consisted of a weekly dose of 280 mg over a period of two years. Since the risk of heart disease in the above subjects was small, it could be postulated that AAS played a significant role in the development of their respective cardiovascular abnormalities.

An adult male body builder of international level, who decided to complement his training by self-administering androgenic hormones at doses of 53 mg/day volunteered as a subject in a study by Alen and Hakkinen (1985). They investigated his physical health and fitness over a training period of 1 year. This included only a 4-week abstinence from drug use in the middle of the year. Results showed that the subject had atrophic testicles and low luteinising hormone, follicle stimulating hormone and testosterone levels after drug withdrawal. These findings indicate that sustained anabolic steroid use leads to long-term impairment of testicular endocrine function, and consequently leading to azoospermia and gynegomastia. The observed decrease in serum HDL-cholesterol levels also increased the subject's risk for atherogenesis.

2.1.2 Possible explanations for increases in cardiovascular risk

AAS stimulate cellular protein synthesis through androgenic receptors and promote growth of all organs that have receptors similar to those for the androgens (Di Bello *et al*, 1999). Athletes and body builders who seek a competitive advantage in sports require increased strength and muscle mass and frequently abuse AAS. The use of anabolic steroids has numerous adverse side effects that include altered endocrine function leading to conditions such as testicular atrophy, oligospermia and decreased testosterone levels. Hyperlipoprotenemia, hypertension, hypercachexia, peliosis hepatis, jaundice, cholestasis and hepatonoma are some of the additional side effects reported in athletes using anabolic steroids (Loughton and Ruhling, 1977; Lamb, 1984; Lenders *et al.*, 1988). Although Krieg and

colleagues (1978) demonstrated a specific androgen receptor in the rat heart muscle, the effect of chronic use of anabolic steroids on the cardiovascular system, and in particular, the myocardium is still to a great extent unclear.

2.1.2.1 Effects of AAS on lipoproteins

Leeds and colleagues (1986) conducted experiments to investigate the effects of anabolic steroid administration on plasma total cholesterol (TC) concentration and plasma lipoprotein cholesterol profile in both sedentary and anaerobically exercised male and female rats. Rats subjected to the anaerobic exercise program demonstrated changes in their lipid profile parallel to those observed in aerobic exercise-training studies. Male and female rats, when grouped together, showed significant increases in HDL-C (high-density lipoprotein cholesterol) and a significant decrease in LDL-C (low-density lipoprotein cholesterol) concentration. When grouped together, male and female rats treated with anabolic steroids, demonstrated a significant atherogenic trend in their lipoprotein profiles (decreased HDL-C and increased LDL-C). The exercise induced decrease in LDL-C in both male and female rats was completely reversed with steroid administration. It is clear from their study that exercise has a definite anti-atherogenic effect on the HDL-C and LDL-C concentrations. One possible explanation for this phenomenon involves two enzymes that play a role in lipoprotein metabolism, namely endothelial post hepatic lipoprotein lipase (PHLPL) and hepatic lipase (HL) (Peltonen *et al.*, 1981; Stubbe *et al.*, 1983). It has been suggested that AAS increase hepatic triglyseride lipase (HTGL), which catabolises HDL (Figure 2.1). Indeed, it was found that the use of AAS increased HL activity

and decreased PHLPL. This would lead to detrimental changes in the plasma lipid profile placing subjects at an increased risk for atherosclerosis and other associated cardiovascular diseases (Glazer, 1991). Differences seen between male and female rat control groups seem to follow the same trend as humans: Males tend to have higher TC and LDL-C, and lower HDL-C than their female counterparts and this is thought to be due to the female hormone estrogen (Hazzard *et al.*, 1984). In another study Weyrich and his colleagues (1991) examined the relationship between exogenous testosterone and blood lipids in a non-human primate model. Results showed that the use of exogenous testosterone not only decreased HDL-C, but also increased the plasma concentration of thromboxane$_2$. The increase of plasma LDL-C by AAS could cause fatty streaks to occur in the coronary endothelium with subsequent endothelial injury. By both increasing platelet derived growth factor and cell proliferation, it is possible that AAS could produce an advanced atherosclerotic lesion (see Figure 2.1).

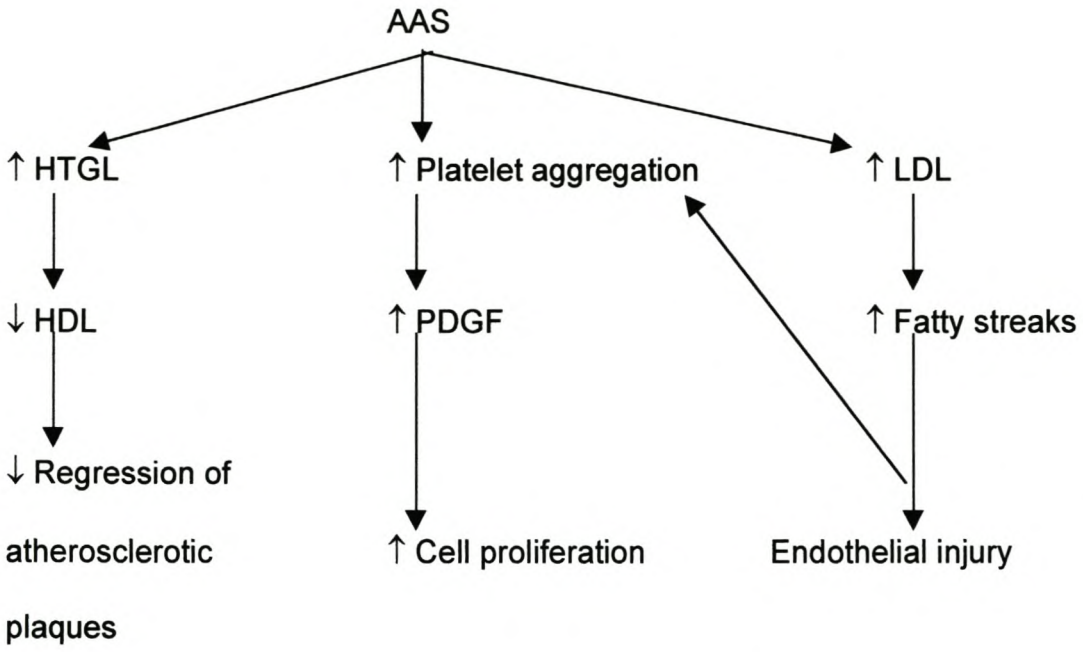

Figure 2.1: Hypothetical model of atherogenesis induced by androgenic-anabolic steroids HTGL=hepatic triglyseride lipase; HDL=high-density lipoprotein; PDGF=platelet derived growth factor; LDL=low-density lipoprotein (Melchert and Welder, 1995)

2.1.2.2 Cardiac hypertrophy

Cardiac hypertrophy is a fundemental adaptation to an increased workload due to hemodynamic overload (Cooper, 1987; Mondry and Swynghedau, 1995). The development of cardiac hypertrophy appears to be beneficial at first, since it increases the number of contractile units and reduces left ventricular wall stress. This adaptation is limited however, and heart failure may follow the initial beneficial response.

Several changes are associated with cardiac hypertrophy:

- The rapid induction of proto-oncogenes and heatshock proteins
- Quantitative and qualitative changes in gene expression
- Increased rates of protein synthesis

The primary stimulus for cardiac hypertrophy appears to be mechanical stress with an accompanying increase in neural and humoral factors (Ruwhof and van der Laarse, 2000). Growth factors and hormones may be involved indirectly in hemodynamic overload-induced cardiac hypertrophy. In hemodynamic overload induced hypertrophied hearts and stretch induced hypertrophied cardiomyocytes, the expression and release of several of these factors have been reported and are seen as markers of cardiac hypertrophy. These include:

- endothelin-1 (Arai *et al.*, 1995; Yamazaki *et al.*, 1996, Seko *et al.*, 1999; Serneri *et al.*, 1999)
- Angiotensin II (Miyata *et al.*, 1996; Tamura *et al.*, 1998)
- Transforming growth factor-β (Villarreal and Dillmann, 1992)
- Insulin-like growth factor-1 (Serneri *et al.*, 1999)
- Myotrophin (Sil *et al.*, 1995)
- Vascular endothelial growth factor (Li *et al.*, 1997; Seko *et al.*, 1999)
- TNF-α (Klein *et al.*, 1995)

There has been much speculation about the link between heamodynamic overload and the induction of intracellular signals that are responsible for the hypertrophic response. Several possible mechanisms have been implicated

belonging to two major groups: the integrins and cytoskeleton; and the sarcolemmal proteins (Ruwhof and Van der Laarse, 2000).

Integrins are a family of cell surface receptors that link the extracellular matrix to the cytoskeleton at places called focal adhesion sites (Hynes, 1992; Juliano and Haskill, 1993; Schwartz *et al.*, 1995). Integrins may integrate a variety of different signaling pathways that are activated by both the extracellular matrix and growth factors to establish the coordinated hypertrophic response. Mechanical stress can cause the deformation of the sarcolemma, which may directly or indirectly, cause conformational changes in proteins (and their subsequent activation) that are situated in the inner surface of cell membranes. Several of these effector enzymes include phospholipases, protein kinase C isoenzymes, ion channels and ion exchangers such as the sodium/hydrogen exchanger (Ruwhof and Van der Laarse, 2000).

There are two main signal transduction pathways for cardiac hypertrophy: the mitogen activated protein kinase pathway (MAPK), and the Janus associated kinase/signal transducers and activators of transcription (JAK/STAT) (Ruwhof and Van der Laarse, 2000).

Resistance training stimulates hypertrophy of the left ventricular wall and interventricular septum independent of exogenous steroid administration (Sullivan *et al.*, 1998). Echocardiograms of experienced male weight lifters showed that those actively consuming AAS had greater left ventricular mass, increased interventricular and septal thickness compared with those either not

taking steroids at all or having stopped 2 months previously (Sachtleben *et al.*, 1993). Supplementation with AAS therefore pathologically alters physiological adaptations to exercise (Sullivan *et al.*, 1998). Sachtleben and colleagues (1993) compared 11 male weight lifters that used AAS with 13 controls 8 weeks after finishing a cycle of AAS treatment, and then again at the peak of their next cycle. Echocardiograms showed that AAS users at peak cycle have left ventricular septal thickness during diastole that is significantly greater than that of either non-steroid users or steroid users 8 weeks after termination of a cycle. In contrast to the control group, both the peak cycle and off-cycle AAS subjects had greater left ventricular posterior wall thickness and internal diameter during diastole.

The actual cellular mechanism by which AAS promotes myocardial hypertrophy is still inconclusive. In a recent study, Woodiwiss and colleagues (2000) investigated the effects of androgenic anabolic steroids on exercise-induced cardiac remodeling in rats. Habitual exercise results in a rightward shift in the left ventricular end diastolic pressure volume relationship. However, exercise mediated left ventricular hypertrophy produces an increase in left ventricular wall thickness despite left ventricular remodeling. They found that high dose androgenic steroid administration alters exercise induced left ventricular remodeling and subsequently reduced the beneficial effect of physiological left ventricular hypertrophy on left ventricular wall thickness to internal radius. Tagarakis and colleagues (2000) compared sedentary female mice (with or without anabolic steroid administration) to treadmill exercised female mice (with or without anabolic steroid administration). They found that

anabolic steroids combined with exercise, induced mild hypertrophy of the cardiac myocytes, and impaired the cardiac microvascular adaptation to physical conditioning. This impairment might cause a detrimental alteration in the myocardial oxygen supply, especially during muscular exercise. This may also make the heart more prone to ischaemic injury as one would expect with compromised myocardial perfusion. McAinsh and colleagues (1995) investigated whether impaired coronary flow reserve associated with cardiac hypertrophy could exacerbate prolonged episodes of ischaemia. They found that the hypertrophied heart was more vulnerable to brief periods of ischaemia because of an impaired reactive hyperaemic response, which results in a delayed metabolic recovery.

2.1.2.3 Thrombosis, vasospasm and direct injury

There are several other detrimental consequences associated with the use of AAS that may negatively affect the heart. The absence of classical risk factors for heart disease, the youth of patients taking anabolic steroids and the lack of arterosclerotic lesions, raised several questions on the prothrombotic effects of high dose androgens (Ferenchick, 1991). It has been suggested that an increase in platelet aggregability with adenosine diphosphate and collagen can be significantly increased in abusers of anabolic steroids compared to their fellow male weight lifters that do not use anabolic steroids. It would seem that high dosages of anabolic steroids are capable of increasing vascular tone and reactivity, i.e. blood pressure and platelet aggregability (Ferenchick *et al.*, 1992).

Several possible models have been suggested as possibilities for the AAS induced exacerbation of cardiovascular injury. The first model is called the *thrombosis model* where AAS are thought to facilitate thrombosis by altering vascular reactivity, enhancing platelet aggregation, and increasing the concentration and activity of particular procoagulant factor proteins (Ferenchick ; 1991 and Ferenchick *et al.*, 1992).

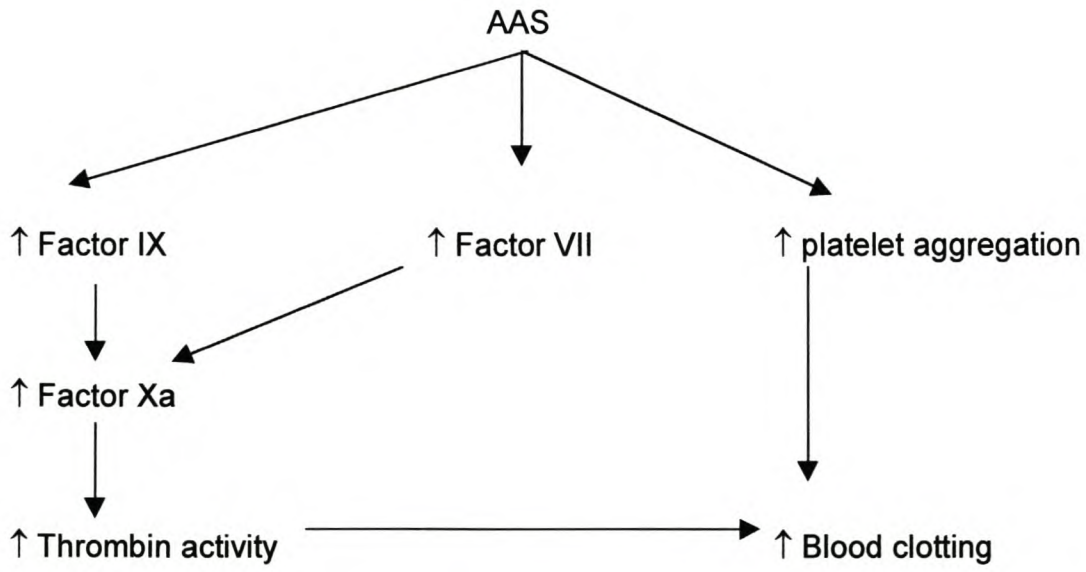

Figure 2.2: Hypothetical model of thrombosis induced by androgenic anabolic steroids (AAS). Factor X and VII probably play a lesser role. (Melchert *et al.*, 1995)

The second model is called the coronary artery *vasospasm model*, which is a hypothetical explanation for AAS induced myocardial infarction in the absence of both artherosclerosis and thrombosis. Nitric oxide acts as an endothelial derived relaxing factor in coronary arteries by acting on guanylyl cyclase to increase cyclic guanosine monophosphate (cGMP) which in turn leads to vascular smooth muscle relaxation (Peach *et al.*, 1985; Ignaro, 1989). Chronic

administration of nandrolone to rabbits resulted in decreased thoracic aorta relaxation possibly by inhibiting guanylyl cyclase (Ferrer *et al.*, 1994).

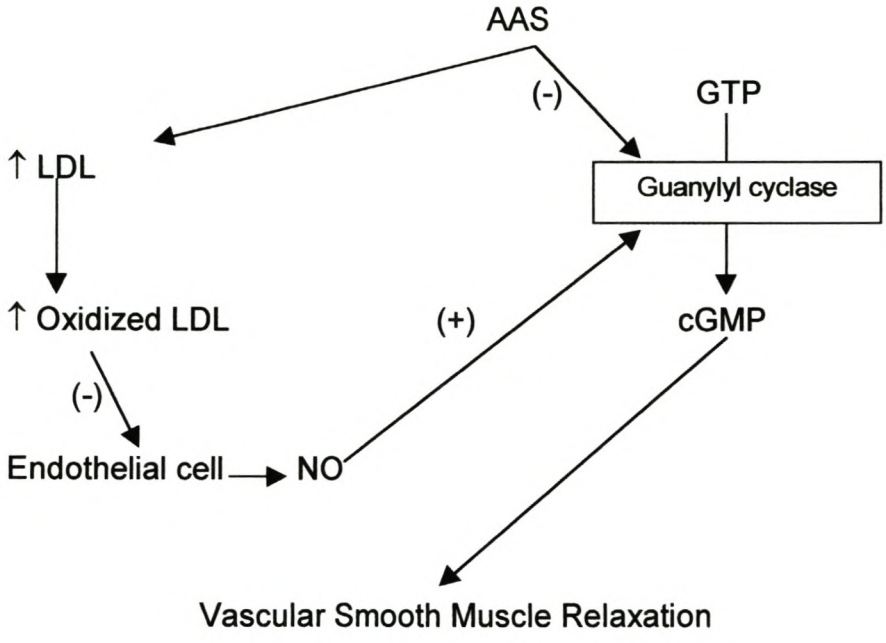

Figure 2.3: Hypothetical model of inhibited vascular smooth muscle relaxation, or potential vasospasm induced by androgenic anabolic steroids (AAS). LDL = low density lipoproteins; GTP = guanosine triphosphate; cGMP = cyclic guanosine monophosphate; NO = nitric oxide; (+) = stimulatory; (-) = inhibitory (Melchert *et al.*, 1995)

The third model is the direct injury model where anabolic steroids directly injure myocardial cells.

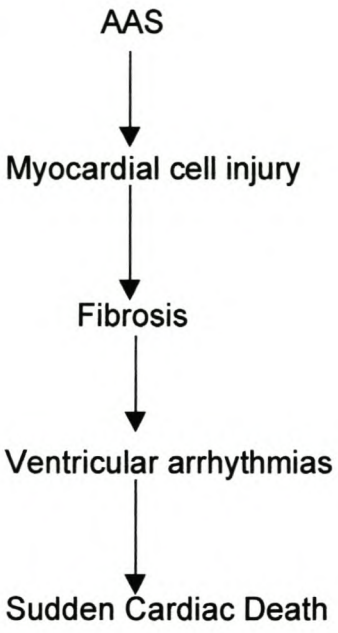

Figure 2.4: Hypothetical model of direct myocardial injury induced by androgenic anabolic steroids (AAS) (Melchert *et al.*, 1995)

Behrendt and colleagues (1977) have found that after exposure to methandrosstenolone, the mitochondria within the rat left ventricle enlarge, become rounded with membrane defects and an electron lucent matrix, and then elongate, leaving a sparse matrix material and few cristae. Melchert and colleagues (1992) also demonstrated the potential cardiac toxicity of anabolic steroids. They exposed primary myocardial cell cultures to testosterone cypionate, stanozolol and fluxymesterone. Myocardial cellular destruction was associated with depressed contractile activity, increased lysosomal fragility, and depressed mitochondrial activity in myocardial cells.

2.1.3 Estrogens versus androgenic anabolic steroids

Woman seems to be protected against heart disease, and only after the drop in estrogen levels during and after menopause, do they experience an increase in risk for coronary artery disease (Henderson *et al.*, 1988 and Stampfer *et al.*, 1991). It has long been postulated that estrogen gives females a cardioprotective advantage. One of the main hypotheses for this cardioprotective effect is that there is a link between NO production and estrogens (Rosselli *et al.*, 1994). It has been suggested and experimentally proven that estrogens stimulate the formation of NO in endothelial cells, which in turn stimulates the production of cGMP in the coronary artery and smooth muscle cells. These events will then lead to a lowering of intracellular calcium, which leads to vasodilation (Wellman *et al.*, 1996). A study investigating 17β - estradiol and cardiac leukocyte accumulation in myocardial ischaemia and reperfusion injury in rats was conducted by Squadrito and colleagues (1997). They found that 17β-estradiol significantly lowered TNF-α production. This cytokine is an important role player in ischaemia and reperfusion injury. In another study, Van Eickles and colleagues (2001) showed that 17β-estradiol attenuated the hypertrophic response to pressure overload in mice. In a study conducted by Webb and colleagues (1998), they found that estrogen also improved exercise-induced myocardial ischaemia in woman.

Thus, in contrast to the beneficial effects of estrogen, it would seem that androgenic anabolic steroid hormones are harmful to the cardiovascular system, while estrogens play a beneficial role.

2.2 Myocardial ischaemia and reperfusion injury

The exact definition of ischaemia in the heart is still under debate, but it essentially refers to a condition of inadequate blood supply to the myocardium. This happens when coronary blood flow restriction to the myocardium is so severe that the oxygen demand of the tissue outweighs the oxygen supply in the tissue (Opie, 1995). By definition, ischaemia exists "*whenever the flow of arterial blood through the diseased vessels is reduced to a volume below that required by the myocardium for adequate function*" (Jennings, 1970). Acute ischaemia induces a wide variety of derangements, from reversible myocardial stunning to irreversible abnormalities such as myocardial infarction. These abnormalities are usually accentuated upon reperfusion of the myocardium (Boyle *et al.*, 1997).

By reintroducing blood flow, the myocardial tissue can be rescued. However, reperfusion of the myocardial tissue not only reoxygenates the tissue, but also triggers a range of complicated cascades and reactions that can also lead to the dysfunction of the myocardium (Vermieren *et al.*, 2000). Two mechanisms of defense against ischaemia in the myocardium have been proposed: 1) a short-term acute adaptation defense and 2) a long term rescue (Hochcachka *et al.*, 1996).

There is an almost immediate energy imbalance with the onset of ischaemia. The lack of blood flow and hence, the lack of oxygen delivery to the myocardium reduces oxidative phosphorylation leading to failure in the resynthesis of high energy phosphates (Maxwell and Lip; 1997), especially

phosphocreatine which acts as a reservoir for adenosine triphosphate (ATP) levels in the myocardium. This depletion is one of the dominant signals for a decrease in heart contractility and the onset of increased anaerobic glycolysis. There are two main hypotheses for ischaemic contractile dysfunction. The first involves the depletion of the high-energy phosphates, with special emphasis on creatine phosphate. The second hypothesis is based on the accumulation of metabolic products that causes pump failure (Dennis *et al.*, 1991). A controversial explanation for the reduction in myocardial contractility during ischaemia is a reduction in β-adrenergic signal transduction caused by reduced β-adrenergic receptor density and adenylate cyclase activity (Dupuis *et al.,* 1997). Following reperfusion, the depressed contractility is less severe and is accompanied by an increase in β-adrenergic signal transduction, indicated by an increase in cAMP. This appears to be the result of increased β-adrenergic receptor density and adenylate cyclase activity (Dupuis *et al.*, 1997).

Cellular leakage of ions plays a key role in myocardial disruption during ischaemia. Once tissue is subjected to an ischaemic environment, the electron transport system becomes impaired, leading to a reduction in available energy to the tissue. Several cellular and subcellular ATP-dependent mechanisms become inactive, leading to a general leakage of cellular ions and substrates into the extracellular environment (Sobel and Shell, 1972; Vatner *et al.*, 1978). The cell can then no longer maintain or restore its homeostatic equilibrium and will fail (Sobel and Shell, 1972). Ion

homeostasis is lost with a leakage of potassium ions into the extracellular environment and calcium ions into the cytoplasm (Maxwell and Lip, 1997).

2.2.1 Effects of ischaemia/reperfusion on pH

The combination of carbon dioxide and proton accumulation decreases the pH of the myocardial tissue (Cross *et al.*, 1995). This cellular acidosis can be argued as either beneficial or detrimental to the ischaemic myocardium. A low pH inhibits the enzyme 5'-nucleotidase, which breaks down adenosine monophosphate. The inhibition of this breakdown ensures a more rapid ATP synthesis during the reperfusion period following ischaemia (Back and Ingwall, 1994). On the other hand it is possible that a very low pH might activate lysosomes, which may lead to the irreversible destruction of the ischaemic myocardial tissue (Bing *et al.*, 1973).

2.2.2 Effects of ischaemia reperfusion on cytosolic Ca^{2+}

Another very important consequence of ischaemia is the disruption of the calcium homeostasis, which leads to several detrimental changes in the myocardium.

In the normal myocardium there is a close relationship between oxygen consumption and contractile performance in the heart and although myocardial oxygen consumption recovers to baseline rapidly after reperfusion, there is a continued depression of regional systolic shortening (Huang and Liedtke, 1989). Cytosolic calcium is increased during prolonged ischaemia (Marban *et al.*, 1998a). This consistent increase in calcium happens, either

before, or concurrently with the onset of ischaemic contracture, or just after the maximal depletion of ATP. The increased calcium has several diverse consequences including damage to contractile proteins, activation of phospholipases, increased depolarization, ischaemic contracture and mitochondrial damage (Myers *et al.*, 1995).

As mentioned before, reperfusion of ischaemic tissue initiates a cascade of processes. In order to limit the so-called reperfusion injury, it is essential to understand the underlying mechanisms and triggers of this reperfusion phenomenon (Vermieren *et al.*, 2000). Reperfusion injury is a syndrome of related conditions that follows reperfusion of the ischaemic myocardium. The mechanisms for reperfusion injury is two-fold, one being cytosolic calcium overload and the other being the formation of free radicals. Other events linked to myocardial reperfusion injury include endothelial dysfunction, reintroduction of neutrophils into the ischaemic area (Forde and Fritzgerald 1997) and microvascular damage (Opie, 1998).

The increase in cytosolic calcium during ischaemia is often exacerbated during reperfusion (Nayler *et al.*, 1980). The source of calcium can be external or internal. The uptake of calcium during the early stages of reperfusion can result from sodium-calcium exchange activation (Du Toit and Opie, 1993) or from calcium entry via the L-type Ca^{2+} channels (Du Toit and Opie, 1992).

2.2.3 Free radicals and ischaemia/reperfusion

Free radicals are highly reactive chemical species. They differ from standard compounds by having unpaired electrons in their outer orbital. Oxygen-derived free radicals include the superoxide radical or superoxide anion. The danger exists that the superoxide anion can react with hydrogen peroxide to form a more reactive hydroxyl ion (Opie, 1995). A potential source of reactive oxygen-derived free radicals in ischaemic and reperfused tissues is the enzyme xanthine dehydrogenase/oxidase (Maxwell and Lip, 1997). Peroxidation of membrane lipids in the myocardium can be caused by free radical formation and can contribute to reperfusion injury (Meerson *et al.*, 1982). The free radical and calcium hypotheses can be integrated. Free radicals may promote calcium overload by:

- Interfering with myocardial calcium transport, either at sarcoplasmic reticulum or cell membrane level
- Impairing mitochondrial oxidative phosphorylation and directly inhibiting glyceraldehyde-3-phosphate dehydrogenase
- Irreversible inhibition of anaerobic glycolysis

Calcium overload may in turn promote the production of free radicals by damaging the electron transport chain, which may lead to the leakage of free radicals. This can activate calcium dependant proteases leading in turn to the activation of xanthine oxidase (Maxwell and Lip, 1997).

2.2.4 Endothelial dysfunction and reperfusion injury

Endothelial dysfunction may play an important role in reperfusion damage. Superoxide anions can, in fact be generated in endothelial cells during reoxygenation. It also seems that endothelial cells are targets for free radicals (Lefer *et al.*, 1991; Mcfalls *et al.*, 1991; Sobey *et al.*, 1992). Endothelin-1 (ET-1) is a potent vasoconstrictor peptide that is derived from the vascular endothelial cells. Other sources include smooth muscle cells and cardiomyocytes themselves. During ischaemia and myocardial reperfusion, the production and release of ET-1 is stimulated and the coronary constrictor response towards this substance is enhanced. Experimental evidence suggests that ET-1 plays an important role in the pathophysiological state of the reperfused myocardium, enhancing the ischaemia-reperfusion injury of the heart (Pernow and Wang, 1997; Brunner *et al.*, 1992).

An inflammatory response has been observed with reperfusion by activation of a wide variety of cells, including monocytes, macrophages, endothelial cells and T-cells. These cells lead to the activation of cytokines such as tumor necroses factor (TNF) and interleukins (IL). IL-8 is known to be a neutrophil activating substance (Opie, 1998). Neutrophil activation can be divided into several stages. First, the neutrophils accumulate in the vasculature of the ischaemic area, mainly facilitated by the secretion of chemotactic factors, such as leukotrines and other cytokines. Once the neutrophils are established in the vasculature, the neutrophil acts directly with endothelium to enter the tissue. During the next phase, the neutrophils penetrate the interstitium of the tissue and release the contents of their granules (Vermieren *et al.*, 2000).

Once inside the vascular interstitial space, the neutrophils are activated and thought to liberate free radicals and leukotrines (Opie, 1998)

2.3 Exercise and the cardiovascular system

Exercise is associated with extensive alterations in the circulatory and respiratory systems as part of the homeostatic responses that make moderate to severe exercise possible (Ganong, 1995). The skeletal system provides the basic framework through which muscles act, the cardiovascular system delivers nutrients to the body's various cells and in return removes waste products. The cardiovascular and respiratory systems work together to provide oxygen to cells and remove carbon dioxide. The integumentary system helps to maintain body temperature by allowing the exchange of heat between the body and its surroundings. The urinary system maintains fluid and electrolyte balance and provides long-term blood pressure regulation. The nervous system and endocrine systems in turn coordinate and direct all these activities to meet the needs of the body (Willmore and Costill, 1994).

The heart itself goes through structural changes during prolonged exercise. The myocardium undergoes hypertrophy, and to a lesser degree hyperplasia. Hypertrophy refers to cells becoming larger, whereas hyperplasia refers to the increase in cell numbers. The latter happens to a lesser extent in response to exercise. With extensive exercise hypertrophy is beneficial, because of the increase in contractile units. This type of hypertrophy can progress to a state of pathophysiological hypertrophy. In the hypertrophied myocardium there is an increase in myocyte size. Capillaries and interstitial cells, containing

collagen, increase via hyperplasia, although this happens to a lesser extent. The capillary surface area remains the same though, leading to a decrease in the surface area to total volume area. When the cell volume increases by approximately 60%, beta-receptor density decreases by about 33%. The sodium-calcium exchange rate decreases as well as the density of the calcium-ATPase pump in the sarcoplasmic reticulum. There is however, a relative increase in the T-tubules and the surface area of the sarcoplasmic reticulum. This is a possible compensatory mechanism in response to the decreased density of the sarcolemmal calcium pump. All these changes could contribute to the abnormalities of contraction observed in the hypertrophied pathologically heart (Opie, 1998).

The myocardium also undergoes an accumulation in collagen, termed fibrosis. Initially there is an increase in interstitial collagen dubbed reactive fibrosis. This leads to a general increase in stiffness of the myocardium both in systole and diastole. Stiffness during systole might improve force generation (Weber *et al.*, 1989) while, during diastole, it might lead to impaired relaxation rates (Brilla *et al.*, 1991).

2.3.1 The blood supply and oxygen utilization of the heart

There are no direct circulatory channels within the hearts' chambers, so the heart relies on coronary circulation for its oxygen and nutritional needs. Even at rest, oxygen utilization of the myocardium is high in relation to the blood flowing through the coronary vessels. The increase in myocardial oxygen demand during exercise can only be met by a proportional increase in

coronary blood flow. It has been noted that during vigorous exercise, coronary blood flow can increase up to four to five times that of the normal resting level. This increase is achieved in two ways:

1) An increase in myocardial metabolism has a direct effect on the coronary vessels, causing them to dilate via local factors and hormones of the sympathetic nervous system.
2) Exercise results in an increase in aortic pressure, which forces a proportionately greater quantity of blood into the coronary circulation.

Adequate oxygen supply is critical for the myocardium, because its tissue has a limited ability to generate energy anaerobically (Tune *et al.*, 2002)

2.3.2 Cardiovascular adaptations to exercise

Numerous cardiovascular adaptations occur in response to training, which will be discussed below.

2.3.2.1 Heart size

Endurance training leads to an increase in heart weight, specifically an increase in left ventricle wall thickness and chamber size. This is a biological adaptation to an increased workload. (Frenzel *et al.*, 1988). Earlier researchers called this adaptation "athletes heart", because experts believed that the enlargement of the heart to be a pathological state. Currently it is recognized as a normal result of chronic exercise training. Endurance training leads to an increase in chamber size as well as an increase in myocardial thickness previously believed only to occur with resistance training. This

results in a stronger heart that can generate a relatively larger stroke volume (Oakley, 2001).

It is important to distinguish between physiological hypertrophy versus pathological hypertrophy. Left ventricular hypertrophy can be classified as 1) physiological hypertrophy or 2) pathological hypertrophy. The first is induced due to long term exercise (De Maria *et al.*, 1978) while the latter is induced by abnormalities such as volume overload, insulin resistance (Steinberger, 2001), obesity (Lauer *et al.*, 1991), aortic stenosis, or anabolic steroid abuse (Ferrer *et al.*, 1994; Melchert and Welder, 1995). Exercise induced LV hypertrophy is associated with normal or improved cardiac function while pathological LV hypertrophy is associated with impaired systolic contraction, diastolic relaxation and an increase in diastolic stiffness (Lecarpentier *et al.*, 1987, Doering *et al.*, 1988;). Evidence would also suggest that elevated myocardial cytokine levels (TNF-α, IL and Cardiotrophin-1) might cause decompensation, dilatation, and heart failure in the hypertrophied heart (Kubota *et al.*, 2000; Latchman, 2000).

2.3.2.2 Stroke volume

There is an overall increase in stroke volume in response to endurance training. A stronger heart and the availability of a larger blood volume appear to account for the increase in rest, submaximal, and maximal stroke volumes following an endurance exercise program (Eshani *et al.*, 1991). Training increases blood plasma volume, which leads to a greater end diastolic volume. The larger volume of blood entering the ventricle leads to stretching

of the walls, which may result in more elastic recoil (Frank-Starling law). At the same time, there is hypertrophy of the posterior and septal walls of the left ventricle, leading to stronger contractility. This means that more blood enters the left ventricle, and a larger percentage of this blood is ejected with each contraction (Ginszton *et al.*, 1989; Jensen-Urstad *et al.*, 1998).

2.3.2.3 Heart rate

Heart rate and oxygen consumption are linearly related for both trained and untrained individuals. Resting heart rate decreases considerably as the result of endurance training. Highly trained athletes often have a resting heart rate of only 40 beats/minute or even less (Wayne *et al.*, 1998). Heart rate during submaximal exercise also decreases proportionately with the training completed. Maximal heart rate either remains unchanged or decreases slightly with training. This slight decrease in maximal heart rate is probably to allow for a proper stroke volume to maximize the cardiac output (Jones and Carter; 2000).

2.3.2.4 Cardiac output

Cardiac output, at rest or during sub maximal exercise, remains unchanged or decrease slightly, but cardiac output at maximal levels of exercise, increases considerably. This is mainly because of the substantial increase in the maximal stroke volume in the trained individual (Charlton and Crawford; 1997).

2.3.2.5 Blood flow

Training leads to an enhanced blood supply to skeletal muscles (Kalliokoski *et al.*, 2001).

Three factors account for this:

i) Increased capillarization of trained muscles

ii) Larger opening of existing capillaries in trained muscles

iii) More effective blood distribution

These factors lead to an increase in the cross sectional area of the vascular bed as well as a decrease in the distance between blood and active cells for the diffusion of oxygen and metabolites (Brevetti *et al.*, 2001).

2.3.2.6 Blood pressure

Resting blood pressure is usually reduced by endurance training in people with borderline to moderate hypertension. Endurance training has, however, little or no effect on blood pressure during standardized submaximal or maximal exercise (Lewis *et al.*, 1999).

2.3.2.7 Blood volume

Endurance training increases blood volume. This increase in blood volume is the result of an exercise-induced increase in the blood plasma volume. Blood plasma volume is increased by two mechanisms:

1) Exercise increases the release of antidiuretic hormone (ADH) and aldosterone. These two hormones act on the kidneys to promote water retention, thus resulting in an increase in blood plasma volumes (Willmore and Costill; 1994).

2) Exercise increases the amount of plasma proteins and in particular albumin in the blood. With the increase in plasma protein concentration, there is an increase in the osmotic pressure leading to the retention of more fluid in the vascular compartment (Green *et al.*, 1991).

2.4 Cyclic nucleotides in the normal, ischaemic, and hypertrophied heart

Accumulating evidence suggests that ventricular pathophysiological hypertrophy is associated with contractile dysfunction, impaired tolerance to ischaemia and increased risk during cardiac surgery. Allard and colleagues (1994) compared calcium overload and morphologically evident myocardial injury in hypertrophied hearts and normal hearts subjected to ischaemia. They found that calcium overload was two and a half times greater in hypertrophied hearts than in their normal counterparts. During myocardial ischaemia these hearts also exhibited an accelerated loss of high-energy phosphates, larger accumulation of tissue lactate and an earlier onset of ischaemic contracture. Studies have also reported increased dependence on glycolysis for energy metabolism and changes in intracellular calcium regulation and excitation-contraction coupling (Chang *et al.*, 1996).

In eukariotes the cyclic nucleotides 3', 5' cyclic adenosine monophosphate (cAMP) and 3', 5'- cyclic guanosine monophosphate (cGMP) help transmit information from extracellular hormones to intracellular enzymes. (Horton et.al., 1996). Many hormones that regulate intracellular metabolism exert their effects on target cells by activating the adenylate cyclase pathway. Binding of

a hormone to a stimulatory receptor causes the conformational change of the receptor, which promotes the interaction between the receptor and a G protein named protein G_s that in turn binds to an effector enzyme named adenylate cyclase and activates it (Horton et al., 1996).

2.4.1 cAMP

Adenylate cyclase is an integral membrane enzyme with an active site facing the cytosol. It catalyses the formation of the second messenger, cAMP, from ATP. Cyclic AMP diffuses from the membrane surface into the cytosol and activates the enzyme protein kinase A, a serine, threonine protein kinase. Protein kinase A catalyses the phosphorylation of the hydroxyl groups of specific serine and threonine residues on target enzymes. Phosphorylation of the amino acid side chains is reversed by the action of protein phosphatases (Ganong, 1995).

Hormones that bind to stimulatory receptors activate adenylate cyclase and raise cAMP levels. Hormones that bind to inhibitory receptors inhibit adenylate cyclase activity. The ultimate response of a cell to a hormone depends on the type of receptor present in the cell and the type of G protein to which they are coupled (Ganong, 1995).

There is strong evidence linking myocardial ischaemic and reperfusion injury to calcium overload (Marban *et al.*, 1989b). The cardiac L-type Ca^{++} channel is an important determinant of myocardial contractility. Its regulation via neural and hormonal factors contributes to the control of cardiac output. A large

number of these extracellular first messengers act on specific membrane receptors in cardiomyocytes and regulates the activity of adenylate cyclase. Adenylate cyclase in turn controls the intracellular levels of cAMP, the activity of the cAMP dependent protein kinase, and the degree of phosphorylation and stimulation of L-type Ca^{++} channels (McDonald *et al.*, 1994; Hove-Madsen *et al.*, 1996; Striessnig, 1999). Increased cAMP levels associated with ischaemia (Wollenberger *et al.*, 1969; Depré and Hue, 1994) would therefor increase Ca^{++} levels and exacerbate reperfusion injury.

Osaki and colleagues (1997) found that in perfused adult rat hearts, c-fos expression is induced by acute aortic pressure overload and is coupled to increases in cAMP content. The pressure-induced induction of c-fos may play a transducing role in the cAMP-dependant protein synthesis system by which stretch of the left ventricular wall, consequent to acute aortic pressure, increases rates of protein synthesis. There is therefore evidence linking abnormal cAMP levels to both myocardial hypertrophy and ischaemia/reperfusion injury.

2.4.2 cGMP

Cyclic GMP is a second messenger that regulates physiological processes in various cell types, such as retinal rods, renal tubular cells, platelets and muscle cells (Schmidt *et al.*, 1993). In the heart, cGMP may increase or decrease cAMP by its effect on cAMP phosphodiesterases, and stimulate PKG, which inhibit inward Ca^{++} currents (Lohman *et al.*, 1991). The cardiac L-type Ca^{2+} is especially important in this regard, since the channel current is an

important determinant of myocardial contractility. Its regulation by neurotransmitters, hormones, and paracrine factors contributes to the control of cardiac output (Hartzell, 1988; McDonald *et al.*, 1994).

Guanylyl cyclases are a family of enzymes that catalyses the formation of cGMP. There are two types: the particulate form which is stimulated by ANP, and the soluble form which is stimulated by NO (Walter, 1989). These isoforms are of special interest since it would seem that NO is involved in the modulation of cardiac contractility and rhythm, in part through its ability to control the amplitude if the L-type Ca^{++} current (Shah and MacCarthy, 2000). This regulation is mediated through the generation of cGMP by NO-stimulated soluble guanylyl cyclase (Chesnais *et al.*, 1999). Besides its effects on myocardial contractility, the NO-cGMP pathway stimulation during ischaemia may protect the heart against ischaemia/reperfusion induced calcium overload (Du Toit *et al.*, 2001). It is believed that elevated cAMP levels in the heart would exacerbate ischaemia/repefusion injury by elevating the cytosolic calcium levels (Du Toit and Opie, 1992). In this regard it is possible that cGMP may attenuate this type of injury by inhibiting the cAMP induced slow inward calcium current thus leading to a decrease in cytosolic calcium levels (Hartzell and Fischmeister, 1986; Summi and Sperelakis, 1995). Another protective pathway might be the NO-cGMP dependent pathway in which the sarcolemmal K_{ATP} channels are opened and the cystolic calcium levels are lowered (Figure 2.5).

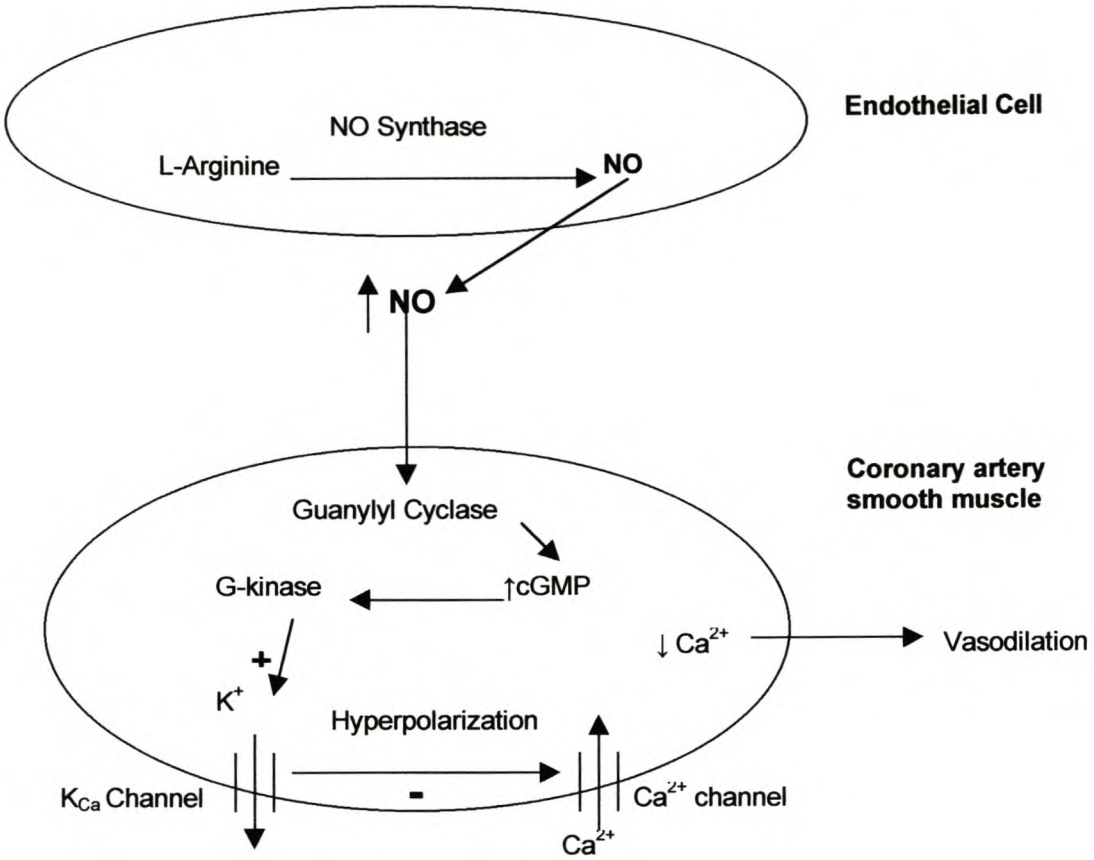

Figure 2.5: A proposed model to explain the role of NO in reducing myogenic tone in coronary arteries (Adapted from Wellman *et al.*, 1996)

2.5 Mitogen-activated protein kinases

Mitogen activated protein kinases (MAPK) are present in all eukaryotes and play an important role in signal transduction from the membrane to the nucleus (Herskowitz 1995; Lewis *et al.*, 1998; Cobb, 1999). During a cell's lifetime, it needs to respond to various stress stimuli, to which the cell will either adapt or undergo cell death. These stresses include inflammatory cytokines, ischeamia, ATP depletion, endotoxin and genotoxic stress (Nagarkatti and Sha'afi, 1998). Cells respond to these extracellular signals by transmitting intracellular instructions to coordinate the correct responses.

The mitogen activated protein kinase pathway and the extracellular signal regulated protein kinase (ERK) cascades are often used to transduce these signals. Three pathways lead to the activation of three different sets of MAP kinases. Two of these pathways can participate in overlapping stress responses and are referred to as stress pathways. Activation of these pathways lead to growth arrest and apoptosis. The other pathway stimulates proliferation and differentiation and is subsequently named the mitogenic pathway (Tibbles and Woodgett, 1999).

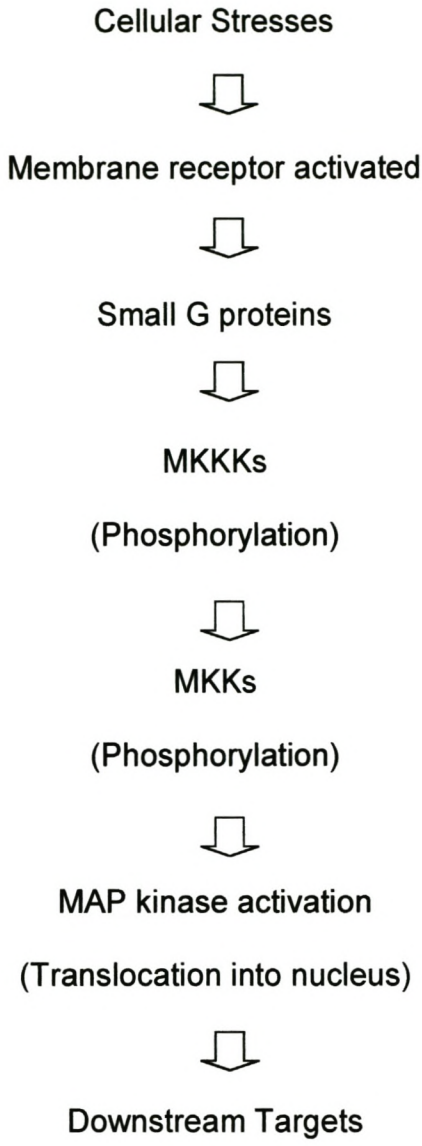

Figure 2.6: The cytoplasmic-signalling cascade (Adapted from Sudgen and Clerk, 1998)

The three best-characterized MAPK pathways are the extracellular regulated protein kinases (ERKs), the c-jun NH_2-terminal kinases (JNKs) and the p38 MAPK cascade (Robinson and Cobb, 1997).

All MAPKs phosphorylate Serine/threonine residues in proteins within a (serine/threonine) Pro consensus motif, but additional factors dictate the precise substrate specificity (Yao *et al.*, 1995).

The MAPK cascade consist of a three kinase module that includes a MAPK, which is activated by a MAPK/ERK kinase (MEK), which in turn is activated by a MEK kinase (MEKK) (Schlessinger, 1994; Blenis, 1993). The MEKs are substrates for Raf-1 which can be activated through either receptors involved in Ras or a protein kinase C (PKC)-dependent pathway. These MAPK activators cause translocation from the cytosol to the nucleus, where transcription factors such as c-Jun, Elk-1 and c-Myc are substrates for MAP kinase (Pulverer *et al.*, 1991; Seth *et al.*, 1991; Janknecht *et al.*, 1993). In response to stress via the small GTP binding protein MAPK kinase, MEKK1 and dual specificity protein kinases MKK3, MKK4 and MKK6 are activated (Force *et al.*, 1996).

At least four isoforms of p38 have been identified:

- P38α
- P38β
- P38γ
- P38δ

P38 α and p38 β can both be blocked with the blocker SB203580. P38 has cellular functions that are not only related to stress responses, but also unrelated to stress responses as illustrated in Figure 2.7 (Nebreda and Porras, 2000).

Figure 2.7 summarises the very diverse actions of p38 in various systems. This ranges from proliferation and differentiation to stress, inflammation and apoptosis.

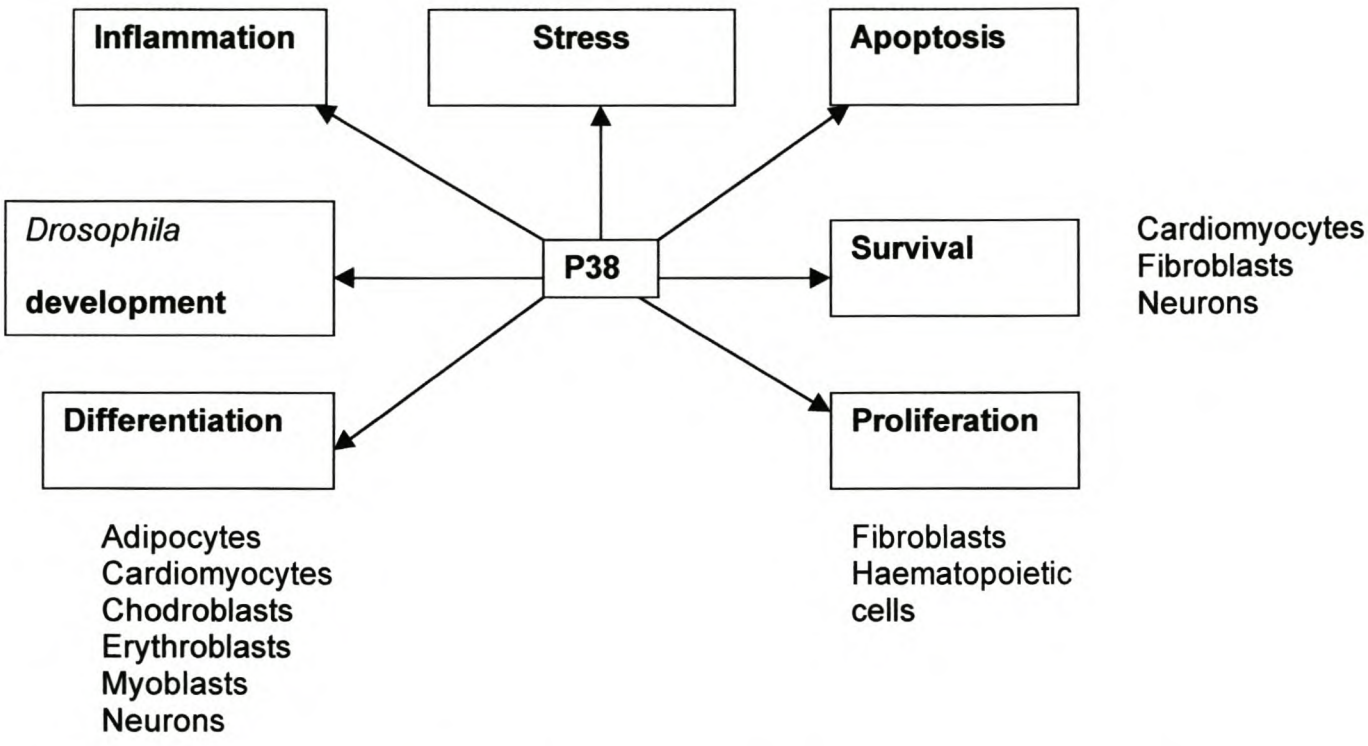

Figure 2.7 Cellular functions of p38 that are related and unrelated to stress (Nebreda and Porras; 2000)

2.5.1 P38 and ischaemia

2.5.1.1 Protective role of p38

On the one hand, p38 kinase can activate mitogen-activated protein kinase-2, which phosphorylates small heat shock proteins (hsp25/27) (Rouse *et al.*, 1994). These small proteins act as an F actin cap binding protein and modulate actin filament dynamics. Activation of the heat shock proteins leads to the protection of cells to ischaemic injury (Martin *et al.*, 1997). When p38 was blocked, the cardioprotective effect was abolished (Maulik *et al.*, 1998;

Armstrong *et al.*, 1999 and Nakano *et al.*, 2000). This apparent protection against ischaemic injury is referred to as ischaemic preconditioning, where brief episodes of ischaemia or pharmacological agents protect the myocardium against subsequent ischaemic damage (Downey and Cohen, 1997).

2.5.1.2 Deleterious role of p38

On the other hand, several studies have reported that the blocking of p38 with SB203580 and SB202190 lead to improved post-ischaemic functional recovery (Saurin *et al.*, 1999; Schneider *et al.*, 1999; Barancik *et al.*, 2000). The activation of p38 generally leads to myocardial damage and myocyte apoptosis. Ma and colleagues (1999) have indeed shown that the inhibition of p38 MAPK decreased cardiomyocyte apoptosis and that the inhibition also led to improved cardiac function after myocardial ischeamia and reperfusion. Nagarkatti and Sha'afi (1998) also found that the activation of p38 MAPK was decreased in preconditioned cells, further implicating p38 MAPK in a deleterious role in the heart. In response to oxidative stress, cardiac injury might also be mediated by a p38 MAPK-mediated production of TNFα (Meldrum *et al* 1998).

2.5.2 P38 and hypertrophy

The activation of p38-MAPK and c-Jun NH_2 -terminal kinase (JNK) has also been implicated in alpha-1 adrenergic stimulation induced hypertrophic growth (Wang *et al.*, 1998). It is possible that the p38 MAPK pathway is involved in mechanical stress-induced hypertrophy. Evidence supporting this hypothesis

was found in a mouse model of pressure overload where p38 activity was increased (Wang *et al.*, 1998). As mentioned before, markers of cardiac hypertrophy include the so-called early response genes (e.g. *erg-1, hsp-70, c-fos, c-jun and c-myc*) (Hefti *et al.*, 1997). MEKK3 and MEKK6 both activate p38-MAPKs preferentially. Transfection of neonatal cardiac myocytes with these two constitutively activated upstream activators stimulate the expression of ANF and skeletal muscle α-actin and is associated with the hypertrophic response (Sudgen and Clerk, 1998).

The seemingly contradictory activation of p38, which leads to cell proliferation and growth versus apoptosis, might be due to the different isoforms of p38. It would seem that p38β activation leads to hypertrophy while the activation of p38α leads to the induction of myocyte apoptosis (Wang *et al.*, 1998).

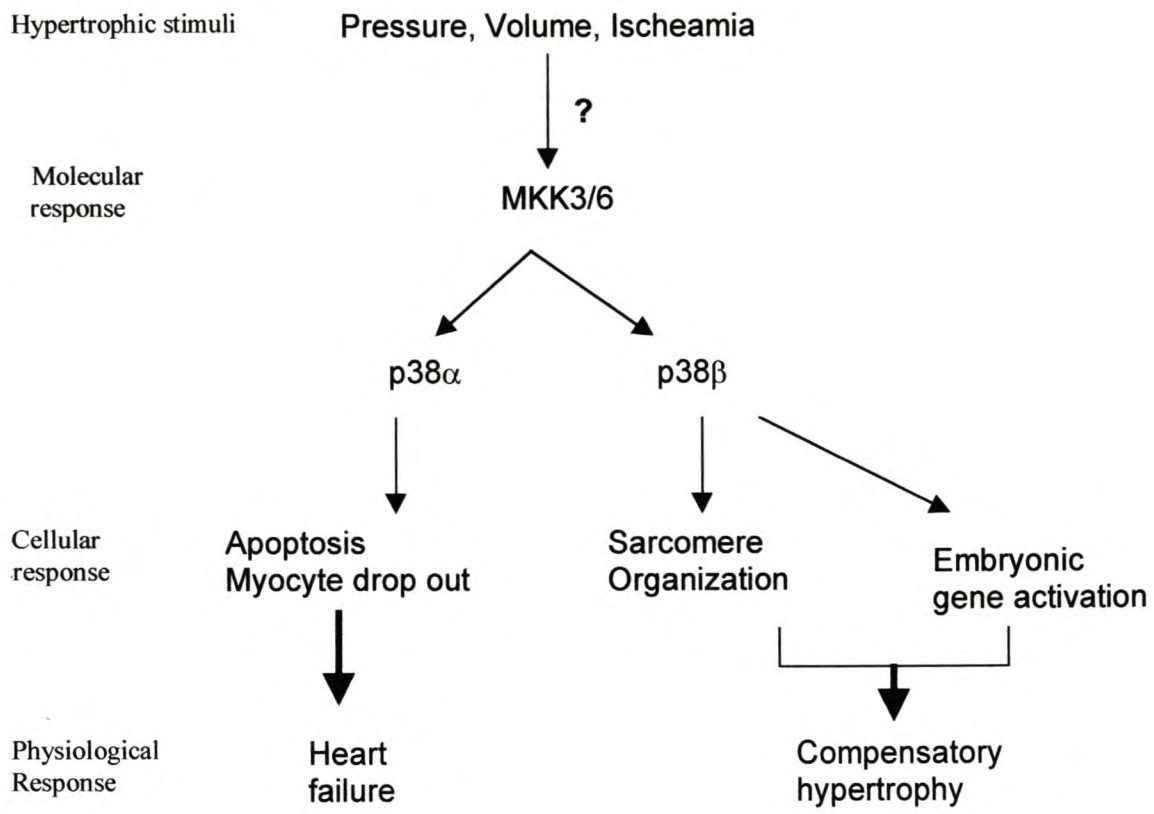

Figure 2.8: Proposed role of p38 in cardiac hypertrophy and heart failure in response to haemodynamic stress (Wang *et al.*, 1998)

P38 can also phosphorylate C/EBP homology protein (CHOP), also known as growth arrest and DNA damage-153 (Wang and Ron; 1996). Expression of MKK6 enhanced the ability of etopically expressed MyoD, an essential transcription activator in myogenesis, to differentiate 10T1/2 fibroblasts to muscle cells. The stimulation of muscle gene expression by p38 is apparently mediated by the myocyte enhancer factor-2C (MEF2C) transcription factor. This suggests the p38 might regulate MyoD activity via phosphorylation and increases the transcriptional activity of MEF2C (Galbiati *et al.*, 1999). Whether the cell is committed to death or survival will depend on the stimulus and cellular context (Nagarkatti and Sha'afi, 1998).

2.6 Tumor Necrosis Factor alpha

Tumor Necrosis Factor (TNF) is a pro-inflammatory cytokine, existing in both the alpha (α) and beta (β) form, but TNF-α is the cytokine associated with ischaemia and reperfusion injury. TNF-α is implicated in several cardiovascular diseases and abnormalities. These include acute myocardial infarction, chronic heart failure, atherosclerosis, viral myocarditis, cardiac allograft rejection and sepsis-associated cardiac dysfunction (Levine *et al.*, 1990; Yokoyama *et al.*, 1992; Latini, 1994; Neumann *et al.*, 1995; Torre-Amione *et al.*, 1995; Biasucci *et al.*, 1996; Torre-Amione *et al.*, 1996a; Torre-Amione *et al.*, 1996b; Oral *et al.*, 1997; Yokoyama *et al.*, 1997). It has been established that resident cardiac macrophages can produce TNF-α (Neumann *et al.*, 1995; Kapadia *et al.*, 1995; Kapadia *et al.*, 1995; Biasucci *et al.*, 1996; Kumar *et al.*, 1996;) but surprisingly cardiac myocytes themselves are a major source of TNF-α (Kapadia *et al.*, 1995). It has also been shown that endotoxin-induced TNF-α production is evenly distributed between the resident cardiac macrophages and cardiac myocytes (Kapadia *et al.*, 1997). Several scenarios stimulate TNF-α production, including ischaemia and reperfusion (Herskowitz et al., 1995; Gurevitch *et al.*, 1996), burn trauma (Horton, 1996), clinical myocardial infarction (Latini *et al.*, 1994, Neumann *et al.*, 1995; Biasucci *et al.*, 1996), cardiopulmonary bypass (Hattler *et al.*, 1995; Cameron, 1996) and chronic heart failure (Torre-Amione *et al.*, 1996a; Torre-Amione *et al.*, 1996b). Locally produced TNF-α may be an important contributor to myocardial dysfunction, apoptosis and hypertrophy (Finkel *et al.*, 1987; Krown *et al.*, 1996; Kumar *et al.*, 1996; Yokoyama *et al.*, 1997).

The wide range of activities of TNF-α can be explained by the presence of TNF receptors in almost all nucleated cell types (Ferrari, 1998). Two distinct types of receptors for TNF-α have been identified: TNF-RI or TNF-R55 with a molecular mass of 55-KDa, and TNF-RII or TNF-R75 with a molecular mass of 77-KDa. The extracellular portion of these two receptors show a significant sequence homology, but there is a difference between the receptors when looking at the cytoplasmic domains. This would hint towards the activation of different intracellular pathways by the two receptors. The ligand-passing model might explain the different bioactivities of the receptors. This model suggests that TNF-RII has a much higher affinity and dissociation for TNF than TNF-RI, binding preferentially TNF at a low ligand concentration. The ligand is then passed on to the neighbouring TNF-RI, monopolises all TNF mediated signalling (Vandenabeele *et al.*, 1995; Habib *et al.*, 1996). TNF itself is a 17-KDa polypeptide. It is expressed as a 26-KDa integral trans-membrane precursor protein from which the 17-KDa unit is released after proteolytic cleaving (Vassalli, 1992).

2.6.1 TNF-α and ischaemia-reperfusion injury

It is thought that locally produced TNF-α can induce post-ischaemic cardiac dysfunction via two distinct processes: contractile dysfunction and myocyte apoptosis. Contractile dysfunction can be accomplished either through a nitric oxide dependent pathway or a nitric oxide independent pathway, the latter is thought to be a sphingosine dependent pathway (Meldrum, 1998).

Since calcium homeostasis is of such importance to the normal functioning myocardium, the relationship between TNF-α and calcium have been investigated. Yokoyama and colleagues (1992) determined that TNF-α administration led to decrease calcium transient amplitude during systole. It would seem that TNF-α depresses systolic function by interfering with the calcium-induced calcium release by the sarcoplasmic reticulum. It has also been shown that TNF-α disrupts the L-type channel-induced influx (Krown *et al.*, 1995). In addition to this Oral and colleagues (1997) demonstrated that the early effects of TNF-α on the calcium transient and systolic function are mediated by sphingosine. The role of NO in this regard would seem to be the desensitization of the myofilaments to intracellular calcium (Goldhaber *et al.*, 1996). It has been suggested that TNFα-induced sphingosine mediated disruption of the calcium homeostasis occurs early and that NO mediates the later desensitisation of the myofilaments to calcium (Figure 2.9) (Gross and Auchampach, 1992; Kelly and Smith, 1997; Oral *et al.*, 1997). It has also been suggested by some investigators that NO may serve in a protective capacity during ischaemia and reperfusion (Nakanishi *et al.*, 1992; Du Toit *et al.*, 2001). This apparent discrepancy might be due to the different quantities

of NO produced. It is thought that low levels of NO produced by the calcium-dependent constitutive form of NOS (cNos) might be protective, whereas the relatively large amounts of NO produced by calcium-independent, cytokine-inducible iNOS might be injurious (Nussler and Billiar, 1993; Lyons, 1995).

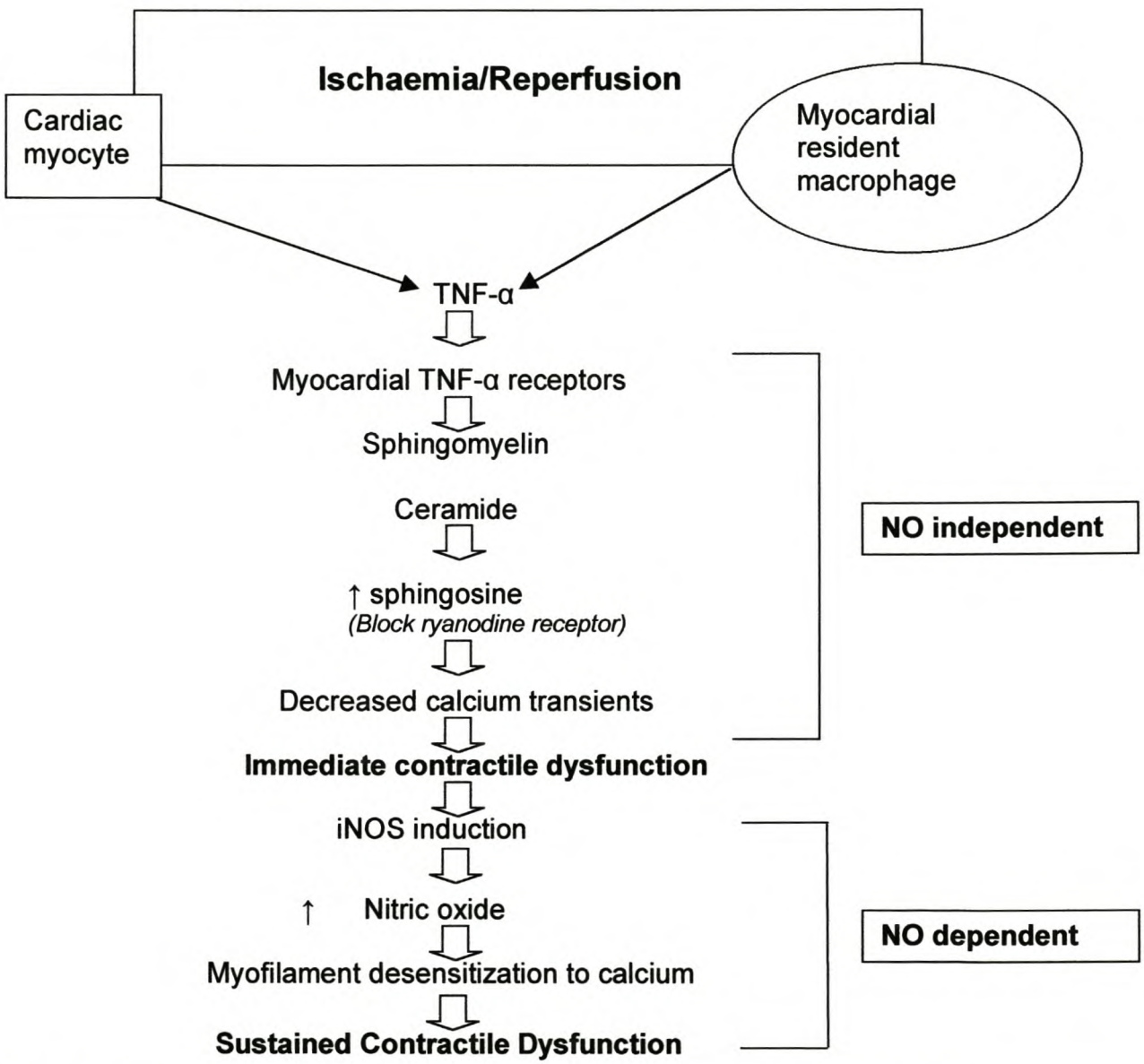

Figure 2.9: Myocardial TNF-α production by both cardiac myocytes and resident cardiac macophages leads to contractile dysfunction. The immediate phase is NO independent and sphingosine mediated. The late phase is NO dependent and due to NO-induced desensitisation to calcium (Adapted from Meldrum, 1998)

2.6.2 TNF-α and cardiac hypertrophy

When looking at the current literature, it is clear that TNF-α is a mediator of cardiac pathology exerting its effects, at least in part, through the inflammatory pathways and via apoptosis. In addition to this it is critical to realise that TNF-α plays a crucial role in the maintenance and control of signalling pathways. It is possible that TNF-α is a mediator in cardiac cell growth and in the remodelling of the cardiac extracellular matrix in response to biochemical stress (Sack *et al.*, 2000). In this regard Mann (1996) proposed that short-term expression of TNF-α within the heart might provide an adaptive response to stress, whereas long-term expression of the cytokine may be maladaptive by producing cardiac decompensation. The association of TNF with its homotrimeric cogenate receptor results in the formation of multimolecular signal transduction complexes, which in turn can activate several different downstream signalling pathways. Although the coupling of the adaptor Fas-associated death domain protein (FADD) to TNF-RI is associated with the activation of apoptotic proteases and programmed cell death, TNFR-I activation is not usually associated with apoptosis. This is due to the induction of a complex cytoprotective response that requires TNFR associated factor 2 (TRAF2), a signal transducer that couples to TNFR-1. This complex then activates both NFκB-dependent and independent transcriptional pathways leading to the induction of cytoprotective genes involved in cellular growth, survival an proliferation (Natoli *et al.*, 1998). These cytoprotective pathways include hypertrophic growth programmes downstream of protein kinase C (PKC), stress activated protein kinases (SAPK) and the c-Jun NH_2-terminal kinase (JNK) (Wallach, 1997; Haunstetter and Izumo, 1998; Natoli *et*

al., 1998). TNF-α expression and production are upregulated in response to pressure overload in the adult heart. Kapadia and colleagues (1997) also demonstrated that simple passive stretch of cat papillary muscle induced the expression of TNF-α mRNA. In addition to this it has also been shown that physiologically relevant concentrations of TNF-α provoke a hypertrophic response by increasing the synthesis of structural and contractile proteins in adult feline cardiocytes (Yokoyama *et al.*, 1997).

With regard to the effects of anabolic steroids on TNFα, Hughes and colleagues (1995) conducted a study in which they investigated the effect of anabolic steroids on the immune system. They found that the steroids nandrolone decanoate and oxmethenelone directly induced the production of inflammatory cytokines IL-1β and TNFα from human peripheral blood lymphocytes. Their data seem to indicate that high doses of anabolic steroids can have significant effects on immune responses and circulating cytokine levels (Hughes *et al.*, 1995). Whether AAS have an effect on myocardial TNFα production is unknown.

It would thus seem that the effects of TNF could be considered either beneficial or harmful (Figure 2.10), depending on the timing, extent of release and stability of circulation. In this regard, TNF is either a killer or protector of its host.

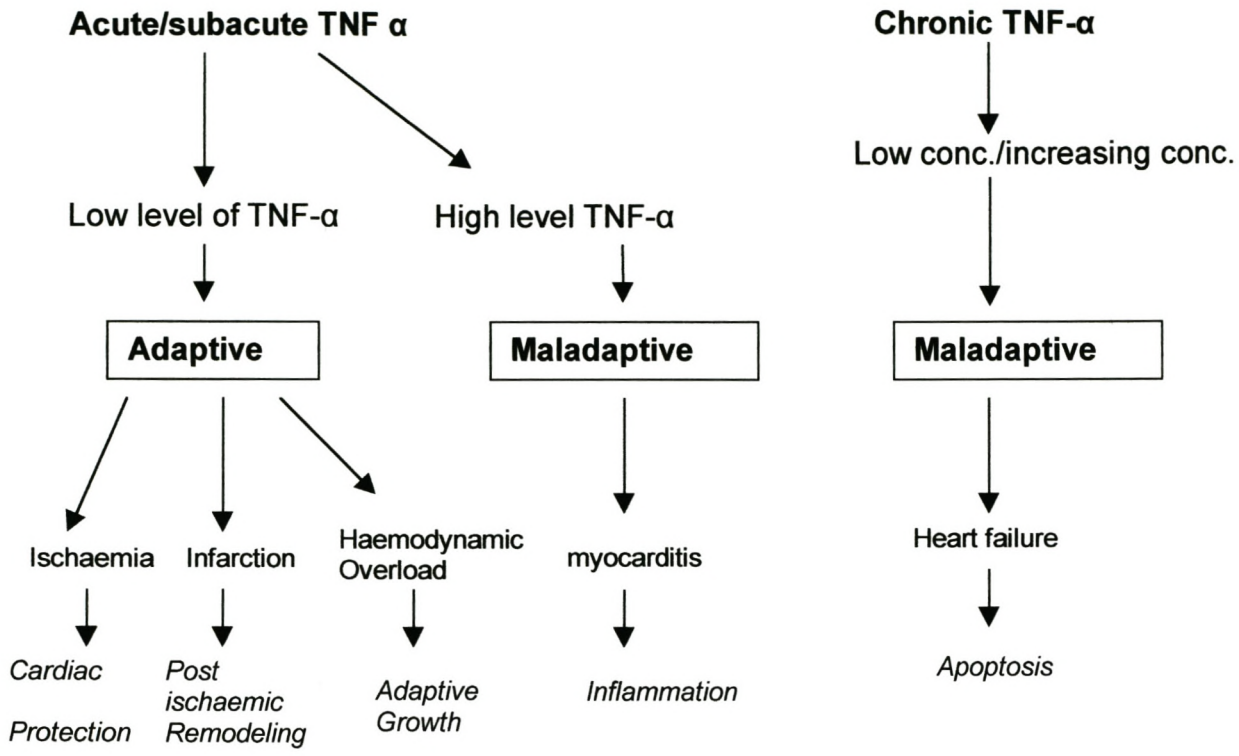

Figure 2.10: Proposed model for the adaptive and maladaptive roles of TNFα (Adapted from Sack *et al.*, 2000)

Chapter 3

Materials and methods

3.1 Animals

Male Long-Evans rats were used throughout the study. Rats were received at approximately 4 weeks of age weighing between 80 g -100 g. The rats had free access to food (standard lab chow) and water. They were maintained in animal quarters at a constant temperature (22 °C) and humidity (40%) with a 12-hour artificial day-night cycle.

3.2 Study design

For the purpose of collecting cardiac mechanical function data, rats were randomly assigned to one of four experimental groups with ten animals allocated to each group:

- A sedentary placebo group (control)
- A sedentary steroid treated group
- A exercise placebo group
- A exercise steroid treated group

For the collection of tissue for biochemical analyses, a sedentary placebo and sedentary steroid treated group was used. Here 25 rats were randomly assigned to each group.

3.2.1 Experimental procedures

The rats were anaesthetized with diethyl ether (B and M Scientific, Cape Town). Once anaesthetized, the heart was quickly excised and placed in 4 °C Krebs-Henseleit solution. The isolated rat heart was then mounted on the

aortic cannula of the Langendorff perfusion system and perfused within 60 seconds of excision. Excess non-cardiac tissue was removed while the hearts were retrogradely perfused with an oxygenated Krebs Henseleit bicarbonate buffer. The perfusion solution contained 118,46 mM NaCl; 24,995 mM $NaHCO_3$; 4,748 mM KCl; 1,185 mM KH_2PO_4; 1,19 mM $MgSO_4.7H_2O$ and 1,25 mM $CaCl_2.2H_2O$ and 10 mM glucose (Merck Pty.Ltd., Darmstadt, Germany). The perfusion solution was gassed with 95% O_2, 5% CO_2 for 20 minutes before and throughout the experiment. The buffer was prepared on the day of use and filtered through a 0,45 μm Millipore filter.

The initial perfusion period lasted 20 minutes during which time the heart was allowed to stabilize and blood and enzymes were washed out. A Watson Marlow 313 peristaltic pump (Watson Marlow Ltd, UK) kept the meniscus of the buffer in the Langendorff reservoir at a constant level so as to maintain a perfusion pressure of 100 cm H_2O. The myocardial temperature was maintained at 37°C throughout the experiment by means of a waterbath (Grant Instruments, Cambridge, England), that circulated warm water through the water-jacketed glass reservoirs. A thermistor probe was inserted into the right coronary sinus to monitor myocardial temperature throughout the experiment.

3.2.2 Training program

The training program consisted of six swimming sessions per week for 6 weeks. The rats in the exercise groups swam in large concrete baths. The water was warmed and maintained at a temperature of between 30 to 32 °C

using submersion heaters. Rats swam in groups of ten rats per bath. At the start of the training programme, the rats swam for a minimum of 5 minutes per day. The exercising times were systematically increased by 10 minutes a week until a maximum training time of 50 minutes per day was reached.

3.2.3 Steroid administration

Nandralone laurate (Laurabolin 25, Intervet, SA) was diluted 1:10 in peanut butter oil. The oil was filtered through a 0.22 μm filter to eliminate the possibility of bacterial contamination. Control rats received pure peanut butter oil. Rats receiving steroids were injected with 0.375 mg nandrolone laurate per kg body weight intramuscularly into the hind leg. The initial injection volume was 100 μl per rat. This volume was increased by approximately 50 μl each week in accordance with the increase in body mass in order to maintain the same weekly nandrolone dosage. In the last week of training each rat was injected with ±400 μl nandrolone laurate. After every weekly injection, each animal was weighed to calculate percentage body weight gain.

3.3 Experimental design

The protocol consisted of three sequential periods lasting 20 minutes, 20 minutes and 30 minutes respectively, starting with a 10 minute stabilisation period.

- 0 – 20 minutes: During the pre-ischaemic phase, hearts were perfused under standard conditions at a perfusion pressure of 100 cm H_2O. Mechanical function and coronary flows were monitored during this phase.

- 20 – 40 minutes: During the ischaemic phase hearts were subjected to global ischaemia. Hearts were submerged in Krebs-Henseleit buffer to maintain myocardial temperatures of 37°C. A thermistor probe was inserted into the right coronary sinus to monitor myocardial temperature.
- 40 – 70 minutes: With the onset of reperfusion hearts were perfused with 1% Lignocane Hydrochloride Monohydrate (Peterkaïen, Intramed) for two minutes in order to reduce the incidence of reperfusion arrhythmias. Hearts were subsequently perfused with a standard Krebs-Heinsleit buffer for the remainder of the reperfusion phase.

3.4 Mechanical function parameters measured

3.4.1 Left ventricular developed pressures

The systolic and diastolic pressure was monitored through the insertion of a compliant balloon into the left ventricle. The balloon consisted of plasticised PVC ("Glad Wrap") and was inflated with distilled water to a diastolic pressure of 4 mmHg. Pressure applied on the balloon by ventricular contraction was monitored with a pressure transducer and data recording system (Powerlab/200, AD Instruments, Australia). Left ventricular pressure and heart rate was recorded by means of the software package, Chart for Windows (Powerlab, AD Instruments, Australia). The balloon's volume was maintained constant throughout the experiment.

3.4.2 Coronary flow

Coronary flow was measured by collecting the hearts effluent for one minute every 10 minutes. Coronary flow rates varied between 10-15 ml/min. during normoxic perfusion at a perfusion pressure of 100 cm H_2O.

3.4.3 Calculations used

Functional recovery was expressed as the percentage rate pressure product (RPP) recovery using the following formula:

Where left ventricular developed pressure (LVDP)= Systolic pressure (SP) - Diastolic pressure (DP)

RPP = Heart rate (HR) x LVDP

% RPPrecovery = $(RPP_{reperfusion}/RPP_{pre\text{-}ischaemia}) \times 100$

3.5 Exclusion criteria

Hearts with coronary flow rates outside the range of 8-20 ml/min, and heart rates outside of 180-340 beats/min during the stabilisation phase, were excluded from the study during this stabilisation phase.

3.6 Tissue samples

Tissue samples were collected for biochemical and molecular biological analysis. Fifty hearts were freeze clamped with Wollenburger metal tongs, which were pre-cooled in liquid nitrogen. Samples were collected at 20 minutes perfusion (just before the onset of ischaemia), after 10 minutes ischaemia, after 20 minutes into ischaemia and 30 minutes after reperfusion.

The ventricular tissue was stored in an -80°C freezer until analyses were performed.

3.7 Biochemical analyses

3.7.1 cGMP determination

3.7.1.1 Assay description

Cyclic GMP levels were determined using a RIA kit purchased from Amersham International plc, Amersham, UK.

This assay is based on the competition between unlabeled cGMP and a fixed quantity of ^{125}I labelled cGMP for a limited number of binding sites on a cGMP specific antibody. With known amounts of antibody and reactive ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added non-radioactive ligand. The Amerlex-M secondary antibody reagent contains secondary antibody that is bound to magnetizable polymer particles. The antibody bound cGMP is reacted with this secondary antibody reagent and either magnetic separation or centrifugation of the suspension and decantation of the supernatant results in the separation of the antibody bound fraction. The amount of labelled cGMP in the bound fraction can then be calculated by measuring the radioactivity in the pellet. The concentration of unlabeled cGMP can be determined by interpolation of a standard curve.

3.7.1.2 Tissue extraction

Approximately 0.1 g of ventricular tissue was fully pulverised in a pre-cooled metal homogeniser at 4 °C. Tissue was extracted using 1.2 ml 6% trichloric acid (TCA) in Greyward tubes on ice. The extracts were centrifuged at 2000 g for 15 seconds at 4 °C. The supernatant was recovered and the pellet discarded. The supernatant was then washed 4 times with 5 volumes of water-saturated diethyl ether, discarding the upper ether layer by aspiration after each wash. Samples were stored overnight to allow for the remaining ether to evaporate.

3.7.1.3 Radioimmuno assay

Standards, ranging from 2-128 fmol/tube, and assay buffer were prepared and all reagents were equilibrated at room temperature. The acetylation reagent was prepared by mixing 1 volume of acetic anhydrate with 2 volumes of triethylamine. Then 500 µl of the assay buffer (0.5 M acetate buffer, pH 5.8 with 0.01% w/v sodium azide) was pipetted into the zero standard acetylation tube and 500 µl of each unknown and standard was added to the tubes. The acetylation reagent (25 µl) was added to all the acetylation tubes containing standards and unknowns and vortexed immediately. Duplicate aliquots (100 µl) were pipetted from all acetylation tubes into the corresponding assay tubes and 100 µl of the antiserum was added to all the assay tubes except to the total count tube (TC). The assay tubes were vortexed thoroughly, covered and incubated at room temperature for 1 hour.

After incubation, 100 µl of [125I] cGMP was pipetted into all the assay tubes and the TC was stoppered and set aside for counting. The tubes were covered and incubated for 15 to 18 hours at 2-8°C.

The Amerlex-M secondary antibody reagent was mixed to ensure a homogenous suspension and 500 µl of the secondary antibody was added to each tube except the TC.

All tubes were then vortexed and incubated for 10 minutes at room temperature. The antibody bound fraction was separated by means of centrifugation for 10 minutes at a minimum of 1500 g or greater. After centrifugation, the tubes were placed into suitable decantation racks and the supernatant liquid were poured off and discarded. The tubes were kept inverted and placed on a pad of absorbent tissues and allowed to drain for 5 minutes. On completion of centrifugation separation, the rims of the tubes were blotted and any adhering droplets of liquid were carefully removed. Radioactivity present in each tube was determined by counting for at least 60 seconds in a gamma scintillation counter (Cobra^{TM}Auto-Gamma, Packard). A standard curve was obtained and values were expressed as fmol/g ww.

3.7.2 cAMP determination

3.7.2.1 Assay description

Cyclic AMP levels were determined by Amersham's AMP [H^3] radioimmuno assay kit (Amersham International plc, Amersham, UK).

This assay is based on the competition between unlabelled cAMP and a fixed quantity of the tritium labelled compound. The amount of labelled protein-cyclic AMP complexes formed is inversely proportional to the amount of unlabelled cAMP present in the sample. The protein bound cAMP is separated from the unbound nucleotide by means of adsorption of the free nucleotides on to coated charcoal, followed by centrifugation. An aliquot of the supernatant is then removed for liquid scintillation counting. The concentration of the unlabelled cAMP in the sample can then be determined from a linear standard curve.

3.7.2.2 Tissue extraction

Approximately 0.1 g of ventricular tissue was fully pulverised in a pre-cooled metal homogeniser. Tissue was extracted using 1.2ml 6% perchloric acid (PCA) in Greyward tubes on ice. Tubes were vortexed, placed on ice for 2 hours with occasional vortexing. The tubes were then centrifuged at 4000 r.p.m for 20 minutes and 1ml of the supernatant was transferred into an Eppendorf tube containing 5 µl of universal indicator. The supernatant was neutralised with two parts 40% saturated KOH –saturated KCL and three parts 0.2 M Tris-HCL, pH 7.5 (4 °C). These neutralising volumes were

recorded. Tubes were then centrifuged for 2 minutes and supernatant transferred into a new set of Eppendorf tubes.

3.7.2.3 Radioimmuno assay

Standards were prepared by means of serial dilutions. To each of 4 glass tubes 0.5 ml Tris EDTA buffer was added. Then 0.5 ml of the cyclic AMP standard was added to the first tube. This was mixed thoroughly and 0.5 ml of this dilution was added to the next tube and mixed again. This procedure was repeated successively and a fresh pipette tip was used making each dilution. The standards ranged from 0.125 to 16 pmol/tube.

Fourteen assay tubes and additional tubes for unknowns, in duplicate, were placed in a rack and kept on ice. Reagent 1 (0.05 M Tris EDTA buffer, pH7.5) was pipetted (150 µl) into assay tubes 1 and 2 for the determination of blank counts per minute of the assay and 50 µl of the Tris/EDTA buffer was pipetted into assay tubes 3 and 4 for the determination of binding in the absence of unlabelled cAMP. Starting at the lowest level of standard cAMP, 50 µl of each dilution were added into each successive pair of assay tubes (tubes 5 to 14) for the standards ranging from 0.125 to 16 pmol/tube. Then 50 µl of each unknown, in duplicate, was added to the additional assay tubes. The labelled cAMP (50 µl) was added to each assay tube and 100 µl of the binding protein was added to tubes 3 to 14 and to each of the unknowns. All tubes were vortexed, placed on ice and incubated for two hours in a cold room (2 to 8°C). At least 15 minutes before the end of the incubation time, 20 ml of ice cold distilled water was added to the charcoal reagent and this was then stirred

continuously during use. After incubation, 100 µl the charcoal suspension was added to all tubes and tubes were vortexed briefly and replaced on ice. Tubes were then spun down in a refrigerated centrifuge. Without disturbing the sediment, a 200 µl sample was removed from each tube and placed in scintillation vials, containing 5 ml scintillation fluid, for counting (Beckman Instruments, US). Time elapsed from adding the charcoal suspension to centrifugation was not less than 1 minute, but not more than 6 minutes. A standard curve was obtained and values were expressed in pmol/g ww.

3.7.3 P38 Determination

3.7.3.1 Preparation of lysates

Freeze-clamped tissue (approximately 200 mg) was added to sorvall tubes, each containing 900 µl of lysis buffer containing (in mM); Tris 20; p-nitrophenylphosphate 20; EGTA 1; NaF 50; sodium ortovanadate 0.1; phenylmethyl sulphonyl fluoride (PMSF) 1; dithiotreitol (DTT) 1; aprotinin 10 µg/ml and leupeptin 10 µg/ml. The tissue and lysis buffer was then homogenized and spun down in a microfuge to remove the particulate matter. Protein determination for each sample was conducted using the Bradford protein determination method (Bradford, 1976). Of each sample, 10 µl was taken and 90 µl of distilled water was added. For the second dilution, 20 µl of the previous dilution was taken and 80 µl of distilled water was added. These samples were prepared in duplicate.

The lysate protein content was determined by using the Bradford technique. For the standard curve 5 mg/ml BSA stock solution ([4.76 mg/ml]) was used.

The dilutions were as follow:

BSA (µl)	H_2O	Bradford reagent (µl)
0	100	900
10 × 2	90	900
20 × 2	80	900
40 × 2	60	900
60 × 2	40	900
80 × 2	20	900

The mixtures were vortexed and the absorptions were read at 595 nm within 30 minutes of incubation.

According to the Bradford determination, each sample was diluted in Laemmli Sample buffer and boiled for 5 minutes.

3.7.3.2 Gel Electrophoreses

After boiling each sample for 5 minutes, 10µg of protein was separated on a 12% polyacrylamide gel, using the standard Bio-Rad Mini-PROTEAN II system (Biorad, Life Science group, US). (Separating gel: 3.35 ml Millipore H_20; 2.5 ml 1.5M Tris-HCl, pH 8.8; 100 µl 10% SDS; 3 ml Acrylamide; 50 µl APS (0.1 g/ml) and 20 µl Temed. Stacking gel: 3.05 ml Millipore H_20; 1.25 ml 0.5 M Tris-HCl; 50 µl 10% SDS; 500 µl Acrylamide; 50 µl 10% APS (0.1 g/ml); 10 µl Temed.) The separated proteins were then transferred to a PVDF membrane (Immobilin^{TM} -P, Millipore, UK). After staining the membranes with Ponceau S red (reversible staining), the quality and quantity of the separated

proteins were determined with a laser scanner (UN-SCAN-IT, Silkscience, US).

Membranes were then washed with TBS Tween to remove Ponceau S red and non-specific binding sites on the membrane were blocked with 5% fat-free milk in Tris-buffered saline and 0.1% Tween 20 (TBST) for two hours. Membranes were then washed again with TBS Tween and blocked with the appropriate primary antibody (phospho-p38 MAPK [Thr 180/Tyr 182] Antibody, New England Biolabs, UK) for at least five hours. After washing the membranes with TBS Tween, the immobilized primary antibody conjugated with a diluted horseradish peroxidase-labelled secondary antibody (Amersham LIFE SCIENCE, UK) for one hour. The membranes were then washed thoroughly with TBS Tween and covered with ECL™ detection agents and quickly exposed to an autoradiography film (Hyperfilm ECL, RPN 2103) to detect light emission. (ECL™ Western blotting from Amersham PhArmacia Biotech, UK). Films were densitometrically analyzed for results.

3.7.4 Myocardial Tumor Necrosis Factor alpha determination

3.7.4.1 Assay Description

An OptEIA™ Rat TNFα ELISA kit (PharmMingen, USA, Lot number M065951) kit was used for the determination of rat tumour necrosis factor-α. The Cytokine sandwich ELISA is a sensitive enzyme immunoassay that can specifically detect and quantitate the concentration of soluble cytokine.

3.7.4.2 Preparation of lysates

Freeze-clamped hearts were homogenized with 5 volumes of ice-cold lysis buffer containing in mM/l: imidazole acetate 50, magnesium acetate 10, potassium hydrogen phosphate 4, EDTA 2, in µM/l: *N*-acetylcysteine 50 and sulphur 12.5 (pH 7.6).

Protein determination for each sample was conducted with the Bradford protein determination method. Of each sample, 10 µl were taken and 90 µl of distilled water was added. For the second dilution, 20 µl of the previous dilution was taken and 80 µl of distilled water was added. These samples were prepared in duplicate.

The lysate protein content was determined by using the Bradford technique as before (see p 60).

3.7.4.3 TNFα ELIZA

The microwells were coated with 100 µl of anti rat TNFα monolconal capture antibody diluted in Coating Buffer (0.1 M Carbonate; 8.4 g $NaHCO_3$ and 3.56g Na_2CO_3 made up to 1L, pH 9.5) and incubated overnight at 4 °C.

The lyophilized standard was warmed to room temperature and reconstituted with 1 ml deionized water for a stock standard. The standard was allowed to equilibrate for a minimum of 15 minutes and vortexed gently before making dilutions. A 2000 pg/ml standard was prepared from the stock standard and vortexed. After the standard was mixed, 300 µl of the assay diluent (animal

serum in buffered solution with 0.15% ProClin-150 as preservative, PharMingen, USA) was added to 6 tubes and serial dilutions were performed by adding 300 µl of each standard to the next tube, vortexing in between. The assay diluent served as the zero standard.

After incubation, wells were aspirated 5 times with 300 µl or more Wash Buffer-PBS 0.05% Tween-20 (16 g NaCl, 2.32 g Na_2HPO_4, 0.4 g KH_2PO_4 and 0.4 g KCL made up to 2 l, pH 7.0). After the last wash, the plate was inverted and blotted on absorbent paper to remove any residual buffer. Plates were then blocked with 200 µl/well assay diluent and incubated at room temperature for 1 hour. After incubation, wells were again aspirated as described earlier. A 100 µl of each standard, sample and control were pipetted into the appropriate wells, the plate was sealed and incubated at room temperature for 2 hours.

Wells were then aspirated again for a total of 5 washes. After the plates were washed, 100 µl of the detection antibody *(Biotinylated anti-rat TNFα)*, diluted in assay diluent (1:250), was pipetted into each well and the plate was sealed and incubated for 1 hour at room temperature.

After incubation, wells were washed and aspirated for 5 times. After aspiration, a 100 µl of enzyme reagent *(Avidin-horseradish peroxidase conjugate)* diluted in assay diluent (1:250), was added to each well, the plate sealed and incubated at room temperature for 1 hour.

Wells were again aspirated, but this time for a total of 7 washes and wells were soaked in wash buffer for 30 seconds to 1 minute with each wash. A 100 µl TMB substrate solution (3,3', 5,5' tetramethylbenzidine and hydrogen peroxide in organic solvent, PharMinge, USA) was added to each well and the plate was incubated in the dark at room temperature for 30 minutes.

After the incubation 50 µl of the stop solution (1 M H_3PO_4 or 2 N H_2SO_4) was added to each well to terminate the reaction. The absorbance was read at 450 nm within 30 minutes of stopping the reaction. A standard curve ranging between 31.3 pg/ml to a 1000 pg/ml was obtained. Values obtained were presented as pg/gram ww.

3.8 Statiscal Methods

The Unpaired Students t-test was used to do statistical analysis of the biochemical and biological data. The ANOVA with Bonferroni corrections for multiple comparisons was used to determine the statistical significance of all other data. Data are presented as means± standard error of the mean (SEM). A p value equal to or smaller than 0.05 was considered as significant. The statistical program GPIS was used to perform all statistical analyses.

Chapter 4

Results

4.1 Biometric data

4.1.1 Rat weights before and after treatment

Comparisons of rat body weights before treatment did not show any significant differences between the four groups. Similarly no differences were found in rat weights after treatment.

Table 4.1: Rat weights before and after treatment in the sedentary (control) placebo group, the sedentary steroid treated group, the exercise placebo group and the exercise steroid treated group

Sedplac		**Sedster**	
Before (g) n = 10	*After (g)* n = 8	*Before (g)* n = 10	*After (g)* n = 8
89.32 ± 4.79	310.33 ± 10.88	86.65 ± 4.85	298.35 ± 8.66
Exerplac		**Exerster**	
Before (g) n = 10	*After (g)* N = 10	*Before (g)* n =10	*After (g)* n = 8
82.57 ± 4.56	300.35 ± 13.20	88.27 ± 4.45	294.12 ± 16.33

All values are expressed as mean ± SEM.

Abbreviations: Sedplac- sedentary placebo group; sedster- sedentary steroid group; exerplac- exercise placebo group; exerster- exercise steroid group

4.1.2 Heart weight to body weight ratio

The heart weight to body weight ratio was different when comparing the sedentary steroid (4.0 ± 0.19 mg) and exercise steroid groups (4.68 ± 0.13 mg) ($p < 0.05$). The heart weight to body weight ratio was similar in all the other groups.

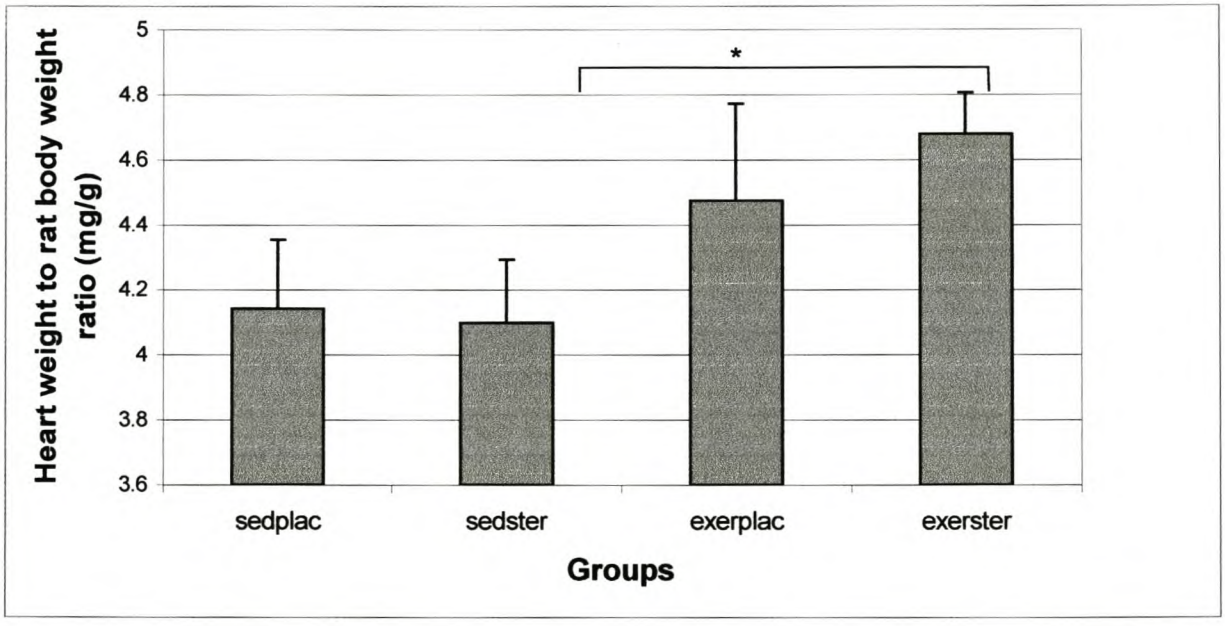

Figure 4.1: Heart weight to body weight ratio for the sedentary placebo group (control), the sedentary steroid treated group, the exercise placebo group and the exercise steroid treated group.

n= 8-14

All values are expressed as mean ± SEM.

Abbreviations: sedplac- sedentary placebo group; sedster- sedentary steroid group; exerplac- exercise placebo group; exerster- exercise steroid group

* $p< 0.05$ sedster vs. exerster

4.1.3 Plasma cholesterol, HDL cholesterol and triglyceride levels

A difference was found when the plasma cholesterol levels of the sedentary placebo (2.04 ± 0.09 mmol/l) and sedentary steroid groups (1.81 ± 0.05 mmol/l) were compared ($p < 0.05$). No differences were found in the HDL cholesterol and TAG levels between the sedentary placebo and sedentary steroid groups.

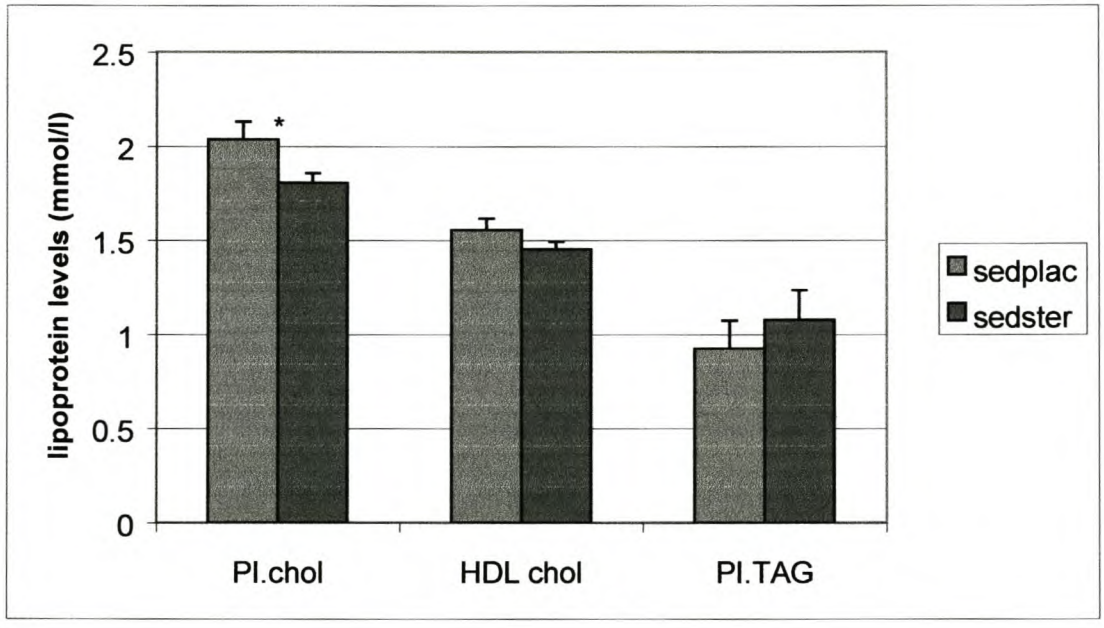

Figure 4.2: Plasma cholesterol, HDL cholesterol and triglyceride levels in the sedentary (control) placebo group and the sedentary steroid treated group.

n = 9 for each respective group

All values are expressed as mean ± SEM.

Abbreviations: sedplac- sedentary placebo group; sedster - sedentary steroid group; Pl.chol - plasma cholesterol; HDL chol - high-density lipoprotein cholesterol; Pl.TAG - plasma triglyceride

* $p < 0.05$ for sedplac vs. sedster

4.1.4 Haemodynamic function

There were no significant statistical differences in coronary flows when comparing the four groups.

4.1.4.1 Pre-ischaemic heart rate and left ventricular developed pressures

There were statistical differences in heart rate when comparing sedentary placebo group (282 ± 9.61 beats/min) and the exercise placebo group (241 ± 12.10 beats/min) ($p < 0.05$) at 30 minutes pre-ischaemia. All of other groups had similar heart rates.

There were no differences between left ventricular developed pressure when comparing the four groups.

4.1.4.2 Reperfusion heart rate and left ventricular developed pressure

There were no statistical differences in heart rate during reperfusion when comparing the four groups.

A difference was found for left ventricular developed pressure when comparing the sedentary placebo (62.75 ± 4.27 mmHg) and exercise placebo (89.57 ± 9.28 mmHg) groups at 20 minutes reperfusion. ($p < 0.05$). At 20 minutes reperfusion there was also a difference between the exercise placebo group (89.57 ± 9.28 mmHg) and the exercise steroid group (57.67 ± 9.36 mmHg) ($p < 0.05$). This difference persisted at 30 minutes of reperfusion

between the exercise placebo group (91.29 ± 8.94 mmHg) and the exercise steroid treated group (58.89 ± 5.00 mmHg) ($p < 0.05$).

Table 4.2: CF, HR, and LVDP for the sedentary (control) placebo group, the sedentary steroid treated group, the exercise placebo group and the exercise steroid treated group

Groups	*Functional parameters*	**Pre-ischaemia**	**Reperfusion**	
		30 minutes	*20 minutes*	*30 minutes*
Sedplac n = 8	*CF (ml/min)*	16.50 ± 0.53	15.68 ± 0.78	14.78 ± 1.12
	HR (beats/min)	282 ± 9.61	255 ± 16.68	259 ± 19.96
	LVDP (mmHg)	113.75 ± 6.05	62.75 ± 4.27	70.37 ± 5.21
Sedster n = 9	*CF (ml/min)*	14.16 ± 0.66	12.93 ± 0.78	11.64 ± 0.55
	HR (beats/min)	278 ± 14.10	240 ± 25.30	216 ± 17.56
	LVDP (mmHg)	111.11 ± 4.25	52.22 ± 8.78	55.89 ± 6.43
Exerplac n = 14	*CF (ml/min)*	17.88 ± 0.62	16.83 ± 0.73	16.63 ± 0.57
	HR (beats/min)	241 ± 12.10#	239 ± 16.29	233 ± 19.51
	LVDP (mmHg)	128.79 ± 5.87	89.57 ± 9.28#	91.29 ± 8.94
Exerster n = 9	*CF (ml/min)*	14.16 ± 0.64	13.40 ± 1.22	12.24 ± 1.37
	HR (beats/min)	247 ± 21.20	215 ± 26.32	220 ± 18.62
	LVDP (mmHg)	115.11 ± 4.12	57.67 ± 9.36*	58.89 ± 5.00*

All values are expressed as mean ± SEM.

Exerplac - exercise placebo group; exerster - exercise steroid group; HR- heart rate; CF - coronary flow; LVDP - left ventricular developed pressure

* $p < 0.05$ for exerplac vs. exerster

# $p < 0.05$ for sedplac vs. exerplac

4.1.5 Left ventricular developed pressure before and after ischaemia

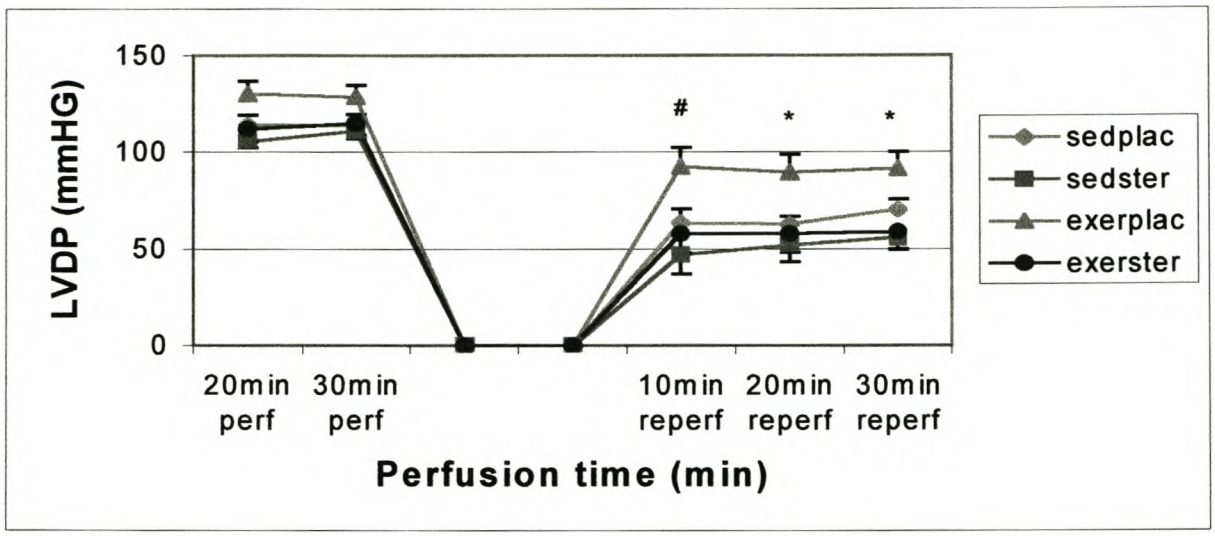

Figure 4.3: Left ventricular developed pressure before and after ischaemia for the sedentary placebo group (control) ,the sedentary steroid treated group, the exercise placebo group and the exercise steroid treated group.

n = 8-14

All values are expressed as mean ± SEM.

Abbreviations: sedplac - sedentary placebo group; sedster - sedenary steroid group; exerplac - exercise placebo group; exerster - exercise steroid group; LVDP - left ventricular developed pressure

* $p < 0.05$ for exerplac vs. exerster

# $p < 0.05$ for sedplac vs. exerplac

4.1.7 Rate pressure product before and after ischaemia

Because heart rates were different in certain groups before ischaemia, rate pressure product values were also determined to quantify mechanical function of the hearts.

Pre-ischaemic rate pressure products were different at 20 minutes perfusion (34582.00 ± 1778.13 mmHg/min vs. 28868.67 ± 2446.02 mmHg/min) when comparing the exercise placebo and exercise steroid groups and this difference persisted at 30 minutes perfusion (34582.00 ± 1778.13 mmHg/min vs. 28868.67 ± 2446.02 mmHg/min), This difference persisted at (22196.29 ± 2904.93mmHg/min vs. 10128.89 ± 1447.00 mmHg/min), 20 (21753.36 ± 2917.17 mmHg/min vs. 11275.33 ± 1941.19 mmHg/min) and 30 minutes reperfusion (21892.21 ± 2912.14 mmHg/min vs. 12838.67 ± 1536.50 mmHg/min) ($p < 0.05$). When comparing the sedentary placebo and sedentary steroid treated groups, there was a difference at 10 minutes (14811.38 ± 1825.61 mmHg vs. 7950 ± 1441.66 mmHg/min) ($p < 0.05$), 20 minutes (16169.63 ± 1772.88 vs. 11243.67 ± 1516.49) ($p < 0.05$) and 30 minutes (18276.38 ± 2026.10 mmHg/min vs. 12018.67 ± 1725.27 mmHg/min) ($p < 0.05$) reperfusion.

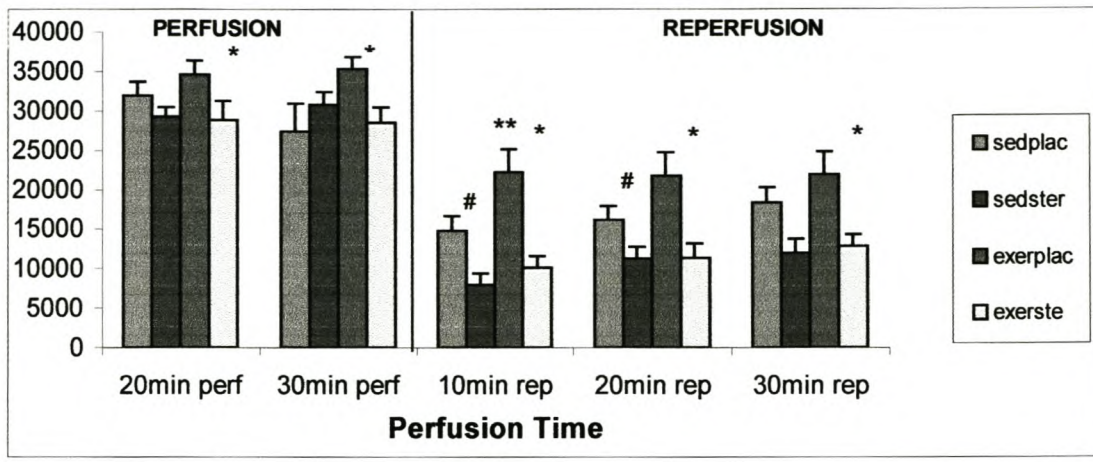

Figure 4.4: Rate pressure product before and after ischaemia for the sedentary (control) placebo group, the sedentary steroid treated group, the exercise placebo group and the exercise steroid treated group.

n = 8 - 14

All values are expressed as mean ± SEM.

Abbreviations: sedplac - sedentary placebo group; sedster - sedentary steroid group; exerplac - exercise placebo group; exerster - exercise steroid group; RPP - rate pressure product

* $p < 0.05$ for exerplac vs. exerster

# $p < 0.05$ for sedplac vs. sedster

** $p < 0.05$ for sedplac vs. sedster

4.1.8 Percentage rate pressure product recovery

When comparing the rate pressure product recoveries of the sedentary placebo and sedentary steroid treated groups, there was a difference at 10, 20 and 30 minutes reperfusion (46.0 ± 5.37% vs. 26.09 ± 4.56%, 50.90 ± 5.04% vs. 36.93 ± 4.79% and 58.01 ± 6.47% vs. 39.89 ± 5.94%) ($p < 0.05$). At 10 minutes (61.12 ± 6.39% vs. 37.32 ± 5.96%) and at 20 minutes (59.04 ± 6.56% vs. 39.87 ± 6.22%) of reperfusion differences was found between the exercise placebo and exercise steroid groups ($p < 0.05$).

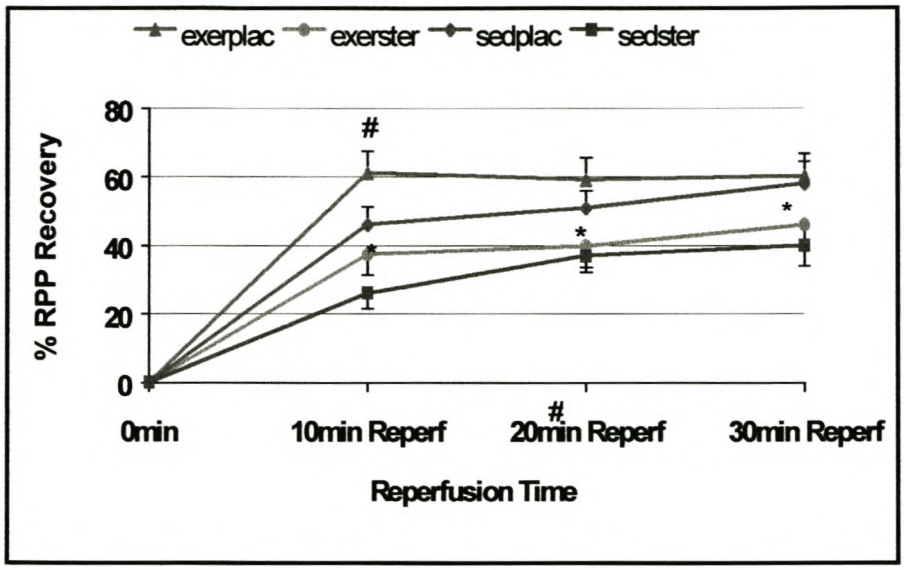

Figure 4.5: Percentage rate pressure product recovery for the sedentary placebo group (control), the sedentary steroid treated group, the exercise placebo group and the exercise steroid treated group.

n = 8-14

All values are expressed as mean ± SEM

Abbreviations: sedplac - sedentary placebo group; sedster - sedentary steroid group; exerplac - exercise placebo group; exerster - exercise steroid group; RPP - rate pressure product.

* $p < 0.05$ for sedplac.vs. sedster

# $p < 0.05$ for exerplac vs. exerster

4.2. Biochemical results

4.2.1 Tissue cGMP values

When comparing the sedentary placebo group (control) with the sedentary steroid group, a difference was found between the two groups at 30 minutes pre-ischaemia (7.381 ± 0.280 pmol/g vs. 9.159 ± 0.815 pmol/g), 10 minutes ischaemia (9.977 ± 2.476 pmol/g vs. 20.814 ± 3.457 pmol/g) and 20 minutes of ischaemia (20.547 ± 3.034 pmol/g vs. 11.633 ± 2.807 pmol/g) ($p < 0.05$).

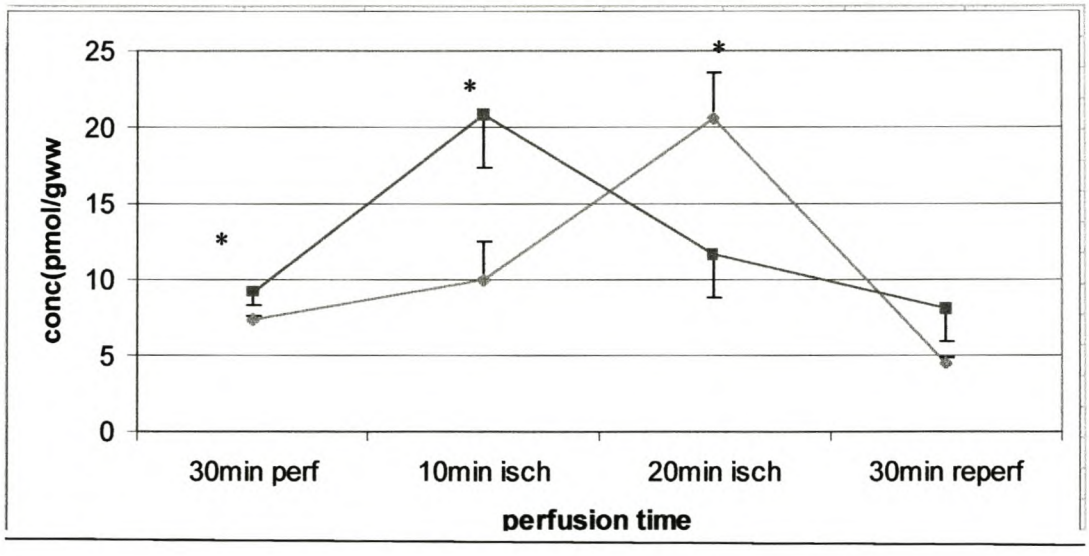

Figure 4.6: Tissue cGMP levels in the sedentary placebo group (control) and the sedentary steroid treated group.

n = 5 - 7

All values are expressed as the mean ± SEM.

Abbreviations: sedplac - sedentary placebo group; exerster - exercise steroid treated group

* = $p < 0.05$ for sedplac vs. sedster

.2.2 Tissue cAMP levels

cAMP levels were elevated in the non-ischaemic steroid treated hearts (406.04 ± 18.41 pmol/g) when compared to the placebo treated rats(235.6 ± 43.26 pmol/g) ($p < 0.05$). There was no difference in cAMP levels during ischaemia and reperfusion in the two groups.

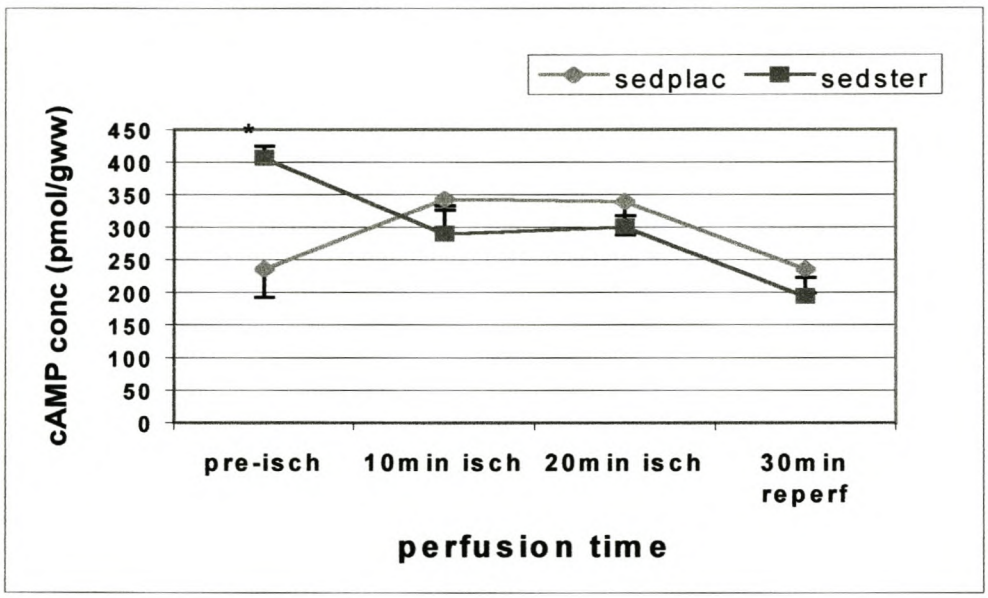

Figure 4.7: Tissue cAMP levels in the sedentary placebo group (control) and the sedentary steroid treated group.

n = 5 - 6

All values are expressed as the mean ± SEM.

Abbreviations: sedplac - sedentary placebo group; sedster - sedentary steroid group

* $p < 0.05$ for sedplac vs. sedster

4.2.3 P38 Mitogen activated protein kinase activity

There was no difference between the sedentary placebo and sedentary steroid treated group when comparing the degree of p38 MAPK activation.

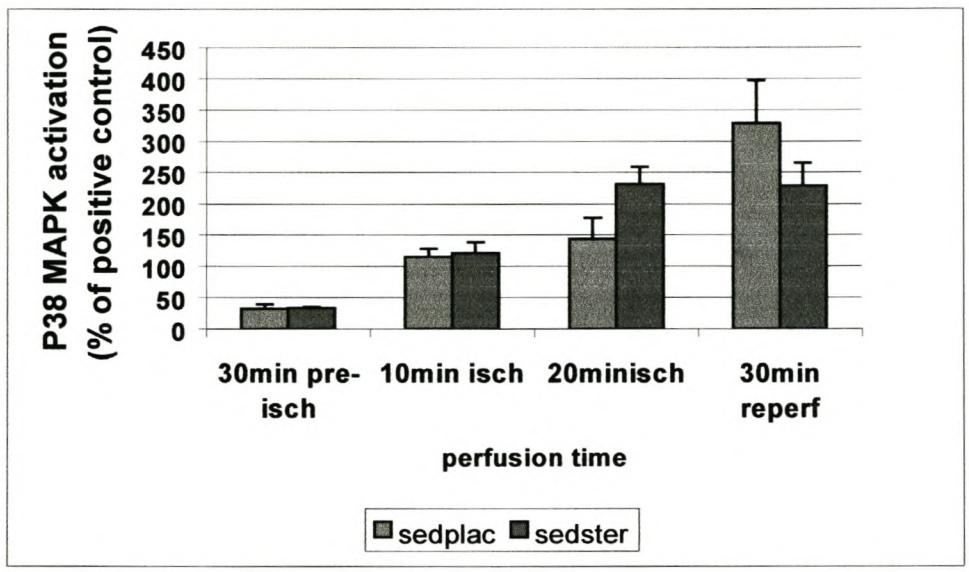

Figure 4.8: Degree of p38 MAPK activation for sedentary placebo group (control) and steroid treated group.

n = 4 for the respective groups

All values are expressed as mean ± SEM.

Abbreviations: sedplac - sedentary placebo group; sedster- sedentary steroid group

4.2.4 Myocardial TNFα values

There was a difference in myocardial TNFα levels at 30 minutes perfusion between the sedentary placebo group (190.00 ± 15.75 pg/g) and the sedentary steroid group (267.75 ± 44.25pg/g) ($p < 0.05$). No statistical difference between the two groups was observed during the ischaemic period. However, at 30 minutes reperfusion the steroid treated group showed higher levels of TNFα (511.13 ± 13 pg/g vs. 287.63 ± 59.95 pg/g).

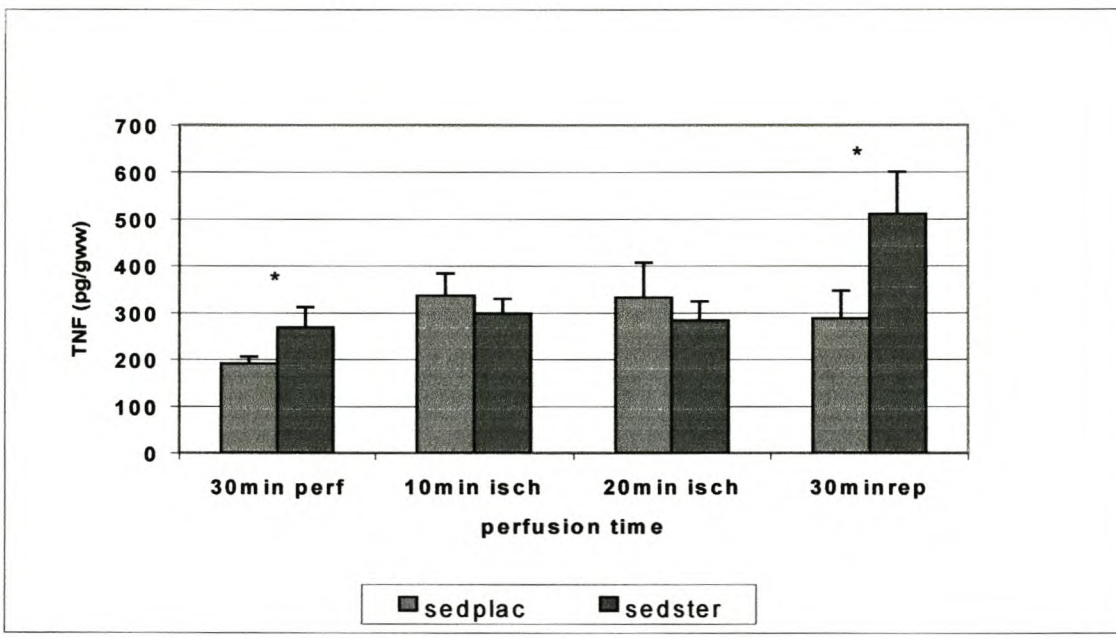

Figure 4.9: Myocardial TNFα levels for the sedentary placebo group (control) and the sedentary steroid treated group.

n = 3 - 6

All values are expressed as the mean ± SEM

Abbreviations: sedplac - sedentary placebo group; sedster - sedentary steroid treated group

* = $p < 0.05$ for sedplac vs. sedster

Chapter 5

Discussion

It was investigated whether androgenic anabolic steroid administration has a detrimental effect on the hearts of sedentary and exercised rats. The purpose of this study was to determine whether there is a link between anabolic steroid induced cardiac hypertrophy and the severity of myocardial ischaemia/reperfusion injury and to possibly gain some insight into the mechanisms for the detrimental effects of the steroids on the heart.

The biometric data indicated that there were no significant differences in rat weights among the four groups before treatment. Similarly, no differences were found after treatment and/or exercise training. Heart weight to body weigh ratio was used as an index of cardiac hypertrophy. In this regard the exercising steroid treated animals exhibited greater cardiac hypertrophy when compared to their sedentary steroid treated counterparts. Plasma cholesterol, HDL cholesterol and TAG were measured and a significant difference in plasma cholesterol was found when comparing the sedentary steroid treated group with the sedentary control group, the latter having higher cholesterol levels.

Haemodynamic parameters measured included heart rate, coronary flow rate and left ventricular developed pressure. As far as coronary flow rate is concerned, no statistical differences were observed among the four groups. The pre-ischaemic heart rate of the exercise-trained animals was significantly lower than that of their sedentary counterparts. There was no statistical

difference in left ventricular developed pressure for the four groups when measured before ischaemia. Left ventricular developed pressure differed significantly during reperfusion for the exercise placebo group compared with sedentary placebo group (10 minutes reperfusion) and for the exercise steroid treated group compared with the exercise placebo group (20 and 30 minutes of reperfusion).

As an index of mechanical function of the hearts, rate pressure product was used, while rate pressure product recovery was used as an index of post-ischaemic recovery.

Pre-ischaemic function of the exercising placebo treated group was significantly better when compared to the exercising steroid treated animals and the sedentary control group. Post-ischaemic function of the exercising placebo treated group consistently showed a better function when compared to the exercise steroid treated animals. Another consistent statistical difference was seen between the sedentary control group and the sedentary steroid treated group with the latter showing the poorer functional recovery of the two groups. At the beginning of reperfusion, there was a difference between the sedentary steroid treated and exercise steroid treated animals. The exercise-trained group displayed the better RPP of the two. Lastly, statistical differences were also found between the sedentary placebo and exercise placebo (control) groups during this period. The trained animals again displayed better reperfusion function recovery than their sedentary counterparts.

For the purpose of biochemical investigation, myocardial cGMP, cAMP, TNFα and p38 levels were measured in the sedentary placebo treated and in the sedentary steroid treated animals. The control animals had significant higher cGMP levels during the pre-ischaemic phase and early during the ischaemic phase, displaying no statistical differences during the reperfusion phase. Myocardial cAMP levels were statistically greater in the steroid treated animals during the pre-ischaemic phase, which may indicate that steroid treatment increased non-ischaemic myocardial cAMP levels. The normoxic, non-ischaemic hearts of the steroid treated rats had significantly higher TNFα levels than their non-treated counterparts. At 30 minutes reperfusion, the steroid treated animals again displayed signifiantly higher myocardial TNFα levels. No statistical differences were found between the two groups when measuring p38 activity before, during and after ischaemia.

5.1 Rat weights before and after treatment

There were no significant differences between the weights of the four experimental groups prior to treatment. Any differences in the animals' end weight after treatment and/or exercise would be ascribed to the treatment administered. The study did not yield any differences in body weights between the four groups. In contrast to this, Liang and colleagues (1992) found larger weight gains in steroid treated and exercise steroid treated groups compared to control and exercise groups. These conflicting results are probably due to a difference in experimental protocol. Liang and colleagues (1992) injected rats with a starting dose of 1.5 nandrolone decanoate mg/kg body weight for between 42 to 63 days ending with a dosage of 3.0 mg nadrolone decanoate

per kg body weight. This is a much higher dosage than that used in this study (0.375 mg/kg body weight) and the duration of treatment was also longer in the study performed by Liang and colleagues (1992).

In agreement with our results, Philips and colleagues (2000) found no evidence of a generalised anabolic effect of nandrolone in their unexercised animals. Kinson and colleagues (1991) found that neither castration nor treatment with AAS produced any significant change in body weight.

5.2 Heart weight to body weight ratio

Cardiac hypertrophy is a fundamental process of adaptation to an increased workload due to haemodynamic overload (Cooper 1987; Mondry and Swynghedauw, 1995). This adaptation would appear to be beneficial at first by increasing the number of contractile units, thus compensating for the increased workload and hence reducing left ventricular wall stress. This beneficial adaptation is limited however, because at a certain point the adaptational value is replaced by detrimental cardiac hypertrophy, even leading to heart failure (Ruwhof and Van der Laarse, 2000). Several stimuli exist to induce cardiac hypertrophy of which the most important seems to be mechanical stress.

The use of androgenic anabolic steroids usually goes hand in hand with exercise training. Exercise induced cardiac hypertrophy has been studied extensively and studies have been done to investigate the effects of the combination of exercise and anabolic steroid treatment. Little conclusive data is however available with regard to the relationship between AAS and cardiac

hypertrophy in untrained subjects. Findings in these studies have been numerous and diverse.

Exercise training results in physiological left ventricular hypertrophy associated with increases in left ventricular end diastolic internal dimensions (de Maria *et al.*, 1978). It has been shown that high-dose androgenic steroid administration produces a leftward shift in the left ventricular end diastolic pressure internal dimension relationships (Trifunovic *et al.*, 1995; Trifunovic *et al.*, 1998). In these two studies it has also been reported that high-dose androgenic steroid administration resulted in a reduction in left ventricular weight in sedentary animals. Takala and colleagues (1991) have reported that simultaneous anabolic steroid administration and exercise training prevents cardiac hypertrophy observed after endurance training in dogs.

In the present study the heart weight to body weight ratios of the four groups were compared as an indirect index of cardiac hypertrophy. Although the exercise group showed signs of possible cardiac hypertrophy, the exercise steroid treated group had distinct cardiac hypertrophy. A significant difference in heart weight to body weight was found between the sedentary steroid treated group and the exercised steroid treated animals, with the latter having larger hearts relative to their body weights.

It is a well known fact that exercise training leads to cardiac hypertrophy. The greater hypertrophy displayed in the steroid treated rat hearts would suggest a cumulative effect of exercise and androgenic anabolic steroids on cardiac hypertrophy. Indeed, several studies have verified that the use of anabolic

steroids lead to an increase in peripheral vascular resistance and cardiac hypertrophy when combined with exercise (Appel *et al.*, 1983; Karhunen *et al.*, 1988; Dickerman *et al.*, 1997). Cardiac hypertrophy was not observed with sedentary steroid treated animals. The mechanism by which AAS causes hypertrophy of the cardiac myocytes is still unclear. Androgen receptors are present in cardiac myocytes, which may allow androgens to induce myocardial hypertrophy by direct receptor specific mechanisms.

5.3 Lipid profiles

The relationship between androgenic anabolic steroid use and changes in lipid profiles has been investigated in both non-primate and primate models and is currently controversial. In many primate models, the use of anabolic steroids not only leads to an increase in LDL cholesterol, but also to a concomitant decrease in HDL cholesterol (Peltonen *et al.*, 1981; Stubbe *et al.*, 1983; Lenders *et al.*, 1988; Weyrich *et al.*, 1991).

Present results did not show any significant changes in the HDL cholesterol or triglyceride levels. It did however show a statistical difference in the plasma total cholesterol with the controls displaying the higher levels. In a study done by Glazer and Suchman (1994) in which they injected 21 men and 3 women with nandrolone decanoate intramuscularly once a week for six weeks. No significant change was noted in high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, total cholesterol, triglycerides, the total cholesterol to HDL-C ratio, or the LDL-C/HDL-C ratio from nandrolone treatment. In a recent study done by Wolffenbuttel and colleagues (1999) they

injected 12 male weight lifters with 200 mg nandrolone decanoate per week (intramuscularly) for 8 weeks. Their results suggested that, perhaps unlike other anabolic steroids, nandrolone decanoate, even in high doses, does not have a detrimental effect on the subjects' lipid profiles. These studies concur with our findings, perhaps due to the fact that we also used a nandrolone derivative.

5.4 Heamodynamic function

5.4.1 Heart rate

An important difference was seen when comparing the pre-ischaemic heart rate of the sedentary placebo group and the exercise placebo group. The exercise group exhibited the lower heart rate of the two, indicating adaptation to exercise in the trained animals. This adaptation is usually referred to as training-induced bradycardia where the resting heart rate of the trained individual is lowered. In a study done as early as 1977, Sigvadsson and colleagues used Sprague-Dawley rats, normal and chemically sympathectomized with 6-hydroxy-dopamine and then trained by treadmill running. The normal rats showed a reduction in exercising heart rate after the training period. Compared to a sedentary control group, the sympathectomized rats showed no statistical difference in intrinsic heart rate after pithing and denervation and no increase in heart weight. These findings suggest that endurance training induces enhanced vagal activity and attenuated sympathetic tone, which may in part contribute to the resting bradycardia and an increase in the spontaneous bradycardia in athletes

(Shin *et al.*, 1995). How the isolated heart is affected by this mechanism is not clear and remains to be elucidated.

5.4.2 Pre-ischaemic cardiac function

The exercise-trained animals displayed the highest pre-ischaemic (as well as post-ischaemic) rate pressure product, i.e. function, throughout the experiment compared to the other three groups. The cardiovascular benefits of exercise are well established and have been studied extensively (Morris *et al.*, 1980; Siscovick *et al.*, 1984). It has been suggested that exercise training improves cardiac function without evidence of cardiac apoptosis and produces a pattern of cardiac gene expression distinct from the pathological cardiac adaptation to exercise (Jin *et al.*, 2000). When comparing the trained animals to those receiving AAS, it would appear that the anabolic steroids reduced the training induced beneficial effects on cardiac function. These negative changes may be related to AAS induced changes in basal cyclic nucleotide levels in the heart (see later).

5.4.3 Post-ischaemic function

The role of ischaemia and ischaemia/reperfusion injury in the heart has been studied extensively. However, no literature could be found on the effects of AAS on function of the post-ischaemic heart. Acute ischaemia induces a wide variety of detrimental effects in the heart, which is usually exacerbated during reperfusion (Boyle *et al.*, 1997). In the present study reperfusion function, i.e. recovery of heart function after an episode of global ischaemia, was used in order to determine the severity of the ischaemia/reperfusion injury. In this

regard we wanted to determine whether hypertrophy and/or anabolic steroids would increase or decrease myocardial susceptibility to ischaemic/reperfusion injury.

The detrimental changes ischaemia induced in the myocardium are brought about by complex mechanisms. These include the depletion of energy stores (Jennings *et al.*, 1983; Maxwell and Lip; 1997), cellular leakage with accompanied disruption of cellular homeostasis (Maxwell and Lip, 1997) and the accumulation of metabolic waste products such as lipid metabolites (Corr *et al.*, 1993). Although data on anabolic steroids and the direct role they play in ischaemia/repefusion injury are scarce, there is a substantial amount of evidence implicating AAS in myocardial infarction and related heart diseases (Kennedy and Lawrence, 1993; Huie, 1994; Appelby *et al.*, 1994).

To our knowledge the present study is the first to investigate the effects of AAS on myocardial susceptibility to ischaemic/reperfusion injury. It is generally thought that AAS users/abusers are at greater risk of atherosclerosis- thought to be secondary to increased concentrations of LDL-C and decreased concentration of HDL-C (Hurley *et al.*, 1984).

To study the possible detrimental effects of androgenic anabolic steroids on the heart and its resistance to ischaemia/reperfusion injury, a sedentary group and a sedentary steroid treated group was compared. Although there was no statistical difference in function between the two groups during the pre-ischaemic phase, there was a definite difference in function during the

reperfusion phase, with the untreated group of hearts consistently recovering better than the steroid treated group. This can be seen in both the rate pressure product values and the percentage recovery data. From the data gathered in the present study, it can be concluded that the lowering of cardiac performance, before and after an ischaemic incident, can also be attributed to other factors besides the lipid profiles, since we did not find the lipid profiles to be altered negatively in the treated animals.

This would suggest that AAS make the heart more prone to ischaemia/reperfusion injury by mechanisms independent of its effects on the lipid profiles. Possible explanations for this, disregarding an atherogenic model due to detrimental lipid profiles, include AAS-induced thrombosis, inhibited vascular smooth muscle relaxation and perhaps direct injury of the myocardial cells (Melchert and Welder, 1995).

5.5 Effect of AAS on selected biochemical parameters

5.5.1 cyclic GMP

Activation of the NO-cGMP pathway is thought to protect the heart against ischaemia/reperfusion injury. It has also been proposed that estrogen, known for its cardiovascular benefits, might protect the heart through activation of this pathway (Wellman *et al.*, 1996). Since one of the possible mechanisms of AAS-induced cardiac injury is mediated through the inhibition of guanylate cyclase, leading to the lowering of cGMP levels (Melchert and Welder, 1995), Cyclic GMP levels were measured in the hearts of the two sedentary groups of animals. Previously it was seen that chronic administration of nandrolone to

rabbits resulted in decreased thoracic aorta relaxation possibly by inhibiting guanylyl cyclase (Ferrer *et al.*, 1994). In the present study the untreated animals displayed lower levels of myocardial cGMP under normoxic conditions when compared with steroid treated hearts. This may indicate that myocardial guanylyl cyclase is stimulated in the steroid treated hearts. During ischaemia there were no statistical differences in myocardial cGMP levels. What cannot be disregarded are the higher cGMP levels with the onset of the experiments in the treated animals. It would be reasonable to suggest that treatment with steroids possibly stimulated guanylyl cyclase activity under normoxic conditions. What we could not establish from this work is whether the beneficiary effects of steroid induced elevations in myocardial cGMP are not being overshadowed by the effects of AAS on cAMP levels.

5.5.2 Cyclic AMP

As mentioned earlier, evidence has accumulated over the years implicating cytosolic Ca^{2+} overload in myocardial ischaemic/reperfusion injury (Marban *et al.*, 1989; Du Toit *et al.*, 1992). Since factors leading to increased cytosolic calcium include adrenergic stimulation leading to cAMP activation, it was investigated whether androgenic anabolic steroids had an effect on myocardial cAMP levels. To our knowledge, this is the first attempt to do so. Indeed, a significant difference was found in pre-ischaemic myocardial values with the steroid treated animals displaying higher myocardial cAMP levels. These elevated levels of cAMP prior to ischaemia could make AAS treated hearts more susceptible to ischaemic/reperfusion injury compared to their untreated counterparts. One would expect these hearts to have elevated

cytosolic Ca^{2+} levels at the onset of ischaemia which would be expected to worsen ischaemic Ca^{2+} overload and, as a consequence, ischaemic/ reperfusion injury.

5.5.3 P38 Mitogen activated protein kinase

The mitogen activated protein kinase, p38, is thought to play a dual role with regard to cardiovascular injury. On the one hand, it can engage in a protective role, activating small heat shock proteins (Rouse *et al.*, 1994), protecting the heart against ischaemia/reperfusion injury. On the other hand, several studies have shown it to be deleterious, exacerbating ischaemia/reperfusion injury (Ma *et al.*, 1999; Saurin *et al* 1999). To the best of our knowledge we are the first to investigate whether there is a correlation between anabolic steroid treatment and myocardial p38 activity. Although no significant statistical differences were found between the steroid treated and control groups, a tendency seems to exist for elevated levels of p38 in the steroid treated animals, particularly during ischaemia. A clearer indication of the effects of AAS on P38 activity may be obtained if the number of hearts for these determinations were to be increased.

5.5.4 Myocardial TNFα

Tumour necrosis factor alpha is a mediator of several processes in the heart. Several conditions can stimulate TNFα production, including ischaemia and reperfusion (Gurevitch *et al.*, 1996). TNFα produced locally in the heart may be important in the induction of myocardial dysfunction, apoptosis and hypertrophy (Finkel *et al.*, 1987; Krown *et al.*, 1996; Kumar *et al.*, 1996;

Yokoyama *et al.*, 1997). On the other hand, it has been suggested that low acute or subacute exposure of the heart to TNFα may be adaptive, while chronic exposure to low or increasing concentration of TNFα is maladaptive and leads to heart failure (Sack *et al.*, 2000).

There are indications that AAS may stimulate TNFα formation in lymphocytes. We propose that AAS may also stimulate myocardial TNFα synthesis. We, to our knowledge, are the first to investigate whether TNFα may be linked to the exacerbation of ischaemic/reperfusion injury in AAS treated hearts. Pre-ischaemic myocardial TNFα levels were higher in the AAS treated animals than their untreated controls. This would suggest that AAS led to elevated levels of myocardial TNFα in the animals during treatment. These elevated TNFα levels may have contributed to both the poorer mechanical function of the normoxic heart and to the exacerbation of ischaemic/reperfusion injury. The elevated TNFα levels during the 30 minute reperfusion phase may contribute to suppressing myocardial function as it is known that elevated myocardial TNFα can suppress mechanical function through sphingomyelin and NOS-cGMP pathways.

Again, it might be helpful to increase the group numbers. Another possible suggestion would be to collect blood while treating the animals to also determine circulating serum TNFα levels.

Chapter 6

Conclusions

Animals subjected to androgenic anabolic steroid treatment together with an exercise training program developed myocardial hypertrophy. It was also found that AAS reduced the beneficial myocardial effects of exercise training as seen in the lowered heart function of the steroid treated group. There was also a reduction in reperfusion function in the steroid treated animals indicating that AAS may make the heart more susceptible to ischaemia/reperfusion injury.

It is also possible that the detrimental pre-ischaemic cyclic nucleotide ratio, i.e. extremely high cAMP levels and marginally elevated cGMP levels, in steroid treated sedentary animals could make these hearts more prone to ischaemia/reperfusion injury. The higher pre-ischaemic and reperfusion levels of TNFα could also contribute to this.

Future investigation and study reservations

The collection of blood samples during the exercise-training program and steroid treatment would confirm whether AAS increases circulating TNFα levels during the treatment program. There was an indication that AAS treatment affected the MAPK pathway, with special reference to P38. To establish whether this is the case, greater sample numbers must be obtained.

Lastly, we found literature indicating that nandrolone laurate had little effect on lipid profiles and body weight gain. It might be advisable to use an AAS also affecting these parameters. With regard to the training program, we advise that tail weights be added to increase resistance training during swimming.

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