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The Effects of Exogenous Human Growth Hormone and Insulin-Like Growth Factor-1 Administration on Mitochondrial Function.

Keane, James

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The Effects of Exogenous Human Growth Hormone and Insulin-like Growth Factor-1 Administration on Mitochondrial Function.

Thesis presented in fulfilment of the degree of Doctor of Philosophy (PhD)

James Keane (BSc Hon)

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I, James Keane, declare that this thesis is a presentation of my original research work, that any data presented is accurate, was collected and analysed by myself and that the appropriate credit has been given where reference has been made to the work of others.

James Keane

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LIST OF ABBREVIATIONS

1RM	One Repetition Maximum
.ОН	Hydroxyl Radical
ADC	Analog-to-Digital Converter
ADP	Adenosine Diphosphate
AGO	Argonaute Protein
AIF	Apoptosis Inducing Factor
Akt	Protein Kinase B
ALS	Acid-Labile Subunit
ANOVA	Analysis of Variance
ANT	Adenine Nucleotide Translocator
AP-1	Activator Protein-1
ATF-2	Activating Transcription Factor-2
ATP	Adenosine Triphosphate
AU	Arbitrary Units
Bad	Bcl-2 Antagonist of Cell Death
Bak	Bcl-2 Antagonist Killer-1
Bax	Bcl-2 Associated X Protein
BCA	Bicinchoninic Acid
Bcl-2	B-cell CLL/Lymphoma Protein-2
Bcl-xL	Bcl-2 Related Gene – Long Isoform
Bid	Bcl-2 Interacting Domain Death Agonist

Bim	Bcl-2 Interacting Mediator of Cell Death
BSA	Bovine Serum Albumin
CAF-1	CCR4-Associated Factor 1
cAMP	Cyclic Adenosine Monophosphate
СССР	Carbonyl Cyanide 3- Chlorophenylhydrazone
CCR4	Carbon Catabolite Repression 4
cDNA	Complementary Deoxyribonucleic Acid
СНО	Chinese Hamster Ovary
CoQ	Coenzyme_Q
COX	Cytochrome C Oxidase
CREB	cAMP-Response-Element-Binding Protein
CS	Citrate Synthase
CsA	Cyclosporine A
CsH	Cyclosporine H
СТ	Cycle Threshold
Cu,Zn-SOD	Copper, Zinc-Superoxide Dismutase
Cyp-D	Cyclophilin-D
Cyt c	Cytochrome C
DAG	Diacylglycerol
DC	Differential Centrifugation
DCF	Dichlorofluorescin
DCP	mRNA Decapping Enzyme

$\text{DilC}_1(5)$	1,1',3,3,3',3'-Hexamethylindodicarbo - Cyanine Iodide
DR	Death Receptor
dsDNA	Double Stranded Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
EIF-4E	Eukaryotic Translation Initiation Factor 4E
ERK	Extra-cellular Signal-Regulated Kinase
ETC	Electron Transport Chain
ETF	Electron Transferring Flavoprotein
ETF-QO	Electron Transfer Flavoprotein-Ubiquinone Oxidoreductase
Ets	E-Twenty Six
FADH	Flavin Adenine Dinucleotide (Reduced Form)
FFA	Free Fatty Acid
FGF-2	Fibroblast Growth Factor-2
FoxO	Forkhead Box O
FSC	Forward Scatter Channel
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
gDNA	Genomic Deoxyribonucleic Acid
GH	Growth Hormone
GHBP	Growth Hormone Binding Protein
GHD	Growth Hormone Deficiency
GHIH	Growth Hormone Inhibiting Hormone
GHR	Growth Hormone Receptor

GHRH	Growth Hormone Releasing Hormone
GPX-1	Glutathione Peroxidase-1
Grb-2	Growth Factor Receptor-Bound Protein-2
GW182	182-kDA Glycine-Tryptophan Protein
H ₂ DCFDA	2',7'-Dichlorodihydrofluorescein Diacetate
H_2O_2	Hydrogen Peroxide
HAD	L-3-Hydroxyacyl-CoA Dehydrogenase
HBSS	Hanks Balanced Salt Solution
HCAEC	Human Coronary Arterial Endothelial Cell
HE	Hydroethidine
hGH	Human Growth Hormone
HO ₂ .	Hydroperoxyl Radical
HPF	3'-p-Hydroxyphenyl Fluorescein
hROS	Highly Reactive Oxygen Species
HRP	Horseradish Peroxidase
HRPT1	Hyperparathyroidism 1
HSL	Hormone-Sensitive Lipase
IGF-1	Insulin-like Growth Factor 1
IGF-1R	Insulin-like Growth Factor-1 Receptor
IGFBP-3	Insulin-like Growth Factor Binding Protein-3
IMM	Inner Mitochondrial Membrane
IR	Insulin Receptor

IRS	Insulin Receptor Substrate
JAK2	Janus Kinase 2
JC-1	5,5',6,6'Tetrachloro-1,1',3,3'-Tetraethylbenzimidazol-Carbocyanine Iodide
kDa	Kilo-Dalton
LPL	Lipoprotein Lipase
LPS	Lipopolysaccharide
MACS	Magnetic Activated Cell Sorting
MANOVA	Multivariate Analysis of Variance
МАРК	Mitogen-Activated Protein Kinase
Mcl-1	Myeloid Cell Leukemia-1
MEF	Mouse Embryonic Fibroblast
MEK	Mitogen-Activated, ERK Activating Kinase
MGF	Mechano Growth Factor
mIGF-1	Local Muscle Specific IGF-1
miRISC	miRNA-Induced Silencing Complex
miRNA	Micro Ribonucleic Acid
Mito-miRNA	Mitochondrial Associated miRNA
MLB	Major League Baseball
Mn-SOD	Manganese-Superoxide Dismutase
MOPS	Potassium Morpholinopropane Sulphonate
mRNA	Messenger Ribonucleic Acid
mtCK	Mitochondrial Creatine Kinase

mtHK Mitochondrial Hexokinase mtPTP Mitochondrial Permeability Transition Pore mtROS Mitochondrial-Derived Reactive Oxygen Species Nicotinamide Adenine Dinucleotide (Reduced Form) NADH NADPH Nicotinamide Adenine Dinucleotide Phosphate (Reduced Form) NCAA National Collegiate Athletic Association NDUFA1 NADH Dehydrogenase (Ubiquinone) 1 Alpha Subcomplex, 1 NDUFA10 NADH Dehydrogenase (Ubiquinone) 1 Alpha Subcomplex, 10 NDUFB11 NADH Dehydrogenase (Ubiquinone) 1 Beta Subcomplex, 11 NEFA Non-Esterified Fatty Acid NF-ĸB Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells NO Nitric Oxide NOT1 Negative on TATA-less NOXA Phorbol-12-Myristate-13-Acetate-Induced Protein 1 NRF Nuclear Respiratory Factor Nuclear Factor (Erythroid-derived 2)-like 2 Nrf-2 Oligo-dT Poly-T Oligonucleotides OMM Outer Mitochondrial Membrane ONOO-Peroxynitrate PBMC Peripheral Blood Mononuclear Cell PBS Phosphate Buffered Saline PDK1 3-Phosphoinositide-Dependent Kinase

Mitochondrial Deoxyribonucleic Acid

mtDNA

PGC-1a	PPAR Co-Activator-1α
Pi	Inorganic Phosphate
PI3-K	Phosphoinositide 3-Kinase
РКА	Protein Kinase A
РКС	Protein Kinase C
РКСб	Protein Kinase Cδ
РМТ	Photomultiplier Tube
PPAR	Peroxisome Proliferator-Activated Receptor
PUMA	p53 Up-Regulated Modulator of Apoptosis
REE	Resting Energy Expenditure
rhGH	Recombinant Human Growth Hormone
rIGF-1	Recombinant Insulin-like Growth Factor-1
RNAi	RNA interference
RO [.]	Alkoxyl
RO ₂ ·	Peroxyl
ROS	Reactive Oxygen Species
RPE	Rate of Perceived Exertion
RT-PCR	Reverse Transcription – Polymerase Chain Reaction
SAMP	Senescence-Accelerated-Prone Mice
SGK1	Serum- and Glucocorticoid-Inducible Kinase-1
SIRT	Silent Information regulator 2 / Sirtuin 1
SOD-PEG	Superoxide Dismutase-Polyethylene Glycol
Sp1	Specificity Protein 1

SSC	Side Scatter Channel
STAT	Signal Transducers and Activators of Transcription
Taq	Thermus aquaticus
tBid	Truncated Bcl-2 Interacting Domain Death Agonist
TCA Cycle	Tricarboxylic Acid Cycle
Tfam	Mitochondrial Transcription Factor A
TMB	3,3',5,5'-Tetramethylbenzidine
TNFR1	Tumor Necrosis Factor Receptor 1
TOM22	22-kDa Translocase of Outer Mitochondrial Membrane
TRAIL	TNF-Related Apoptosis-Inducing Ligand
UC	Ultra Centrifugation
UCP	Uncoupling Protein
UTR	Untranslated Region
VDAC	Voltage-Dependant Anion Channel
WADA	World Anti-Doping Agency
XRN	5'-3' Exoribonuclease
$\Delta \psi_m$	Mitochondrial Membrane Potential

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LIST OF EQUATIONS

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This project was designed to investigate the mechanisms by which recombinant human growth hormone (rhGH) and recombinant insulin-like growth factor-1 (rIGF-1), over a range of physiological and supra-physiological concentrations, regulate key components of mitochondrial function in addition to determining the subsequent impact of this regulation on cellular viability. The project was divided into three studies.

Study One investigated whether rhGH and rIGF-1 exert a direct effect on the function of mitochondria over a range of physiological and supra-physiological concentrations in peripheral blood mononuclear cells (PBMCs) from healthy male subjects. PBMCs were incubated for 4 hours with either rhGH (Range = $0.25 - 100\mu g/L$) or IGF-1 (Range = $100 - 600\mu g/L$) and subsequently analysed for the determination of cellular viability, mitochondrial membrane potential ($\Delta \psi_m$), mitochondrial superoxide (O_2^-) generation and mitochondrial transition pore activity (mtPTP) activity. Levels of mitochondrial superoxide generation were found to be significantly reduced compared to control samples (lymphocytes – $21.5\pm1.6AU$ / monocytes – $230.2\pm9.8AU$) following rhGH treatment at concentrations of $5\mu g/L$ ($13.5\pm1.3AU$, $P\leq0.05$) and $10\mu g/L$ ($12.3\pm1.5AU$, $P\leq0.05$) in lymphocytes and at concentrations of $10\mu g/L$ ($153.4\pm11.4AU$, $P\leq0.05$) in monocytes, while no significant effect was found at higher concentrations or following treatment with IGF-1. Treatment with either hormone was not found to have any significant effect on $\Delta \psi_m$, mtPTP activity or percentages of cellular viability.

Study Two investigated the mitochondrial effects exerted by physiological and supraphysiological concentrations of rhGH (Range = $0.5 - 50\mu g/L$) and rIGF-1 (Range = 100 – 500 $\mu g/L$) under various respiratory conditions of substrate saturation in PBMCs from healthy male subjects. The level of mitochondrial highly reactive oxygen species (hROS) production and $\Delta \psi_m$ were determined in digitonin permeabilized cells, in the presence of the following respiratory substrates 1) pyruvate / malate 2) succinate / rotenone 3) pyruvate / malate / succinate and 4) octanoate / malate. Neither rhGH nor rIGF-1 exerted any significant effect on hROS levels in either lymphocyte (P=0.90) or monocyte (P=0.85) sub-populations at any concentration administered. Similarly, neither rhGH nor rIGF-1 exerted any significant effect on $\Delta \psi_m$ in either lymphocyte (P=0.97) or monocyte (P=0.78) sub-populations at any concentration administered. Study Three was designed to determine the effect of the administration of rhGH for one week on the regulation of mitochondrial mediated apoptosis in PBMCs from healthy male subjects who undertake regular physical activity. Blood sampling was undertaken before rhGH administration and over a period of three weeks (Days 8, 15, 22 & 29) following the final injection of rhGH. PBMCs were isolated for the determination of miRNA, mRNA and protein expression levels in both placebos and cases. Results showed that rhGH had no effects on the mRNA and protein expression of the anti-apoptotic Bcl-2. However, post rhGH administration showed a significant effect on the pro-apoptotic Bak which exhibited decreased protein concentrations compared to baseline and following correction with placebos. This effect was observed up to 8 days following the last rhGH treatment. Cytosolic miRNA expression of miR-125b, a known target of Bak, in mitochondrial fractions showed a significant down-regulation 8 days post rhGH administration.

The evidence presented in this thesis points towards the mitochondrial effects elicited by rhGH being beneficial at physiological concentrations. However, it appears that these benefits are negated following rhGH treatment within the supra-physiological range. In addition, rhGH was found to be incapable of countering mitochondrial dysfunction induced in the presence of saturating substrate conditions at any administered concentration. These findings could have important health implications for any individual intent on improving their athletic capabilities through the administration of rhGH at supra-physiological dosages.

1. Introduction

1.1 – Project Introduction

The aim of this project was two-fold, firstly to investigate how recombinant human growth hormone (rhGH), the genetically engineered form of GH, and insulin-like growth factor-1 (IGF-1), over a range of physiological and supra-physiological concentrations, regulate key components of mitochondrial function, and secondly to determine the consequences of this regulation for cellular viability. The physiological role of growth hormone (GH), a peptide growth factor produced in the anterior pituitary, involves the regulation of cell growth via activation of proliferation and differentiation pathways in multiple tissue types, including bone, connective, adipose and muscle tissue [1, 2]. Its biological actions consist of metabolic effects, including a stimulation of protein synthesis and a promotion of lipolysis [3]. IGF-1, a downstream mediator of many of GH's anabolic effects is released from the liver in response to GH signalling [4, 5].

Growth, protein synthesis and many components of fuel metabolism require the utilization of cellular energy that is obtained through the dephosphorylation of the high energy phosphate nucleotide adenosine triphosphate (ATP) [6]. ATP is primarily generated in mitochondria via oxidative phosphorylation [6-8]. Mitochondria are oval shaped intracellular organelles consisting of an outer membrane and an inner membrane which folds to form cristae in the mitochondrial matrix [8]. Enzymes of the Krebs cycle and oxidative phosphorylation are located in the mitochondrial matrix and within the inner mitochondrial membrane (IMM) respectively allowing for the generation of ATP from the substrates adenosine diphosphate (ADP) and inorganic phosphate (Pi) [6-8]. Oxidative phosphorylation utilizes the potential energy across the IMM, termed the mitochondrial membrane potential ($\Delta \psi_m$), which is generated via proton pumping powered by the transfer of electrons along the electron transport chain (ETC), to drive the phosphorylation of ADP [6-8]. A consequence of this process is the generation of reactive oxygen species (ROS), metabolites of molecular oxygen (O₂) formed as a result of the leakage of electrons from the ETC [9]. If not countered by the cell's antioxidant defences, ROS will react with key cellular components, causing damage to intracellular lipids, proteins and nucleic acids [9, 10]. This has negative implications for optimum cellular function in terms of increased oxidative damage to key mitochondrial components and mitochondrially mediated activation of apoptosis, a regulated form of cell death [9-11].

In higher organisms, cells must adapt to constantly changing metabolic environments where the demand for energy can fluctuate widely depending on the functional activity of the cell [6]. Any stimulation of cellular activity that changes either the anabolic or catabolic environment of the cell requires an adjustment in the rate of energy production in order to match physiological demand [6]. Thus, the rate and efficiency of oxidative phosphorylation has to be tightly regulated [6]. It has been proposed that this regulation is principally mediated by the same extracellular agonists that modulate cellular activity via nuclear directed signalling pathways, through the parallel activation of cell signalling cascades targeted towards mitochondria [6]. Research has demonstrated that while some hormones such as epidermal growth factor (EGF) act through the targeting of their receptor to the organelle via endocytosis, the effects of other hormones, such as insulin and IGF-1, are mediated via the downstream activation of protein kinases [6, 7, 12, 13].

Under optimum conditions mitochondria function in an efficient manner, maintaining a tight coupling between O₂ consumption and ATP synthesis, down-regulating the activity of the ETC in order to avoid the development of high $\Delta \psi_m$ values and keep the rate of ROS production at a low level [14]. This is proposed to be achieved through signal transduction mediated phosphorylation of ETC complexes, under which allosteric feedback inhibits key components of the ETC through elevations in the ATP/ADP ratio [14]. This allows $\Delta \psi_m$ values to be adjusted in line with the demand for ATP [14]. Under conditions of high ATP utilization, increased rates of ATP synthase activity will decrease $\Delta \psi_m$ to values insufficient for the rate of ATP production to be maintained [14]. Thus, the activity of the ETC must be raised in order to prevent a decrease of $\Delta \psi_m$ below values necessary to match the required rate of ATP production [14]. Signal transduction mediated de-phosphorylation of ETC complexes is suggested to both, inactivate allosteric ATP inhibition and, up-regulate the activity of individual ETC complexes [14]. However, excessive stimulation of ETC activity can result in hyperpolarization of $\Delta \psi_m$ to values that do not further accelerate ATP production but at which elevated levels of ROS are generated [14].

The project consisted of three studies which examined the impact rhGH and IGF-1 have on mitochondrial function in peripheral blood mononuclear cell's (PBMCs). PBMCs, which consist of lymphocyte and monocyte cell sub-populations, are easily isolated from whole blood, exhibit both GH and IGF-1 cell surface receptors, and have previously been demonstrated to incur changes in gene and protein expression in response to rhGH and IGF-1 (15-19). Study One investigated whether rhGH and IGF-1 over a range of physiological and supra-physiological concentrations exerted a direct effect on $\Delta \psi_m$ and levels of mitochondrial ROS production in PBMCs *in-vitro*. The implications of this regulation in terms of its impact on mitochondrial mediated apoptosis and levels of cellular viability were also addressed. Mitochondrial responses to rhGH and IGF-1 administration in PBMCs at physiological and supra-physiological concentrations *in-vitro* were further addressed in Study Two, again through the analysis of $\Delta \psi_m$ and levels of mitochondrial ROS production. The examination of these variables was conducted under several respiratory conditions, which to varying degrees, gave control over the sites of electron entry into the ETC, allowing for interpretation of the role played by individual complexes in mediating the effects of both rhGH and IGF-1. These two hormones are suspected to induce an up-regulation of oxidative phosphorylation in order to meet the energy demands associated with their anabolic effects. It is proposed that, at supra-physiological concentrations, this increment in the rate of ATP production is produced at the expense of the efficiency of electron transport along the respiratory chain leading to an augmented production of ROS and that this has negative implications for cellular viability [20].

While rhGH and IGF-1 exhibit well documented anti-apoptotic affects *in-vitro*, whether long-term effects persist *in-vivo* following the cessation of treatment is currently unknown. Furthermore, GH is associated with the development of pro-apoptotic conditions at supra-physiological concentrations *in-vivo* [21-23]. Study Three investigated the effect of rhGH administration on the regulation of mitochondrial mediated apoptosis in PBMCs *in-vivo* for up to three weeks post-treatment with the intent of determining how long purported anti-apoptotic benefits of elevated GH concentrations persist following the cessation of treatment. Potential implications for cellular viability were addressed through analysis of the GH mediated effects on the expression of apoptotic regulatory proteins. It is proposed that the initial anti-apoptotic effects induced by GH are attenuated following the cessation of treatment, leaving the cells susceptible to oxidative insult. Finally, potential mechanisms for the regulation of mitochondrial mediated apoptosis following rhGH administration *in-vivo* were investigated through the examination of the hormones effects on the expression of a class of regulatory RNA molecules, termed microRNA (miRNA), which target specific

apoptotic-regulatory genes [24]. This included the investigation of a novel mitochondrial associated miRNA signalling pathway. In light of the significant interest that peptide growth factors, such as GH and IGF-1, garner in athletic populations, the overriding aim of this research project was to elucidate any potential consequences at the molecular level from the administration of these hormones in healthy populations [25, 26].

1.2 – Project Rationale

Despite the lack of any scientific evidence from peer reviewed journals in regard to the performance enhancing effects of rhGH, its use is reported to be widespread among athletes as well as health and fitness orientated populations [25, 26]. Individuals thought to abuse rhGH consist of elite competitive athletes in addition to both competitive and recreational bodybuilders who often self-administer large doses without supervision and in combination with other anabolic substances [27]. Evidence from the United States indicates that the use of rhGH as well as other anabolic substances such or androgenic steroids is not confined to professional athletes [26]. A report from the National Collegiate Athletic Association (NCAA) in 2001 claimed that 3.5% of college level athletes used GH that year [28], while Rickert *et al.* [29] estimated that 5% of male American high school students are either using or have previously used GH in order to induce an anabolic effect.

The practice of administering rhGH for the purposes of enhancing athletic performance has been banned by most professional sports leagues and associations including the International Olympic Committee, as well as Major League Baseball and the National Football League in America [30]. The World Anti-Doping Agencies (WADA) classifies both GH and IGF-1 as prohibited substances (Class S2 – Peptide hormones, growth factors and related substances), both in and out of competition [31]. In addition the NCAA, the organization representing college athletes in America has also banned the use of rhGH by its members citing the dangerous nature of the drug's many side effects [32]. Noted adverse effects include swelling of the hands and feet, coarsened facial features, dentition abnormalities, arthralgia (joint pain) and fluid retention, in addition to an increased risk of the development of diabetes mellitus, hypertension, cardiomyopathy and osteoporosis [33]. Although it is believed that the use of rhGH is widespread in sports, evidence of its abuse is largely anecdotal [25]. However athletes from a wide range of sports, including baseball, cycling and athletics have been implicated in, or have confessed to, illicit rhGH use [30]. At the 1998 Tour de France, a large number of vials of rhGH were detected in the possession of cycling teams [25]. In 2006 the commissioner of Major League Baseball (MLB) recruited former US senator George Mitchell to conduct an independent investigation into the illegal use of steroids and other performance enhancing substances in MLB [34]. The subsequent investigation reported on former MLB club employees and personal trainers of Major League players who allegedly used performance enhancing drugs, with some of these players having subsequently admitted to using rhGH [34-36].

A limitation of the clinical trials conducted to date into the performance enhancing effects of rhGH is that the number of subjects who have participated in those trials has been small [37]. In addition, clinical trials are developed in order to detect relatively large changes in variables that effect athletic performances and would need many study participants to detect even a 1% change in performance [37]. Thus the enhancement required by professional athletes in order to make up the difference between winning and losing may not be adequately evaluated by such studies [37]. Doping regimes reported in rhGH abusing athletes can range from 3.3 to 8.3mg per day (10 – 25 IU/day), 3 to 4 days per week, which is far in excess of the 0.3 to 0.6mg per day (1-2 IU/day) usually prescribed to adult patients with growth hormone deficiency (GHD) [38]. Liu *et al.* [30] claim that reported rhGH doses used by athletes can be up to five times higher than those used in clinical studies. Such high doses could have negative health implications.

Acromegaly, a disorder which is commonly associated with a GH producing tumour known as a pituitary adenoma, is related to excessive production of GH and is characterized by marked abnormalities in protein and carbohydrate metabolism [21, 22]. The available data indicates that acromegaly consists of an initial anabolic phase leading to an increase in net protein synthesis and lean body mass and a later phase where protein mass is sustained but is subject to a negative effects of protein remodelling [21]. Long standing acromegaly is characterized by impairment of strength, aerobic exercise capacity and cardiac performance [21]. The development of cardiomyopathy and heart failure, conditions associated with a deregulated apoptosis, are also prevalent in acromegalic patients [22]. Acromegaly is associated with increased mortality with data indicating that about 60% of patients die from cardiovascular disease, 25% from

respiratory complications and 15% from cancer [22]. If left untreated, patients with acromegaly would die about 10 years earlier than healthy subjects [22]. In addition, life-span studies on rodents have shown that growth hormone receptor (GHR) knockout mice and Ames dwarf mice, both exhibiting GHD, live longer than their wild-type siblings [39-41]. In contrast, transgenic mice that over express GH live only half has long as normal wild type siblings [40].

Thus, interpretation of how exogenous GH administration affects the rate of cellular energy production will have implications in terms of the relevance of using rhGH as a performance enhancing aid. In addition, determining whether or not GH negatively impacts on mitochondrial function, by enhancing rates of ROS production and augmenting oxidatively induced cellular damage will enhance knowledge in regard to the risks involved in administering rhGH to individuals who exhibit normal rates of GH secretion. This research will also provide insight into the implications of long term rhGH abuse by athletes or anyone looking to benefit from the purported performance enhancing and anti-aging effects of the hormone.

2. Review of the Literature

2.1 – The Roles and Regulation of Mitochondrial Function

Mitochondria play a key role in the production of cellular energy, the generation of ROS and the regulation of apoptosis [6, 42]. Several studies have demonstrated that skeletal muscle mitochondria which tightly couple ATP production to oxygen consumption and which maintains a low rate of ROS generation are necessary for optimum physiological function and a prolonged life expectancy [9, 43, 44]. In contrast, a lack of cellular energy, excessive ROS production and deregulated apoptosis are exhibited either as isolated or combined features in many human diseases including neurodegenerative diseases, stroke, cardiovascular disorders, ischemia/reperfusion injury and cancer [6].

2.1.1 – Regulation of Oxidative Phosphorylation

Oxidative phosphorylation provides up to 90% of cellular ATP with the remainder derived from substrate level phosphorylation [45]. Oxidative phosphorylation is the formation of ATP from the substrates ADP and Pi in association with the transfer of electrons from fuel molecules along a group of trans-membrane protein complexes known as the ETC which is located in the IMM [45]. Carbohydrate and lipid substrates are metabolized in order to provide reducing equivalents in the form of the reduced coenzymes, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂) to the ETC. Through a series of redox reactions, electrons are transported along the enzyme complexes of the ETC, stimulating the activity of the three complexes which function as proton pumps (NADH dehydrogenase - Complex I, Cytochrome c reductase - Complex III and Cytocrome c oxidase - Complex IV) [6, 20]. The activity of these enzymes generates a proton motive force across the IMM which is composed of both an electrical ($\Delta \psi_m$) and chemical (ΔPH) component [14]. Cytochrome c oxidase (COX) accepts electrons from the reduced co-enzyme cytochrome c (cyt c) and transfers them to molecular oxygen which is subsequently reduced to water (H_20) in a reaction that involves four electrons, four hydrogen ions and a single O₂ molecule [6]. The transmembrane potential that is generated by the active pumping of H^+ out of the mitochondrial matrix facilitates the generation of ATP by Complex V of the ETC, ATP synthase [6]. ATP synthase is composed of an F_1 matrix bound subunit that contains the catalytic ATP / ADP / Pi binding sites and a trans-membrane F₀ subunit that couples the

backflow of protons into the mitochondrial matrix with the catalytic activity of the F_1 subunit through a peripheral and rotating mechanical mechanism [6].



Figure 2.1: Electron Transport Chain, image adapted from Nelson et al. [46].

Whether an increase in the rate of oxygen consumption results in a concomitant increase in the yield of ATP is dependent on the efficiency of oxidative phosphorylation [47]. The efficiency of ATP synthesis is highly regulated and several mechanisms may affect it, including the nature of the substrate provided to the respiratory chain, proton leak across the IMM and the "slipping" of the coupling between redox reactions and proton pumping [48]. The free energy of electron transport within the proton pumps is sufficient to generate a proton motive force (ΔP) of approximately 240 millivolts (mV) [14]. However, the proton permeability of biological membranes increases exponentially above 130mV, leading to a waste of energy at high values [14]. Uncoupling, through proton leak, results in a dissociation of the rate of oxidation from that of phosphorylation and thus to a decrease in the yield of oxidative phosphorylation, with the additional energy being dissipated as heat [48]. Electron transport and oxygen consumption are accelerated when control exerted by ADP is lost and ATP utilization exceeds ATP synthesis [7]. In addition, at $\Delta \psi_m$ above 140mV, production of the ROS superoxide anion (O₂⁻) at complexes I and III of the respiratory chain has been reported to increase exponentially with increasing $\Delta \psi_m$ [14].

2.1.2 – Generation of Reactive Oxygen Species

The accumulation of oxidative damage caused by ROS contributes to impaired physiological function, increased incidence of disease and a reduction in maximum lifespan potential [10]. In healthy tissue, mitochondria are the principal organelles responsible for the generation of ROS [9]. ROS are metabolites of molecular O₂ that have a higher reactivity than O₂ [10]. While the process of oxidative phosphorylation ultimately involves a four-electron reduction of O₂ to H₂O, during this process one or two electron reductions of O₂ can occur, leading to the formation of O₂⁻, from which other ROS can be generated [10]. These include hydroperoxyl (HO₂⁻), hydroxyl ('OH), peroxyl (RO₂) and alkoxyl (RO) radicals, molecules containing unpaired electrons, as well as non-radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen [49]. In addition peroxynitrate (ONOO-) is formed from the reaction of O_2^- with nitric oxide (NO) [49]. H₂O₂ may be converted into the highly reactive 'OH, resulting from a reaction with transition metals, namely with ferrous iron (Fe²⁺) [49]. OH is an extremely reactive oxidizing radical that will react with most biomolecules at diffusion controlled rates [49]. ROS can react directly with DNA at the sugar phosphate backbone or at the bases, producing many different oxidatively modified purines and pyrimidines [9]. Mitochondrial DNA (mtDNA) is especially prone to oxidative damage due to its proximity to this primary source of ROS generation and its deficient repair capacity compared with nuclear DNA [10]. The direct consequence of oxidative stress is damage to various intracellular constituents. Changes in cellular membrane permeability and even membrane leakage can be manifested when lipid peroxidation occurs [10]. Protein oxidation has many important physiological consequences that affect normal cellular functions, while oxidative damage to both nuclear and mtDNA has detrimental effects, ranging from uncontrolled cell proliferation to accelerated cell death [10].

The source of ROS generation along the ETC has classically been attributed to the coenzyme of complex III, ubiquinone or Coenyme_Q (CoQ) which produces the free radical semiquinone through the acquisition of a single electron; however recent research also implicates complex I as a key site of electron "slippage" [9]. Flavin mononucleotide, semiquinones or iron-sulphur (Fe-S) clusters have been proposed as sites of ROS generation in complex I [9]. Both flavin and Fe-S clusters are situated in the hydrophilic domain of complex I facing the mitochondrial matrix compartment where mtDNA is located [9]. In contrast, ROS generated at complex III seem to be mainly directed to the cytosolic side of the IMM [9]. The prolonged exposure of these sites to electrons occurs when the ETC is inhibited, leading to an augmentation in the production of O_2^{-} [42]. Jaburek *et al.* [49] reported that significant elevations in the generation of ROS occur when mitochondria approach a non-phosphorylating state, such as when the efficiency of the coupling between ATP production and oxygen consumption is decreased. Under optimum physiological conditions, the oxidized form of the co-enzyme CoQ⁻, arising when complex III non-haem iron is reduced, is a shortterm intermediate that is rapidly reduced to CoQ as its electron is passed on to the cytochrome b₁ subunit of complex IV [50]. In the absence of respiratory control, exerted by the supply of ADP, cytochrome b₁ is highly reduced since its oxidation is prevented by high trans-membrane electrochemical proton potential, which leads to CoQ⁻ becoming a long lasting component [50].

2.1.3 – Mitochondrial Regulation of Cell Death Pathways

Programmed cell death, termed apoptosis, functions as a homeostatic mechanism responsible for the ordered removal of superfluous, aged or damaged cells [51]. Apoptotic cell death is characterized by cell shrinkage, chromatin condensation, nuclear defragmentation, plasma membrane blebbing and the separation of cell fragments into apoptotic bodies [51]. There is essentially no inflammatory reaction associated with the process of apoptosis as apoptotic cells do not release their cellular constituents into the surrounding interstitial fluid and they are quickly phagocytised by surrounding macrophages [51]. The mechanisms that initiate apoptosis are highly complex, involving an energy-dependent cascade of signalling events which can be mediated via both intrinsic and extrinsic pathways [51]. Intrinsic signalling pathways involve a diverse range of non-receptor mediated stimuli that produce signals in response to intracellular damage caused by radiation, toxins, hypoxia, viral infections and oxidative stress [51]. The extrinsic death receptor pathway is characterised by ligand activation of trans-membrane receptors, such as the Fas receptor, tumour necrosis factor receptor 1(TNFR1), TNF-related apoptosis-inducing ligand (TRAIL) receptor and death

receptors (DR) 3-6, which mediate caspase activation in response to extracellular stimuli [51].

Mitochondria have been demonstrated to participate in intrinsic apoptotic signalling and to play an important role in extrinsic-receptor mediated apoptosis and non-apoptotic forms of cell death [52, 53]. In recent years, research investigating the role that mitochondria play in cell death pathways has focused attention on the regulation of mitochondrial membrane permeability as the potential mechanism linking key cellular signalling pathways to the execution of cell death [54]. Permeability transition is regulated by a unique supra-molecular complex known as the mitochondrial permeability transition pore (mtPTP), whose 3nm diameter increases the permeability of the IMM to solutes with a molecular mass of up to 15kDa [54]. While formation of the mtPTP is thought to arise from the apposition of multiple mitochondrial protein components at IMM and outer mitochondrial membrane (OMM) contact sites, at present the precise molecular composition of the pore is unresolved [54, 55]. However, a restricted set of proteins have been proposed to play a role in the composition of the mtPTP [54]. These include the OMM protein, voltage-dependent anion channel (VDAC), the IMM protein, adenine nucleotide translocator (ANT), the mitochondrial matrix protein, cyclophilin-D (Cyp-D) and the inter-membrane proteins, mitochondrial hexokinase (mtHK) and mitochondrial creatine kinase (mtCK) [54, 55]. Opening of the mtPTP has dramatic consequences for mitochondrial physiology, including the collapse of the mitochondrial trans-membrane potential, uncoupling of the respiratory chain, hyper-production of O_2^{-} , disruption of mitochondrial biogenesis, the efflux of small molecules such as calcium and glutathione from the mitochondrial matrix and the release of soluble inter-membrane proteins into the cytosol [11, 55]. Among the peptides released from mitochondria upon mtPTP opening are proteases and protease activators with key apoptogenic functions such as cyt c and apoptosis inducing factor (AIF) [11, 54]. The subsequent activation of down-stream caspase enzymes leads to a cytosolic cascade of endonuclease activity targeted towards to nucleus [11].

The mtPTP complex has been found to be subject to modulation by numerous endogenous physiological effectors including ions (Ca²⁺), the proton gradient ($\Delta \psi_m$), the matrix concentration of adenine nucleotides (ATP/ADP), the redox state of electron carriers (NAD⁺/NADH), the redox state of matrix thiols (which is in equilibrium with the redox status of gluthathione – an indicator of the overall oxidative state of the cell),

ROS, NO and the concentration of lipoids (such as ceramide) [11]. From the nature of the factors that have been demonstrated as capable of inducing mtPTP opening, it is apparent that any major change in energy balance (e.g.: absence of oxygen, decrease in ATP/ADP, depletion of NADH and dissipation of the trans-membrane potential) or changes in the redox balance (e.g.: oxidation, leading to depletion of reduced glutathione concentrations or hyper-production of ROS) can provoke opening of the mtPTP [11]. This implies that the mtPTP complex integrates stress responses and that major damage of cells will invariably cause permeability transition [11].

The regulation of mitochondrial apoptotic events is mediated through members of the B-cell CLL/Lymphoma protein 2 (Bcl-2) family of proteins which exhibit control over mitochondrial membrane permeability [51, 56]. To date, a total of 25 genes have been identified in the Bcl-2 family, which are divided into three groups based on the presence of up to four Bcl-2 homology domains (BH1-4 domains) [51, 56]. The anti-apoptotic Bcl-2 proteins, such as Bcl-2, Bcl-2 related gene - long isoform (Bcl-xL), and myeloid cell leukemia-1 (Mcl-1) contain all four BH domains and function to preserve mitochondrial integrity, preventing cyt c efflux in response to apoptotic stimuli [51, 56]. The pro-apoptotic Bcl-2 proteins are functionally divided into two groups [56]. The multi-domain (BH1-3) Bcl-2 associated x protein (Bax) and Bcl-2 antagonist killer 1 (Bak) are pro-apoptotic effector molecules responsible for inducing mitochondrial permeabilization via the formation of proteolipid pores on the OMM [56]. The third class of Bcl-2 proteins, BH3-only molecules such as Bcl-2 antagonist of cell death (Bad), Bcl-2 interacting domain death agonist (Bid), Bcl-2 interacting mediator of cell death (Bim) and p53 up-regulated modulator of apoptosis (PUMA), function as activator molecules in distinct cellular stress pathways via interactions with other Bcl-2 family members [56].

Upon activation, Bax and Bak form higher order oligomers in the OMM which are implicated in the induction of mitochondrial permeabilization [57-59]. Bax exists as a monomer in the cytosol with its C-terminal α -9 helix occupying the dimerization pocket to prevent mitochondrial targeting and homo-oligomerization [59]. In contrast, Bak is perennially located in the OMM, where its activity is restricted by the VDAC isoform, VDAC2, which occupies its dimerization binding site [59]. The pro-apoptotic activity of Bax and Bak is triggered by the BH3 activator proteins whose activity in turn falls under the regulation of upstream death signalling cascades [57-59]. For example,
following activation of Fas or TNFR1 receptors, Bid (21kDa) is cleaved by caspase 8 to its active truncated form tBid (15kDa) which subsequently interacts with the proapoptotic effector molecules [57]. Furthermore, the phosphatidylinositol 3-kinaseprotein kinase B (PI3K-Akt) signalling pathway, which plays a critical role in mediating cell survival signals, has been implicated in the regulation of BH3 activators [60]. Akt has been shown to phosphorylate serine residues on Bad leading to its sequestration and inactivation by 14-3-3, a phosphoserine binding molecule [60]. Withdrawal of survival signals have been demonstrated to result in the translocation of monomeric Bax from the cytosol to the mitochondria via activation by de-phosphorylated Bad [60]. The BH3 activators, tBid, Bim and PUMA have been demonstrated to bind to the α -1 helix on the N-terminal of Bax, inducing conformational changes which expose the dimerization binding site and allow the C-terminal trans-membrane domain to become available for insertion into the OMM [59]. Bak also requires BH3 mediated activation to trigger its homo-oligomerization by inducing conformational changes that disrupt Bak-VDAC2 interactions [59]. Conversely, anti-apoptotic Bcl-2 proteins sequester BH3 activators into inert complexes, preventing Bax/Bak activation [59]. Thus, the ratio of expressed anti-apoptotic to pro-apoptotic Bcl-2 proteins is acknowledged to dictate the immediate fate of a cell in response to apoptotic stimuli [57].

2.1.4 – The Regulation of Mitochondrial Respiration through Cell Signalling Pathways

The most basic level of regulation of oxidative phosphorylation, termed respiratory control, is dependent upon substrate availability and $\Delta \psi_m$ [61, 62]. Oxidative phosphorylation is limited by the rate at which substrates can supply reducing equivalents to complexes I and II of the ETC and the availability of ADP and phosphate to allow for ATP synthase activity, while increases in $\Delta \psi_m$ inhibits proton pumping across the respiratory complexes at high values, slowing the rate of ATP synthesis under resting conditions [61, 62]. While respiratory control serves as the principal form of regulation in bacteria and lower eukaryotic organisms, this level of control is insufficient in higher organisms where the rate of ATP utilization ranges widely (up to 10 fold) depending on functional activity [14]. Thus, a hierarchy of additional regulatory mechanisms have developed, allowing for the adjustment of energy production to physiological demand [6, 14, 61, 62]. Further levels of control include allosteric regulation and post-translational modifications of ETC proteins [6, 61, 62]. Allosteric regulation represents an intracellular process allowing for the fine tuning of

energy production within the cell [6]. In contrast, regulation of the level of phosphorylation of respiratory complexes is mediated via hormonal and electrical signals that activate signal transduction cascades targeting mitochondria [6]. This represents a higher order of regulation, allowing for communication to take place between cells and organs [6].

Allosteric regulation allows for fine tuning of the kinetic properties of metabolic enzymes through the binding of respiratory substrates, products and intermediates [61]. In particular, cyt c and COX represent key targets for this mode of regulation with the activity of both being adjusted via the binding of adenine nucleotides [14, 61]. At physiological concentrations ATP binds to cyt c leading to an inhibition of electron transfer between cyt c and COX [14, 61]. In addition, an ATP/ADP binding site is located on the matrix side of COX subunit IV leading to allosteric inhibition of COX in the presence of ATP and allosteric stimulation at high concentrations of ADP [14].

Optimal rates of ATP synthesis are dependent upon $\Delta \psi_m$ values above 100mV, with maximal rates of ATP synthase activity observed at approximately 120mV [14]. At higher $\Delta \psi_m$ values the efficiency of oxidative phosphorylation decreases while the rate of ROS production is enhanced owing to an increase in basal proton leak across the IMM [14]. Allosteric inhibition of cyt c and COX in the presence of high intracellular ATP/ADP ratios serves to prevent an increase in $\Delta \psi_m$ to values above 120mV, maintaining tightly coupled rates of oxidative phosphorylation and keeping ROS production low under resting conditions [14]. It should be noted however that cell signalling mediated phosphorylation of ETC components is considered to be the overarching regulatory mechanism preventing the development of high $\Delta \psi_m$ values [6]. Indeed, the ability of ATP to allosterically inhibit COX has only been demonstrated in phosphorylated forms of the enzyme [14]. While $\Delta \psi_m$ values are kept low under resting conditions, under conditions of increased ATP utilization augmented rates of ATP synthase activity will attenuate resting $\Delta \psi_m$ values [14]. This is due to an increased flow of protons back into the mitochondrial matrix through its F₀ subunit, resulting in submaximal values (< 120mV) for further ATP synthesis [14]. The mechanism of $\Delta \psi_m$ hyperpolarization serves to counteract this process [14]. However, in order for hyperpolarization of $\Delta \psi_m$ values to occur, cell signalling mediated de-phosphorylation of ETC components must first negate allosteric ATP mediated inhibition, further demonstrating the higher order of control elicited on mitochondrial respiration by extracellular effector molecules such as hormones [14].

While phosphorylation sites have been identified on all ETC complexes, in most cases the signalling pathways involved and the functional consequences of specific phosphorylation events have not been elucidated [6, 14, 61, 62]. To date, four serine (Ser) residues have been identified as phosphorylation sites on complex I, all from bovine heart mitochondria, including Ser20 of the NDUFB11-18kDa subunit, Ser55 of the 10kDa-NDUFA1 subunit, Ser59 of the NDUFA10 (42kDa) subunit and Ser95 of the B14.5a subunit [6]. Furthermore, seven additional complex I subunits (75kDa Fe-S protein 1, flavoprotein 1, 39kDa 1α subcomplex, 24kDa subunit, 23kDa subunit, 22kDa 1β subcomplex and 15kDa subunit) have been identified as phosphorylated proteins through mass spectrometry on isolated porcine mitochondria, although no phosphate groups were assigned to specific amino acids [63]. Tissue specific differences in the efficiency of respiratory rates have been attributed to the activity of complex I, with Cocco et al. [64] observing variable P/O ratios between rat brain, heart and liver mitochondria with complex I substrates while no differences were observed with complex II substrates. Thus, while no study to date has examined the functional consequences of the phosphorylation of any of the identified complex I sites, it is likely their regulation plays a role in mediating changes in the efficiency of respiratory control [64]. Identification of phosphorylated proteins via mass spectrometry on porcine mitochondria has also indicated the flavoprotein precursor subunit and the iron sulphur cluster containing B subunit of complex II in addition to the core protein I precursor, core protein II, Riesk iron sulphur protein precursor and the ubiquinone binding protein of complex III as targets for phosphorylation [6]. However, the specific amino acids phosphorylated, together with the relevant signalling pathways and mediated effects remain elusive [6].

Cyt c and COX represent the most extensively characterized of the ETC components to date, with four and 14 phospho-epitopes having been identified, respectively [61]. Tyrosine (Tyr) residues 48 and 97 are confirmed phosphorylation sites on cyt c, from bovine liver and heart mitochondria respectively, whose phosphorylation has been demonstrated to inhibit the activity of the electron carrier [65, 66]. Maximal rates of turnover for Tyr48-phosphorlated cyt c was found to be more than 50% reduced compared to unphosphorylated cyt c [65]. Half maximal turnover was demonstrated at a

substrate concentration of 5.5µM for Tyr97-phosphorylated cyt c compared to 2.5µM for unphosphorylated cyt c, indicating that phosphorylation inhibited the reaction with COX by shifting the K_m of COX for cyt c [66]. In addition, threonine (Thr) 28 and serine (Ser) 47 have also been identified as phosphorylation sites on cyt c from human skeletal muscle mitochondria, however their specific functions remain unknown [61]. Several phosphorylation sites have been identified on COX subunits which likely play a pre-eminent role in regulating its enzymatic activity [61]. One site of phosphorylation, identified from bovine liver tissue as Tyr residue 304 on COX subunit I, is located adjacent to the oxygen binding site on the complex [67]. Tyr304-phosphorylated COX was found to be strongly inhibited with cyt c substrate concentrations of up to 10µM, even in the presence of the allosteric activator ADP [67]. While it was demonstrated that high cyclic adenosine monophosphate (cAMP) levels lead to Tyr304phosphorylation of COX subunit I, it is evident that this phosphorylation cannot be directly mediated through the cAMP dependent protein kinase A (PKA) which is a Ser/Thr-specific protein kinase [67]. It is suggested that PKA activates an as yet unidentified down-stream tyrosine kinase which in turn is responsible for the phosphorylation of COX subunit I [67]. COX inhibition through phosphorylation has also been demonstrated following the induction of ischemic stress in leporine heart tissue with identified phosphorylation sites including Ser115 and Ser116 of subunit I, Thr52 of subunit IV and Ser40 of subunit Vb [68]. Although phosphorylation of these sites was originally attributed to PKA owing to the abolishment of phosphorylation in the presence of alleged PKA inhibitor H89, the identified sites do not exhibit consensus phosphorylation site motifs for PKA, indicating that it is unlikely this kinase is directly involved in mediating their phosphorylation [61]. Thus, the specific kinases involved in the phosphorylation's observed following ischemia remain undetermined [61]. Additional phosphorylation sites mapped on COX include Tyr11 of subunit IV-1 in bovine liver COX, Ser126 of subunit II, Ser34 of subunit IV-1, Ser4 and Thr35 subunit Va and Thr11 of subunit VIa in bovine heart COX, and Ser67 and Ser136 of subunit IV-1 and Thr38 of subunit Va in human HeLa cells, although the signalling pathways involved in their regulation and their functional consequences remain unknown [61]. In regard to ATP synthase, identified phosphorylated sites include Ser76 of the α subunit, Thr213 of the β subunit and Tyr75 of the δ subunit, however once again their mode of regulation by cell signalling and the impact of their phosphorylation remains to be determined [6].

It is apparent that cell signalling regulates mitochondrial metabolism through direct phosphorylation and de-phosphorylation of mitochondrial proteins, however little is known about the mitochondrial targeting of signal transduction pathways or the specific mitochondrial kinases and phosphatases which mediate these effects [6, 61, 62]. While an increasing number of effector molecules including PKA, Akt, and protein kinase $C\delta$ (PKC\delta), all of which are Ser/Thr specific kinases, and the non-receptor Tyr kinase c-Src have been demonstrated to be translocated into mitochondria upon their activation, the mechanism behind their subcellular relocation remains unelucidated [6]. Translocation of PKA to the mitochondria is associated with an inhibition of the activity of COX while the translocation of PKC δ is followed by the loss of $\Delta \psi_m$ and the release of cyt c [6]. Cellular stimulation with IGF-1 resulted in the translocation of phosphorylated Akt into the mitochondria where it was found to phosphorylate the β subunit of ATP synthase, although the functional consequences of this effect were not determined [12]. Meanwhile, the localization of c-Src to the mitochondrial inter-membrane space has been shown to result in the phosphorylation of COX at subunit II in osteoblasts, leading to an increase in COX activity [69]. The mitochondrial localization of kinases which target ETC components necessitates the presence of mitochondrial phosphates to counter their activity [61]. The protein tyrosine phosphatase Shp-2 was the first identified tyrosine phosphatase that localizes to the mitochondrial inter-membrane space and the OMM [70]. Although mitochondrial targets of Shp-2 are currently unknown, it is hypothesized to target cyt c and COX, the only two components of the ETC with mapped tyrosine phosphorylation sites [6]. Calcium (Ca^{2+}) signalling represents another mechanism by which extracellular signals have been demonstrated to target mitochondria [6, 61]. Indeed, Ca^{2+} has been demonstrated to play an important role in signal transduction between cytosol and mitochondria, with spikes in intramitochondrial Ca²⁺ concentrations leading to an up-regulation of mitochondrial oxidative metabolism [6, 61, 62]. Mammalian COX is known to contain a calciumsodium exchange site in subunit I which is speculated to affect COX activity, although the function of reversible Ca^{2+} binding at this site is unknown [6, 61, 62]. In addition, Ca²⁺ has been found to cause de-phosphorylation of most mitochondrial proteins, an effect which is widely attributed to the activation of as yet unidentified calciumdependent mitochondrial phosphatases [6, 61, 62]. In addition to affecting the activity of individual ETC complexes it has been suggested that cell signalling mediated phosphorylation plays a role in organizing the ETC proteins into hetero-oligomeric supercomplexes termed respirasomes [6, 62]. The arrangement of ETC components into higher order complexes makes possible a more efficient electron transfer due to substrate channelling which is especially beneficial under conditions that require increased rates of ATP production [6, 62]. Thus, elucidation of the signalling pathways responsible for regulating respirasome stoichiometry represents an important direction for future research [6, 62].

2.1.5 – Regulation of Mitochondrial Gene Expression

While the majority of mitochondrial proteins are encoded by the nucleus, key elements of the ETC are also encoded by the mitochondrial genome [71, 72]. Co-ordinated regulation of the expression of both nuclear and mitochondrial encoded genes is required to give rise to a fully operational ETC [71, 72]. The expression of mitochondrial proteins is regulated at both the transcriptional and post-transcriptional level.

2.1.5.1 – Transcriptional Regulation

While transient changes in mitochondrial activity are regulated through post-translation modifications of mitochondrial proteins, in the long term cell signalling affects mitochondria through the regulation of changes in the gene expression of proteins that localize to the organelle [6]. It is well established that Ca^{2+} mediated signalling plays an active role in this regulation [73]. Increases in the concentration of cytosolic Ca^{2+} leads to the activation of a series of kinases, including Ca^{2+} dependent protein kinase C (PKC) isoforms [74], c-Raf [75], the extra-cellular signal-regulated kinase (ERK)-1 and ERK-2 [76], mitogen-activated protein kinase (MAPK), mitogen-activated protein kinase kinase (MEK) [77] and the p90 ribosomal S6 kinase [78], which translocate their signals to the nucleus to alter the rate of gene transcription [7]. Freyssenet et al. [73] found that increases in intracellular Ca^{2+} concentrations trigger the activation of a PKC/MEK/MAPK dependent pathway which leads to an up-regulation of the expression of cyt c. Additionally, modifications in cytosolic Ca^{2+} concentrations have been reported to enhance the expression of a number of genes encoding mitochondrial proteins, including malate dehydrogenase and the β subunit of ATP synthase [7]. However, not all mitochondrial proteins are transcribed via Ca^{2+} signalling [7]. Indeed it has been reported that increases in cytosolic Ca^{2+} concentrations fail to induce changes in messenger RNA (mRNA) levels encoding COX subunits (IV, Vb and VIc),

indicating that a broader range of signalling molecules likely play a role in mediating changes in the synthesis of mitochondrial components [7].

Nuclear genes encoding mitochondrial proteins possess upstream responsive elements to which proteins termed "transcription factors" bind in order to induce transcriptional activation [7]. Transcription factors implicated in up-regulating the expression of nuclear encoded mitochondrial proteins include nuclear respiratory factor (NRF)-1 and NRF-2 [71], peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ [72], c-Jun, c-Fos and Sp1 [79]. Nuclear binding of NRF-1 and NRF-2 is critical to the transcriptional activation of multiple genes that encode components of the respiratory chain [71]. In addition to nuclear encoded mitochondrial proteins, mitochondria contain their own genome from which genes are transcribed, including 13 mRNA molecules that all encode protein components of the respiratory chain [7]. Mitochondrial DNA (mtDNA) also encodes 22 tRNA and 2 rRNA molecules that are essential for mitochondrial function [7]. The transcription of genes from mtDNA requires the import of nuclear encoded transcription factors into the organelle [7]. NRF-1 is known to bind to and activate the promoter of the nuclear encoded mitochondrial transcription factor A (Tfam) which localizes to the mitochondria to regulate the expression of mtDNA [7]. Tfam expression correlates strongly with alterations in mitochondrial transcriptional activation and oxidative capacity and its loss has been demonstrated to result in either partial or total depletion of mtDNA [80, 81].

A coordinated increase in the transcription of nuclear and mitochondrial encoded genes requires the activation of higher order regulatory proteins capable of simultaneously adjusting transcriptional activation at multiple sites [7]. Induction of the regulatory factor PPAR co-activator 1α (PGC- 1α) is known to play a critical role in coordination of the activation of genes involved in oxidative metabolism [82]. PGC- 1α does not bind DNA directly, instead mediating its effects through interactions with transcription factors already bound to the promoter region of select genes [82]. The transcriptional co-activator enhances the overall efficiency of transcriptional activation by binding to specific transcription factors and recruiting additional co-activators, RNA polymerase II and various splicing factors [82]. Up-regulation of PGC- 1α has been demonstrated to cause dramatic increases in the expression of NRF-1, NRF-2 and Tfam in addition to nuclear and mitochondrial encoded COX subunits and cyt c [82].

The expression of Bcl-2 family proteins falls under the regulation of numerous transcription factors, demonstrating the complex mechanisms that underlie the control of mitochondrial mediated apoptotic pathways [83-88]. The nuclear factor kappa-lightchain-enhancer of activated B cells (NF-kB) protein complex is a transcription factor which rapidly translocates to the nucleus upon activation via growth factor, cytokine, viral or stress mediated pathways [83]. Nuclear localization of NF-KB is associated with up-regulated expression of anti-apoptotic Bcl-2 proteins [83]. In particular, NF-KB contains specific motifs for both Bcl-2 and Bcl-xL transcription [83, 84]. Intriguingly, NF- κ B has also been shown to play with positively associated with expression of the pro-apoptotic Bax protein, further demonstrating the complex nature of the interplay involved in the regulation of apoptotic pathways [83]. Bcl-2 expression is also found to be under the control of activating transcription factor-2 (ATF-2) and the cAMPresponse -element-binding protein (CREB), transcriptional regulators whose effects are mediated through the PI3K/Akt and ERK signalling pathways [85, 86]. Furthermore, nuclear factor (erythroid-derived 2)-like 2 (Nrf2) has recently been demonstrated to upregulate Bcl-2 levels in response to high levels of oxidative stress [87]. The regulation of Bcl-xL expression appears to diverge from that of Bcl-2 with the activation of Etwenty six (Ets), signal transducers and activators of transcription (STATs) and the activator protein-1 (AP-1) transcription factors, following stimulation with a variety of extracellular signalling molecules, positively correlating with the proteins up-regulation [84]. The p53 tumour suppressor protein, upon exposure to stress mediated stimuli such as DNA damage and oxidative stress, is phosphorylated via MAPK dependent pathways, resulting in its activation as a transcription factor [89]. Activation of p53 triggers a number of signalling pathways that lead to cell cycle arrest, cellular senescence, DNA repair and apoptosis [89]. The Bcl-2 pro-apoptotic effector proteins, Bax and Bak, are both known transcriptional targets whose expression is induced upon activation of p53 [88]. In contrast, the expression of Bcl-2 is found to be negatively influenced [88]. Furthermore, expression of the BH3-only activator proteins, PUMA and phorbol-12-myristate-13-acetate-induced protein 1 (Noxa) have been found to be induced following p53 activation in response to DNA damage [88].

2.1.5.2 – Post-Transcriptional Regulation

Post-transcriptional regulation of gene expression falls under the control of a class of short non-coding RNAs, 18 to 24 nucleotides in length, termed miRNAs [90-92]. Mature single-stranded miRNA molecules regulate protein synthesis by base-pairing with imperfect complementarity to sequences in the 3'-untranslated region (3'-UTR) of target mRNA [90-92]. Indeed, the interactions between miRNA and mRNA are reported to be predominately restricted to the 5'-proximal "seed" region (positions 2-8) of the miRNA [90-92]. While it is established that only 1% of genome transcripts encode miRNA in mammalian cells, nearly 30% of all encoded genes are regulated by miRNA with each mRNA being targeted by multiple sources [93, 94]. The inhibition of protein synthesis by miRNA is mediated either by repressing translation or by inducing deadenylation and subsequent degradation of mRNA targets [90, 92]. miRNAs function in the form of ribonucleoprotein complexes termed miRISCs (miRNA-induced silencing complexes), the core components of which are argonaute (AGO) proteins and the 182-kDa glycine-tryptophan (GW182) protein [90]. It has been experimentally demonstrated that GW182 represses protein synthesis when artificially connected to the 3'-UTR of mRNA indicating that it functions as an effector molecule of translational repression while miRNAs mainly serve as the identifiers of mRNA targets [95, 96]. Meanwhile AGO proteins are reported to act as scaffolds to anchor miRNA and recruit the silencing effect GW182 to the target mRNA [90]. While the exact molecular mechanisms behind miRISC mediated translational repression have yet to be fully elucidated, interference of mRNA 5'-cap recognition by the eukaryotic translation initiation factor 4E (eIF-4E) and prevention of 80S ribosomal complex assembly are known to play a role [90]. GW182 also plays a crucial role in miRNA-mediated deadenylation and decapping associated with mRNA degradation, recruiting the CAF1 (CCR4-associated factor 1) – CCR4 (carbon catabolite repression 4) – NOT1 (negative on TATA-less) deadenylase complex and the DCP1 (mRNA decapping enzyme 1) -DCP2 decapping complex to the target mRNA [90, 92]. The body of the mRNA is subsequently degraded by XRN1, a 5'-3' exonuclease [90, 92].

While little is known regarding the control of mitochondrial gene expression by miRNA owing to the small percentage of validated targets elucidated, a number of miRNAs which play a crucial role in the regulation of mitochondrial function have been identified [97, 98]. MiR-210 has been shown to target iron-sulphur cluster assembly

proteins and its up-regulation decreases the activity of prototypical iron-sulphur proteins controlling mitochondrial respiration [97]. MiR-696 has been experimentally validated to target PGC-1 α , with its up-regulation shown to result in significant reductions in fatty acid oxidation and mtDNA content [97]. Down-regulation of miR-23 was also found to be associated with significant increases in PGC-1 α mRNA expression and protein content, which coincided with up-regulation in the expression of the mitochondrial metabolic proteins citrate synthase (CS) and cyt c [98]. Specific miRNAs have also been implicated in the regulation of miR-181a with their up-regulation found to induce apoptosis via mitochondrial mediated pathways [24, 97, 99]. MiR-34a has also been found to target Bcl-2 following its transcriptional activation by p53 [24]. Conversely, miR-26a, miR-29b and miR-125b have all been demonstrated to directly target Bak mRNA, conferring a potent anti-apoptotic effect upon their induction [100-102].

Mounting evidence suggests that many components of the miRNA machinery and the repression process itself may not be localized to the cytosol but occur in association with different cellular organelles and structures [90]. Both the components of miRISC and repressed mRNA are found to accumulate in processing bodies (P-bodies), cytoplasmic structures implicated in the storage and degradation of translationally repressed mRNA [90, 103]. Though P-body integrity is not essential for miRNA function, many of the p-body components are found to play an important role in miRNA-mediated repression [90, 103]. P-bodies are also enriched in proteins required for mRNA degradation, including CCR4-NOT1, DCP1-DCP2 and XRN1 [90]. In addition to P-bodies, several studies have recently demonstrated that unique miRNAs are enriched in mitochondria independent of total cellular abundance [104-107]. To date, mitochondria-associated_miRNA (mito-miRNA) have been identified in rat liver [105], mouse liver [104], human HeLa epithelial cells [106] and human skeletal muscular cells [107], with expression profiles found to be heavily dependent on organism and cell type [103]. For many of these mito-miRNAs in-silico analysis has identified potential gene targets from the mitochondrial genome indicating that they are involved in regulating gene expression within the organelle [105]. Indeed, it has recently been demonstrated that miR-181c is capable of translocating into the mitochondria where it was found to bind to, and translationally repress, mitochondrial

encoded COX subunit I (mt-COX1) mRNA [108]. However, a number of mito-miRNAs have also been identified which are complementary to neither the mitochondrial genome nor nuclear RNAs encoding mitochondrial proteins [105]. Instead these miRNAs exhibited predicted targets in the cytoplasm which are known to mediate cellular processes such as cellular proliferation, differentiation and apoptosis [105]. It has been hypothesized that mitochondria may serve as a storage site for these miRNAs with their sequestration and release acting as a mechanism for intracellular signalling [105]. While the mechanism behind such a mode of action remains unclear, it has been proposed that P-bodies, which are known to have dynamic interactions with mitochondria, could play a role in facilitating the trafficking of miRNAs in and out of the organelle [103].

2.1.6 – Factors that Affect Mitochondrial Function: Aging, Exercise and Insulin Resistance

It has been hypothesized that the decline in mitochondrial function associated with aging results from an increase in mtDNA mutations and a decrease in mtDNA abundance, with oxidative damage induced by ROS being the underlying cause [43]. The mitochondrial theory of aging hypothesizes that oxidative stress initiates a positive feedback cycle whereby the accumulation of damage induced by ROS over time impairs mitochondrial function which subsequently leads to an augmentation in the generation of ROS [10]. In support of the proposed role that mitochondria play in the process of aging, research indicates that an increase in damage to mtDNA is associated with aging [10], while mitochondrial antioxidant capacity is also reported to be diminished, as evidanced by significant reductions in CoQ levels with cellular aging [109]. Oxidative damage to mitochondrial and nuclear nucleic acids is significantly increased in all major tissues in aged organisms including mice, hamsters, rats and humans [43, 110, 111].

While many studies provide evidence in support of the role that declining mitochondrial function plays in the process of aging, research from the laboratory of Short and colleagues [43, 112] suggests that the decline seen in mitochondrial function may result from age associated lifestyle changes rather than as a direct consequence of aging. Schrauwen and Hesselink [113] suggest that mitochondrial dysfunction arises in underutilized skeletal muscle when the supply of nutrients to the mitochondrial matrix exceeds the organelle's capacity to oxidize these substrates. Pathological conditions associated with a senescent lifestyle such as obesity and diabetes mellitus are associated with elevated plasma free fatty acid (FFA) concentrations [113]. Elevated fatty acid concentrations, together with a diminished capacity to oxidize these substrates results in the accumulation of FFA and their metabolites, such as diacylglycerol (DAG) and ceramide, in the mitochondrial matrix of skeletal muscle and other non-adipose tissue including pancreatic β cells, heart and liver [113]. FFAs and their metabolites are very sensitive to attack from ROS, forming highly reactive lipid peroxides, which can negatively influence mitochondrial function, by reacting with mtDNA and proteins [113].

Conversely mitochondrial biogenesis, induced by contractile activity in skeletal muscle associated with regular aerobic exercise, results in a greater sensitivity of mitochondrial respiration to free ADP levels in the mitochondrial matrix, since smaller increments in concentrations of the metabolite are required to maintain the same level of oxygen consumption, thus improving the respiratory control over ATP production and increasing the efficiency of oxidative phosphorylation [7]. Biogenesis lowers the rate of respiration required per mitochondrion for any given workload, reducing the level of ROS, and subsequently attenuating the potential for ROS mediated lipid, protein or DNA damage [7].

Insulin is recognized as an important regulator of mitochondrial function [44, 114-117]. Intravenous insulin infusion was found to increase mitochondrial capacity for oxidative phosphorylation and augment the activity of oxidative enzymes in skeletal muscle [114, 115]. In addition, insulin has been shown to increase the rate of mitochondrial protein synthesis and is known to induce mitochondrial biogenesis [115-117]. In contrast, insulin resistant states, such as type II diabetes, are associated with mitochondrial dysfunction [118]. Boushel et al. [118] examined rates of oxidative phosphorylation in the skeletal muscle of diabetic patients and while they did not establish an impairment of the function of isolated mitochondria, they did observe a diminished oxidative capacity in skeletal muscle which was attributed to a decrease in mitochondrial content and volume. In support of this, several studies have found a down-regulation of genes responsible for the induction of mitochondrial biogenesis, including PGC-1a, NRF-1, NRF-2 and Tfam, in insulin resistant subjects [118, 119]. Elevated plasma FFA concentrations have long been associated with the development of insulin resistance, which results from the intracellular accumulation of pro-inflammatory lipid metabolites, such as fatty acyl CoA and DAG, and the subsequent activation of stress sensitive kinases including AP-1 and NF- κ B, that antagonize insulin signalling [120-123]. The mechanism of interference with normal insulin signalling involves an augmented phosphorylation of serine and threonine residues on key elements of the insulin signalling pathway, including insulin receptor substrate-1 (IRS-1), which prevents the activation of downstream molecules such as PI3-K's [124]. Recent studies have provided evidence supporting the role of oxidative stress as the cause underlying the induction of insulin resistance by high concentrations of FFA's [120, 124]. St-Pierre et al. [125] demonstrated that the rate of mitochondrial H₂O₂ emission is significantly greater when basal respiration is supported by fatty-acid compared to carbohydrate based substrates, while Anderson et al. [120] found that treatment of rats, kept on a high-fat diet, with the antioxidant SS31 completely blocked the development of insulin resistance. In addition, 50µM H₂O₂ added to cultured 3T3-L1 adipocytes inhibited glucose uptake and glycogen synthesis in the presence of insulin, indicating that increased levels of oxidative stress can induce insulin resistance as an isolated factor [124]. Anderson et al. [120] concluded that the reducing potential of the respiratory chain provides a means for the cell to sense metabolic imbalance, with the generation of ROS from the mitochondria providing a means of initiating a counter-balancing response, by shifting the redox state of the cell and decreasing insulin sensitivity in an attempt to restore metabolic balance. However, Bloch-Damti and Bashan [124] point out that prolonged stimulation of this mechanism can lead to a chronic cellular desensitization of insulin and the development of pathological conditions.

2.2 – Growth Hormone and IGF-1

Growth hormone, which is also known as somatotropin, is a protein hormone that is secreted by the somatotroph cells of the anterior pituitary [3]. Hypothalamic secretion of growth hormone releasing hormone (GHRH), withdrawal of somatostatin, also known as growth hormone inhibiting hormone (GHIH), and the presence of GH releasing peptides, such as ghrelin, mediate its release [126]. GH exists in several different isoforms, the most abundant of which is a 22kDa molecule that contains 191 amino acids [127]. Other biologically active forms of growth hormone include a 20kDa isoform, a 17kDa isoform and a 5kDa isoform [127]. While some of its effects are mediated directly via its actions on the GHR's of target cells, many of the noted anabolic and metabolic effects of GH are mediated indirectly through its activation of another peptide hormone, IGF-1, a 70-amino acid containing peptide that is released

primarily from the liver in response to stimulation by GH [2, 3, 127]. IGF-1 acts to stimulate cellular proliferation and differentiation in numerous cell types including, myoblasts, osteoblasts, adipocytes, oligodendrocytes, neurons and haemopoietic cells [4, 128]. It has been concluded from several studies that IGF-1 also acts in an autocrine or paracrine fashion, rather than solely acting at a distance via blood borne transport [2, 3, 129]. Tissue types shown to be capable of IGF-1 production include muscle, bone and adipose tissue [5, 129]. Human leucocytes have also been shown to produce IGF-1 in response to *in-vitro* stimulation with GH [1]. The importance of the role that IGF-1 plays in conveying the anabolic effects of GH is evident from several studies which demonstrated that IGF-1 can stimulate both DNA and protein synthesis [5, 130-132].

GH is released from the anterior pituitary in 6 to 12 discrete pulses per day in response to several physiological stimuli [133]. Factors which cause an increase in GH secretion include exercise, stress, sleep, hypoglycaemia and amino acid uptake, with sleep and exercise being able to induce the strongest response [3, 133]. In contrast, hyperglycaemia and an increase in plasma FFA levels are capable of inducing an inhibition of GH release [3]. The daily GH secretion rate has been negatively correlated with age [134, 135]. It has been estimated that for men with a normal body mass index (BMI) for each decade of increasing age there is a reduction in the GH production rate of approximately 14% and the GH half-life of approximately 6% [134]. It is suggested that with increasing age there is a disruption in the pathways directing GH secretion, possibly as a result of a reduction in the co-ordination of the secretion of GHRH and somatostatin [126, 135].

Although circulating GH concentrations fluctuate widely due to the pulsatile nature of growth hormone secretion, basal plasma concentrations of $0.32 \pm 0.77\mu g/L$ have been recorded in young adult males (21-26 years old) following overnight fasting [3, 136]. Sleep is known to be a principal stimulator of GH secretion with peak pulsatile GH secretions of $30\mu g/L$ reported [22]. Exercise is also a potent stimulus for GH release with the magnitude of the GH response varying according to the type intensity and duration of exercise [126]. Table 2.1 outlines the effects of different exercise conditions on peak serum GH concentrations, which vary over a range of $5.5 - 44\mu g/L$ [137-142]. Exogenous rhGH administration has also been shown to augment circulating GH concentrations [138, 143, 144]. Studies conducted by Hansen *et al.* [143] and Lange *et al.* [144] found that an acute dose of 2.5mg rhGH elevated serum GH to concentrations

of approximately $15\mu g/L$ two hours following administration in physically active male subjects. In addition, Wallace *et al.* [138] recorded serum GH concentrations of approximately $33\mu g/L$ following 7 days of rhGH administration at a dose of 0.05mg/kg in endurance trained male athletes.

Туре	Subjects	Intensity	Duration	Peak GH (µg/L)	Reference
Aerobic	Ten active males [mean age = 26 ± 1.1 yrs]	62% VO ₂ max	30 minutes	5.5	Pritzlaff <i>et al.</i> [137]
		76% VO₂max	30 minutes	9.5	
		90% VO ₂ max	30 minutes	14	
	Seventeen elite endurance male athletes [mean age = 26.9 ± 1.5 yrs]	80% VO₂max	30 minutes	24.3±3.6	Wallace <i>et al.</i> [138]
Anaerobic	Ten healthy males [mean age = 24.5 ± 1.1 yrs]	Maximum intensity sprint (Cycle Ergometer)	30 seconds	13.6±2.7	Stokes <i>et al.</i> [139]
	Six active male sprinters	Maximum intensity sprint (Treadmill)	30 seconds	44	Nevill <i>et al.</i> [140]
Resistance	Ten strength trained males [mean age = 22 ± 2 yrs]	10RM loads	4 sets 1 min rest	26.5±2.0	Boroujerdi & Rahimi [141]
	, .	10RM loads	4 sets 3 min rest	22.9±1.6	
	Eight strength trained males	10 RM loads	1 set	8	Gotshalk <i>et al.</i> [142]
	[mean age = 25.4 ± 4.14 yrs]	10 RM loads	3 sets	15	

Table 2.1: Effects of exercise on circulating GH concentrations in physically active young males.

It has been suggested that IGF-1 may be a more useful indicator of the status of the GH/IGF-1 pathway, because of its low diurnal variability compared to GH [2]. Resting serum IGF-1 concentrations have been recorded over a range of 123-280µg/L in healthy male subjects between the ages of 20 years and 32 years [3, 145]. The influence of exercise on circulating IGF-1 concentrations has been shown to be dependent on the duration of exercise [3, 146]. Nguyen et al. [146] demonstrated that while serum IGF-1 levels increased by 11.9% from basal concentrations in healthy male athletes following an incremental exercise test to exhaustion (mean duration 21 ± 1 mins), concentrations were observed to decrease by approximately 15% in the same group of athletes following a Nordic ski race (mean duration $3hr 12 \pm 3 mins$). Endurance exercise training has been shown to significantly affect circulating IGF-1 levels with Roelen et al. [147] observing a rise in serum IGF-1 concentrations from $252 \pm 56 \mu g/L$ pretraining to $344 \pm 61 \mu g/L$ following two weeks of intense endurance exercise training. While exogenous administration of rhGH has not been shown to influence circulating IGF-1 levels in studies administering an acute dose [143, 144], continuous daily rhGH administration has been found to exert a significant effect [148]. Berggren et al. [148] demonstrated in ten healthy subjects who were administered 0.033mg/kg of rhGH for 28 consecutive days that serum IGF-1 concentrations were augmented from 316 \pm $103\mu g/L$ at baseline to $678 \pm 267\mu g/L$ post-treatment. In addition a higher dosage of 0.067mg/kg of GH yielded post-treatment serum IGF-1 concentrations of 769 \pm 218µg/L [148].

2.2.1 – Physiological Effects of Growth Hormone and IGF-1

The growth promoting effects of GH and IGF-1 are mediated primarily via an anabolic effect on protein metabolism [149]. Several studies have found that there is an increase in lean body mass when rhGH is administered to healthy subjects [30, 150, 151]. It has been demonstrated that GH stimulates nitrogen retention [152] and increases protein synthesis at a whole body level [153, 154]. However, controversy exists over which tissues benefit from this protein conservation [152]. While pathological conditions of GH excess are associated with an increase in lean body mass, skeletal muscle hypertrophy is not evident and patients typically present with muscle weakness [155, 156]. Studies carried out by Fryburg et al. [157, 158] demonstrated that short term infusion of rhGH increases muscle protein synthesis as indicated by an increase in the rate of disappearance of phenylalanine across the forearm during isotope dilution protocols. However, other studies [149, 154, 159] have been unable to show any effect of GH on muscle protein synthesis. Yarasheski et al. [154] treated healthy male subjects with rhGH for 12 weeks. While they found an increase in whole body protein synthesis as a result of rhGH administration, the rate of quadriceps muscle protein synthesis was found to be no greater in rhGH treated subjects compared to the control group.

Skeletal muscle is a predominantly post-mitotic tissue, the myocytes of which are incapable of proliferating in response to growth factor stimulation [160]. It is the proliferation of satellite cells, which are small mono-nucleated cells located between the basal lamina and the sarcolemma of muscle fibres, that leads to overload induced, skeletal muscle hypertrophy [160]. IGF-1 has been shown to stimulate the proliferation and differentiation of satellite cells, which subsequently fuse with existing myotubes, resulting in an increase in the nuclear DNA content of skeletal muscle fibres and an enhanced capacity for muscle protein synthesis [160]. Several authors have suggested that skeletal muscle IGF-1 isoforms play a role in the muscle hypertrophy and tissue remodelling that occurs with resistance training [2, 127]. Mechanical loading induces production of a specific isoform of IGF-1 within skeletal muscle which is termed "mechano growth factor" (MGF) [160]. It is possible that local muscular production of IGF-1 may be increased in response to endurance and resistance exercise, but not be represented through changes in serum IGF-1 levels in exercise studies [2]. It has been reported that five days of endurance training in rats resulted in increases in MGF without any corresponding changes in circulating IGF-1 [161].

A marked increase in resting energy expenditure (REE) is seen with GH administration [162]. Lean body mass is a positive determinant of REE and it has been suggesting that the calorigenic actions of GH are secondary to increments in lean body mass [163]. However a stimulation of energy expenditure has been recorded after only 5 hours of intravenous rhGH infusion in healthy subjects, and a significant decline in REE has been noted after short term (24hr) discontinuation of rhGH treatment in GH-deficient adults, both of which imply that GH may stimulate REE independent of changes in body composition [163].

GH is known to increase the circulation of fatty acids in the blood. The biological mechanisms of the lipolytic effects of GH remain to be determined but involve a reduced lipoprotein lipase (LPL) activity, stimulation of hormone-sensitive lipase (HSL) and suppression of the antilipolytic effects of insulin [164]. GH was found to reduce adipose tissue LPL activity by 65% and muscle LPL activity by 20%, while exogenous administration of rhGH has been found to significantly enhance HSL activity [165]. Hormone sensitive lipase regulates the breakdown of triglycerides in adipose tissue to yield non-esterified fatty acids (NEFAs) and glycerol [162]. It is stimulated by catecholamine activation of ß-adrenergic receptors on cell membranes which leads to an increase in intracellular cAMP levels through the increased activation of adenylate cyclase [133, 162]. This leads to an increased activation of protein kinase-A which phosphorylates HSL at a single serine residue [133, 162]. Stimulation of lipolysis is instrumental for the protein conserving actions of GH during fasting [149]. Inhibition of lipolysis during rhGH administration using acipimox, a niacin derivative that lowers blood lipid concentrations, increases muscle protein breakdown by 50%, neutralizing growth hormone's ability to restrict protein breakdown, while restoration of high FFA levels decreased whole body phenylalanine degradation by 10-15% [166].

Increased lipid availability is suggested to be responsible for the insulin resistance observed with elevated GH levels [149]. GH and FFA concentrations were positively correlated with peripheral and hepatic insulin resistance [167], while lowering FFA levels pharmacologically with acipimox improves insulin sensitivity. However, it has been reported that GH induced insulin resistance precedes increases in circulating lipid intermediates suggesting that GH also induces insulin resistance in a manner independent of its lipolytic action [168].

2.2.2 – The Signal Transduction Pathways of Growth Hormone and IGF-1

The underlying mechanisms of the metabolic and anabolic effects of GH have been studied through elucidation of its signalling pathways in target tissues [169, 170]. The initial step in GH signalling is dimerization of the GHR, which brings together two Janus kinase 2 (JAK2) molecules [149]. When brought together these molecules phosphorylate tyrosine residues on the cytoplasmic side of the receptor and other molecules adjacent to the receptor-JAK2 complex, such as STAT proteins, which translocate to the nucleus and activate gene transcription by binding to responsive DNA elements of specific genes within DNA [149]. Administration of rhGH in-vivo has been found to induce stimulation of STAT 1, 3 and 5 in hypophysectomised rats [171]. GH also activates the Ras/Raf-1/mitogen activated protein (MAP) kinase pathway which is involved in cell proliferation [149]. Protein kinase C also plays a role in GH signalling as treatment with inhibitors of protein kinase C has been shown to decrease GH induction [172]. GH leads to phosphorylation of IRS-1 and IRS-2, opening binding sites for phosphoinositide 3-kinase (PI-3 kinase), a class of enzyme that, in addition to having roles in cellular proliferation, differentiation and intracellular trafficking are also a key component in the insulin signalling pathway [149].

Two distinct cytoplasmic regions of the GH receptor have been identified as being important for GH receptor mediated signal transduction, i) a proline rich region, termed Box I, which is located close to the trans-membrane domain and ii) the C-terminal 184 amino acid sequence [170]. The Box I region is required for the binding of the tyrosine kinase JAK2 and the activation of all subsequent signal transduction proteins associated with this molecule [170]. The C-terminal region has been demonstrated to be involved in a GH induced increase in intracellular Ca²⁺ [170]. Ca²⁺ signalling has been found to regulate gene expression in several cases [173, 174]. In addition, studies performed by Billestrup *et al.* [170] on GH receptor transgenic mice found that GHR's lacking the C-terminal 184 amino acid sequence were unable to induce a transcriptional response to GH of the SPI 2.1 gene, which codes for a serine protease inhibitor, indicating that this specific amino acid sequence is required for stimulation of SPI 2.1 transcription. From this data it is evident that activation of the JAK2 kinase and increases in intracellular Ca²⁺ are both essential for growth hormone's ability to stimulate transcription of certain genes [170].

The physiological effects of IGF-1 are predominantly mediated through interaction of the hormone with the IGF-1 receptor (IGF-1R) [128]. IGF-1 may also mediate some of its effects by binding to the closely related insulin receptor (IR); however this interaction occurs at a much lower affinity than that of insulin with its own receptor [128, 175]. Both the IR and the IGF-1R are cell surface receptors that belong to a family of receptor tyrosine kinases [176]. The IGF-1R is expressed in almost all cell types, with a few notable exceptions including hepatocytes and mature B lymphocytes [4]. The receptor is a tetramer consisting of two extracellular α subunits and two primarily intracellular β subunits that are linked together by disulphide bonds [176]. The binding of IGF-1 to the extracellular domain of the IGF-1R activates the intracellular tyrosine kinase, resulting in receptor autophosphorylation which is facilitated by the binding of ATP to the receptor's intracellular domain [128, 176]. Activation of the receptor causes the phosphorylation of tyrosine residues on several intracellular substrates, resulting in the activation of a number of down-stream signalling cascades. Tyrosine phosphorylation of IRS-1 and Shc following IGF-1R activation allows these proteins to phosphorylate the SH-2 domain of the growth factor receptor-bound protein-2 (Grb-2), which leads to the activation of the Ras /Raf-1 / MAP kinase pathway [128, 177]. IRS-1 phosphorylation also leads to the activation of PI-3 kinase, which indirectly activates the serine/threonine kinase p70^{s6k} through the activation of Akt [177].

Valentinis and Baserga [4] have noted that IGF-1 transmits two contradictory signals to cells, which will induce either cellular proliferation or terminal differentiation. They suggest that whether a cell undergoes proliferation or differentiation in response to IGF-1 is dependent on the cell type and the level of expression of signalling proteins, such as IRS-1, within the cell [4]. Coolican *et al.* [177] have shown that inhibition of the MAP kinase signalling pathway using PD098059, which is a non-competitive inhibitor of MEK, leads to an inhibition of IGF-1 stimulated proliferation in L6A1 myoblasts. The same group also found that inhibition of p70^{s6k} activation, through administration of the immunosuppressant drug rapamycin, completely abolished IGF-1 stimulated differentiation in L6A1 myoblasts [177]. Their results indicate that the MAP kinase pathway plays a primary role in the proliferative response to IGF-1 while the PI-3 kinase signalling pathway is essential for IGF-1 stimulated differentiation [177]. However, the PI-3 kinase signalling pathway has also been implicated in the

proliferation of cells in response to IGF-1 and in mediating the hormone's antiapoptotic effects [4, 128].

2.2.3 – The effects of rhGH Administration on Athletic Performance

The use of rhGH for the purposes of enhancing athletic performance has been extensively reported in the literature [25, 26, 33, 37]. Exaggerated claims about the anabolic and metabolic effects of GH have been posted on websites promoting the use of rhGH as a rejuvenating agent for the elderly or as a muscle building agent for athletes and body builders [178]. In contrast, there is a lack of evidence published in peer reviewed literature that supports the performance enhancing effects of rhGH [178].

Only a limited number of studies have evaluated the effects of supra-physiological doses of rhGH, a genetically engineered peptide that is identical to the 22kDa endogenous growth hormone isoform in amino acid sequence and three-dimensional structure, on exercise capacity outcomes in healthy subjects [30]. Evidence from these studies does not support the existence of a performance enhancing effect of GH and has found that in some cases rhGH administration may have an adverse effect on exercise capacity [30]. Berggren et al. [148] assessed the exercise capacity of a group of young healthy male and female subjects who performed an incremental exercise test to exhaustion on a cycle ergometer following one month of receiving supra-physiological doses of rhGH (0.067 mg/kg/day). GH was found to have no significant effect on maximum oxygen consumption or power output during exercise compared with placebo treated controls [148]. Irving et al. [179] evaluated the effects of time after acute rhGH administration on metabolic and performance measures during 30 minutes of aerobic exercise conducted at an intensity above the subjects lactate threshold and concluded that GH does not alter exercise performance regardless of the timing of rhGH administration in relation to exercise. rhGH administration was not seen to have any significant effect on physiological parameters related to performance during exercise, including total work output, caloric expenditure, heart rate response, blood lactate response or the rate of perceived exertion (RPE) [179].

Only two studies to date have evaluated the effects of rhGH administration on strength parameters in healthy young subjects undergoing a resistance training protocol [154, 180]. Yarasheski *et al.* [154] provided subjects with either rhGH (0.04 mg/kg) or a placebo, 5 days a week while they underwent a 12 week resistance training program,

while in a study conducted by Deyssig *et al.* [180] all subjects performed between 8 and 14 hours of high intensity resistance training per week for 6 months prior to self-administering rhGH (0.03 mg/kg) nocturnally for a 6 week period. In both studies rhGH administration was not found to result in significant improvements in either muscle biceps strength or quadriceps strength as assessed by 1 repetition maximum (1RM) strength testing [30, 154, 180]. Deyssig *et al.* [180] concluded that any improvements in strength from baseline measurements were the result of a training effect and not due to rhGH treatment. Despite having induced no significant effect on strength parameters, GH is known to significantly increase lean body mass [30, 154, 180]. Yarasheski *et al.* [154] hypothesized that resistance training stimulates muscle protein synthesis to a rate above which the GH stimulus cannot further advance.

It is argued that clinical studies examining the performance enhancing effects of GH have not used rhGH doses large enough to induce any significant anabolic effects [178]. Liu *et al.* [30] reported that the average daily rhGH dose administered in clinical studies was 0.036mg per kilogram of body weight, with treatment regimes in these studies ranging from acute rhGH doses to between 7 and 42 days of continuous therapy. Rennie [178] claims that these treatment regimens fail to match the supra-physiological doses which athletes and bodybuilders who abuse rhGH are purported to use. However, the rhGH doses used in clinical trials have been sufficient to elevate plasma IGF-1 concentrations 3 to 6 times above normal baseline levels and to induce significant increases in lipolysis, water retention and the rate of lipid oxidation [148, 178]. Rennie [178] argues that it is unlikely that these same IGF-1 levels would be insufficient to have any effect on rates of protein metabolism.

2.3 – Growth Hormone and IGF-1: Impact on Mitochondrial Function

Early animal studies, conducted on isolated hepatic mitochondria, used manometry to examine the effects of GH administration on rates of oxidative phosphorylation, in the presence of carbohydrate and lipid substrates [181, 182]. Melhuish and Greenbaum [181] found that acute administration of GH (1mg), 4 hours prior to sacrifice in rats, increased the rate of oxidation in isolated liver mitochondria by 40% when β -hydroxybutyrate was used as a substrate. However, the rate of oxidative phosphorylation when GH was administered was not found to differ significantly to that of control samples and a lower P/O ratio (ratio of ATP produced to oxygen consumed)

was recorded in GH trials, indicating that GH impacts negatively on the efficiency of oxidative phosphorylation [181]. Sordahl et al. [182] demonstrated that lowered P/O ratios from isolated mitochondria in hypophysectomized cats were restored to values seen in healthy mitochondria following 3 days of *in-vivo* GH administration. In contrast, in-vitro GH administration exerted no effect on the P/O ratios of isolated mitochondria, suggesting that GH mediates its effects on oxidative phosphorylation indirectly, possibly though the actions of IGF-1 [182]. In addition, the positive effect of insulin on the efficiency of mitochondrial function was found to be inhibited in the presence of GH in-vitro [182]. Several studies have hypothesized that the ability of GH to restore function to the mitochondria of hypophysectomized animals is mediated through its effects on the rate of synthesis of mitochondrial proteins [183, 184]. Maddaiah et al. [183] demonstrated that the incorporation of radio-labelled leucine into rat liver mitochondria was significantly elevated by GH administration, while a subsequent study from this group [184] found that a reduced content of cytochrome proteins in mitochondria following hypophysectomy was restored to normal levels after two weeks of GH treatment. A significant effect of GH on mitochondrial cytochrome content might explain observed decreases in the efficiency of oxidative phosphorylation following GH administration in healthy animals [181], as elevated cytochrome content has been associated with high rates of mitochondrial respiration [47]. In particular, COX is recognized as the location of a "slippage" between redox reactions and proton pumping and an increment in its concentration is associated with a decrease in the overall efficiency of the respiratory chain [47].

Conversely, studies that measured oxygen consumption rates from isolated mitochondria following *in-vivo* GH administration in rats failed to establish an effect of GH on oxidative phosphorylation [185, 186]. DiMarco and Hoppel [185] demonstrated that GH had no effect on the P/O ratio of isolated liver mitochondria in the presence of either lipid (hexanoate) or carbohydrate (pyruvate) substrates, while Peyreigne *et al.* [186] found that 14 days of GH treatment in male Wister rats did not affect the respiratory states of isolated skeletal muscle mitochondria, measured in the presence or absence of ADP and Pi. A limitation of these studies is that the well-defined conditions used during *in-vitro* experiments, such as State 3 (optimum phosphorylating conditions) and State 4 (non-phosphorylating conditions) respiration never occur *in-vivo*, while the saturating concentrations of substrates used do not reflect physiological conditions [48].

Perret-Vivancos et al. [187] found that in-vitro administration of GH induced an increase in the oxygen consumption of cultured Chinese hamster ovary (CHO) cells, indicating a direct effect of the hormone on mitochondrial function. Exposure of cells to GH concentrations of 200nM produced optimum stimulation of oxygen consumption which was associated with an increased oxidation of reducing equivalents as determined through the analysis of the intrinsic fluorescence of reduced pyridine nucleotides (NADH) [187]. However, the effect of GH on these variables is not evident in CHO cells transfected with GHR's lacking the BOX-1 intracellular domain, which is required for the binding of JAK2 to the GHR and the subsequent activation of GH intracellular signal transduction pathways [187]. This indicates that signalling molecules downstream of the GHR mediate the hormone's effects on mitochondrial oxidative capacity [187]. GH and its receptor are known to be internalized in cells via the clathrin coated pit pathway, to be degraded by lysosomes and via the caveolae pathway, which translocates the hormone into sub-cellular compartments [188, 189]. In analysing the subcellular distribution of radio-labelled human growth hormone (hGH) in female rat hepatocytes, Postel-Vinay et al. [190] found no radioactivity associated with mitochondria. In agreement a study by Mutvei et al. [191] concluded that no high affinity binding sites for GH were present on rat liver mitochondria. However, Perret-Vivancos et al. [187] demonstrated, in cells where internalization of the GHR was impaired, that there were perturbations in the cell's respiratory state, suggesting an implication of the internalization process of GH on the regulation of the hormone's effects on mitochondrial function. In addition, permeabilization of the mitochondrial membrane has been found to abolish any effect induced by GH on either oxygen consumption or rates of oxidative phosphorylation suggesting that the primary effect of GH on the state of cellular respiration is mediated on the mitochondrial membrane [181].

Few studies have examined the *in-vivo* effects of rhGH administration on healthy human tissue [192, 193]. Lange *et al.* [193] found that combining 12 weeks of rhGH administration with aerobic exercise in elderly women significantly increased the activity of oxidative enzymes involved in the tricarboxylic acid (TCA) cycle (CS (P < 0.02)) and in β -oxidation pathways (L-3-hydroxyacyl-CoA dehydrogenase (HAD) (P < 0.02)) of skeletal muscle mitochondria. In agreement, Short *et al.* [192] found significant increases in the activity of these enzymes (CS = + 16% / HAD = + 13%) in

skeletal muscle of healthy human subjects in response to 14 hours of intravenous rhGH infusion. In addition, rhGH infusion resulted in an 8-35% increment in the rate of ATP production in isolated skeletal muscle mitochondria [192].

While these authors successfully demonstrated increased mitochondrial oxidative activity in response to GH, attempts at establishing the hormone's effects on mitochondrial biogenesis have yielded inconclusive results [192, 193]. Analysis of the fractional synthetic rate of muscle proteins using C¹³-labeled leucine, found that GH had no effect on the rate of synthesis of muscle mitochondrial proteins [192]. However, these measurements only represent average rates of synthesis and do not reflect changes in specific proteins [192]. Although increases were seen in mRNA levels of Tfam, a transcription factor responsible for inducing the replication and transcription of mtDNA, and both individual mitochondrial encoded (COX3) and nuclear encoded (COX4) mitochondrial proteins, the expression of key transcription factors involved in the regulation of mitochondrial biogenesis, including PGC-1 α , NRF-1 and NRF-2, were not affected by rhGH infusion [192]. Short *et al.* [192] suggest that an additional stimulus, such as exercise, may be necessary for GH to induce a significant effect on mitochondrial biogenesis.

In contrast to studies which have demonstrated a positive effect of GH on mitochondrial oxidative enzyme activity, micro-array analysis on the expression of genes in skeletal muscle of hypopituitary rats and humans treated with rhGH have found a down-regulation of gene transcripts expressing enzymes involved in β -oxidation (3-hydroxyacyl-CoA dehydrogenase), the TCA cycle (oxoglutarate dehydrogenase) and the ETC (subunits of COX and ATP synthase), suggesting a reduction in oxidative capacity in response to GH treatment [194, 195]. Deficiencies in the plasma concentrations of other pituitary hormones, in light of the hypopituitary nature of the subjects used in these trials may have negatively impacted on the actions of GH. In addition, it is possible that GH induced insulin resistance might negate any positive effects which the hormone may induce on the expression of mitochondrial oxidative enzymes was observed in the liver of aged rats, which highlights a tissue-specific effect of GH on gene expression and suggests that the liver may play a key role in mediating the effects of GH on energy expenditure [197].

While the research of Lange et al. [193] and Short et al. [192] demonstrates an in-vivo effect of rhGH administration on skeletal muscle mitochondrial function, their work fails to establish the mechanism behind this effect. To our knowledge, studies examining the effects of IGF-1 on skeletal muscle mitochondrial function or rates of gene expression and mitochondrial protein synthesis have, to date, not been conducted [192]. Garcia-Roves et al. [198] demonstrated that elevated FFA concentrations also induce an increment in the expression of enzymes involved in β-oxidation (medium chain acyl-CoA dehydrogenase), the TCA cycle (CS) and the respiratory chain (cyt c), suggesting that GH may mediate its effects indirectly through the actions of lipolysis. In opposition to this hypothesis Leung and Ho [199] have demonstrated a direct effect of GH on rates of mitochondrial fatty acid oxidation in cultured human fibroblast cells, while in-vitro IGF-1 administration was found to have no effect. In addition, the temporal extent of changes induced by GH on mitochondrial oxidative capacity and gene expression persist following cessation of rhGH administration is unknown as studies to date have only analysed variables within a post treatment window of 24 hours [192, 193], while the effects of long term rhGH administration on mitochondrial function, to date, have not been studied [192].

Studies conducted on cultured human cell-lines have demonstrated anti-oxidant effects following rhGH and IGF-1 administration in-vitro [200, 201]. Csiszar et al. [200] found that rhGH, at concentrations of 3.3 and 33µg/mL and IGF-1, at concentrations of 10, 100 and 1000 μ g/L, significantly reduced cellular O_2^- and H_2O_2 production, in addition to mitochondrial O_2^- production, following 24 hours of treatment in human coronary arterial endothelial cells (HCAECs). In agreement, Thum et al. [201] observed cellular ROS levels to be significantly reduced in cultures of a human endothelial cell-line (EAhy126 cells) at rhGH concentrations of 100 and 1000µg/L after 24 hours. Both studies attributed the observed effects to a GH/IGF-1 elicited improvement in antioxidant status, with Csiszar et al. [200] noting a significant up-regulation of the antioxidant enzymes manganese-superoxide dismutase (Mn-SOD), copper, zincsuperoxide dismutase (Cu, Zn-SOD), and glutathione peroxidise 1 (GPX-1). In contrast, Brown-Borg et al. [202] observed that rhGH and IGF-1 significantly decreased antioxidant capacity in cultured murine hepatocytes after 24 hours. Catalase and GPX-1 activity were found to be significantly suppressed in the presence of GH at 1 and 10µg/mL and in the presence of IGF-1 at 3.8, 38 and 380µg/L [202]. Indeed, GH studies conducted on animals have demonstrated that excessive concentrations of the hormone can impact negatively on mitochondrial function by modifying levels of oxidative stress [39-41, 203, 204]. Seiva et al. [39] demonstrated that high doses of rhGH (2mg/kg) exerted detrimental effects related to energy metabolism and oxidative stress in rat myocardium, while a lower dose (1mg/kg) exerted beneficial effects on energy production and reduced levels of oxidative damage. This was attributed to the capacity of the antioxidant glutathione to handle the increase in superoxide production following administration of the low rhGH dose, while this capacity was exceeded at the higher dose [39]. Studies performed on transgenic animal models exhibiting an excess production of GH found that these animals possessed higher rates of oxygen consumption and free radical production [40, 204]. In contrast to animals overexpressing GH, Ames dwarf mice, which are characterized by a deficiency in GH secretion, exhibit less oxidative damage to mtDNA in liver, brain and heart tissue compared to wild type mice [40, 204]. Of note however, fibroblasts from Lewis dwarf rats, an animal model which exhibits normal pituitary function except for a selective genetic GH deficiency, were observed to have similar rates of cellular O_2^- and H_2O_2 production compared to wild type controls [205]. In addition, the expression of important anti-oxidant enzymes, Mn-SOD, Cu, Zn-SOD and catalase were not found to be down-regulated in comparison with wild type rats [205]. Indeed, thirty days of GH replacement therapy was found to significantly increase antioxidant capacity in these animals through up-regulation of the expression of GPX-1 [205]. To date, how in-vivo rhGH administration in healthy human subjects affects the generation of ROS or the level of oxidative stress in mitochondria has not been evaluated. However, in patients with acromegaly, ATP production has been shown to be reduced by 25%, indicating that excessive GH concentrations can induce prolonged mitochondrial dysfunction [206].

It has been demonstrated that both GH and IGF-1 confer protection against cellular apoptosis, mediated via an effect on the permeability transition pore of the IMM [17, 18]. Yamamura *et al.* [17] demonstrated that Ca^{2+} induced opening of the mtPTP, as assessed from identification of large amplitude swelling of mitochondria and measurement of levels of cyt c release, was significantly inhibited by IGF-1 pre-treatment in rat myocardium, while Mitsunaka *et al.* [18] found that rhGH treatment prevented apoptosis following activation of plasma membrane bound Fas receptors,

which initiate intracellular death signalling pathways, in both CEM/L7 and IM-9 lymphocytes. The mechanism behind these noted anti-apoptotic effects was attributed to GH/IGF-1 induced up-regulation and down-regulation of respective, anti-apoptotic and pro-apoptotic Bcl-2 family proteins, which are hypothesized to regulate mtPTP opening by interacting with the VDAC on the OMM [17, 18, 207]. Indeed, several studies have demonstrated that GH induces an up-regulation of Bcl-2 gene and protein expression in peripheral blood lymphocytes and monocytes in-vitro [18, 19, 208]. In addition, Bcl-xL mRNA levels in cultured insulin producing cells (INS-1 cells) were observed to be significantly up-regulated following only four hours of exposure to rhGH at concentrations of 500µg/L [209]. In contrast, one month of rhGH administration, at a dosage of 2mg/kg/day, was not found to significantly affect the expression of Bcl-2 in male senescence-accelerated prone mice (SAMP8 mice) [210]. The anti-apoptotic effects induced following rhGH administration in this study were instead attributed to a significant down-regulation of Bad, a pro-apoptotic signalling protein, and Bax, its down-stream effector [210]. Similarly, rhGH administration was not found to impact Bcl-2 expression in a human gastric cancer cell-line (BGC823 cells) in-vivo, following the inducement of carcinoma xenografts in nude mice [211]. In regard to IGF-1, the anti-apoptotic affects observed by Yamamura et al. [17] following systemic IGF-1 treatment in male Sprague-Dawley rats were attributed to significantly elevated Bcl-xL and significantly decreased Bax protein concentrations in isolated cardiomyocyte mitochondria. In addition, Kang et al. [212] observed that cultured mesangial cells exhibited a significant decrease in the Bax/Bcl-2 protein ratio in the presence of 100µg/L IGF-1, although individual changes in the expression of these proteins was not reported. Increased phosphorylation of Bad at Ser112 and Ser136 was also observed following IGF-1 administration in this study, leading to inactivation of the proteins proapoptotic activity [212]. At present it is unknown what effect increases in cellular stress levels in response to long term rhGH use in subjects with a healthy GH/IGF-1 axis would have on the mitochondria mediated induction of cell death pathways.

3. Outline of Project Studies: Aims and Hypotheses

The two major aims of this research project were firstly, to investigate whether rhGH and IGF-1, over a range of physiological and supra-physiological concentrations, regulates key components of mitochondrial function and secondly, to determine the implications of any potential regulation in terms of its impact on cellular viability. This included the investigation of a novel mitochondrial associated signalling pathway as a potential mechanism for the regulation of mitochondrial mediated apoptotic effects by GH and IGF-1. The project consists of three studies, the individual aims and hypothesis for each of which are outlined below.

3.1 – Outline of Study One

The purpose of this study was to determine whether rhGH and rIGF-1 exert a direct effect on the function of mitochondria over a range of physiological and supraphysiological concentrations in PBMCs from healthy male subjects. Physiological GH concentrations are defined as serum GH concentrations which fall within the range seen following the stimulation of peak endogenous GH responses in normal subjects. Through the measurement of serum GH concentrations following administration of an insulin tolerance test, Hoffman *et al.* [213] determined this range to be $5.3 - 42.5 \,\mu$ g/L in normal subjects. Normal physiological serum IGF-1 concentrations for healthy male between 20 – 35 years of age have been reported to be within the range of 137 - 257μ g/L [214]. For the purposes of this study supra-physiological concentrations of GH and IGF-1 are defined as any concentration which exceeds these reference ranges. The effects of GH were investigated over a concentration range of 100 - 600\mug/L. The concentrations used are cited in table 3.1 below.

GH	0.25	0.5	1	2.5	5	10	25	50	100
IGF-1	100	200	300	400	500	600			

Table 3.1: Concentrations of GH and IGF-1 used for in-vitro analysis in Study One $(\mu g/L)$.

Ten healthy male subjects between the ages of 18 and 35 years were recruited to participate in the study. Subjects had a 30mL blood sample taken in the morning following an overnight fast. PBMCs were isolated from whole blood by density gradient centrifugation and resuspended in RPMI-1640 cell culture medium.

Once isolated, PBMCs were incubated for 4 hours in the presence of either rhGH or rIGF-1 at pre-determined concentrations as outlined in Table 3.1. All samples were subsequently analysed by flow cytometry for the determination of $\Delta \psi_m$, mitochondrial O_2^- generation, mtPTP activity and cellular viability.

3.1.1 – Outline of Variables Analysed in Study One

3.1.1.1 *Mitochondrial Membrane Potential* $(\Delta \psi_m)$

The $\Delta \psi_m$ is the electrical component of the proton motive force (Δp_m) that exists across the IMM [62]. It exhibits a negative charge inside the mitochondrial matrix which is normally maintained at approximately 100-120 mV [14]. The creation of this electrical gradient is driven by the activity of the ETC which pumps H⁺ from the mitochondrial matrix across the IMM to the inter membrane space [8]. The membrane potential in turn drives the flow of H⁺ back to the mitochondrial matrix through the enzyme protein complex ATP-synthase where ATP is synthesised by the phosphorylation of ADP [8]. As noted previously, at excessively high trans-membrane potentials, the efficiency of mitochondrial ATP synthesis decreases due to an increase in proton leak across the IMM and a decrease in the stoichiometry between electron flux and proton pumping along the ETC [14]. Analysis of $\Delta \psi_m$ allows for interpretation of how the functional status of mitochondria responds to rhGH and rIGF-1 and in conjunction with levels of mitochondrial O₂⁻ generation provides an insight into how these hormones affect the efficiency of energy production within the organelle.

3.1.1.2 Mitochondrial Superoxide Generation

Under normal physiological conditions it is estimated that approximately 1-3% of electrons carried by the ETC leak out of the pathway and transfer directly to oxygen to produce O_2^- [215]. Complex I and Complex III of the ETC are thought to be the most important sites of mitochondrial superoxide production [215, 216]. The O_2^- undergoes a reaction catalysed by the antioxidant enzyme Mn-SOD in the mitochondria to produce H_2O_2 , which in the presence of intracellular antioxidants such as catalase and gluthathione is reduced to H_2O [215]. However if superoxide production exceeds the capacity of the antioxidant enzymes, then highly reactive oxygen species (hROS), such as the 'OH, will accumulate in the mitochondria and lead to damage of mitochondrial DNA, proteins and lipid membranes [10, 216]. Analysis of superoxide levels within

mitochondria allows for determination of whether the functional response of the organelle to rhGH and rIGF-1 has negative implications in terms of an increased risk of structural damage to key mitochondrial components [215].

3.1.1.3 Mitochondrial Permeability Transition Pore Activity

As mentioned previously, the mtPTP is activated in response to a variety of physiological stresses, including excessive intracellular Ca²⁺ concentrations, depleted intra-mitochondrial substrate levels and an augmentation of the oxidative status of the cell [11, 54, 55]. In contrast, conditions of optimum cellular function associated with the adequate supply of ADP and Pi to the mitochondrial matrix and a low rate of ROS generation keep the pore in a closed conformational state [11, 54, 55]. Opening of the mtPTP initiates cellular apoptotic pathways via the release of cyt c and AIF [11, 54, 55]. Thus, analysis of the open / closed status of the mtPTP provides an indication of the rate of mitochondrial mediated cellular apoptosis, allowing for interpretation of how the mitochondrial response to rhGH and rIGF-1 affects functional conditions at a cellular level.

3.1.1.4 Cellular Viability

Cellular viability, the capacity of a cell to maintain its physiological function, is a defining characteristic of healthy living cells which are distinguishable from non-viable cells undergoing one of either two forms of cell death: apoptosis and necrosis [11, 51, 217]. As mentioned previously, apoptosis is a programmed form of cell death responsible for the removal of damaged or diseased cells without incurring an inflammatory reaction [51]. Apoptosis is a controlled process which is dependent upon the availability of ATP and is mediated by the activation of extracellular and intracellular signalling pathways which can affect cells at an individual level [11, 51]. In contrast, necrosis is defined as a pathological form of cell death resulting in the liberation of chemical factors from the cell which lead to a localized inflammatory response [51, 217]. Necrosis is an uncontrolled and passive process that can affect large numbers of cells and whose mediation is triggered either through interference of the cells energy supply or as a direct result of damage to the cell membrane [51, 217]. Identification of the percentages of viable, apoptotic and necrotic cells within a sample allows for a direct interpretation of the functional consequences of rhGH and rIGF-1 effects at a cellular level.

3.1.2 – Study One Aims

The primary aims of Study One were as follows:

- 1. To investigate whether rhGH and IGF-1 exert any direct effect on the efficiency of mitochondrial function, through the examination of relative changes in $\Delta \psi_m$ and mitochondrial O_2^- levels, following the *in-vitro* administration of rhGH and rIGF-1 in PBMCs from healthy male subjects, over a range spanning both physiological and supra-physiological concentrations.
- 2. To investigate the implications for cellular viability imposed by such rhGH and IGF-1 mediated effects on the mitochondrial function of PBMCs' at both physiological and supra-physiological concentrations, through the examination of relative changes in the level of activity of the mtPTP in addition to the direct assessment of changes in the percentages of viable, apoptotic and necrotic cells.

The hypotheses of Study One were as follows:

- Neither rhGH nor rIGF-1 will exert any significant effect on $\Delta \psi_m$ values in samples treated at physiological concentrations compared to untreated control samples. In contrast, $\Delta \psi_m$ values will be significantly increased by both rhGH and rIGF-1 in samples treated at supra-physiological concentrations compared samples treated at physiological concentrations and untreated samples.
- Both rhGH and rIGF-1 will either significantly decrease or exert no significant effect on mitochondrial O₂⁻ levels in samples treated at physiological concentrations compared to untreated control samples. In contrast, mitochondrial O₂⁻ levels will be significantly increased by both rhGH and rIGF-1 in samples treated at supra-physiological concentrations compared to samples treated at physiological concentrations and untreated samples.
- Neither rhGH nor rIGF-1 will exert any significant effect on the level of mtPTP activity in samples treated at physiological concentrations compared to untreated control samples. In contrast, the level of mtPTP activity will be significantly increased by both rhGH and rIGF-1 in samples treated at supra-physiological concentrations and untreated samples.
- Neither rhGH nor rIGF-1 will exert any significant effect on the percentages of cellular apoptosis and necrosis in samples treated at physiological concentrations compared to untreated control samples. In contrast, these percentages will be significantly increased by both rhGH and rIGF-1 in samples treated at supraphysiological concentrations compared to samples treated at physiological concentrations and untreated samples.

3.2 – Outline of Study Two

Study Two was designed for the purposes of investigating the mitochondrial effects exerted by physiological and supra-physiological concentrations of rhGH and rIGF-1 under various respiratory conditions of substrate saturation in PBMCs from healthy male subjects. The effects of GH were investigated over a concentration range of 0. 5 - $50\mu g/L$ while the effects of IGF-1 were investigated over a concentration range of 100 - $500\mu g/L$. The concentrations used are cited in table 3.2 below.

GH	0.5	5	50
IGF-1	100	300	500

Table 3.2: Concentrations of GH and IGF-1 used for in-vitro analysis in Study Two $(\mu g/L)$.

Cellular respiration is dependent upon the continuous supply of endogenous substrates into the mitochondrial matrix *in-vivo* [218]. However, in isolated mitochondria or permeabilized cells *in-vitro*, the endogenous supply of respiratory substrates is lost [219]. The artificial supply of specific combinations of respiratory substrates under these conditions allows for control of electron supply to the ETC, where electron entry can either be mediated exclusively through complex I or complex II or via the convergent supply of electrons to both complexes simultaneously [218, 220, 221]. The transfer of electrons to the ETC via the β -oxidation pathway can also be controlled through the supply of specific fatty acid respiratory substrates [221].

Ten healthy male subjects between the ages of 18 and 35 years were recruited to participate in the study. Subjects had a 30mL blood sample taken in the morning following an overnight fast. PBMCs were isolated from whole blood by density gradient centrifugation and resuspended in RPMI-1640 cell culture medium.

Once isolated, PBMCs were incubated for 4 hours in the presence of either rhGH or rIGF-1 at pre-determined concentrations as outlined in Table 3.2 for the purposes of establishing hormonal effects on mitochondrial function before the various respiratory conditions were induced. Cells were permeabilized following hormonal treatment and subsequently analysed for the determination of $\Delta \psi_m$ values and levels

of mitochondrial hROS production by flow cytometry, in the presence of substrate combinations which give rise to the following respiratory conditions:

1) Complex I mediated respiration – Administration of a substrate combination of pyruvate and malate (Pyr/Mal) results in the activation of isocitrate dehydrogenase, α -ketoglutarate dehydrogenase complex and malate dehydrogenase within the TCA cycle, giving rise to the reduction of NAD⁺ to form NADH [218]. These reducing equivalents feed electrons into complex I of the ETC, which subsequently travel down a thermodynamic gradient, through the Q-cycle and complex III, to complex IV where they are involved in the reduction of O₂ to H₂O [218]. Complex II is not involved in mitochondrial respiration in the presence of Pyr/Mal *in-vitro*, as malate equilibrates with fumarate at concentrations above 2mM [218]. This inhibits the conversion of succinate to fumarate by succinate dehydrogenase, preventing the formation of FADH₂ [218].

2) Complex II mediated respiration – Administration of rotenone inhibits the transfer of electrons from iron-sulfur centres in complex I to ubiquinone [220]. NADH-linked dehydrogenases within the TCA cycle subsequently become inhibited by the redox shift from NAD⁺ to NADH [220]. Under conditions of rotenone induced complex I inhibition, administration of succinate (Succ/Rot) supplies electron to the ETC exclusively through complex II via the activation of succinate dehydrogenase leading to the reduction of FAD to form FADH₂ [220]. Succinate administration, in the absence of rotenone inhibition, results in elevated mitochondrial concentrations of oxaloacetate, a potent competitive inhibitor of succinate dehydrogenase [220]. Reverse electron transfer from complex I to complex II also occurs if complex I is not inhibited, stimulating the production of ROS [220].

3) Combined Complex I and Complex II mediated respiration – The convergent transfer of electrons via complex I and complex II into the Q-cycle corresponds with the operation of the TCA cycle and mitochondrial respiration *in-vivo* [221]. Administration of a combination of pyruvate, malate and succinate (Pyr/Mal/Succ) allows for the complete operational function of the TCA cycle in permeabilized cells *in-vitro* [221]. The complex I substrate combination of Pyr/Mal alone does not allow for the transfer of electrons to complex II, owing to a substrate mediated inhibition
of succinate dehydrogenase [221]. The additional administration of succinate at high concentrations overcomes this inhibition and drives the activity of succinate dehydrogenase to produce FADH₂, complementing the formation of NADH via the activity of the NADH-linked dehydrogenases [221].

4) Fatty acid mediated respiration – Administration of octanoate, a medium chain FFA the oxidation of which by mitochondria is not carnitine dependent, supplies electrons produced during β -oxidation to the ETC via the reducing equivalent, electron-transferring flavoprotein (ETF) [221]. While the capacity to oxidize fatty acids has been found to be relatively low in isolated mitochondria, respiration rates have been demonstrated to improve significantly in the presence of malate [222, 223]. Substrate oxidation under these conditions (Oct/Mal) *in-vitro*, give a measure of mitochondrial respiration owing to β -oxidation together with the oxidation of acetyl-CoA through the TCA cycle [221, 223]. Indeed, it has been shown that respiratory rates are higher under substrate conditions that support the convergent transfer of electrons into the Q-cycle through complex I and ETF, than with electron input via either complex I or complex II alone [221].

3.2.1 – Outline of Variables Analysed in Study Two

3.2.1.1 *Highly Reactive Oxygen Species Production*

ROS are defined as any oxygen-containing species that exhibits a higher reactivity than O_2 [224]. The redox status of these molecules can range from relatively stable species such as O_2^- and H_2O_2 to what are termed hROS which include 'OH and ONOO- [224]. While many biochemical reactions are responsible for the generation of O_2^- and H_2O_2 *in-vivo*, hROS are mainly generated by non-enzymatic processes involving the mediation of transition metal cations [224]. Indeed, 'OH is generally produced through the reactions of mitochondrial produced O_2^- and H_2O_2 with transition metals such as iron or magnesium [224]. In addition, decomposition of ONOO- can also yield 'OH which is reported to have a half-life in the nanosecond range [224]. While direct tissue damage has only been attributed to O_2^- and H_2O_2 at supra-physiological concentrations, oxidative stress associated with the presence of 'OH is well established, owing to its high non-specific reactivity with a variety of biomolecules [224]. Whereas O_2^- and H_2O_2 are neutralized through the activity of catalase, peroxidases and superoxide dismutases, there are no known antioxidant enzymes responsible for the removal of hROS [224]. Analysis of hROS levels within mitochondria allows for determination of whether the functional response of the organelle to rhGH and rIGF-1 under conditions of substrate saturation has negative consequences in terms of an increased risk of oxidative damage to key mitochondrial components [215].

3.2.1.2 Electron Transport Chain Activity

The activity of ETC complexes, in terms of their ability to induce phosphorylation of ADP via the generation of an electrochemical potential across the IMM, has important implications for cellular function [219]. As noted previously, complex I and complex III of the ETC are the principal sites of ROS generation within mitochondria [215, 216]. In particular, the functional activity of complex I has been identified to play a key role in the regulation of ROS generation and in the determination of the efficiency of mitochondrial ATP production [64, 225]. Defects in respiratory chain activity, resulting in a decline in levels of cellular ATP production and an augmentation of the rate of ROS generation, are associated with an initiation of apoptotic pathways and are implicated in the age-associated decline of mitochondrial function [219]. Analysis of ETC activity, through the examination of $\Delta \psi_m$ values and hROS levels under various conditions of mitochondrial respiration, will provide an indication of how mitochondrial responses to rhGH and rIGF-1 are implemented and allow for determination of the principal source of ROS generation along the ETC.

3.2.2 - Study Two Aims

The primary aims of Study Two were as follows:

- 1. To investigate the role played by individual respiratory complexes in mediating the mitochondrial effects of rhGH and rIGF-1 through the examination of relative changes in $\Delta \psi_m$ and mitochondrial hROS levels under the respiratory conditions outlined in section 3.2 which to varying degrees allowed for control over the sites of electron entry into the respiratory chain at complexes I and II.
- 2. To investigate how rhGH and rIGF-1 mediated mitochondrial responses are affected under conditions of substrate saturation at both physiological and supra-physiological concentrations.

3.2.3 – Study Two Hypotheses

The hypotheses of Study Two were as follows:

- Both $\Delta \psi_m$ and hROS values will be significantly increased under conditions of respiration utilizing the activity of complex I at saturating substrate concentrations compared to conditions of respiration utilizing the activity of complex II at saturating substrate concentrations following the inhibition of complex I.
- Under conditions of respiration utilizing the activity of complex I at saturating substrate concentrations, neither rhGH nor rIGF-1 will exert any significant effect on $\Delta \psi_m$ in samples treated at physiological concentrations compared to untreated control samples. However, hROS values under these conditions will either not change or be significantly decreased. In contrast, both $\Delta \psi_m$ and hROS values will be significantly increased by both rhGH and rIGF-1 in samples treated at supra-physiological concentrations compared samples treated at physiological concentrations and untreated samples.
- Under conditions of respiration utilizing the activity of complex II at saturating substrate concentrations following the inhibition of complex I, neither rhGH nor rIGF-1 will exert any significant effect on $\Delta \psi_m$ or hROS values in samples treated at either physiological or supra-physiological concentrations compared to untreated control samples.

3.3 – Outline of Study Three

Study Three was designed to determine the effect of the administration of recombinant human growth hormone for one week on the regulation of mitochondrial mediated apoptosis in PBMCs from healthy male subjects who undertake regular physical activity.

Ten healthy male subjects between the ages of 18 and 35 years who had engaged in resistance training at least three times a week for a period of at least 12 months were recruited to participate in the study. Subjects were randomly assigned to receive either recombinant human growth hormone (n=5) (1mg – Genotropin, Pfizer, Australia) or a placebo (n=5) in the form of a solution of physiological saline (0.9%) for seven consecutive days. Treatment was administered subcutaneously in a double blind manner. The selected dosage was chosen in order to minimize the occurrence of adverse side effects observed in previous studies where rhGH was administered at higher dosages [148, 154, 180]. Such side effects include transient fluid retention, increased rates of sweating, carpel tunnel compression and joint edema and pain [148, 154, 180].

Blood was collected from each subject 24 hours prior to treatment in addition to 1, 8, 15 and 22 days post treatment. Subjects were instructed to fast for 12 hours prior to sample collection. PBMCs were isolated from whole blood by density gradient centrifugation. Once isolated, cytosolic and mitochondrial fractions were extracted from PBMCs using magnetic activated separation techniques. Total RNA and protein were subsequently extracted from both cytosolic and mitochondrial fractions using column based isolation techniques. Following synthesis of complementary DNA (cDNA) from total RNA, the level of gene expression for Bcl-2 and Bak in cytosolic fractions and miR-181a and miR-125b in both cytosolic and mitochondrial fractions was analysed by real-time polymerase chain reaction (RT-PCR). Protein concentrations of Bcl-2 and Bak in mitochondrial fractions were analysed using enzyme-linked immunosorbant assays.

3.3.1 – Outline of Variables Analysed in Study Three

3.3.1.1 Bcl-2 and Bak Expression

The Bcl-2 protein is an anti-apoptotic member of the Bcl-2 family whose association with apoptotic effector molecules on the mitochondrial outer membrane prevents proteolipid pore formation and the subsequent release of apoptotic proteases from the inter-membrane space [56]. Bak is a pro-apoptotic member of the Bcl-2 family, termed an effector molecule, which is localised to the mitochondrial outer membrane [59]. Following activation by BH-3 only proteins, homo-oligomerization of Bak leads to permeabilization of the mitochondrial outer membrane, an event which represents the irreversible execution phase of mitochondrial mediated apoptosis [59]. Analysis of the gene and protein expression of these apoptotic regulatory proteins allows for the determination of how rhGH administration affects the regulation of mitochondrial mediated apoptosis in PBMCs.

3.3.1.2 Cytosolic and Mitochondrial miRNA Expression

Both of the miRNAs investigated in Study Three were chosen as they had previously been demonstrated to target either Bcl-2 or Bak mRNA [99, 102, 226-228]. MiR-181a has been demonstrated to act as a potent inhibitor of cellular proliferation and has been experimentally validated to down-regulate the expression of Bcl-2 [99, 227, 229]. MiR-125b, which has been shown to provide protection against cellular senescence and apoptosis, has been experimentally validated to down-regulate the expression of Bak [102, 226, 228, 230]. Analysis of the effects of rhGH on the expression of these miRNA in both cytosolic and mitochondrial fractions allows for the investigation of potential pathways by which rhGH could mediate the regulation of mitochondrial mediated apoptotic effects.

3.3.2 – Study Three Aims

The primary aims of Study Three were as follows:

- 1. To investigate the potential implications of the administration of rhGH in healthy male subjects for cellular viability through the analysis of the hormone mediated effects on the expression of the apoptotic regulatory proteins, Bcl-2 and Bak.
- 2. To determine whether the protection provided by rhGH's putative antiapoptotic effects remains sufficient, in the long term, to counter the development of pro-apoptotic stimuli that are associated with prolonged elevations in GH concentrations *in-vivo*.
- 3. To examine whether both cytosolic and mitochondrial associated miRNA signalling could be responsible for the rhGH induced changes observed in the expression of Bcl-2 associated regulatory proteins.

3.3.3 – Study Three Hypotheses

The hypotheses of Study Three were as follows:

- Bcl-2 expression will be significantly up-regulated while Bak will be significantly down-regulated following rhGH administration. However, the significance of these initial anti-apoptotic effects will become attenuated by 22 days post-treatment.
- The cytosolic expression of miR-181a will be significantly decreased while expression of cytosolic miR-125b will be significantly increased following rhGH administration. However, the significance of these initial anti-apoptotic effects will become attenuated by 22 days post-treatment.
- The mitochondrial expression of miR-181a will be significantly increased while expression of mitochondrial miR-125b will be significantly decreased following rhGH administration. However, the significance of these initial anti-apoptotic effects will become attenuated by 22 days post-treatment.

4. Methods of Analysis

4.1 – Isolation Techniques

4.1.1 – Cell Isolation: Density Gradient Centrifugation

Density gradient centrifugation, using Ficoll-Paque PLUS, allows rapid and efficient isolation of mononuclear cells from human peripheral blood without altering either the phenotype or function of the mononuclear cell population [231]. Ficoll-Paque PLUS, a solution consisting of a mixture of the high molecular weight polysaccharide Ficoll with sodium diatrizoate, exhibits a low viscosity and has density of 1.077g/mL [231]. Anticoagulated blood (5mL) is diluted with an equal volume of phosphate buffered saline (PBS), layered over 3mL of Ficoll-Paque PLUS and centrifuged at 900*g, $18^{\circ} - 20^{\circ}$ C, for 30 minutes [231]. The specific viscosity, osmolarity and density of Ficoll-Paque PLUS allows for the differential separation of mononuclear cells during centrifugation from other elements found in the blood [231, 232]. Ficoll acts as an erythrocyte aggregant at room temperature, causing these cells to sediment through the Ficoll-Paque PLUS layer at an accelerated rate [232]. In addition, the osmotic pressure placed on granulocytes by the hypertonic Ficol-Paque PLUS increases their density above that of the separation medium resulting in their sedimentation at the bottom of this layer [231]. Lymphocytes, monocytes, and platelets all have a lower density than the separation medium and accumulate at the interface between the plasma and the Ficoll-Paque PLUS layer [231]. These cells are then recovered from the interface while platelets are separated from the mononuclear cells by subsequent washing in a balanced salt solution [231]. Sample yields are reported to range between one and two million mononuclear cells per mL of blood in health individuals [231]. Approximately 60% to 70% of isolated mononuclear cells are reported to be lymphocytes with a viability of > 95%[231]. Monocytes and macrophages make up the remainder of the population and are reported to have a viability of > 95% [231].

4.1.2 – Mitochondrial Isolation: Magnetic Cell Sorting

Magnetic activated cell sorting (MACS) is an isolation technique which utilizes superparamagnetism to separate cells through the magnetic labelling of specific cell surface antigens [233]. While originally developed for the purification of specific cell populations, the technique has been successfully applied to the isolation of cellular organelles including Golgi vesicles [234], endosomes [235], lysosomes [236], nuclei [237], and mitochondria [107, 238]. Indeed, it has been demonstrated that mitochondrial isolation using the MACS approach provides a high yield of functionally intact mitochondria in a fast, reproducible and standardized procedure which can be performed with as few as $1*10^6$ cells [233].

The procedure involves the labelling of mitochondria in cell lysate with anti-TOM22 antibody conjugated microbeads [107, 233]. These monoclonal antibodies bind specifically to the 22-kDa translocase of outer mitochondrial membrane (TOM22) on the cytoplasmic surface of human mitochondria [107, 233]. Microbeads are an evenly distributed suspension of super-paramagnetic nanoparticles, 50nm in diameter, which allow for the efficient isolation of labelled mitochondria as they are passed through high gradient magnetic columns [107, 233]. Cellular contaminants are washed from the columns which are subsequently removed from the magnetic field, allowing for the elution of isolated mitochondrial fractions [107, 233].

As demonstrated by Hornig et al. [233], mitochondrial isolation by MACS compares favourably with traditional and frequently used differential centrifugation (DC) protocols as well as more sophisticated density gradient ultra-centrifugation (UC) procedures. Flow cytometric analysis of mitochondrial fractions showed that mitochondria isolated using the MACS protocol were found to be significantly enriched, with 89% of isolated events found to be TOM22 positive, compared with 59% of the mitochondrial fraction prepared by DC [233]. In addition, the functional quality (assessed through the determination of respiratory control ratios; a measure of the tightness of mitochondrial respiration) and purity (defined as the absence of contaminants from the endoplasmic reticulum and nucleus) of mitochondrial fractions was shown to be significantly enhanced using MACS compared with DC [233]. In contrast, the UC method exhibited levels of mitochondrial enrichment (88% of events were TOM22 positive) which did not significantly differ from the MACS approach [233]. Furthermore, the quality and purity of MACS isolated mitochondria was comparable to mitochondria isolated using UC, a procedure which is both more time consuming and costly than MACS [233].

4.1.3 – Total RNA Purification: Solid Phase Extraction

Commercially available kits for RNA purification utilize either a conventional organic phase extraction, a solid phase extraction or a combination of these procedures [239]. Organic phase extraction is a single step technique whereby RNA is isolated from DNA and other organic biomolecules following the biphasic separation of a guanidinium thiocyanate-phenol-chloroform solution into organic and aqueous phases under acidic conditions [240, 241]. Guanidinium thiocyanate is a chaotropic agent which denatures proteins, disrupting protein-nucleic acid interactions and inactivating nuclease enzymes [240, 241]. Total RNA is collected from the aqueous phase of the solution and recovered by alcohol precipitation [240, 241]. While organic phase extraction has been demonstrated to deliver high yields across all sizes of RNA [240], the process is time consuming and samples isolated by the method are reported to be frequently contaminated with proteins, genomic DNA (gDNA) and organic solvents owing to incomplete phase separation during liquid-liquid extraction [240]. In contrast, solid phase extraction has been demonstrated to be efficient protocol which allows for the recovery of high quality RNA exhibiting low levels of contamination from proteins and other organic biomolecules without the required use of toxic organic solvents [241, 242].

Solid phase extraction of nucleic acids involves the passing of a dissolved sample through the solid phase material of what are termed "spin-columns" under centrifugal force [241, 243]. Specific nucleic acids (DNA or RNA) selectively bind to the solid phase material under defined pH and ionic conditions [241, 243]. Solid phase extraction is a four step process which involves cell lysis, nucleic acid adsorption to solid phase material, washing and elution [241, 243]. Cells are first homogenised in a buffer containing detergents and high concentrations of chaotropic salts, resulting in cell lysis, protein denaturation and nuclease inactivation [241, 243]. A silicon-based affinity medium is commonly used for the adsorption of nucleic acids, which is dependent on the presence of chaotropic ions at high concentrations [241, 243]. Under conditions of high ionic strength, the hydration shells surrounding nucleic acids and the silicon-based medium become destabilized, resulting in the dehydration of both [241, 243]. Positively charged ions subsequently saturate the silicon-based molecules which allows for the formation of cationic salt bridges between the affinity medium and the negatively charged phosphate backbone of nucleic acids [241, 243]. Ionic conditions can be optimized to favour RNA adsorption over that of DNA [241, 243]. Although DNA contamination of the affinity medium does occur during RNA purification, even under optimized conditions, this can be removed by digestion through the application of DNase directly onto the solid phase medium [244]. Subsequent washing of the affinity

medium allows for the removal of salt, protein and other cellular contaminants [241, 243]. Purified RNA molecules become rehydrated following the application of either TE buffer or distilled water to the spin-column and are finally eluted under conditions of low ionic strength [241, 243].

Most commercially available spin-columns utilize silica (SiO₂) based materials such as glass fibre or silica gel as their RNA affinity medium [241, 242]. However, silicon carbide (SiC) was utilised as the chromatographic medium of RNA purification in Study Three. It has been reported that commercially available kits, designed for the purification of total RNA, which utilize a silica-based separation medium selectively exclude RNAs that are less than 200 nucleotides in size and do not efficiently recover the miRNA fraction [245]. Indeed miRNA yields have been demonstrated to be between 3.7 - 7.9 times higher using SiC-based RNA purification over silica-based RNA binding columns [246]. In addition, SiC-based RNA binding columns were found to exhibit the highest RNA yield and the broadest RNA size distribution when compared to phenol-based RNA purification, silica-based RNA binding column extractions and combined phenol / silica-based column approaches [239].

4.2 – Flow Cytometry

Flow Cytometry is a technology that utilizes light scattering and fluorescence emission to quantitatively analyse the properties of individual particles, such as cells, as they flow in a fluid stream through a beam of light [247]. A flow cytometer consists of several systems which work in unison in order to provide a quantitative measurement of cellular parameters [247, 248]. The fluidics system uses hydrodynamic focusing to transport a single stream of particles through a laser beam for interrogation [247]. The biological sample is injected into a stream of sheath fluid in the flow chamber, where differences in the density and velocity of the two fluids allows them to form a two-layer stable flow (laminar flow) that does not interact [247]. The sheath fluid flows at a greater velocity than the sample fluid, restricting it to the centre of the flow chamber. The optical system consists of a laser and a series of focusing lenses which generate light signals when they interact with particles in the flow stream, and a series of dichroic mirrors and bandpass filters which direct specific wavelengths of light to the appropriate photo-detector for signal processing [247, 248]. Light emitted from sample particles in the flow stream generates a small current when it comes into contact with a photo-detector, the

amplitude of which is proportional to the total number of light photons received by the detector [247]. The electrical current is amplified by a series of linear and logarithmic amplifiers and is subsequently converted to a voltage pulse, which is assigned a digital value by the Analog-to-Digital Converter (ADC). The ADC converts a pulse ranging from 0-1000 mV to a digital number representing 0-1000mV channels, with each voltage pulse generated expressing data from a single particle [247]. Finally, computer software is used to store the data, display results graphically and generate statistics [247, 248].

4.2.1 – Measurement Parameters

4.2.1.1 Light Scatter

Sample particles deflect laser light as they pass through the beam, scattering light in all directions. Light scattered in a forward direction is measured approximately 20° offset from the axis of the laser beam and is collected in the forward scatter channel (FSC) by a type of photo-detector called a photodiode [247]. Forward scatter light is proportional to a particle's surface area and equates to a particle's size [247]. Side scatter light, which is measured perpendicular to the axis of the laser beam, is collected in the side scatter channel (SSC) by a photomultiplier tube (PMT) detector [247]. Side scatter light reflects cellular granularity or the internal complexity of sample particles [247].

4.2.1.2 *Fluorescence*

Light is a form of electromagnetic energy that travels in waves [249]. The energy content of a light beam is directly proportional to its wave frequency and inversely proportional to wavelength, which also determines the colour of light [249]. A fluorescent compound is capable of absorbing light energy within a specific wavelength range that is characteristic of that compound, leading to excitation of the electrons within that compound which raises them to a high energy unstable state [247]. The compound subsequently undergoes conformational changes and returns to a more stable state by releasing some of the absorbed energy as heat [247]. Fluorescence refers to the subsequent release of the remaining energy as light which is emitted at a longer wavelength to the light source which originally excited the compound [249]. The

difference in wavelength between the excitation light source (E $_{\text{excitation}}$) and the fluorescent light source (E $_{\text{emission}}$) is called Stokes shift [249].

A flow cytometer uses lasers to focus light consisting of a single wavelength on the individual particles of a sample [247-249]. In order to analyse specific cellular parameters, fluorescent probes called fluorochromes that are maximally excited within the specific wavelength utilized by a flow cytometer are used to target specific cellular antigens or intracellular molecules. This allows for the biological and biochemical properties of the target molecule to be quantified by the subsequent emission of fluorescent light, as the amount of fluorescent signal detected is proportional to the number of fluorochrome molecules on the particle [247, 249]. Fluorescent probes have a wide range of applications including, quantifying distinct cellular populations, cell surface receptors or intracellular organelles, measuring enzyme activity and quantifying cellular ion concentrations [247]. Flow cytometers use separate fluorescence (FL-) channels to detect the specific wavelength of light emitted from fluorochromes [247]. A list of the fluorochromes used in this thesis and their respective excitation and emission wavelengths are presented in table 4.1 below.

Fluorochrome	Assay Parameter	Excitation Peak	Emission Peak
JC-1 (Monomer)	Mitochondrial Membrane Potential	514nm	529nm
JC-1 (Aggregate)		514nm	590nm
DilC ₁ (5)	Mitochondrial Membrane Potential	638nm	658nm
MitoSOX Red	Mitochondrial Superoxide Production	510nm	580nm
Annexin V-Cy5	Apoptotic Cells	625nm	670nm
Sytox Green	Dead Cells	504nm	523nm
Calcein	Mitochondrial Permeability Transition Pore Activity	495nm	515nm
HFP	Highly Reactive Oxygen Species	490nm	515nm

 Table 4.1: Summary of the excitation / emission wavelengths of the fluorochromes

 utilized and the associated parameters analysed in Studies One and Two.

4.2.2 – Data Analysis

Measurements made on each photo-detector of a flow cytometer provide data on a single parameter or variable [247]. Computer software allows this data to be graphed and analysed statistically [247, 248]. Single parameters can be displayed on a histogram with the parameters signal value in channel numbers displayed on the x-axis and the number of events recorded represented on the y-axis [247]. Two measurement parameters can be displayed on a dot plot, with one parameter represented on the x-axis and the other on the y-axis [247]. Contour plots and density plots also provide a two parameter display, while using contour lines and colour changes respectively to provide a three dimensional representation of the event count [247]. Statistics generated can provide the arithmetic mean, the geometric mean, or the median channel values for any given parameter, which allow for interpretation of the central tendency of the data [250].

During data collection the conversion of a light scatter or fluorescent signal into a digital value can be processed by either linear or logarithmic amplification of the electrical current produced by a photo-detector [247]. Linear data is reported on a 1024 channel scale in proportion to the intensity of collected light and displayed graphically on axes with linear values [251]. Logarithmic amplified data is reported on a 1024 channel scale with the digital channel number proportional to the log of the original light intensity [251]. Linear amplification displays data over a limited range of intensities (1-1024) and subsequently is used for analysis of parameters in which a narrow range of signal intensity is expected, such as forward scatter and side scatter light [251]. Logarithmic amplification is normally used to display data of fluorescent parameters as it compresses strong signals which range up to hundreds or thousands of times the intensity the intensity of weak signals, thus allowing such data to be displayed graphically [247, 251]. Flow cytometry software allows for analysis of the relative intensities of logarithmically amplified signals by converting the channel values to a number that corresponds to the fluorescent intensity of the original light signal [252]. These values are displayed graphically on a four log decade scale $(10^{0} - 10^{4})$ and correspond to linear values in the range of 1 - 10,000 [252].

A frequent goal of flow cytometric analysis is to classify cells as positive or negative for a given marker or to determine the precise ratio of positive to negative cells [253]. In order to accurately distinguish between positive and negative data sets, the use of negative control samples is required [253]. Commonly used control samples in flow cytometry include negative isotype controls that provide non-specific staining for an antibody of a particular isotype conjugated to a particular fluorochrome and biological comparison controls, which provide relevant comparison conditions to determine the degree of positivity or negativity for a particular marker [253]. For example, assays examining the functional activity of a given cellular parameter by stimulating the activity of that parameter may use an unstimulated sample as a means of distinguishing between positive and negative events [253]. The use of histogram markers on single parameter graphs and quadrant markers on dual parameter graphs can identify positive and negative data sets, the percentages of which are analysed statistically [247].

Computer software allows for subsets of collected data to be analysed in isolation through the use of a "gate" [247]. The purpose of creating gates is to identify cells of interest while excluding data from unwanted cells [247]. A gate creates numerical or graphical boundaries based on the parameters displayed on the graph which is being analysed [247]. These boundaries define the characteristics of particles to be included in further analysis [247]. The most common application of gating is the targeting of specific cell populations when analysing blood samples [247]. Graphing forward scatter and side scatter parameters on a dot plot allows for visualization of specific cellular populations, such as granulocytes, monocytes and lymphocytes due to differences in the physical properties of these cells, which makes it possible to create boundaries around the population of interest [247].

4.2.3 – Analysis of Variables

4.2.3.1 *Mitochondrial Membrane Potential* $(\Delta \psi_m)$

In Study One, $\Delta \psi_m$ was analysed on a FACS Calibur flow cytometer using the MitoProbeTM JC-1 Assay Kit (Life Technologies, Australia). JC-1 (5,5',6,6'tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide) is a lipophilic cationic dye that exhibits potential dependent accumulation in the mitochondrial matrix [254, 255]. The fluorescence emission of JC-1 changes depending on $\Delta \psi$, due to the reversible formation of JC-1 aggregates upon membrane polarization [256]. When concentrations of the dye in the mitochondria are low, the dye exists in its monomeric form, emitting light at an emission maximum of 530nm (green) when excited at 488nm [254]. When concentrations of the dye in the mitochondria are high, the dye aggregates to form Jcomplexes which emit light at an emission maximum of 590nm (orange) when excited at 488nm [254]. Aggregate formation begins at potential values corresponding to approx 80 - 100 mV and reaches a maximum at approx 200 mV, as illustrated in figure 4.1 [256]. Measurements provided by JC-1 are qualitative, based on the ratio of fluorescence emission at 590nm and 530nm, and quantitative, based on the absolute values of both green and red fluorescence emission [256]. The orange / green fluorescence intensity ratio gives an indication of $\Delta \psi_m$ that is independent of factors such as mitochondrial volume, shape or density, allowing comparative measurements of membrane potential and determination of the percentage of mitochondria within a population that respond to an applied stimulus [254, 257, 258].

In Study Two, $\Delta \psi_m$ was analysed by flow cytometry using the $\Delta \psi_m$ indicator, 1,1',3,3,3',3'-hexamethylindodicarbo - cyanine iodide (DilC₁(5)) (Life Technologies, Australia). DilC₁(5) is a cationic dye that, at concentrations below 100nM, accumulates primarily in mitochondria with active membrane potentials [259]. It emits a peak fluorescence emission of 658nm when excited at 633nm [259].

Negative control samples were treated with the mitochondrial uncoupler carbonyl cyanide 3- chlorophenylhydrazone (CCCP), a protonophore that carries H⁺ ions across lipid membranes, to confirm that the JC-1 and DilC₁(5) responses were sensitive to $\Delta \psi_m$ [255]. Positive control samples were treated with oligomycin, an antibiotic that inhibits oxidative phosphorylation by binding to ATP synthase and which has previously been utilised as a positive control for $\Delta \psi_m$ in isolated organelles [258]. By

blocking the flow of protons into the mitochondrial matrix through the F_0 subunit of ATP synthase, oligomycin maximizes the proton gradient as it has no direct effect on electron transport [258]. Figure 4.1 illustrates the effects exerted by CCCP and oligomycin on JC-1 fluorescence.



Figure 4.1: Flow cytometric analysis of mitochondrial membrane potential. **A)** Cellular and mitochondrial loading of JC-1, *image adapted from Cossarizza [260]*. **B)** Dot plot displaying green vs. orange mean channel fluorescence values for JC-1 from gated lymphocytes in the presence of CCCP. C) Dot plot displaying green vs. orange mean channel fluorescence values for JC-1 from gated lymphocytes in the presence of oligomycin. **D)** Dot plot displaying green vs. orange mean channel fluorescence values for JC-1 from gated lymphocytes in the presence of oligomycin. **D)** Dot plot displaying green vs. orange mean channel fluorescence values for JC-1 from gated lymphocytes in untreated cells.

4.2.3.2 Mitochondrial Superoxide Generation and Cellular Viability

In Study One, mitochondrial superoxide production was analyzed by flow cytometry using MitoSOXTM Red Mitochondrial Superoxide Indicator (Invitrogen, Australia). MitoSOX Red is a derivative of the flourochrome hydroethidine (HE) that contains a positive charge on its phosphonium group, allowing it to accumulate in mitochondria as a function of $\Delta \psi_m$ [261]. When the probe is oxidized, it leads to the fluorescent product 2-hydroxy-ethidium, which stains the DNA of mitochondria and emits light with a peak emission of 567nm when excited at 488nm [261]. MitoSOX Red is readily oxidized by O_2^- but exhibits a limited response to other ROS and oxidation of the probe is prevented by superoxide dismutase [261]. The use of MitoSOX Red by flow cytometry has been validated for the selective detection of mitochondrial superoxide production in a wide range of cell types [262-264].

The advantage of this method of analysis over other cell based techniques is that it provides quantitative measurements of mitochondrial superoxide generation in live cells without compromising cellular function [261, 262]. During data analysis it is necessary to exclude fluorescent values from dead cells as dissipation of the $\Delta \psi_m$ during cell death causes release of the fluorophore from the mitochondria and leads to staining of nuclear DNA, resulting in large increments in fluorescence emissions which do not accurately indicate superoxide levels [261]. Annexin V and Sytox Green are fluorometric markers of apoptotic and dead cells respectively [261]. Annexin V is a 35–36 kDa phospholipid binding protein that has a high affinity for phosphatidylserine, a phospholipid that is externalised to the surface of plasma membranes in cells undergoing apoptosis [265]. When conjugated to the cyanine dye, Cy5, the probe emits light at a peak value of 660nm when excited at 633nm [266]. Sytox Green binds to the DNA of dead cells and emits fluorescence at an emission peak of 523nm when excited at 488nm, as it can only enter cells that have lost their plasma membrane integrity, as illustrated in figure 4.2 [261].

Antimycin A, which inhibits mitochondrial respiration and augments mitochondrial superoxide production by inhibiting electron transfer at complex III of the ETC, was used as a positive control in order to confirm the sensitivity of MitoSOX Red for the detection of mitochondrial superoxide generation [261]. Figure 4.2 illustrates the effects exerted by antimycin A on MitoSOX Red fluorescence. Negative control

samples were treated with superoxide dismutase-polyethylene glycol (SOD-PEG), an antioxidant protein and superoxide scavenger conjugated to the polyether compound polyethylene glycol which prolongs the circulatory half-life of the native enzyme and enhances its ability to diffuse across plasma membranes [261].



Figure 4.2: Flow cytometric analysis of mitochondrial superoxide production **A**) Depiction of how AnnexinV-Cy5 and Sytox Green label apoptotic and dead cells respectively, *image adapted from Imgenex [267]*. **B**) Dot plot displaying mean channel fluorescence values for Sytox Green vs. AnnexinV-Cy5 from gated lymphocytes in untreated cells. **C**) Dot plot displaying mean channel fluorescence values for Sytox Green vs. AnnexinV-Cy5 from gated lymphocytes in the presence of antimycin A. **D**) Histogram displaying mean channel fluorescence values for MitoSOX Red from gated live cell population. **E**) Histogram displaying mean channel fluorescence values for MitoSOX Red from gated live cell population in the presence of antimycin A.

4.2.3.3 Mitochondrial Permeability Transition Pore Activity

In Study One, mtPTP activity was assessed by flow cytometry using the MitoProbeTM Transition Pore Assay Kit (Life Technologies, Australia)), which allows for direct examination of mtPTP opening through monitoring of the mitochondrial fluorescence of calcein, a fluorescent dye that emits light at a peak intensity of 517nm when excited at 488nm [268]. Although the probe itself is cell impermeant, calcein can be loaded into cells and accumulate in cytosolic compartments, including mitochondria, by using its acetoxymethyl ester form (calcein-AM) [268]. Once the acetoxymethyl ester is cleaved by intracellur esterases the dye present in the mitochondria becomes trapped [268]. In order to detect changes in mitochondrial calcein fluorescence in intact cells, the fluorescent signal is quenched by the addition of cobalt chloride (CoCl₂) in both cytosolic and nuclear compartments [268]. Petronilli et al. [268] used confocal microscopy to confirm the localization of calcein fluorescence to mitochondrial compartments in MH1C1 cells. Opening of the mtPTP results in a spontaneous decrease in the mitochondrial fluorescence of calcein which is mediated by either the entry of CoCl₂ into the mitochondrial matrix or the mitochondrial release of calcein, as illustrates in figure 4.3 [268].

Ionomycin, a Ca^{2+} ionophore that allows excess Ca^{2+} to enter mitochondria, was used as a positive control as mitochondrial Ca^{2+} overload is known to induce mtPTP activation [269]. Nicolli *et al.* [269] confirmed that the decrease observed in the mitochondrial fluorescence of calcein in the presence of Ca^{2+} ionophores is induced by mtPTP opening because the response is significantly reduced by cyclosporine A (CsA), a polypeptide that is known to prevent mitochondrial transition pore formation by binding to Cyp D. In addition, cyclosporine H (CsH), an analog of CsA that does not inhibit mtPTP activation was found to have no effect on mitochondrial calcein fluorescent emissions [269]. Figure 4.3 illustrates the effects exerted by ionomycin and CsA on calcein fluorescence.

Early research into the activity of the mtPTP *in-vivo* provided indirect measurements based on events that are secondary to the induction of mtPTP opening, such as mitochondrial depolarization and mitochondrial matrix swelling [268, 270-272]. Such events are subject to a variety of influences and may not accurately reflect mtPTP activity [268]. Flow cytometric analysis using the calcein – CoCl₂ method allows for

direct measurement of mtPTP opening in living cells [268]. A limitation of this technique is that Co^{2+} can adversely affect cellular respiration by inhibiting mitochondrial Ca^{2+} uptake, however Petronilli *et al.* [268] note that most of the Co^{2+} present in cells is bound to calcein in the cytosol and that calcein fluorescent emissions respond normally to mtPTP agonists and antagonists.



Figure 4.3: Flow cytometric analysis of mitochondrial permeability transition pore activation. A) Cellular and mitochondrial loading followed by cytosolic quenching of calcein, *image adapted from Salisbury [273]*. B) Histogram displaying mean channel fluorescence values for calcein from gated lymphocytes following cobalt chloride quenching in the presence of ionomycin. C) Histogram displaying mean channel fluorescence values for calcein from gated lymphocytes following cobalt chloride quenching in untreated cells. D) Histogram displaying mean channel fluorescence values for calcein from gated lymphocytes following cobalt chloride quenching in untreated cells. D) Histogram displaying mean channel fluorescence values for calcein from gated lymphocytes following cobalt chloride quenching in untreated cells. D) Histogram displaying mean channel fluorescence values for calcein from gated lymphocytes following cobalt chloride quenching in the presence of CsA.

4.2.3.4 *Highly Reactive Oxygen Species Production*

In Study Two, hROS production was analysed by flow cytometer using the ROS indicator, 3'-p-hydroxyphenyl fluorescein (HPF) (Life Technologies, Australia). HPF is a hROS indicator with a high specificity for 'OH and the ONOO– [274-276]. It emits a peak fluorescence emission of 515nm when excited at 488nm [274]. HPF offers greater specificity and a higher resistance to light induced oxidation than commonly used ROS indicators such as dichlorofluorescin (DCF) [274, 275]. Positive control samples were treated with H_2O_2 in the presence of ammonium iron II sulphate in order to induce 'OH production via the Fenton reaction. Figure 4.4 illustrates the effects exerted by H_2O_2 and ammonium iron II sulphate on HPF fluorescence.



Figure 4.4: Flow cytometric analysis of highly reactive oxygen species production. **A**) Histogram displaying mean channel fluorescence values for HPF from gated lymphocytes in untreated cells. **B**) Histogram displaying mean channel fluorescence values for HPF from gated lymphocytes in the presence of H_2O_2 and ammonium iron II sulphate.

4.3 – Real-time RT-PCR

The processes of reverse transcription (RT) and polymerase chain reaction (PCR) (RT-PCR) provide a powerful combination which is applicable to the detection and quantification of both mRNA and miRNA [277, 278]. However, PCR efficiency becomes compromised as amplification product, which are termed amplicons, accumulate owing to a depletion of reaction components and the re-association of the amplified target strands with each other, which inhibits primer annealing [279]. As a result, the exponential rate at which template is generated following each cycle of the

PCR reaction eventually declines, making the end point quantitation of amplicons following conventional PCR unreliable [279]. Real-time PCR uses fluorescent reporter molecules to measure the products generated during each cycle of the PCR reaction, combining nucleic acid amplification and amplicon detection into one homogenous assay [280]. Identification of amplification products by fluorescence detection in real-time is highly accurate compared with classical end point detection [280]. In addition, a wide dynamic quantification range allows for the analysis of samples that differ in their target abundance by orders of magnitude (> $10^7 -$ fold) [281]. Thus, real-time RT-PCR represents the standard for the detection and quantification of gene expression levels, in particular for the detection of RNA from limited amounts of sample and for the elucidation of small changes in RNA expression levels [277].

4.3.1 – Assay Parameters

4.3.1.1 *Reverse Transcription*

The production of a single-strand cDNA copy from isolated RNA represents the first phase of the RT-PCR process [280, 281]. Oligonucleotide primers anneal to the RNA template, permitting its elongation through the RNA-dependent DNA polymerase activity of the retroviral enzyme, reverse transcriptase [280]. The choice of primers used to initiate cDNA synthesis can be either target gene-specific or non-specific [280]. Target gene-specific primers allow RT reactions to take place at elevated temperatures which increases specificity and decreases the background noise associated with unwanted transcripts [280]. However, the use of gene-specific primers necessitates that a separate RT reaction be carried out for each gene of interest which can result in high inter-assay variability [280]. In contrast, the use of non-specific primers results in the synthesis of a cDNA "pool" from which separate target specific PCR reactions can be assayed [280]. Non-specific primers include random hexamers, which contain all possible nucleotide combinations of a 6-base oligonucleotide allowing for binding at multiple origins along every RNA template and poly-T oligonucleotides (Oligo-dT), which consist solely of 16-25 deoxythymidine residues allowing for annealment to polyadenylated 3' (poly-A) tails found on most mRNAs [280]. In addition, mature miRNAs can be reversed transcribed into cDNA through the use of oligo-dT primers following their polyadenylation through the activity of poly (A) polymerase [282]. RT reactions primed by random hexamers and oligo-dT primers maximize the number of genes that can be assayed from small RNA yields [280].

4.3.1.2 Polymerase Chain Reaction

PCR is the amplification of a specific target sequence through repeated cycles of temperature manipulation which facilitates enzymatic replication of the target amplicon [277, 278]. The process utilizes the ability of the thermostable enzyme *Thermus aquaticus* (Taq) DNA polymerase to synthesize DNA strands which are complementary to the template available [278]. However, DNA polymerase can only add free nucleotides to a pre-existing 3'-OH group, necessitating that oligonucleotide primers which exhibit unique complementarity to the target amplicon be base paired to the template sequences [283]. Each cycle of the reaction consists of a three step process which is initiated through heating the reaction to 94°C resulting in the denaturation of all ds-DNA present [284]. The reaction is subsequently cooled to the specific annealing temperature (in the range of $40 - 60^{\circ}$ C) of the two primers present, allowing them to hybridize to their complementary sequences on opposite strands of the target amplicon [284]. The cycle is completed by heating the reaction to 72°C to allow for elongation of the template strands by DNA polymerase [284].

As a PCR progresses, the reaction goes through three distinct phases of product amplification; the exponential phase, the linear phase and the plateau phase [285]. During exponential amplification there exists a high degree of efficiency surrounding amplicon production where the amount of DNA synthesized is theoretically doubled following each successive cycle [285]. This is followed by the linear phase of amplification where the efficiency of the reaction slows down owing to a decrease in the critical concentrations of one or more reaction components [285]. As a result increases in amplified product are observed on an arithmetic rather than logarithmic scale [285]. As reaction components become increasingly limited, the rate of target amplification increases until the PCR reaches its plateau phase where there is little or no increase in PCR product [285]. The amount of amplified target is directly proportional to its original concentration in the starting template only during the exponential phase of PCR amplification [285].

4.3.1.3 *Amplicon Detection*

Real-time RT-PCR measures the progress of amplification through the monitoring of changes in fluorescence within the PCR reaction [279]. This is achieved through the addition of non-sequence specific fluorescent intercalating double stranded DNA (dsDNA) binding agents, such as SYBR Green, to the reaction mix [279]. SYBR Green, which has an excitation maxima of 497nm and an emission maxima of 520nm, exhibits an extremely low intrinsic fluorescence [286]. However, its fluorescence quantum yield is increased by up to 1000 fold when intercalated into dsDNA [286]. Thus, the intensity of the fluorescent signal is dependent on the quantity of dsDNA present in the reaction [286]. The level of fluorescence is measured at the end of the elongation phase of each PCR cycle, when the quantity of dsDNA reaches its maximum level [286].

A limitation to the use of SYBR Green is that the dye binds to all dsDNA formed during the PCR reaction and is therefore not specific to the target amplicon, allowing for the presence of non-specific PCR products and "primer-dimers" to generate artefact within the fluorescent signal [277, 287]. Melt curve analysis is therefore used to monitor the specificity of the reaction [277, 287]. At the end of the PCR run, fluorescence is constantly measured as the temperature of the reaction is gradually raised at a constant rate from a low temperature where all amplified products are stable to a point at which all dsDNA present has become fully dissociated [287]. Melt curves, as shown in figure 4.5, are subsequently generated through plotting the negative first derivative of the change in fluorescence as a function of temperature (-dF/dT) against temperature (Tm) of the amplicon (which corresponds to the point at which 50% of target DNA is denatured) will distinguish it from amplification artefacts that melt at lower temperatures and exhibit broader peaks [277, 287].



Figure 4.5: Melt curve displaying the negative first derivative of the change in fluorescence as a function of temperature (-dF/dT) against temperature ($^{\circ}C$)

4.3.2 – Data Analysis

4.3.2.1 *Cycle Threshold*

Amplification plots, as shown in figure 4.6, are generated using the fluorescence emission data collected during the PCR protocol [278]. A baseline reading is recorded during the initial PCR cycles in which a fluorescent signal is being accumulated but is beneath the detection capabilities of the instrument [278]. Quantification in real-time RT-PCR is achieved through the measurement of the number of cycles required for the fluorescent signal to cross a threshold level [278]. This arbitrary threshold must be set at a value at least three standard deviations above the baseline noise level and occur in the exponential phase of target amplification in order to produce accurate and reproducible data [278]. The cycle number at which a fluorescent signal is detected above this chosen threshold value is defined as the "cycle threshold" (Ct) [278]. The Ct of a sample is inversely proportional to the concentration of target sequence in the starting template [278]. Thus, the more target copies present at the beginning of a reaction, the fewer cycles of amplification that are required to generate the number of amplicons which can be detected reliably [278].



Figure 4.6: RT-PCR amplification plot displaying fluorescence emissions against cycle threshold.

4.3.2.2 *Relative Quantification*

Relative quantification uses the comparative threshold method $(2^{-\Delta\Delta Ct} \text{ method})$ to determine approximate changes in the steady state expression of a gene across multiple samples relative to the expression of an internal control RNA [278]. The method compares the Ct values from target RNAs to those from one or more endogenous reference or 'housekeeping' genes with the results expressed as ratios of the target specific signal to that of the endogenous reference [278]. Mathematical equations are used to calculate the expression of 'experimental' target samples in relation to a reference 'control' sample such as a non-treated or placebo treated sample [278]. The amount of target RNA in an experimental sample, normalized to an endogenous housekeeping gene and relative to the normalized control sample is thus given by equation 4.1:

 $2^{-\Delta\Delta Ct}$,

where $\Delta\Delta Ct = \Delta Ct$ (experimental sample) – ΔCt (control sample), and $\Delta Ct = Ct$ (target gene) – Ct (reference gene) [278].

4.3.2.3 Data Normalization

The accuracy of calculated expression results in real-time RT-PCR experiments are significantly affected by minor differences in the quantity and quality of template RNA or variation in the efficiency of cDNA synthesis and PCR amplification [279]. It is therefore essential to apply a normalization strategy to control for the significant error introduced by these parameters [279]. The reliability of any relative real-time RT-PCR experiment can be improved through the use of a housekeeping gene which serves as an endogenous references control in the assay to correct for sample to sample variation in RT-PCR efficiency and errors in sample quantification [279].

The most commonly used housekeeping genes include β -actin – a cytoskeletal protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) – a glycolytic enzyme and 18s or 28s – ribosomal RNAs [278, 279]. All of these genes are necessary for basic cell survival and should theoretically be expressed at a constant level among different cell types at all stages of development [278, 279]. Their expression level should also remain relatively constant under different experimental conditions [278, 279]. However, no single gene is capable of meeting the criteria required of a universal reference gene as all are regulated in some capacity and none are constitutively expressed under all conditions in all cell types [281]. Therefore, the expression stability of selected housekeeping genes must be experimentally validated for the cell population under investigation as the reliability of the expression data depends on the choice of the most relevant housekeeping gene for the cells of interest [281].

Furthermore, it is imperative that the amplification efficiencies of target and reference samples are similar as even small differences directly affect the accuracy of any calculated expression result [279]. A difference in amplification efficiency of 3% between target and reference gene amplicons is reported to generate error in the calculated expression ratio of 47% when target amplification efficiency exceeds reference amplification efficiency and 209% when reference amplification efficiency exceeds target amplification efficiency after 25 cycles of the PCR run [279]. It is therefore essential that selected primers are validated to achieve a directly comparable level of efficiency for the target and housekeeping gene amplicons under investigation [279].

5. Study One:

The Effect of Recombinant Human Growth Hormone and Insulin-like Growth Factor-1 on the Mitochondrial Function and Viability of Peripheral Blood Mononuclear Cells *In-Vitro*

5.1 – Abstract

This study proposes that the benefits induced by both hormones at a cellular level are countered at supra-physiological concentrations due to an augmentation in the production of mitochondrial-derived free radicals with a subsequent increase in oxidative damage compromising mitochondrial function. In order to test this hypothesis, PBMCs were incubated for 4 hours with either rhGH (Range = $0.25 - 100 \mu g/L$) or IGF-1 (Range = $100 - 600 \mu g/L$) and subsequently analysed by flow cytometry for the determination of cellular viability, $\Delta \psi_m$, mitochondrial superoxide (O₂⁻) generation and mtPTP activity. Levels of mitochondrial superoxide generation were found to be significantly reduced compared to control samples (lymphocytes - 21.5±1.6AU / monocytes - 230.2±9.8AU) following rhGH treatment at concentrations of 5µg/L $(13.5\pm1.3AU, P\leq0.05)$ and $10\mu g/L$ $(12.3\pm1.5AU, P\leq0.05)$ in lymphocytes and at concentrations of $10\mu g/L$ (153.4±11.4AU, P ≤ 0.05) in monocytes, while no significant effect was found at higher concentrations or following treatment with IGF-1. In addition, treatment with either hormone was not found to have any significant effect on $\Delta \psi_m$, mtPTP activity or percentages of cellular viability. Results indicate that at physiological concentrations, rhGH elicits a protective cellular effect through the reduction of oxidative free radicals within mitochondria. This effect was diminished at supra-physiological concentrations but not to a level that would elicit disruption of mitochondrial function.

5.2 – Introduction

Both GH and its mediator IGF-1 induce specific anabolic effects via stimulation of cellular proliferation and differentiation in numerous cell types, including myoblasts, osteoblasts, adipocytes, oligodendrocytes, neurons and haemapoeitic cells [2, 4, 128]. They also exhibit a broad range of regulatory actions on cellular metabolism including a stimulation of lipolysis and lipid oxidation, an increased resistance to the actions of insulin and an enhanced level of energy expenditure [23, 128]. When present at normal physiological concentrations ($0.32\pm0.72\mu g/L$ – Resting; 5 - $44\mu g/L$ – Following stimulation by exercise), the actions of GH are regarded as beneficial [136, 137, 140]. Growth hormone deficient adults exhibit impairments in maximum oxygen consumption, ventilatory threshold, cardiac output, lean body mass and lipid metabolism which are all improved by replacement therapy with rhGH [21].

Reported improvements in exercise capacity and muscle strength in growth hormone deficient (GHD) patients following rhGH administration piqued the interest of sporting professionals looking to obtain a performance enhancing effect. The use of rhGH for the purposes of enhancing athletic performance has been extensively reported in the literature with doping regimes estimated to range from 3.3 to 8.3 mg per day (10-25IU/day), 3 to 4 days per week [38]. These supra-physiological concentrations are far in excess of the 0.3 to 0.6 mg per day (1-2 IU/day) usually prescribed to adult GHD patients [38]. However, evidence in the peer reviewed literature fails to support claims that supra-physiological GH concentrations exert performance enhancing benefits, with many studies finding no significant improvement in either muscle strength or endurance capacity following administration of rhGH to healthy subjects [178-180]. In addition, acromegaly, a pathological condition characterized by excessive GH secretion, is associated with impairments of strength, aerobic capacity and cardiac performance [21]. Indeed, patients with acromegaly, if left untreated, have an increased mortality associated with cardiovascular disease, pulmonary complications and cancer which suggests that the administration of rhGH to healthy individuals at high doses could have negative health implications [22].

Growth, protein synthesis and many of the components of fuel metabolism require the utilization of cellular energy, obtained through the dephosphorylation of ATP which is primarily generated via oxidative phosphorylation in the mitochondria. In a recent

publication, Kadenbach et al. [14] postulated a possible mechanism for the direct hormonal regulation of oxidative phosphorylation. Under resting conditions, when cellular ATP requirements are low, oxidative phosphorylation is known to be under the regulation of a high intra-mitochondrial ATP/ADP ratio, where allosteric inhibition of COX by ATP functions to keep $\Delta \psi_m$ low [14]. Bender and Kadenbach [20] found that through a calcium dependent dephosphorylation of serine and threonine residues on subunit 1 of COX, allosteric inhibition of the enzyme by ATP was no longer present, allowing for higher $\Delta \psi_m$ values ($\geq 140 \text{mV}$) under conditions of increased ATP utilization. Although specific effecter molecules and signalling pathways for this mechanism have not yet been elucidated, Kadenbach et al. [14] suggest that any stimulation of cells by hormones, cytokines or neurotransmitters resulting in modified gene expression must lead to the parallel activation of signalling pathways targeted to mitochondria in order to modulate oxidative phosphorylation to cell specific energy requirements. In addition, at $\Delta \psi_m$ above 140mV, the production of ROS is reported to increase exponentially with increasing $\Delta \psi_m$ [14]. Consequential oxidative damage to lipids, proteins and mitochondrial DNA can have detrimental effects for the cell, ranging from uncontrolled cellular proliferation to accelerated cell death [10]. Indeed, oxidative stress is reported to be one of the key activators of the mtPTP, whose opening initiates mitochondrial apoptotic pathways via the release of cyt c and AIF from the mitochondrial inter-membrane space [54].

Few studies to date have examined the effect of rhGH administration on mitochondrial function in healthy human subjects. Lange *et al.* [193] and Short *et al.* [192] found significant increases in the activity of mitochondrial oxidative enzymes in skeletal muscle, in response to rhGH administration, in healthy human subjects. In addition, Short *et al.* [192] found that 14 hours of intravenous rhGH infusion resulted in an 8-35% increment in the rate of ATP production of isolated skeletal muscle mitochondria. To our knowledge, limited research has been conducted into examining the effects of IGF-1 administration on mitochondrial function in tissue from healthy human subjects.

While research investigating the direct effects of GH and IGF-1 on mitochondrial function in human tissue is sparse, studies conducted on animals have shown that excessive concentrations of GH can impact negatively on mitochondrial function by modifying levels of oxidative stress [39, 40, 203]. Sieva *et al.* [39] demonstrated that high doses of rhGH (2 mg/kg) exerted detrimental effects related to energy metabolism

and oxidative stress in rat myocardium, while a lower dose (1 mg/kg) exerted beneficial effects on energy production and reduced levels of oxidative damage. In addition, studies conducted on transgenic animal models exhibiting an excess production of GH found that these animals possessed higher rates of oxygen consumption and free radical production [40, 203].

The purpose of this study was to determine whether GH and IGF-1 exert direct effects on the activity of mitochondria over a range of physiological and supra-physiological concentrations in PBMCs of healthy human subjects. Determination of the functional integrity of mitochondria was interpreted through examination of $\Delta \psi_m$, mitochondrial generation of O_2^- , and the active state of the mtPTP. This occurs in response to physiological stresses including a depletion of intra-mitochondrial substrate levels and any augmentation of the oxidative status of the cell [54]. In addition, the effect of treatment on percentages of viable, apoptotic and dead cells was also determined.

We propose that both GH and IGF-1 induce an up-regulation of oxidative phosphorylation in order to meet the energy demands associated with their anabolic effects and that this increment in the rate of ATP production is mediated at the expense of the efficiency of electron transport along the respiratory chain, which at supra-physiological concentrations leads to an augmented production of ROS. This could have negative implications for optimum cellular function in terms of increased oxidative damage to key mitochondrial components and mitochondria mediated activation of cellular apoptotic pathways.

5.3 – Methods

5.3.1 – Subjects

Ten healthy male subjects (mean \pm SEM: age $=23\pm1$ yrs, height $= 1.78\pm.02$ m, body mass $= 77.1\pm1.9$ kg, BMI $= 24.39\pm.71$ kg/m²) were recruited to participate in the study which was ethically approved by the Bond University Human Research Ethics Committee. All subjects had the nature of the study and the associated risks involved explained to them prior to providing written informed consent. Exclusion criteria included smoking, the use of therapeutic, recreational or performance enhancing drugs, including anabolic steroids and rhGH up to twelve months prior to prescription in the study, the presence of diabetes, cardiovascular disease or the use of prescription medication.

5.3.2 – Reagents Used

Hanks balanced salt solution (HBSS), RPMI Medium 1640, CCCP, JC-1, calcein acetoxymethylester (calcein AM), cobalt (II) chloride (CoCl₂), ionomycin, mitohydroethidine (MitoSOX Red) and SYTOX Green were purchased from Invitrogen (Carlsbad, CA, USA). Oligomycin, CsA, antimycin-A and SOD-PEG were purchased from Sigma Aldrich (St Louis, MO, USA). Ficoll-paque PLUS was obtained from GE Healthcare (Rydalmere, NSW, Australia). Annexin V-Cy5, annexin V binding buffer and recombinant insulin-like growth factor-1 (rIGF-1) were purchased from Biocore (Sydney, NSW, Australia). Finally, rhGH was purchased from Pfizer (Sydney, NSW, Australia).

5.3.3 – Blood Sample Collection and PBMC Isolation

Subjects arrived for sample collection in a post prandial state, in the morning following an overnight fast having not taken part in any form of physical activity in the prior 24 hours. Following insertion of a catheter into the antecubital vein, 30mL of blood was drawn into 6mL lithium heparinised vacutainers (BD, CA, USA). Collected blood was diluted in equal volume HBSS and subsequently layered over Ficoll-paque (PLUS) at a ratio of 2:1. All samples were centrifuged at 450*g for 30 mins for the separation of PBMCs from whole blood. Isolated cells were washed in HBSS and their concentration was determined using a Countess automated cell counter (Invitrogen, Carlsbad, CA, USA). Finally, cells were resuspended in RPMI 1640 cell culture medium at a concentration of 1*10⁶-cells/mL.

5.3.4 - rhGH and rIGF-1 Treatment

Once isolated, cells were divided into aliquots which were either left untreated or administered with rhGH (concentration range = $0.25 - 100\mu g/L$) or rIGF-1 (concentration range = $100 - 600\mu g/L$). All samples were subsequently incubated for 4 hours at $37^{\circ}C$ in the presence of $5\% CO_2$.

5.3.5 – Analysis of Mitochondrial Membrane Potential

Mitochondrial membrane potential was analysed by flow cytometry using the ratiometric lipophilic cation, JC-1. The dye, which exists in monomeric form at low $\Delta \psi_m$ and fluoresces at an emission maximum of 530nm, begins to form aggregates in the mitochondrial matrix at potential values corresponding to approximately 80-100mV, reaching a maximum at approximately 200mV [288]. These aggregates, termed J complexes, have an emission maximum of 590nm. The ratio of the fluorescence emission, between the aggregated and monomeric forms of the dye, provides an indication of $\Delta \psi_m$ that is independent of the size, shape or volume of mitochondria in the cell [288].

Following treatment with rhGH and rIGF-1, 1mL aliquots from each treatment condition were washed and resuspended in 1mL PBS. Positive and negative control samples are incubated at 37° C in 5% CO₂ for 5 minutes in the presence of 5µM of the ATP synthase inhibitor, oligomycin and 50µM of the mitochondrial uncoupler, CCCP respectively. All samples were subsequently incubated at 37° C in 5% CO₂ for 20 minutes in the presence of JC-1 at a concentration of 2µM. Following incubation, all samples were washed and resuspended in 500µL PBS, prior to analysis by flow cytometry.

5.3.6 – Analysis of Mitochondrial Permeability Transition Pore Activity

Following treatment, 1mL aliquots from each treatment condition were washed and resuspended in 1mL HBSS containing Ca²⁺ at a concentration of 1.26mM. Positive control samples were treated with 500nM ionomycin, a Ca²⁺ ionophore which induces opening of the mtPTP by overloading the mitochondrial matrix. Negative control samples were treated with 1 μ M cyclosporin A, which prevents opening of the mtPTP by binding to its matrix component, Cyp-D. Control samples were incubated at 37°C in 5% CO₂ for 5 minutes.

All samples are subsequently loaded with 10nM calein AM, which becomes trapped within cellular conpartments, including mitochondria, following de-esterification of its acetoxymethyl group [268]. The unbound form of the dye emits fluorescence at a peak emission of 517nm. All samples are subsequently treated with 400 μ M CoCl₂ which serves to quench the fluorescent signal from calcein in both cytosolic and nuclear

compartments of the cell. Opening of the mtPTP allows entry of $CoCl_2$ into the mitochondrial matrix resulting in a decrease in the mitochondrial fluorescence of calcein [268]. All samples were subsequently incubated in the dark at 37°C in 5% CO₂ for 15 minutes. Following incubation samples were washed in 2mL HBSS/ Ca²⁺ and resuspended in 500µL PBS prior to analysis by flow cytometry.

5.3.7 – Analysis of Mitochondrial Superoxide Generation and Cellular Viability

Following treatment, 1mL aliquots from each treatment condition were washed and resuspended in 1mL PBS. Negative control samples were incubated at 37° C in 5% CO₂ for 1 hour in the presence of the superoxide scavenger SOD-PEG at a concentration of 200IU/mL. All samples were subsequently incubated at 37° C in 5% CO₂ for 30 minutes in the presence of 5µM MitoSOX Red. Oxidation of the probe by O₂⁻ produces the fluorescent compound L-hydroxy-ethidium, which stains mitochondrial DNA and emits fluorescence at a peak emission of 567nm [261]. Positive control samples were incubated for a further 30 minutes in the presence of 100µM of the cytochrome c oxidoreductase inhibitor, antimycin A.

Following incubation, all samples are washed and resuspended in 100μ L of annexin V binding buffer. For the determination of cellular viability all samples were incubated in the dark at room temperature for 15 minutes with 5μ L of annexin V-Cy5, which binds with high affinity to phosphatidylserine, a phospholipid externalized to the surface of the plasma membranes of the apoptotic cells and 1μ M SYTOX Green, a DNA stain which acts as an indicator of necrosis as it is impermeable to intact plasma membranes [261]. Annexin V-Cy5 emits fluorescent light at a wavelength of 660nm while SYTOX Green emits fluorescent light at a peak emission of 523nm. All samples were resuspended in 400 μ L annexin V binding buffer prior to analysis by flow cytometry.

5.3.8 – Analysis by Flow Cytometry

All samples were analysed using a FACS Calibur flow cytometer (BD, Australia). For the analysis of $\Delta \psi_m$, samples were excited at a wavelength of 488nm with fluorescent emissions detected in channels using bandpass filters of 515-545nm and 564-606nm. For the analysis of mtPTP activity, cells were excited with a wavelength of 488nm while fluorescent emissions were detected in a channel using a 515-545nm bandpass filter. For the analysis of mitochondrial superoxide production and the determination of cellular viability samples were excited by both 488nm and 633nm wavelengths with
emitted light being detected in channels using bandpass filters of 515-545nm, 564-606nm and \geq 670nm. For all samples analysed a total of 10,000 events were collected while lymphocyte and monocyte populations were gated and analysed separately. All data collected was analysed using Cellquest Pro software (BD, Australia).

5.3.9 – Statistical Analysis

All data is reported as mean \pm SEM. A one-way analysis of variance (ANOVA) was used to determine whether significant differences existed within treatment groups for each of the analysed variables, while Dunnett's T3 multiple comparisons were used for post hoc analysis between treatment conditions (SPSS Inc, PAWS Statistics Version 18, USA). Statistical significance was determined at an alpha level of 0.05.

5.4 – Results

5.4.1 – Mitochondrial Membrane Potential

Ratios of the mean channel fluorescence between J-Complexes (Orange) and the monomeric form of JC-1 (Green) for all treatment conditions are presented in Figure 5.1. Treatment with CCCP significantly reduced ratios compared to untreated samples in lymphocyte (mean difference \pm SEM, 95% confidence intervals, p-values: -3.45 \pm 0.26, -4.73 – -2.17, P \leq 0.05) and monocyte (-3.11 \pm 0.30, -4.57 – -1.66, P \leq 0.05) populations. The ratio was significantly increased following treatment with oligomycin compared to untreated samples in lymphocyte populations (0.75 \pm 0.30, -0.58 – 2.08, P \leq 0.05). However, no significant difference was observed following treatment with oligomycin in monocytes (-0.69 \pm 0.32, -2.15 – 0.78, P=0.91). In addition, no significant differences were found between untreated and rhGH or rIGF-1 treated samples at any concentration administered.



Figure 5.1: Mitochondrial Membrane Potential **A**) JC-1 Orange/Green ratio (mean \pm SEM) from PBMCs for negative and positive controls compared to untreated samples. (* *P*<0.05 compared to CCCP, ** P<0.05 compared to untreated). **B**) JC-1 Orange/Green ratio from PBMCs for GH treated samples compared to untreated samples. **C**) JC-1 Orange/Green ratio (mean \pm SEM) from PBMCs for IGF-1 treated samples compared to untreated samples.

5.4.2 – Mitochondrial Permeability Transition Pore Activity

Mean channel fluorescence values, presented in arbitrary units (AU), from intramitochondrial calcein for all treatment conditions are presented in Figure 5.2. Treatment with ionomycin resulted in a significant reduction in the mean channel fluorescence compared to untreated samples for both lymphocyte (mean difference \pm SEM, 95% confidence intervals, p-values: -48.97±4.17AU, -60.98 – -36.96AU, P≤0.05) and monocyte (-270.78±22.90AU, -338.12 – -203.46AU, P≤0.05) populations. Values for mean channel fluorescence were significantly increased following treatment with cyclosporin A compared to untreated samples in both lymphocyte (20.03±7.50AU, 0.10 – 39.96AU, P≤0.05) and monocyte (106.08±39.22AU, 0.94 – 211.22AU, P≤0.05) populations. Values for mean channel fluorescence were not significantly different between untreated and rhGH or rIGF-1 treated samples for any concentration administered.

Figure 5.3 shows the percentage change in fluorescence values compared to negative control samples for all treatment conditions, which provides an indication of the percentage of mitochondria within a sample exhibiting activation of mtPTP. Untreated samples showed significant decreases in fluorescence values compared to negative controls for both lymphocyte ($-18\pm2\%$, -25 - -11%, P \leq 0.05) and monocyte ($-24\pm4\%$, -34 - -13%, P \leq 0.05) populations. In addition, the percentage decrease in fluorescence values from negative controls was significantly higher following treatment with ionomycin compared to untreated samples in both lymphocytes ($-96\pm1\%$, -98 - -94%, P \leq 0.05) and monocytes ($-95\pm1\%$, -97 - -92%, P \leq 0.05). Finally, there was found to be no significant differences between untreated and rhGH or rIGF-1 samples at any treatment concentration in either cell population.



Figure 5.2: Mitochondrial Permeability Transition Pore Activity **A**) Calcein mean channel fluorescence (mean \pm SEM) from PBMCs for negative and positive controls compared to untreated samples. (* *P*<0.05 compared to Ionomycin, ** P<0.05 compared to untreated). **B**) Calcein mean channel fluorescence (mean \pm SEM) from PBMCs for GH treated samples compared to untreated samples. **C**) Calcein mean channel fluorescence (mean \pm SEM) from PBMCs for IGF-1 treated samples compared to untreated samples.



Figure 5.3: Mitochondrial Permeability Transition Pore Activity **A**) Percent of PBMCs (mean \pm SEM) exhibiting active mtPTPs for negative and positive controls compared to untreated samples. (* *P*<0.05 compared to Cyclosporin A, ** P<0.05 compared to untreated). **B**) Percent of PBMCs (mean \pm SEM) exhibiting active mtPTPs for GH treated samples compared to untreated samples. **C**) Percent of PBMCs (mean \pm SEM) exhibiting active mtPTPs for IGF-1 treated samples compared to untreated samples.

5.4.3 – Mitochondrial Superoxide Generation

MitoSOX Red mean channel fluorescence values (AU) are shown in Figure 5.4 for all treatment conditions. Fluorescence values were significantly increased following treatment with antimycin A compared to untreated samples in both lymphocyte (mean difference \pm SEM, 95% confidence intervals, p-values: 48.80 \pm 4.88AU, 35.24 – 62.37AU, P \leq 0.05) and monocyte (220.48 \pm 33.71AU, 126.72 – 314.24AU, P \leq 0.05) subpopulations. Treatment with SOD-PEG resulted in no significant effect in mean channel fluorescence.

Fluorescence values were significantly reduced compared to untreated samples at rhGH concentrations of $5\mu g/L$ (-7.95±2.07AU, -15.81 – -0.10AU, P≤0.05) and $10\mu g/L$ (-9.14±2.20AU, -17.44 – -0.84AU, P≤0.05) in lymphocytes, in addition to at the concentration of $10\mu g/L$ (-76.76±16.29AU, -140.05 – -13.47AU, P≤0.05) in monocytes. No significant differences were observed following rhGH treatment at any other concentration in either cell population. Finally, rIGF-1 did not exert any significant effect on mean channel fluorescence compared to untreated samples in either lymphocyte or monocyte populations.

5.4.4 – Cellular Viability

The percentages of viable cells from analysed samples are presented in Figure 5.5 for all treatment conditions. Cell viability significantly decreased following treatment with antimycin A compared to untreated cells in both lymphocytes (mean difference \pm SEM, 95% confidence intervals, p-values: $-6\pm1\%$, -9 - -2%, P ≤ 0.05) and monocytes ($-8\pm2\%$, -13 - -4%, P ≤ 0.05). In contrast, the percentage of cellular apoptosis, (Figure 5.6), was significantly increased by antimycin A treatment compared to untreated samples in both lymphocytes ($4\pm1\%$, 1 - 7%, P ≤ 0.05) and monocytes ($8\pm2\%$, 3 - 12%, P ≤ 0.05). In addition, the percentage of dead cells, (Figure 5.7), was also found to be significantly increased in lymphocytes treated with antimycin A compared to unstimulated cells ($2\pm1\%$, 1 - 3%, P ≤ 0.05). However, antimycin A was not found to significantly affect the percentage of dead cells in the monocyte sub-population. Following treatment with SOD-PEG no significant differences were found in percentages of viable, apoptotic or dead cells compared to untreated cells. Finally, there were found to be no significant differences between unstimulated and rhGH or rIGF-1 treated samples in the percentages of live, apoptotic and dead cells for any concentration administered.



Figure 5.4: Mitochondrial Superoxide Production **A**) MitoSOX Red mean channel fluorescence (mean \pm SEM) from PBMCs for negative and positive controls compared to untreated samples. (* *P*<0.05 compared to SOD-PEG, ** P<0.05 compared to untreated). **B**) MitoSOX Red mean channel fluorescence (mean \pm SEM) from PBMCs for GH treated samples compared to untreated samples. (* *P*<0.05 compared to untreated). **C**) MitoSOX Red mean channel fluorescence (mean \pm SEM) from PBMCs for IGF-1 treated samples compared to untreated samples.



Figure 5.5: Cellular Viability **A**) Percentage of viable PBMCs (mean \pm SEM) for negative and positive controls compared to untreated samples. (* *P*<0.05 compared to SOD-PEG, ** P<0.05 compared to untreated). **B**) Percentage of viable PBMCs (mean \pm SEM) for GH treated samples compared to untreated samples. **C**) Percentage of viable PBMCs (mean \pm SEM) for IGF-1 treated samples compared to untreated samples.



Figure 5.6: Cellular Viability **A**) Percentage of apoptotic PBMCs (mean \pm SEM) for negative and positive controls compared to untreated samples. (* *P*<0.05 compared to SOD-PEG, ** P<0.05 compared to untreated). **B**) Percentage of apoptotic PBMCs (mean \pm SEM) for GH treated samples compared to untreated samples. **C**) Percentage of apoptotic PBMCs (mean \pm SEM) for IGF-1 treated samples compared to untreated samples.



Figure 5.7: Cellular Viability **A**) Percentage of dead PBMCs (mean \pm SEM) for negative and positive controls compared to untreated samples. (* *P*<0.05 compared to SOD-PEG, ** P<0.05 compared to untreated). **B**) Percentage of dead PBMCs (mean \pm SEM) for GH treated samples compared to untreated samples. **C**) Percentage of dead PBMCs (mean \pm SEM) for IGF-1 treated samples compared to untreated samples.

5.5 – Discussion

The principal finding of this study was that rhGH, when present at concentrations of 5 and $10\mu g/L$, significantly reduced the generation of O_2^- in the mitochondrial matrix of PBMCs' (Figure 5.4). However rhGH, at concentrations of 25µg/L and above, and rIGF-1 at any treatment concentration exerted no significant effect on mitochondrial superoxide levels. In contrast, Csiszar et al. [200] found that mitochondrial superoxide levels were significantly decreased in cultured HCAECs following 24 hours incubation in the presence of IGF-1 (10-1000µg/L) and supra-physiological concentrations of rhGH (0.001 – 0.01 IU/mL – approximately 0.33 – 3.33µg/mL). These changes were largely attributed to the elicitation of significant concentration-dependent increases in the expression of the antioxidant enzymes Mn-SOD, Cu, Zn-SOD, and GPX-1 by GH and IGF-1 [200]. Sanz et al. [41] observed both a significant increase in oxidative damage to mitochondrial DNA and a decrease in the rate of mitochondrial H_2O_2 generation in the liver cells of Wistar rats that were treated with rhGH for two weeks. Their results suggest that an initial pro-oxidative effect induced by the treatment is later countered by an increase in antioxidant protection [41]. Although the expression of antioxidant genes was not investigated in the present study, it is unlikely that GH or IGF-1 exerted any significant effect on the concentration of antioxidant enzymes following only 4 hours of incubation.

In the only study published to date examining the effect of *in-vitro* GH administration on ROS production following only 4 hours of treatment, Thum *et al.* [201] observed significant decreases in intracellular ROS production in human endothelial cells which were attributed to an improved regulation of cellular metabolic and antioxidant status. In contrast to the findings from the present study, these effects were only seen at rhGH concentrations of 1,000µg/L, with concentrations of 10 and 100µg/L inducing no significant effect [201]. However, such discrepancies could be attributed to the use of dichlorodihydrofluorescin diacetate (H₂DCFDA), a non-specific indicator of ROS production, in the study by Thum *et al.* [201], in order to determine changes in oxidative status at a cellular level. This may not have been sensitive enough to detect the comparatively small changes in mitochondrial located O_2^- which were found in the present study, at lower GH concentrations using a specific indicator of O_2^- which was directly targeted at mitochondria (MitoSOX Red). In addition, while effects observed using rhGH concentrations of 1,000µg/L may indicate that such high concentrations are necessary in order to induce the antioxidant effects of the hormone within this time frame, doubt is raised as to whether this indicates a direct effect via mitochondrial regulation due to the failure of Thum *et al.* [201] to distinguish between mitochondrial and cytosolic free radical production. Indeed, it is known that endothelial cells can produce large amounts of O_2^- in their cytosol through activation of NADPH oxidase, a mechanism which has been demonstrated to be inhibited by IGF-1 over expression *invivo* [289-291].

To date no studies have investigated the effects of GH or IGF-1 on the generation of ROS in human tissue in-vivo, while research conducted on animal models examining the impact of the GH/IGF-1 axis on levels of oxidative stress have produced conflicting results. In agreement with their findings on the in-vitro effects of rhGH and IGF-1 on cultured HCAECs, Csiszar et al. [200] observed elevated levels of mitochondrial superoxide together with a reduction in the expression of antioxidant enzymes in endothelial cells from the aortas of hypopituitary Ames dwarf mice, who exhibit low levels of circulating GH and IGF-1, compared to wild type control mice. In contrast, Brown-Berg *et al.* [40] found a reduction in levels of H_2O_2 in mitochondria from the liver of Ames dwarf mice, in addition to a 47% decrease in levels of protein oxidation, compared to wild type mice. Csiszar et al. [200] suggest that such discrepancies may be attributed to differences between plasma and tissue concentrations of IGF-1. While they found that circulating levels of IGF-1 play a central role in regulating oxidative stress in the vasculature, they hypothesized that autocrine/paracrine pathways of IGF-1 expression exhibit a greater control over antioxidant defence in other tissues [200]. This may account for the differences observed in the effects of rhGH and rIGF-1 on mitochondrial superoxide generation in the present study. It must be noted that in addition to GH and IGF-1, Ames dwarf mice also exhibit deficiencies in other pituitary derived hormones, including prolactin and thyroid stimulating hormone, which could have impacted on the results obtained in these studies [292]. In fibroblasts from Lewis dwarf rats, an animal model which exhibits a specific deficiency of GH secretion, Unguari et al. [292] observed similar rates of cellular O₂⁻ and H₂O₂ production compared with control rats, however subsequent treatment with GH was found to significantly increase antioxidant capacities in these animals.

Although in the present study no significant changes were observed in the rate of mitochondrial superoxide production with supra-physiological concentrations of GH *in*-

vitro, how such concentrations, when administered *in-vivo*, will affect oxidative status is currently unresolved. Seiva et al. [39] demonstrated that while administration of 1mg/kg per day of rhGH for 2 weeks in Wistar rats significantly lowered levels of lipid peroxidation in cardiac tissue, a doubling of the treatment concentration resulted in detrimental increases in oxidative damage. They concluded that any beneficial effect on cellular antioxidant capacity exerted by the hormone was overcome by greater prooxidant effects at high doses [39]. GH is known to increase the rate of lipolysis leading to elevated plasma concentrations of FFA's, while administration of rhGH in-vivo has been shown to induce insulin resistance [21, 23]. Such effects themselves are associated with increased levels of oxidative stress [45, 293]. A significant reduction in the expression of genes involved in oxidative phosphorylation was found by Sparks et al. [293] in subjects fed an isoenergetic, high-fat diet for 3 days, while the mitochondrial oxidative capacity of type 2 diabetic patients was reported by Hawley and Lessard [45] to be 40% lower than that of healthy volunteers. As a result, several authors [45, 113] have hypothesized that a subsequent excess supply of reducing equivalents to the respiratory chain cannot be dissipated by oxidative phosphorylation leading to an increase in the mitochondrial proton motive force and an augmentation in the production of ROS. In support of this, Sanz et al. [41] found that while two weeks of rhGH treatment in ad libitum fed Wistar rats increased oxidative damage to mitochondrial DNA in hepatocytes, the same treatment in caloric restricted animals induced no significant effect. However, the limitations in translating the results from animal models to human subjects must be noted here as animal studies do not always accurately predict human outcomes [294].

Although no significant differences were found in $\Delta \psi_m$ values following rhGH administration at any concentration (Figure 5.1), it is possible that the method of analysis utilized in the present study was not sensitive to transient changes in $\Delta \psi_m$ which may significantly impact rates of O_2^- generation. In support of this assumption, Jaburek *et al.* [49] notes that even a slight increase in proton backflow into the mitochondrial matrix may substantially suppress mitochondrial ROS formation. Thus, the possibility cannot be excluded that the decrease in mitochondrial superoxide levels seen with rhGH treatment at physiological concentrations results from an attenuation of the mitochondrial proton motive force and an increase in the efficiency of oxidative phosphorylation.

Kadenbach et al. [14] suggest that any extracellular signalling molecule which induces anabolic effects via intracellular signal transduction pathways must also modulate oxidative phosphorylation via the activation of signalling pathways directed at mitochondria. The maximum rate of activity of ATP synthase occurs at $\Delta \psi_m$ of approximately 120mV but this potential declines as the flow of protons back into the mitochondrial matrix through its F₀ subunit increases [20]. Thus in order to allow for augmentation of the rate of ATP production, the resting $\Delta \psi_m$ must be increased. Recent work conducted by Kadenbach et al. [14] has led to the hypothesis that when specific amino acid residues on COX are de-phosphorylated, the ability of ATP to allosterically inhibit the enzyme is switched off resulting in an augmentation of the rate of activity of the respiratory enzymes and a subsequent increase in $\Delta \psi_m$. COX, the terminal enzyme complex of the respiratory chain, is also the most exergonic of the respiratory chain enzymes (ΔG° ' = -100kJ/mol) and is considered to be the principal regulatory site of oxidative phosphorylation [14]. Through polargraphic analysis of the activity of COX, Kadenbach and Bender [20] demonstrated that de-phosphorylation of the isolated enzyme with protein phosphatase 1 switched off allosteric ATP inhibition, while cAMP dependant activation of protein kinase A switched on the allosteric inhibition of activity at high intra-mitochondrial ATP concentrations. Incubation of isolated mitochondria from bovine liver with Ca²⁺ was also found to switch off the ATP inhibition of COX, suggesting that intra-mitochondrial Ca^{2+} concentrations play a key role in up-regulating the activity of respiratory enzymes in response to extracellular signals [20]. However this did not result from a direct interaction with the enzyme as inhibition of protein phosphatise activity was found to abolish any effect induced by Ca²⁺ on COX activity in isolated mitochondria [295].

Billestrup *et al.* [170] demonstrated that GH induced increases in intracellular free Ca²⁺ concentrations in CHO cells via activation of voltage dependent L-type Ca²⁺ channels in the plasma membrane, although high doses of rhGH were needed before significant differences were observed, with a minimally effective dose of $50\mu g/L$ and a maximum response observed at $500\mu g/L$. However, Perret-Vivancos *et al.* [187] found that CHO cells expressing GH receptors which lacked the 184 amino acids of their c-terminal, the domain known to be responsible for inducing the hormones effects on intracellular free Ca²⁺ oscillations, had the same stimulatory effect on mitochondrial oxygen consumption as cells expressing full length receptors. Their findings indicate that GH induces a direct

effect on mitochondrial function independent of signal transduction induced changes in cellular Ca²⁺ concentrations [187]. Indeed several studies have noted the targeting of GH and its receptor to mitochondria with both Ardail et al. [296] and Perret-Vivancos et al. [187] observing the presence of GH in the mitochondrial inter-membrane space and bound to the mitochondrial membranes using electron microscopy and confocal microscopy, respectively. Additionally Perret-Vivancos et al. [187] demonstrated that disruption of GH internalization via a caveolar-dependent pathway abolished any stimulatory effect induced by the hormone on rates of cellular oxygen consumption. Conversely, Ardail et al. [296] found a dose dependent decrease in the activity of the oxidative enzymes succinate dehydrogenase and COX when GH was targeted directly at isolated mitochondria, with the greatest level of inhibition observed at 100nM. The authors hypothesized that while internalization of the hormone is necessary for stimulation of oxidative phosphorylation due to a synergistic interaction with GHR mediated signal transduction pathways, at high concentrations hormone translocation to the mitochondria also acts as a negative regulatory mechanism, attenuating the rise in $\Delta \psi_{\rm m}$ and the generation of ROS [296].

In relation to observations from the present study, it is possible that at physiological concentrations GH induced an up-regulation of the rate of ATP synthesis which was sufficient to counter any associated augmentation of the activity of oxidative enzymes, resulting in the observed decrease in mitochondrial superoxide levels. At supraphysiological concentrations, where administration of the hormone no longer induced significant decreases in mitochondrial superoxide generation, it is possible that as the rate of ATP synthesis became rate-limiting the up-regulation of the rate of oxygen consumption elevated the $\Delta \psi_m$, while inhibition of the activity of respiratory chain enzymes due to internalization of the hormone may have prevented any significant rise in superoxide levels in comparison to untreated samples.

In addition to GH, IGF-1 has also been found to initiate signal transduction pathways targeting mitochondria. Bijur and Jope [12] observed, following stimulation with IGF-1 in human neuroblastoma and embryonic kidney cells, the translocation of phorphorylated Akt to the mitochondria where it was found to phosphorylate the β subunit of ATP synthase. However, at the concentrations administered in the present study, IGF-1 was found to induce no significant effect on either $\Delta \psi_m$ or the rate of mitochondrial superoxide production.

Neither GH nor IGF-1, at any treatment concentration, was found to have a significant effect on mtPTP (Figure 5.2) or the percentage of cellular apoptosis (Figure 5.6), which is not surprising given that both hormones failed to induce an increase in the level of mitochondrial oxidative stress, even at supra-physiological concentrations. Indeed both hormones have been shown to act as "survival agents", preventing the onset of apoptosis in specific cell types [17, 18, 212, 297]. Both Mitsunaka *et al.* [18] who initiated intracellular death signalling pathways via activation of plasma membrane bound Fas receptors in IM-9 lymphocytes and Han *et al.* [297] who induced hypoxic – ischemic injury in neonatal rat brain neurons attributed the ability of GH to prevent apoptosis to an inhibition of caspase-dependent cell death pathways. In addition, Mitsunaka *et al.* [18] found that GH treatment up-regulated the expression of the anti-apoptotic protein Bcl-2, whose interaction with the VDAC on the OMM is hypothesized to opening of the mtPTP and the subsequent activation of intracellular caspases via the release of AIF and cyt c from the mitochondrial inter-membrane space.

Studies from the laboratories of Yamamura et al. [17] and Kang et al. [212] have also found that pre-treatment with IGF-1 inhibits mitochondrial mediated apoptosis via the release of cyt c following the induction of ischemia-reperfusion injury in rat myocardium and the administration of high glucose concentrations (25mM) in human mesangial cells respectively. Both studies attributed the anti-apoptotic effects of IGF-1 to signal transduction mediated regulation of the expression and activity of the Bcl-2 family of proteins, a large number of which reside on the OMM. While the antiapoptotic regulators of the family, Bcl-xL and Bcl-2 – in their homo-dimeric form, act to prevent mtPTP mediated release of cytotoxic proteins, the pro-apoptotic promoter Bad, acts to neutralize this function by forming heterodimers with Bcl-xL and Bcl-2 [212]. Yamamura et al. [17] found that a 1mg dose of IGF-1 administered in-vivo to male Sprague-Dawley rats resulted in an up-regulation in the expression of Bcl-xL while the expression of the pro-apoptotic effector protein, Bax was found to be attenuated in cardiomyocytes. Furthermore, Kang et al. [212] demonstrated that a decrease in the concentration of Bax, relative to that of Bcl-2, in addition to the inactivation of Bad via the phosphorylation of serine residues is dependent on the IGF-1 mediated activation of both Akt and ERK signalling pathways.

However it must be pointed out that IGF-1 induced changes in the expression of Bcl-2 proteins are not immediate with Wang *et al.* [298] noting that a minimum of 3 hours

incubation was necessary before any changes are observed in rat cardiomyocytes. Despite this Lai *et al.* [299] observed that doxorubicin induced mitochondrial membrane depolarization in rat cardiomyocytes was reversed following only one hour incubation in IGF-1. These findings indicate that IGF-1 is capable of directly modulating $\Delta \psi_m$, favouring an increase in the efficiency of electron transport, although the mechanisms underlying this modulation currently remain unresolved.

In summary, it was found that rhGH concentrations of 5 and 10 µg/L significantly reduced the generation of mitochondrial-derived ROS while higher doses of the hormone or the administration of IGF-1 at any concentration failed to induce a significant effect. These concentrations fall within the range observed following the stimulation of peak endogenous GH responses ($5.3 - 42.5 \mu g/L$), indicating that GH elicits beneficial responses in mitochondrial function at physiological concentrations [213]. That this protection was not found to be present at higher concentrations could have negative implications for the cell under these conditions *in-vivo*, as any additional source of free radical production is likely to induce oxidative damage. Hormonally induced changes to $\Delta \psi_m$ and the rate of ATP synthesis likely play a role in mediating the effects of rhGH on levels of oxidative stress and their subsequent ability to inhibit cellular apoptosis, although no significant effect was observed for either hormone on $\Delta \psi_m$ in PBMCs. While evidence exists that both GH and IGF-1 signalling pathways are directed towards mitochondria, the mechanisms underlying their effects on mitochondrial function require further investigation.

6. Study Two:

The Effect of *in-vitro* administration of Recombinant Human Growth Hormone and Insulin-like Growth Factor-1 on Mitochondrial Vitality and Highly Reactive Oxygen Species Production under differing Respiratory Conditions in Peripheral Blood Mononuclear Cells.

6.1 – Abstract

Growth hormone (GH), but not insulin-like growth factor-1 IGF-1, has been demonstrated to decrease the rate of mitochondrial-derived reactive oxygen species production (mtROS) in-vitro. However at supra-physiological concentrations in-vivo these hormones are associated with the induction of high levels of oxidative stress. The purpose of this study was to investigate the mitochondrial effects exerted by physiological and supra-physiological concentrations of rhGH and rIGF-1 under various respiratory conditions of substrate saturation in PBMCs from healthy male subjects. PBMCs were treated with either rhGH at concentrations of 0.5, 5 and 50µg/L or IGF-1 at concentrations of 100, 300 and 500µg/L for 4 hours for the purposes of establishing the hormonal effects on mitochondrial function under various respiratory conditions at saturating substrate concentrations. Mitochondrial membrane potential $(\Delta \psi_m)$ and the level of mitochondrial hROS production were analysed by flow cytometry in digitonin permeabilized cells, in the presence of the following respiratory substrates 1) pyruvate / malate 2) succinate / rotenone 3) pyruvate / malate / succinate and 4) octanoate / malate. Neither rhGH nor rIGF-1 exerted any significant effect on $\Delta \psi_m$ as indicated by DilC₁(5) mean channel fluorescence values in either lymphocyte (P=0.97) or monocyte (P=0.78) sub-populations at any concentration administered. Similarly, neither rhGH nor rIGF-1 exerted any significant effect on hROS levels as indicated by HPF mean channel fluorescence values in either lymphocyte (P=0.90) or monocyte (P=0.85) subpopulations at any concentration administered. That neither hormone was capable of attenuating levels of oxidative stress mediated via either complex I linked respiration or lipid-derived respiration could have serious health implications for rhGH administration in healthy subjects which is frequently associated with significant increases in the availability of FFA's as oxidisable substrates.

6.2 – Introduction

In light of the risk of cellular oxidative damage associated with mitochondrial O_2^- production [300, 301], attempts at identifying the sites of electron leak from the ETC responsible for free radical generation have received much attention in the literature [302-304]. While it has been widely reported that O_2^- is produced by the NADH dehydrogenase (Complex I) and CoQ: Cytochrome c-oxidoreductase (Complex III), controversy exists over both the specific electron transferring components within these complexes that leak electrons and the site at which the highest rates of O_2^- are generated [125, 302]. While some studies, conducted using specific respiratory substrates and complex inhibitors have reported complex III to be the principal source of O_2^- and H_2O_2 production [304-306], others have identified complex I to be the main contributor [303, 307, 308]. Indeed, the relative rates of free radical production attributed to the different complexes may be tissue-specific with Barja [309] observing the generation of ROS from complex I of both heart and brain mitochondria while complex III was only found to be capable of generating ROS from mitochondria isolated from the heart.

The binding of ubiquinol (QH₂) to the Qo site of complex III is believed to be the principal O₂⁻ generating source of that complex with disruption of electron flow giving rise to increased steady-state concentrations of the semiguinone (.Q-) radical at that site [300, 301, 304]. Structurally, the Qo site of complex III is orientated towards the intermembrane space and O_2^{-} produced at this site has been found to be predominantly released into the cytosol, although evidence exists that some O_2^- produced here may also be released into the mitochondrial matrix [125, 301, 310]. Inhibition of complex I near its ubiquinone binding site by rotenone has been shown in many studies, to increase electron leak to ROS in the presence of complex I respiratory substrates, identifying this complex as a major source of O_2^- production [303, 307, 308]. Complex I contains several redox centres, including 8 or 9 iron-sulfur clusters and flavin mononucleatide (FMN), in addition to the ubiquinone binding site itself, which are all located proximal to the point of inhibition by rotenone [304, 307]. While each of these components have been examined as a potential O_2^- producing centre, at present the identification of specific sites of electron leak from complex I remains unresolved. Despite this it is known that the production of O_2^- by complex I is released on the matrix side of the IMM [300, 301, 310].

In addition to complexes I and III of the ETC, ETF and electron transfer flavoprotein quinine oxidoreductase (ETF-QO) are two enzymes involved in the transfer of electrons from reduced carriers formed during β -oxidation in the mitochondrial matrix to the ubiquinone pool on the IMM, which may produce O_2^- [125, 300]. St-Pierre *et al.* [125] found significant increases in the rate of H₂O₂ production in the mitochondrial matrix from rat skeletal muscle and heart mitochondria in the presence of palmitoyl carnitine with no added respiratory inhibitors. The authors identified ETF and ETF-QO as potential sources of O_2^- generation as they are known to exist in partially reduced states during lipid metabolism.

A decline in the systemic release of GH and its subsequent stimulation of IGF-1 is a well-known effect of aging in both humans and experimental animals [200, 311]. Indeed, it has been demonstrated that circulating concentrations of these hormones plays an important role in modifying intracellular levels of oxidative stress [200, 205, 292, 312]. Evans et al. [312] observed elevated levels of lipid peroxides in blood samples from GHD patients compared to healthy controls which decreased significantly following administration of a low dose GH therapy of 0.03IU/kg/day for 3 months. Csiszar et al. [200] found that, in-vitro, incubation of HCAECs for 24 hours in the presence of IGF-1 (10 - 1000µg/L) and GH (0.001 - 0.01 IU/mL) significantly reduced the mitochondrial production of O_2^- as well as O_2^- and H_2O_2 at a cellular level. The authors also found significant increases in the expression of the antioxidant enzymes Mn-SOD, Cu, Zn-SOD and GPX-1, which likely account for the observed hormonal effect on ROS production. In addition, several authors have also noted that GH is capable of translocating to the mitochondria [187, 296]. Ardail et al. [296] observed that the activity of the ETC enzymes succinate dehydrogenase (Complex II) and COX (Complex IV) were decreased in isolated mitochondria in the presence of high concentrations of GH (100nM) indicating that the hormone can attenuate mitochondrial O_2^- generation via direct modulation of the $\Delta \psi_m$.

However, despite the documented antioxidant effects of these hormones in recent studies, at supra-physiological concentrations *in-vivo*, GH is associated with adverse effects which hold negative implications for normal physiological function [21, 23]. Patients with acromegaly, a pathological disorder associated with a hyper-secretion of GH from a pituitary adenoma, suffer from neurological, cardiovascular, respiratory and metabolic complications which lead to increased mortality if GH and IGF-1

concentrations are not reduced to normal levels [22]. In addition, the prevalence of diabetes in acromegalic patients is reported to range between 20 to 46%, while the inducement of supra-physiological GH concentrations in healthy subjects has been shown in many studies to lead to the development of insulin resistance [21-23]. Increased levels of oxidative stress has been demonstrated to induce insulin resistance directly while it is also widely cited as a causative factor in the development of diabetes [23, 124], suggesting that any observed oxidative defence afforded by either GH or IGF-1 in some studies is negated at supra-physiological concentrations in-vivo. Studies on animals lend support to this hypothesis, with Seiva et al. [39] observing significant increases in oxidative stress, as demonstrated from levels of lipid peroxidation, following two weeks of high dose GH treatment (2mg/kg/day) in male Wistar rats. In contrast, the same authors observed reduced levels of oxidative stress and significant enhancements in the expression of antioxidant enzymes following administration of the same treatment at a lower dosage (1mg/kg/day). In addition, transgenic mice manipulated to over-express GH have been found to have increased levels of oxidative damage to liver proteins and a suppressed antioxidant capacity compared to wild type mice [40].

While current studies examining the effect of GH and IGF-1 on cellular oxidative status have analysed levels of O_2^- and H_2O_2 production [200, 205, 292], to date no study has looked at how these hormones affect levels of highly reactive oxygen intermediates such as 'OH and ONOO- which are produced when electron leak evades cellular antioxidant defences and are directly responsible for oxidative damage to cellular components. In addition, it is not known how physiological concentrations of these hormones influence the rate of free radical production from the principal sites of electron leak along the ETC or whether these rates differ at supra-physiological concentrations. In light of this, the present study aimed to 1) examine mitochondrial levels of hROS and 2) identify the principal complexes involved in ROS production from the ETC in PBMCs following incubation in the presence of either rhGH or IGF-1 at both physiological and supra-physiological concentrations. This was achieved through flow cytometric analysis of hROS (OH and ONOO-) levels and $\Delta \psi_m$ in digitonin permeabilized cells following treatment with saturating concentrations of various respiratory substrate combinations and the use of specific ETC complex inhibitors which allowed for control over the sites of electron entry into the ETC at complexes I and II, in addition to the entry of electrons from reduced carriers involved in β oxidation at the level of ubiqunol.

6.3 – Methods

6.3.1 – Subjects

Ten healthy male subjects (mean \pm SD: age =23 \pm 4yrs, height = 1.78 \pm .06m, body mass = 77.1 \pm 6.2kg, BMI = 24.39 \pm 2.25kg/m²) were recruited to participate in the study which was ethically approved by the Bond University Human Research Ethics Committee. All subjects had the nature of the study and the associated risks involved explained to them prior to providing written informed consent. Exclusion criteria included smoking, the use of therapeutic, recreational or performance enhancing drugs, including anabolic steroids and rhGH up to twelve months prior to prescription in the study, the presence of diabetes, cardiovascular disease or the use of prescription medication.

6.3.2 – Reagents Used

HBSS, RPMI Medium 1640, CCCP, DilC₁(5), HPF, and sodium pyruvate were purchased from Invitrogen (Carlsbad, CA, USA). L-malic acid (malate – pH adjusted to 7.4), sodium succinate, sodium octanoate, ammonium iron (II) sulphate , H_2O_2 , rotenone, digitonin, sucrose, potassium phosphate monobasic (KH₂PO₄), magnesium chloride (MgCl₂), potassium morpholinopropane sulphonate (MOPS), ADP, ethylenediaminetetraactic acid (EDTA) and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St Louis, MO, USA). Phosphate buffered saline was purchased from Kinetic (Burpengary, QLD, Australia). Ficoll-paque PLUS was obtained from GE Healthcare (Rydalmere, NSW, Australia). Recombinant insulin-like growth factor-1 was purchased from Biocore (Sydney, NSW, Australia). Finally, rhGH was purchased from Pfizer (Sydney, NSW, Australia).

6.3.3 – Blood Sample Collection and PBMC Isolation

Subjects arrived for sample collection in a post prandial state, in the morning following an overnight fast having not taken part in any form of physical activity in the prior 24 hours. Following insertion of a catheter into the antecubital vein, 24mL of blood was drawn into 6mL lithium heparinised vacutainers (BD, CA, USA). Collected blood was diluted in an equal volume PBS and subsequently layered over Ficoll-paque PLUS at a ratio of 2:1. All samples were centrifuged at 450*g for 30 mins for the separation of PBMCs from whole blood. Isolated cells were washed in HBSS and their concentration was determined using a Countess automated cell counter (Invitrogen, Carlsbad, CA, USA). Finally, cells were resuspended in RPMI 1640 cell culture medium at a concentration of 1*10⁶-cells/mL.

6.3.4 - rhGH and rIGF-1 Treatment

Once isolated, cells were divided into aliquots which remained untreated or administered with rhGH at concentrations of 0.5, 5 and $50\mu g/L$ or rIGF-1 at concentrations of 100, 300 and $500\mu g/L$. All samples were subsequently incubated for 4 hours at $37^{\circ}C$ in the presence of $5\% CO_2$.

6.3.5 – Treatment with Respiratory Substrates and Inhibitors

Following hormonal treatment, cells had their plasma membranes permeabilized for the analysis of $\Delta \psi_m$ and hROS generation under differing respiratory conditions in a method adapted from the work of Pham *et al.* [219]. Briefly, sample aliquots were treated with digitonin at a concentration of 5µg/mL for 5 mins, washed in PBS and resuspended in a respiratory buffer (0.25M sucrose, 2mM KH₂PO₄, 5mM MgCl₂, 1mM ETA, 0.1% BSA, 1mM ADP and 20mM MOPS – pH adjusted to 7.4). Samples were subsequently treated with either pyruvate (5mM) and malate (5mM) for the initiation of complex I mediated respiration (Pyr/Mal), succinate (10mM) and the complex I inhibitor, rotenone (20µM) to initiate respiration at complex II (Suc/Rot), pyruvate (2.5mM), malate (2.5mM) and succinate (5mM) to mediate the reduction of electron carriers at complexes I and II (Pyr/Mal/Suc) or octanoate (10mM) and malate (2mM) to mediate the transfer of electrons to reducing equivalents via β oxidation (Oct/Mal).

6.3.6 – Analysis of Mitochondrial Membrane Potential

Mitochondrial $\Delta \Psi$ was analysed by flow cytometry using the lipophilic cationic dye, DilC₁(5). The dye accumulations in the mitochondria as a function of membrane potential at concentrations below 100nM and fluoresces at an emission maximum of 658nm when excited at 633nm [313]. Control samples were incubated at 37°C in 5% CO₂ for 5 minutes in the presence of 50µM of the mitochondrial uncoupler, CCCP in order to dissipate $\Delta \psi_m$. Aliquots from each treatment condition (1mL) were subsequently incubated at 37°C in 5% CO₂ for 15 minutes in the presence of DilC₁(5) at a concentration of 10nM. Following incubation, all samples were washed and resuspended in 500µL PBS, prior to immediate analysis by flow cytometry.

6.3.7 – Analysis of Highly Reactive Oxygen Species Generation

The generation of hROS was analysed by flow cytometry using the fluorescein derivative, HPF. HPF is a highly selective ROS indicator, only emitting a fluorescent signal upon reacting with either 'OH or ONOO- [274]. The probe fluoresces at an emission maximum of 515nm when excited at 488nm. Control samples are incubated at 37° C in 5% CO₂ for 5 minutes in the presence of 100µM ammonium iron (II) sulphate and 1mM H₂O₂ to induce 'OH generation via the Fenton reaction. 1mL aliquots from each treatment condition were subsequently incubated at 37° C in 5% CO₂ for 15 minutes in the presence of 100µM. Following incubation, all samples were washed and resuspended in 500µL PBS, prior to immediate analysis by flow cytometry.

6.3.8 – Analysis by Flow Cytometry

All samples were analysed using a FACS Calibur flow cytometer (BD, Australia). For the analysis of $\Delta \psi_m$, samples were excited at a wavelength of 633nm with fluorescent emissions detected in channels using a bandpass filter with a range of \geq 670nm. For the analysis of hROS production samples were excited at a wavelength of 488nm with fluorescent emissions detected in channels using a bandpass filter with a range of 515-545nm. For all samples analysed, a total of 10,000 events were collected while lymphocyte and monocyte populations were gated and analysed separately. All data collected was analysed using Cellquest Pro software (BD, Australia).

6.3.9 – Statistical Analysis

All data is reported as mean \pm SEM. A factorial multivariate analysis of variance (MANOVA) was used to determine whether significant interactions existed between respiratory conditions and hormonal treatments for each of the analysed variables, while Bonferroni's multiple comparisons were used for post hoc analysis between individual treatment conditions (SPSS Inc, PAWS Statistics Version 18, USA). Statistical significance was determined at an alpha level of 0.05.

6.4 – Results

6.4.1 – Mitochondrial Membrane Potential

Treatment with CCCP significantly reduced DilC₁(5) mean channel fluorescence (AU) from samples in the presence of Pyr/Mal (mean difference \pm SEM, 95% confidence intervals, p-values: -129.69 \pm 8.65AU, -149.22 – -110.17AU, P \leq 0.05), Succ/Rot (-181.89 \pm 19.03AU, -224.66 – -139.12AU, P \leq 0.05), Pyr/Mal/Succ (-130.45 \pm 20.17AU, -175.95 – -84.95AU, P \leq 0.05) and Oct/Mal (-162.84 \pm 18.75AU, -205.17 – -120.50AU, P \leq 0.05) compared to untreated samples under the same respiratory conditions in the lymphocyte sub-population (Figure 6.1 A). Fluorescence values from Succ/Rot treated samples were significantly increased compared to all other respiratory conditions analysed (P \leq 0.05), while Oct/Mal treated samples were significantly increased compared to Pyr/Mal (65.21 \pm 9.00AU, 41.27 – 89.14AU, P \leq 0.05) and Pyr/Mal/Succ (66.99 \pm 9.00AU, 43.05 – 90.93AU, P \leq 0.05) treated samples. In addition, no significant difference was observed between Pyr/Mal and Pyr/Mal/Succ treated samples (P=1.00).

In the monocyte sub-population treatment with CCCP also significantly reduced DilC₁(5) mean channel fluorescence from samples in the presence of Pyr/Mal (-854.44±67.44AU, -1006.96 – -701.92AU, P≤0.05), Succ/Rot (-924.91±55.88AU, -1055.58 – -794.24AU, P≤0.05), Pyr/Mal/Succ (-956.69±49.41AU, -1068.34 – -845.04AU, P≤0.05) and Oct/Mal (-930.30±60.47AU, -1066.97 – -793.63AU, P≤0.05) compared to untreated samples under the same respiratory conditions (Figure 6.1 B). Fluorescence values from Succ/Rot treated samples were significantly increased compared to Pyr/Mal (146.40±28.54AU, 70.50 – 222.30AU, P≤0.05) and Pyr/Mal/Succ (122.73±28.54AU, 46.83 – 198.62AU, P≤0.05) treated samples but was not significantly different compared to Oct/Mal treated samples (P=0.73). Oct/Mal treated samples displayed significant increases in fluorescence compared to Pyr/Mal (102.03±28.54AU, 26.13 – 177.93AU, P≤0.05) and Pyr/Mal/Succ (78.36±28.54AU, 2.46 – 154.26AU, P≤0.05) treated samples. In addition, no significant difference was observed between Pyr/Mal and Pyr/Mal/Succ treated samples (P=1.00).

Neither GH (Figure 6.2) nor IGF-1 (Figure 6.3) exerted any significant effect on $\Delta \psi_m$ as indicated by DilC₁(5) mean channel fluorescence values in either lymphocyte (P=0.97) or monocyte (P=0.75) sub-populations at any concentration administered. Finally no significant interaction effect between hormonal treatment and respiratory substrate condition was observed in either lymphocyte (P=0.99) or monocyte (P=0.99) sub-populations.



Figure 6.1: Mitochondrial Membrane Potential **A**) $\text{DilC}_1(5)$ Mean Channel Fluorescence (mean \pm SEM) from lymphocytes for negative control samples treated with CCCP compared to untreated samples under different substrate conditions.(# P<0.05 compared to untreated, * P<0.05 compared to Succ/Rot, ** P<0.05 compared to Oct/Mal, **** P<0.05 compared to Pyr/Mal, **** P<0.05 compared to Pyr/Mal/Succ). **B**) $\text{DilC}_1(5)$ Mean Channel Fluorescence (mean \pm SEM) from monocytes for negative control samples treated with CCCP compared to untreated samples under different substrate conditions. (# P<0.05 compared to untreated, * P<0.05 compared to Succ/Rot, ** P<0.05 compared to Succ/Rot, *** P<0.05 compared to Oct/Mal, **** P<0.05 compared to Pyr/Mal, **** P<0.05 compared to Succ/Rot, *** P<0.05 compared to Succ/Rot, *** P<0.05 compared to Succ/Rot, *** P<0.05 compared to Oct/Mal, **** P<0.05 compared to Pyr/Mal, **** P<0.05 compared to Pyr/Mal/Succ).



Figure 6.2: Mitochondrial Membrane Potential **A**) $\text{DilC}_1(5)$ Mean Channel Fluorescence (mean \pm SEM) from lymphocytes for growth hormone treated samples compared to untreated samples under different substrate conditions. **B**) $\text{DilC}_1(5)$ Mean Channel Fluorescence (mean \pm SEM) from monocytes for growth hormone treated samples compared to untreated samples under different substrate conditions.



Figure 6.3: Mitochondrial Membrane Potential **A**) $\text{DilC}_1(5)$ Mean Channel Fluorescence (mean \pm SEM) from lymphocytes for IGF-1 treated samples compared to untreated samples under different substrate conditions. **B**) $\text{DilC}_1(5)$ Mean Channel Fluorescence (mean \pm SEM) from monocytes for IGF-1 treated samples compared to untreated samples under different substrate conditions.

6.4.2 – Highly Reactive Oxygen Species Production

Treatment with H_2O_2 and ammonium iron (II) sulphate significantly increased HPF mean channel fluorescence (AU) from samples in the presence of Pyr/Mal (mean difference \pm SEM, 95% confidence intervals, p-values: 102.40 \pm 21.15AU, 55.25 – 149.54AU, P \leq 0.05), Succ/Rot (27.29 \pm 2.17AU, 22.47 – 32.11AU, P \leq 0.05), Pyr/Mal/Succ (106.36 \pm 11.51AU, 80.68 – 132.04AU, P \leq 0.05) and Oct/Mal (45.85 \pm 5.51AU, 33.42 – 58.29AU, P \leq 0.05) compared to untreated samples under the same respiratory conditions in the lymphocyte sub-population (Figure 6.4 A). Fluorescence values from Pyr/Mal treated samples were significantly increased compared to all other respiratory conditions analysed (P \leq 0.05), while Pyr/Mal/Succ treated samples were significantly increased compared to Succ/Rot (35.68 \pm 1.03AU, 32.93 – 38.42AU, P \leq 0.05) and Oct/Mal (25.29 \pm 1.03AU, 22.54 – 28.03AU, P \leq 0.05) treated samples. In addition, fluorescence values from Oct/Mal treated samples were significantly increased compared to Succ/Rot (31.4AU, P \leq 0.05).

In the monocyte sub-population treatment with H₂O₂ and ammonium iron (II) sulphate also significantly increased HPF mean channel fluorescence (AU) from samples in the presence of Pyr/Mal (1578.36±237.45AU, 1042.18 - 2114.55AU, P≤0.05), Succ/Rot 1752.24 (2308.22±245.80AU, _ 2864.21AU. P≤0.05), Pyr/Mal/Succ (2306.77±279.60AU, 1675.39 – 2938.15AU, P≤0.05) and Oct/Mal (2458±137.21AU, 2148.31 - 2768.98AU, P ≤ 0.05) compared to untreated samples under the same respiratory conditions (Figure 6.4 B). Fluorescence values from Pyr/Mal treated samples were significantly increased compared to all other respiratory conditions analysed (P≤0.05), while Pyr/Mal/Succ treated samples were significantly increased compared to Succ/Rot (243.25±6.33AU, 226.43 - 260.08AU, P≤0.05) and Oct/Mal (207.29±6.33AU, 190.47 – 224.11AU, P≤0.05) treated samples. In addition, fluorescence values from Oct/Mal treated samples were significantly increased compared to Succ/Rot samples (35.97±6.33AU, 19.15 – 52.79AU, P≤0.05).

Neither GH (Figure 6.5) nor IGF-1 (Figure 6.6) exerted any significant effect on hROS levels as indicated by HPF mean channel fluorescence values in either lymphocyte (P=0.90) or monocyte (P=0.85) sub-populations at any concentration administered. Finally no significant interaction effect between hormonal treatment and respiratory substrate condition was observed in either lymphocyte (P=0.99) or monocyte (P=0.39) sub-populations.



Figure 6.4: Highly Reactive Oxygen Species Production **A**) HPF Mean Channel Fluorescence (mean \pm SEM) from lymphocytes for positive control samples treated with H₂O₂ and ammonium iron (II) sulphate compared to untreated samples under different substrate conditions. (# P<0.05 compared to untreated, * P<0.05 compared to Pyr/Mal, *** P<0.05 compared to Pyr/Mal/Succ, *** P<0.05 compared to Oct/Mal, **** P<0.05 compared to Succ/Rot). **B**) HPF Mean Channel Fluorescence (mean \pm SEM) from monocytes for positive control samples treated with H₂O₂ and ammonium iron (II) sulphate compared to untreated samples under different substrate conditions. (# P<0.05 compared to Untreated samples treated with H₂O₂ and ammonium iron (II) sulphate compared to untreated samples under different substrate conditions. (# P<0.05 compared to Pyr/Mal, ** P<0.05 compared to Pyr/Mal, ** P<0.05 compared to Pyr/Mal, ** P<0.05 compared to Pyr/Mal, *** P<0.05 compared to Pyr/Mal, **** P<0.05 compared to Pyr/Mal, **** P<0.05 compared to Pyr/Mal, **** P<0.05 compared to Pyr/Mal/Succ, **** P<0.05 compared to Succ/Rot).



Figure 6.5: Highly Reactive Oxygen Species Production **A**) HPF Mean Channel Fluorescence (mean \pm SEM) from lymphocytes for growth hormone treated samples compared to untreated samples under different substrate conditions. **B**) HPF Mean Channel Fluorescence (mean \pm SEM) from monocytes for growth hormone treated samples compared to untreated samples under different substrate conditions.



Figure 6.6: Highly Reactive Oxygen Species Production **A**) HPF Mean Channel Fluorescence (mean \pm SEM) from lymphocytes for IGF-1 treated samples compared to untreated samples under different substrate conditions. **B**) HPF Mean Channel Fluorescence (mean \pm SEM) from monocytes for IGF-1 treated samples compared to untreated samples under different substrate conditions.

6.5 – Discussion

In agreement with studies which have evaluated the rate of ROS production in isolated mitochondria, the level of mitochondrial hROS production was found to be significantly increased under conditions supporting NADH-linked respiration in comparison to all other respiratory conditions analysed, indicating that electron leak at the site of complex I is an important contributor to the level of ROS production within the mitochondrial matrix [309, 314, 315]. In addition, $\Delta \psi_m$ was observed to be significantly decreased under conditions of complex I linked respiration. While elevated $\Delta \psi_m$ is known to be associated with increased rates of mitochondrial ROS production, at sufficiently high concentrations mitochondrial derived ROS have been demonstrated to cause the dissipation of $\Delta \psi_m$ prior to the initiation of cellular apoptosis in several studies [219, 316, 317]. Hence, it is likely the saturating concentrations of NADH-linked substrates utilized in the present study induced levels of oxidative stress that were detrimental to cellular viability.

In contrast, under conditions of complex II linked respiration, hROS levels were significantly decreased in comparison to other respiratory conditions, indicating that complexes II – IV of the ETC, and in particular complex III, do not significantly contribute to electron leak on the matrix side of the IMM. Whether cytosolic levels of hROS were affected by electron leak initiated at complex III could not be determined in the present study due to the probable diffusion of such molecules out of the cell following surface membrane permeabilization. Finally, mitochondrial hROS production was significantly increased in the presence of the eight carbon saturated FFA octanoate in comparison to complex II linked respiration, indicating that reducing equivalents derived from β -oxidation play a role in the generation of mtROS. It must be noted that the β -oxidation generates equal amount of NADH, which passes electrons to complex I, and FADH₂ whose electrons are transferred to ubiquinones in the inter-membrane space via ETF and ETF – QOR [125]. Thus, the principal site of electron leak under conditions of fatty acid oxidation remains to be determined.

The principal finding of this study was that neither GH nor IGF-1 exerted any significant effect on the rate of production of the highly reactive free radicals 'OH and ONOO-, under the respiratory conditions analysed, at any administered concentration.
While this is the first study to look at the effects of GH/IGF-1 on the production of these molecules, other studies have found that these hormones exhibit significant effects invitro on the generation of the reactive intermediates, O_2^- and H_2O_2 , whose creation precedes that of hROS [200, 201, 318]. Csiszar et al. [200] found that both mitochondria specific and cellular levels of O_2^- were significantly reduced in HCAECs following treatment at both physiological and supra-physiological concentrations of GH $(0.33 - 33\mu g/mL)$ and IGF-1 $(10 - 1,000\mu g/L)$. Thum et al. [201] also demonstrated significant decreases in intracellular ROS levels, determined using the non-specific ROS probe H₂DCFDA, in cultured human endothelial cells 24 hours post treatment with GH at concentrations of 100 and 1,000µg/L. In contrast, Gustafsson et al. [318] found that no significant change in intracellular ROS levels was induced by the presence of 10nM (76.5µg/L) IGF-1 in cultured human neuroblastoma cells under standard substrate conditions found in N2 culture medium. However IGF-1 was effective in preventing the rise of hyperglycaemic-induced ROS production in these cells at glucose concentrations in the range of 30 – 60mM. While Csiszar et al. [200] related the antioxidant effects of GH/IGF-1 directly to the up-regulated expression of the antioxidant enzymes Mn-SOD, Cu, Zn-SOD and GPX-1, Gustafsson et al. [318] found significant increases in uncoupling protein 3 (UCP3) expression following IGF-1 treatment and attributed the hormone's antioxidant effects directly to a decrease in the rate of electron loss from the respiratory chain as a result of an uncoupling of oxidative phosphorylation under saturating substrate conditions.

It is possible that significant reductions in mitochondrial O_2^- and H_2O_2 were induced by rhGH and IGF-1 under certain respiratory conditions in the present study and that the differences in the levels of these intermediates between untreated and hormone treated cells did not manifest as significant changes in HPF fluorescence due to the antioxidant capacity of untreated cells to prevent a rise in 'OH and ONOO- production. However, significant elevations in HPF fluorescence were observed under conditions of electron flow through complex I, precluding the possibility of reduced levels of O_2^- and H_2O_2 as a result of rhGH or IGF-1 administration under these respiratory conditions. In addition, the antioxidant effects of GH and IGF-1 recorded in most previous *in-vitro* studies were observed following treatment times in the range of 24 – 72 hours [200, 201, 318], while samples were only incubated in the presence of rhGH and IGF-1 for four hours in the present study. Thus, it is possible that the expression of the reported antioxidant effects of these hormones is time dependant and that four hours is insufficient for the manifestation of significant effects.

Despite this, in Study One of the present thesis it was found that rhGH treatment in human lymphocytes in-vitro at physiological concentrations of 5 and 10µg/L significantly decreased levels of mitochondrial derived O₂⁻ following only four hours of incubation, while treatment at higher concentrations up to 100µg/L exhibited no significant effect. It has been hypothesized by Kadenbach et al. [14] that cellular activity stimulated by any hormone, cytokine or neurotransmitter must be associated with a parallel modulation of the rate of electron transfer along ETC complexes in order to compensate for the loss of $\Delta \psi_m$ due to increased ATP synthase (Complex V) activity as a result of the elevated energy requirements imposed on the cell by the signalling molecule. Relating this hypothesis to results from the Study One suggest that GH was exerting its effects on the rate of mitochondrial ROS generation via direct regulation of the organelle at the level of electron transfer within the ETC. Furthermore, while an efficient coupling of oxidative phosphorylation at rhGH concentrations of 5 and 10µg/L may explain the significant reductions in mitochondrial O_2^- observed in that study at these concentrations, a decrease in this efficiency may have occurred at higher concentrations as the production of ATP became rate limiting [14]. In the present study neither rhGH nor IGF-1 exerted a significant effect on $\Delta \psi_m$ under the respiratory conditions analysed at any administrated concentration. The finding that neither hormone induced a change in the rate of hROS production is possibly due to the effect exerted on $\Delta \psi_m$ by saturating concentrations of respiratory substrates. In addition, saturating concentrations of ADP were also utilized in the study in order to initiate state 3 (phosphorylating) respiration following cell permeabilization, which doesn't accurately reflect endogenous respiration in-vivo where cells cycle between state 3 and state 4 (non-phosphorylating) respiration [319]. Thus, any subtle effects induced by GH or IGF-1 on the efficiency of oxidative phosphorylation may have been negated under the respiratory conditions analysed.

In the liver tissue of Wistar rats, following two weeks of GH treatment, Sanz *et al.* [35] found significant increases in the levels of oxidative damage to mitochondrial DNA despite observed decreases in the rate of H_2O_2 generation, indicating that initial prooxidative effects preceded any changes in antioxidant capacity induced by the hormone. Together with the finding by Gustafsson et al. [318] that IGF-1 only influenced the rate of ROS production in human neuroblastoma cells in-vitro following the addition of a stimulus to induce oxidative stress, this suggests that the changes induced by GH/IGF-1 on cellular antioxidant capacity are not mediated via a direct up-regulation of the expression of antioxidant enzymes. Indeed, it has been suggested that the influence exerted by the GH/IGF-1 axis on cellular oxidative capacity is mediated via the mechanism of mitochondrial hormesis [205]. A concept which has recently garnered much attention in the literature [320], mitochondrial hormesis refers to an adaptive response to small sub-lethal increases in mitochondrial derived ROS concentrations, which subsequently act as signalling molecules, leading to an up-regulation of antioxidant enzymes and increased resistance to oxidative stress. Ungvari et al. [205] have demonstrated that the activity of Nrf2, a transcription factor that regulates the expression of a subset of genes that contribute to antioxidant defences in response to oxidative stress, is significantly decreased in GH deficient Lewis Dwarf rats and that this activity is normalised following repletion of physiological GH concentrations, lending support to the hypothetical involvement of GH in the induction of this regulatory feedback pathway. In addition, Lewis Dwarf rats also exhibited a significant down-regulation of the deacetylase enzyme, silent information regulator 2 / Sirtuin1 (SIRT1) which is also reversed following treatment to attain GH repletion [205]. The presence of oxidative insult has been found to significantly increase the expression of SIRT1, which is known to act via a deacetylation of the forkhead box O (FoxO) transcription factor to exert changes in the expression of numerous proteins that regulate mitochondrial metabolism and antioxidant capacity, among which is an up-regulation of the antioxidant enzymes, Mn-SOD and GPX-1 [205, 321, 322]. It is likely that modulation of oxidative phosphorylation by the GH/IGF-1 axis is not the only factor affecting the efficiency of electron transfer in-vivo. Other factors which induce oxidative stress by up-regulating the rate of mitochondrial ROS production likely contribute to an interactive effect on mitochondrial respiration which is necessary for the production of mtROS at concentrations that will maximize their effect as intracellular messengers to induce an antioxidant response. Hence, a pre-existing prooxidative status within the cell may be necessary for GH/IGF-1 to induce any significant changes in antioxidant capacity, possibly explaining the absence of any significant effect on mtROS levels, following pre-treatment with GH and IGF-1 in-vitro in the present study.

In animal studies, cellular oxidative responses to GH and IGF-1 administration have varied depending on the cell type analysed, indicating that any antioxidant effects of the GH/IGF-1 pathway are tissue specific [41, 200, 202]. In contrast to the findings of Csiszar et al. [200] who showed that in-vitro GH and IGF-1 treatment in cardiomyocytes isolated from wild type mice significantly up-regulated the expression of Mn-SOD, Cu, Zn-SOD and GPX-1, Brown-Berg et al. [202] observed significant decreases in the expression of Mn-SOD, GPX-1 and catalase following similar treatment in murine hepatocytes. In addition, Sanz et al. [41] observed significant increases in oxidative damage to liver tissue isolated from Wistar rats following two weeks of GH treatment, while oxidative damage was significantly decreased in isolated cardiac tissue from the same animals. Discrepancies in the GH / IGF-1 effect between tissue types could be attributed to the expression of cell specific isoforms of GH and IGF-1 regulated locally at a tissue level [200]. Indeed, several tissue types have been shown to be capable of locally producing both GH and IGF-1, including subpopulations of human mononuclear leukocytes [323-326]. Thus, the autocrine / paracrine activation of GH / IGF-1 pathways may be more relevant to the hormonal effect on the rate of mtROS production in these cell types, than local concentrations of the systemic isoforms secreted from the anterior pituitary and the liver, respectively [200]. In support of this, Vinciguerra et al. [327] showed that the "local muscle specific" IGF-1 isoform (mIGF-1) in murine cardiomyocytes elicited a protective effect in response to paraquat induced oxidative stress, while increasing concentrations of the systemic IGF-1 isoform itself elicited a pro-oxidative response. The mIGF-1 isoform comprises a class 1 signal peptide sequence on its N-terminal which is 48 amino acids in length and a C-terminal "Ea extension" peptide sequence containing 35 amino acids, which the mature systemic IGF-1 isoform does not possess [327-329]. Both isoforms are known to trigger phosphorylation of the IGF-1R [327]. However, the systemic isoform is found to typically activate PI3K/Akt and MAPK dependent pathways, while mIGF-1 has been shown to instead activate 3-phosphoinositide-dependent kinase (PDK1) and serum- and glucocorticoid-inducible kinase-1 (SGK1) signalling molecules in cardiomyocytes, demonstrating differences in the isoforms respective signalling mechanisms downstream of surface receptor activation [327]. Hence, the activation of autocrine / paracrine pathways by locally produced isoforms may be required for significant effects on antioxidant capacity to be exhibited in the cell populations

analysed in the present study. However, the limitations in translating the results from animal models to human subjects must be noted here as animal studies do not always accurately predict human outcomes [294].

In conclusion, the present study has demonstrated that neither GH nor IGF-1 pretreatment was capable of attenuating the high rates of mitochondrial hROS production induced by complex I linked respiration under saturating substrate conditions. In addition, the oxidation of lipid-derived substrates was found to contribute to mitochondrial hROS levels, irrespective of the absence or presence of either hormone. While rates of mitochondrial ROS production under conditions of lipid-derived respiration did not compare to the high values observed under conditions of complex I linked respiration, St-Pierre et al. [125] demonstrated significant elevations in O₂⁻ levels in isolated mitochondria from rat skeletal muscle and cardiac tissue when respiring on palmitoyl carnitine, a derivative of the 16-carbon long chain FFA palmitate, which they attributed to a prolonged reduction of ETF and ETF-QOR, in addition to an increase in complex I mediated electron leak. Indeed, elevated concentrations of long chain FFA's in-vivo, in excess of the mitochondrial capacity to oxidize them, are known to become trapped in the mitochondrial matrix, elevating the redox state of the organelle and giving rise to the formation of highly reactive lipid peroxides [45, 113]. Thus, long chain FFA's which require active transportation across the IMM may be more prone to inducing ROS production than medium chain FFA's such as octanoate, which can enter and leave the mitochondrial matrix freely [330].

These observations have implications for the administration of GH *in-vivo*, which has been demonstrated to induce two to three fold elevations in serum FFA concentrations in healthy subjects [21, 144, 331]. In addition, Jurand and Oliver [332] have reported that the percentage of the serum FFA, palmitate is significantly higher in acromegalic patients than in healthy controls. Oxidative stress has been shown to be significantly reduced by IGF-1 administration in intact cells exposed to high ambient glucose concentrations *in-vitro*, likely owing to the insulin antagonizing effects of the hormone acting to inhibit the over-supply of substrates to the mitochondria [212, 318]. In contrast, both GH and IGF-1 are known to promote lipid derived oxidation *in-vivo* [21]. Whether either hormone would have the same impact on attenuating levels of mitochondrial ROS induced by the presence of saturating FFA concentrations in intact cells is unknown. In the future, studies analysing the effects of GH and IGF-1 on

mitochondrial oxidative status *in-vitro*, should also be carried out in the presence of FFA at concentrations that mimic the cellular environment *in-vivo*, in order to account for any interaction that occurs between the hormonal and substrate impact on mitochondrial metabolism.

7. Study Three:

The effect of one week's administration of recombinant human growth hormone on the regulation of mitochondrial apoptosis in peripheral blood mononuclear cells *invivo*. An expression study of mitochondrial and cytosolic derived miRNA, mRNA and protein.

7.1 – Abstract

While the anti-apoptotic effects of administration of rhGH are well documented *in-vitro*, the temporal extent of these effects in-vivo following the cessation of rhGH administration is currently unknown. In the present study, healthy resistance trained male subjects, aged 25.4±1.22 years, were subcutaneously injected with either rhGH (1mg) or saline (0.9%) for seven consecutive days (Days 1-7) in a blinded fashion. Blood sampling was undertaken before rhGH administration and over a period of three weeks (Days 8, 15, 22 & 29) following the final injection of rhGH. PBMCs were isolated for the determination of miRNA, mRNA and protein expression levels in both placebos and cases. The purpose of this study was to determine whether PBMCs, post rhGH administration, would show any significant effects on the regulation of Bcl-2 family members. Respective anti and pro-apoptotic, Bcl-2 and Bak genes and proteins were measured, over a period of three weeks following the final rhGH injection. Additionally, post-transcriptional regulation of gene expression by miRNAs associated with apoptotic genes was assessed in two cellular compartments, the cytosol and the mitochondria. The latter mitochondrial presence of miRNA, the functions of which have yet to be elucidated, has only recently been reported in the literature, raising consequently important questions as to whether these mitochondrial associated miRNAs (mito-miRNAs) act as intracellular signals in cellular apoptosis. Special attention was drawn on the expression levels of miR-181a and miR-125b, known as translational inhibitors of Bcl-2 and Bak respectively. These miRNA molecules were analysed in both cytosolic and mitochondrial fractions following rhGH administration. Results showed that rhGH had no effects on the mRNA and protein expression of Bcl-2. However, post rhGH administration showed a significant effect on Bak which exhibited decreased protein concentrations compared to baseline and following correction with placebos. This effect was observed up to 8 days following the last rhGH treatment. Cytosolic miRNA expression was not found to be significantly affected by rhGH. However, measurement of the expression of miR-125b in mitochondrial fractions showed a significant down-regulation 8 days post rhGH administration. In conclusion, these findings suggest that rhGH induces short term anti-apoptotic effects which may be partially mediated through a novel pathway not yet reported that alters the concentration of mitochondrially associated miRNA's.

7.2 – Introduction

Apoptosis or "programmed cell death" is a physiologically favourable form of cell death which in addition to maintaining homeostatic control over cell populations, functions to remove damaged or diseased cells without incurring an inflammatory reaction [51, 333, 334]. Mitochondrial mediated apoptotic signals are also initiated by extracellular stimulation which serves to propagate the caspase response, demonstrating the key and central roles of mitochondrial organelles in apoptosis [335 – 337]. Dysfunctional regulation of these apoptotic pathways is implicated in the development of pathological conditions such as cancer, diabetes, ischemia-reperfusion, autoimmune disorders, neurodegenerative diseases and acute organ failure [11, 333, 336, 338].

Interestingly, *in-vitro* rhGH administration has been well documented to induce antiapoptotic effects following stimulation of both the intrinsically and extrinsically mediated apoptotic pathways. Anti-apoptotic effects were reported and demonstrated in various cell lines including peripheral blood lymphocytes, monocytes and pancreatic β cells [208, 209, 339]. In addition, IGF-1, whose secretion from diverse tissues including the liver is regulated by GH *in-vivo* [4, 128], shows well characterised survival effects both for doxorubicin challenged cardiomyocytes [17, 298] and for neuroblastoma cells under hyperosmotic stress [340]. Additional survival effects mediated by IGF-1 are demonstrated in serum starved granulocytes [341], PC12 cells [342] and for renal mesangial cells exposed to high glucose concentrations [212].

Despite these noted anti-apoptotic effects, supra-physiological *in-vivo* concentrations of GH, arising from either disease states or rhGH administration in healthy individuals, have been shown to cause hyperglycemia, hypertriglyceridemia and in some cases to lead to the development of new onset diabetes [23, 343]. Under such pathological conditions, cells are exposed to oxidative stress and damage which may predispose them to prematurely undergo apoptosis [124]. A recent case study by Geraci *et al.* [343] has presented findings from a 33 year old male bodybuilder with extreme hyperglycaemic and hypertriglycederimic blood concentrations, three weeks after completing an androgenic anabolic steroids program that included the intramuscular injection of supra-physiologic concentrations of bovine growth hormone. Sustained hyperglycemia has previously been reported to induce cardiomyocyte apoptosis in diabetes and animal models [344]. In light of the development of these pro-apoptotic

conditions, even following the cessation of growth hormone abuse, the temporal extent of the anti-apoptotic effects of GH could have important implications for cellular survival.

In both extrinsic and intrinsic pathways, the release of cyt c from the inter-membrane space is widely regarded as the rate-limiting step towards initiation of the execution phase of apoptosis, the phase at which the cell is committed to programmed death [51, 55]. While debate is ongoing as to whether cyt c release is instigated by permeabilization across the IMM and OMM via mtPTP activation or simply by OMM permeabilization, there is a general consensus that the release of cyt c is regulated by the Bcl-2 family of proteins [59, 333]. To date, twenty five Bcl-2 family proteins have been identified, which exhibit either a pro-apoptotic or anti-apoptotic function [51]. It has been extensively demonstrated that the concentration ratio of the anti-apoptotic OMM based Bcl-2 proteins, Bcl-2 and Bcl-xL, and pro-apoptotic Bax and Bak proteins is an important determinant as to whether a cell undergoes apoptosis in response to either intrinsic or extrinsic stimuli [345-349].

Both Bcl-2 and Bcl-xL have been demonstrated to inhibit a wide array of apoptotic agents. Up-regulation of Bcl-2 gene expression in neural cells has been reported to result in decreased levels of intracellular ROS production, in addition to increased resistance to both mitochondrial dysfunction and cell death induced by oxidative stress [51, 350, 351]. The prevention of apoptosis upon death-receptor activation by GH administration in PBMCs *in-vitro* has been previously associated with increased Bcl-2 expression [19, 208, 339]. In addition, GH induced an up-regulation of Bcl-xL expression that prevented apoptosis in pancreatic β cells targeted by the pro-inflammatory cytokines, interleukin-1 β , interferon- γ and tumour necrosis factor- α [209]. Increased expression of Bcl-xL was also observed in IGF-1 treated rats which conferred myocardial protection against ischemia-reperfusion injury [17].

Conformational changes induced upon Bax in the cytosol by pro-apoptotic activator Bcl-2 proteins such as tBID, Bim and PUMA lead to translocation of the protein to the OMM. Subsequent homo-oligomerization activation of Bax in the OMM has been shown to be associated with mitochondrial membrane permeabilization and cyt c release [57-59]. Interestingly, synthesis of Bax is found to be significantly decreased in the presence of GH [210, 352]. In addition, IGF-1 treatment in rats was found to decrease

the cellular concentration of Bax in cardiomyocytes and to attenuate, in the presence of doxorubicin, the induction of Bax into the OMM [298]. Over expression of Bak has been widely documented to accelerate the process of apoptosis in both murine and human cultured cell lines [353, 354]. In a manner similar to Bax, homo-oligomerization of Bak is found to induce cyt c release from the mitochondrial inter-membrane space [57-59]. In contrast to cytosolic located Bax, Bak is perennially located within the OMM [57-59], explaining possibly why in single Bax-/- knockout mouse embryonic fibroblasts (MEFs), Bak-dependent cells are found to exhibit significantly higher rates of apoptosis compared to Bax-dependent cells in Bak-/- MEFs [59]. Surprisingly, the effects of bath GH and IGF-1 administration on the level of Bak gene and protein expression have, to date, not been investigated.

The respective down- and up-regulation of these pro and anti-apoptotic proteins contributes significantly to the survival effects induced by GH and IGF-1. Interestingly, these hormones have also been shown to induce changes in miRNA - molecules in target cells and tissues involved in regulating protein expression [355, 356]. Mature miRNA's are non-coding short single stranded RNA, sequences of approximately 18 -22 nucleotides in length, known to regulate gene expression post-transcriptionally [93, 357, 358]. Through complementary binding, commonly to the 3'UTR of target mRNA molecules, miRNAs are able to induce mRNA degradation. Meanwhile complementary binding of only the 2 - 8 nucleotides of the "5' seed region" of miRNA to its target mRNA is enough to induce down-regulation of gene translation, which reduces the rate of protein synthesis [93, 102]. It has also been shown that miRNA is capable of inducing translational up-regulation of target mRNA under specific conditions, such as cell cycle arrest [359]. To date nearly 1,000 miRNAs have been identified, while a single miRNA has the potential to bind hundreds of mRNA targets [93, 357, 358]. Indeed it is estimated that up to 30% of the gene transcriptome could be regulated by miRNAs [93]. Consequently, the identification and validation of miRNA/mRNA interactions is an ongoing and fundamental area of miRNA research [226]. Bcl-2 and Bak genes have been experimentally validated as targets for translational downregulation by miRNAs miR-181a [99, 227, 360] and miR-125b [102, 226, 228] respectively. In addition to their apoptotic roles, miR-181a has been demonstrated to act as a potent inhibitor of cellular proliferation, while miR-125b has been shown to adversely affect cellular senescence and apoptosis [229, 230]. Interestingly, whether or

not GH of IGF-1 induces changes in the expression of these specific miRNAs has not been reported to date in the literature.

While miRNA have predominantly been shown to exert their post-transcriptional influence in the cytosol, recently several studies have demonstrated the localisation of miRNA in isolated mitochondria [104-107, 361]. Interestingly, the level of expression of mito-miRNA has been shown to be independent of the total cellular miRNA expression profile [104, 105]. Surprisingly, Kren et al. [105] have demonstrated that the mito-miRNA pool isolated from rat liver-derived mitochondria was not predicted to regulate any nuclear derived mRNA transcripts coding for mitochondrial located proteins. While the significance of these findings has yet to be elucidated, it has been speculated that mitochondria may serve as a miRNA reservoir [105]. Furthermore, the trafficking of these short nucleotide sequences between the mitochondrial compartment and the cytosol is thought to be under the regulation of cytosol located mRNAprocessing bodies (P-bodies) [361]. It has been suggested that the storage and subsequent release of selective miRNA from mitochondria may act as a mechanism of intracellular signalling, exerting control over cellular processes such as apoptosis, proliferation and differentiation [104, 105]. Indeed, the predicted gene-targets of some miRNA identified to be localized to mitochondria are consistent with those found to be down-regulated at the onset of cell death [105]. Furthermore, disruption of mitochondrial function has been reported to precede translational inhibition taking part in the apoptotic process [105]. Considering the known anti-apoptotic effects exhibited by rhGH administration, it is possible such anti-apoptotic roles might be mediated in part by mito-miRNA.

The purpose of this study is to determine the effects on the regulation and expression of pro- and anti-apoptotic Bcl-2 family proteins in PBMCs following the cessation of a seven day programme of rhGH administration in healthy trained male subjects for a period of three weeks. Evaluation of Bcl-2 and Bak expression was determined for mRNA levels and proteins from mitochondrial fractions. In addition, cytosolic expression of miR-181a and miR-125b was determined to identify possible effects on Bcl-2 and Bak mRNA and protein expressions. As both miR-181a and miR-125b have previously been found to be significantly expressed in isolated mitochondria from human skeletal primary muscular cells [107], the present study therefore aimed at determining whether these two miRNAs were present in mitochondria isolated from the

subjects PBMCs. Correlation analysis of possible rhGH induced expression of mitomiRNAs and Bcl-2 and BAK protein/mRNA levels was undertaken. We hypothesized that GH's anti-apoptotic role might be mediated through the activation of an intracellular signalling cascade implicating mito-miRNA recruitment.

Of note, the conservative rhGH concentration injected in this study was chosen in order to prevent the development of any adverse effects. Extreme doses of up to 25IU (8.3mg) rhGH per day are reportedly taken in its use as a performance enhancing drug [38]. However, our laboratory has previously demonstrated that at this low concentration, rhGH injection did significantly affect the GH/IGF-1 axis in healthy male subjects [362]. Ramos *et al.* [362], found that 1mg rhGH administration in healthy male subjects was sufficient to result in a significant increase in serum IGF-1 concentrations 24 hours post-treatment compared to baseline measurements (P<0.05), while no significant difference was seen in placebo treated control subjects. Additionally, in these same subjects, the expression of over 3,000 genes in PBMCs were observed to be significantly affected by the rhGH treatment protocol used for up to three weeks following the cessation of treatment (unpublished data).

7.3 – Methods

7.3.1 – Subjects

Ten healthy resistance trained male subjects (mean \pm SEM: age = 25.40 \pm 1.22 yrs, height = 1.80 \pm 0.16 m, body mass = 78.92 \pm 1.52 kg, BMI = 24.32 \pm 0.53 kg/m²) were recruited to participate in the study which was approved by the Bond University Human Research Ethics Committee. All subjects had both the nature of the study and the associated risks involved explained to them prior to providing written informed consent. Resistance trained was defined as the undertaking of a resistance exercise program, 3-5 days per week for a period of at least 12 months. Exclusion criteria included smoking, the use of therapeutic, recreational or performance enhancing drugs, including anabolic steroids and rhGH, up to 12 months prior to participation in the study, the use of prescription medication, the presence of diabetes, cardiovascular disease, acromegaly or any diagnosed condition which would contra-indicate either participation in resistance exercise or the administration of rhGH.

7.3.2 – Reagents Used

MACS Mitochondrial Isolation kits were purchased from Miltenyi Biotec (Auburn, CA, USA). RNase-free DNase I and Total RNA/Protein purification kits were purchased from Norgen Biotek (Thorold, ON, Canada). Miscript II RT kits, SYBR Green PCR kits and Hs-RNU6-2, Hs-MiR-24, Hs-MiR-92, Hs-MiR-125b and Hs-MiR-181a primer assays were purchased from Qiagen (Donchaster, VIC, Australia). Custom primers for Bcl-2, Bak, GAPDH, Hyperparathyroidism 1 (HRPT1) and 18s, as shown in Table 7.1, were synthesised by Geneworks (Thebarton, SA, Australia). iQ SYBR Green supermix was purchased from Biorad (Hercules, CA, USA). Ribonuclease A and protease inhibitor cocktail were purchased from Sigma Aldrich (St. Louis, MO, USA), while RNasin PLUS RNase inhibitor was purchased from Promega (Madison, WI, USA). Human Bcl-2 platinum ELISA kits and human Bak ELISA kits were purchased from eBiosciences (San Diego, CA, USA) and USCN (Wuhen, China) respectively. Pierce BCA protein assay kit was purchased from Thermo Scientific (Rockford, IL, USA). RNase/DNase free water and PBS (pH 7.4) were purchased from Invitrogen (Carlsbad, CA, USA). Ficoll paque PLUS was obtained from GE Healthcare (Rydalmere, NSW, Australia). Finally, Genotropin was purchased from Pfizer (Sydney, NSW, Australia).

Gene	Unigene ID	Forward Sequence (5' – 3')	Reverse Sequence (5' – 3')	Amplicon Length (bp)
18s	100008588	TTCGAGGCCCTGTAATTGGA	GCAGCAACTTTAATATACGCTATTGG	123
GAPDH	2597	CTCTGCTCCTCCTGTTCGAC	ACCAAATCCGTTGACTCCGAC	108
HRPT1	3278	GCTGAGGATTTGGAAAGGGTG	CAGAGGGCTACAATGTGATGG	112
Bcl-2	596	CATCCAGTACCTTAAGCCCTG	CTCAGACAGAGCCAGTATTGG	83
Bak	578	GAGATGGTCACCTTACCTCTG	GCAACATGGTCTGGAACTCTG	117

Table 7.1: Primer sequences used for each mRNA gene product and the corresponding size of the PCR amplicon generated.

7.3.3 – Experimental Design

Subjects were randomly assigned to either a treatment group (rhGH; n=5) who received 1mg of Genotropin, a recombinant human growth hormone which exhibits a complete sequence homology to the 22kDa hGH isoform, or a placebo group (P; n=5) who were administered with a saline solution (0.9% sodium chloride) for a period of seven consecutive days. Daily injections were administered subcutaneously in a double blind manner. Prior to each injection all participants received a standardized meal (protein shake) followed by a 15 minute rest period. Blood samples were collected from each subject 24 hours prior to treatment administration as well as 1, 8, 15 and 22 days post-treatment.

7.3.4 – Blood Sample Collection and PBMC Isolation

Subjects arrived for sample collection in a post-prandial state. Blood samples were taken from subjects resting in a supine position, prior to insertion of a catheter into the antecubital vein with 20mL of blood was drawn into 10mL EDTA vacutainers (BD, CA, USA). Collected EDTA blood was diluted in an equal volume PBS (pH = 7.4) and layered over Ficoll-paque PLUS at a ratio of 2:1. Samples were subsequently centrifuged at 450*g for 30 minutes to achieve separation of PBMCs from whole blood.

7.3.5 – Mitochondrial Isolation and Cytosolic RNA Decontamination

Mitochondria were isolated from PBMCs using a magnetic antibody cell sorting method. PBMCs were resuspended in 100 μ L cell lysis buffer in the presence of 1 μ L of protease inhibitor and homogenised 10 times by passage through a 27 gauge needle. The mitochondria in the cell lysate were subsequently magnetically labelled with microbead conjugated anti-TOM22 antibodies. The antibody labelled cell lysate was then loaded into a column placed in a magnetic field separator. Of note, flow through cell lysate was kept for later RNA extraction and magnetically labelled mitochondria bound to the column were washed as per manufacturer instruction. Mitochondria were finally eluted following the removal of the column from the magnetic field. Isolated mitochondria were further pelleted by centrifugation at 13,000*g for 2 minutes at 4°C and resuspended in 200 μ L RNase A solution (Ribonuclease A concentration: 10 μ g/mL) at 37°C for one hour to remove any possible residual cytosolic RNA molecules residing

outside intact mitochondria. Post RNase incubation, mitochondria were pelleted a second time by centrifugation at 13,000*g for 2 minutes at 4°C and resuspended in 100μ L mitochondrial storage buffer. RNase activity was stopped by the addition of 5μ L RNasin PLUS.

7.3.6 – Total RNA and Protein Extraction from Cytosolic and Mitochondrial Lysates

Stored isolated mitochondria were pelleted by centrifugation at 13,000*g for 2 minutes at 4°C, resuspended in 350µL lysis solution containing high concentrations of chaotropic denaturant, for rapid inactivation of RNases and proteases, and vortexed vigorously to produce a mitochondrial lysate. Meanwhile the collected cell lysate was treated with 2µL RNasin Plus and 1µL protease inhibitor cocktail to protect the cytosolic RNA fraction from degradation. Both cytosolic and mitochondrial lysates were subsequently mixed with isopropanoyl at a ratio of 2.33:1. Total RNA purification of cytosolic and mitochondrial lysates was performed by spin column chromatography using a proprietary silicon carbide (SiC) resin (Norgen Biotek Corporation) which binds nucleic acids in a manner dependent on ionic concentrations. Thus all sizes of RNA from large mRNA down to miRNA and small interfering RNA (siRNA) were separated from sample proteins which were removed in the column flowthrough. The flowthrough from mitochondrial lysate was retained and stored at -80°C for later analysis of mitochondrial proteins. Samples were subsequently treated with RNase free-DNase I for 15 minutes for the removal of all traces of residual DNA from columns prior to column washing and elution of purified RNA as per manufacturer's instructions. Finally, total cytosolic and mitochondrial RNA was quantified on a spectrophotometer (Nanodrop 1000, Thermo Scientific).

7.3.7 – cDNA Synthesis from Cytosolic and Mitochondrial RNA

Reverse transcription of total RNA into cDNA was performed using the miScript II RT kit from Qiagen. Mitochondrial and cytosolic RNA were transcribed separately with 12μ L of purified RNA (20ng/µl) being used in each 20µL reaction volume as per manufacturer's instructions. The supplied reverse transcriptase mix contained optimised concentrations of both poly (A) polymerase and reverse transcriptase which allowed for polyadenylation and reverse transcription to be performed in parallel for each sample. The miScript HiFlex Buffer was used for cDNA synthesis which was used as a template for real-time PCR (RT-PCR) gene expression analysis and quantitation of both miRNA

and mRNA. Mature miRNAs were polyadenylated by poly (A) polymerase and reverse transcribed into cDNA using modified common oligo-dT primers as these primer sequences hold a 3' degenerate "anchor" and a universal tag sequence on the 5' end which allowed for amplification of mature miRNA by RT-PCR. All non miRNA RNA types were converted into cDNA using usual oligo-dT and random primers. These samples were reverse transcribed at 37°C for 60 minutes prior to being heated at 95°C for 5 minutes for inactivation of miScript reverse transcriptase mix.

7.3.8 – Real-time Polymerase Chain Reaction (RT-PCR) Analysis

RT-PCR assays were performed using a Corbett Rotor-gene 6000 (Qiagen). For relative quantification of mRNA gene expression, 5μ L of lysate-derived cDNA was mixed with 6μ L Rnase-free water, 12μ L iQ SYBR Green supermix (Biorad) and various sets of gene specific primers (forward and reverse: 1μ L each at a concentration of 5μ M) in a 25μ L reaction volume. The forward and reverse sequences of the primers used for each gene product are listed in Table 1. GAPDH was used as an endogenous reference gene for normalisation of target mRNA expression. Comparative PCR efficiency between reference and experimental primers was confirmed by running standard curves for each amplicon using the same sample. The change in cycle threshold between reference and experimental genes was plotted against the input amount of cDNA on a log scale, with a slope of ≤ 0.1 on the resulting semi-log regression line indicating comparable efficiency [363]. RT-PCR reactions were conducted at 94°C for 12 minutes, followed by 40 cycles at 94°C for 30 seconds, 59°C for 30 seconds and 72°C for 30 seconds.

For relative quantification of miRNA expression, both lysate and mitochondrially derived cDNA were mixed with Quantitect SYBR Green PCR master mix and miScript universal reverse primer (Qiagen) in combination with target-specific miScript forward primers in a 20µL reaction volume as per manufacturer's instructions. The predesigned forward primers, purchased from Qiagen, were as follows: Hs-RNU6-2 (Cat No: MS00033740), Hs-MiR-24 (Cat No: MS00006552), Hs-MiR-92 (Cat No: MS00006594), Hs-MiR-125b (Cat No: MS00006629) and Hs-MiR-181a (MS00008827). Amplicon lengths of 85-87bp were expected for all mature miRNA PCR products. RNU6-2 served as an endogenous reference miRNA for normalisation of target lysate-derived miRNA expression. Concentrations of mitochondrial derived cDNA from five samples were normalised against sample mitochondrial protein concentrations. These samples were subsequently analysed by RT-PCR as described for determination of the most appropriate endogenous control miRNA for normalisation of target mitochondrial miRNA expression. Among three possible candidates (RNU6-2 [364], Hs-MiR-24 [365] and Hs-MiR-92 [366]), MiR-92 was found to be the most stably expressed. RT-PCR reactions were conducted at 95°C for 15 minutes, followed by 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds and 70°C for 30 seconds.

The relative expression of mRNA and miRNA were calculated using the comparative C_T ($\Delta\Delta C_T$) method. The results are presented as log₂ fold differences of each target mRNA and miRNA in all post-treatment samples relative to baseline samples. All reactions were performed in triplicate, while no template control reactions were run simultaneously with each PCR assay. Melting curve analysis was performed at the end of each PCR run in order to confirm the identity of each PCR amplicon and to rule out the production of non-specific products. Any samples exhibiting either earlier than expected melting temperatures (T_M), indicating the formation of primer-dimers or multiple peaks, indicating the production of non-specific products, were excluded from the data set. In addition, agarose gel electrophoresis was carried out on the PCR product of all amplified mRNA and miRNA targets for verification of the specificity of each amplicon.

7.3.9 – Determination of Bcl-2 and Bak Protein Concentrations

Quantification of total mitochondrial protein concentration was determined using the Pierce bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL, USA). Samples were incubated at 37° C for 30 minutes in an alkaline medium in the presence of BCA and cupric sulphate. The reduction of Cu²⁺ ions from cupric sulphate to Cu⁺ occurs in proportion to the amount of protein present in the sample. Two molecules of BCA chelate each Cu⁺ ion formed. The product exhibits a high absorbance at 562nm, which was read on a Modulus microplate reader (Turner Biosystems). A standard curve, developed from known concentrations of BSA, was used to determine sample protein concentrations. Total protein concentration was subsequently normalised for all samples prior to analysis of Bcl-2 and Bak levels.

Bcl-2 and Bak concentrations from mitochondrial protein samples were assessed by enzyme-linked immunosorbent assay, according to manufacturer's instruction. Briefly, samples were incubated on antibody coated microtitre plates together with biotinconjugated antibodies, both of which were specific to the analyte of interest. Subsequently, streptavidin-conjugated horseradish peroxidase (HRP) was added which binds to biotin-conjugated antibodies. Removal of unbound antibody and enzyme by microplate washing allows for the development of a colour change in proportion to the concentration of analyte present in sample wells, following the addition of 3,3',5,5'-tetramethylbenzidine (TMB), a substrate of HRP. The enzyme-substrate reaction was terminated by addition of sulphuric acid and absorbance was measured at 450nm on a Modulus microplate reader (Turner Biosystems, Sunnyvale, CA, USA). The optical density of analysed samples was used in conjunction with the development of standard curves for the calculation of Bcl-2 and Bak concentrations, according to manufacturers' instructions.

7.3.10 – Statistical Analysis

All data is reported as mean \pm SEM. A two-way ANOVA with repeated measures was used to determine whether significant differences exist between the two treatment groups (rhGH and placebo) for fold changes in mRNA and miRNA expression and mitochondrial protein concentrations. Bonferroni's multiple comparisons were used for *post hoc* analysis to identify the location of significant differences between conditions. Pearson's product moment correlational analysis was used to determine the existence of any significant relationships between the analysed variables (SPSS Inc, PAWS Statistics Version 18, USA). Statistical significance was accepted at the P<0.05 level of confidence.

7.4 – Results

7.4.1 – Bak / Bcl-2 Protein Concentrations

For pre-treatment measurements Bak protein concentrations from mitochondrial fractions in rhGH and placebo groups were not significantly different from each other (Figure 7.1 A). Interestingly, Bak protein concentrations were found to be significantly decreased in rhGH treated samples compared to placebo treated samples for measurements taken 1 (mean difference \pm SEM, 95% confidence intervals, p-values: - 2.22 \pm 0.77µg/L, -4.01 – -0.44µg/L, P \leq 0.05) and 8 (-1.85 \pm 0.70ng.mL, -3.47 – -0.23µg/L, P \leq 0.05) days post-treatment. However, no significant differences were observed between treatment groups at 15 and 22 days post-treatment. No significant differences were observed in Bcl-2 protein concentrations from mitochondrial fractions between rhGH and placebo treated groups at any time point measured (24 hours pre-treatment; 1, 8, 15 and 22 days post treatment) (Figure 7.1 B).

7.4.2 – Bak / Bcl-2 mRNA Expression

Seven days of administration of rhGH (1mg per day) was not found to exhibit any significant effect on Bak mRNA differences in expression from baseline levels for any measured time period (1, 8, 15 and 22 days following cessation of the treatment program) compared to placebo treated controls (Figure 7.2 A). Similarly, rhGH treated samples were not found to have any significant effect on Bcl-2 mRNA changes in expression from baseline levels in comparison to placebo treated samples at any time point analysed post treatment (Figure 7.2 B).



B)



Figure 7.1: A) Protein concentrations (mean \pm SEM) of Bak from PBMC mitochondrial extracts in rhGH treated compared to placebo treated samples. **B**) Protein concentrations (mean \pm SEM) of Bcl-2 from PBMC mitochondrial extracts in rhGH treated compared to placebo treated samples. (* P<0.05 compared to placebo).



B)



Figure 7.2: A) Changes from baseline measurements (mean \pm SEM) in the expression of Bak mRNA levels from PBMCs in rhGH treated compared to placebo treated samples. **B)** Changes from baseline measurements (mean \pm SEM) in the expression of Bcl-2 mRNA levels from PBMCs in rhGH treated compared to placebo treated samples.

7.4.3 – Cytosolic miR-125b / miR-181a miRNA Expression

No significant differences were observed in the level of expression of miR-125b from cellular lysate at any time point compared to baseline measurements between rhGH and placebo treated groups (Figure 7.3 A). In addition, expression changes from baseline measurements for miR-181a from cellular lysate were not found to be significantly different between rhGH and placebo treated groups for any of the post-treatment time points (Figure 7.3 B). No significant correlation was found for the expression differences from baseline levels between cytosolic miR-125b and either Bak mRNA or protein (Table 7.2). Similarly, cytosolic miR-181a fold expression changes did not significantly correlate with changes in the expression of either mRNA or protein Bcl-2 levels (Table 7.2).

Cytosolic_miRNA		Placebo	GH
Mir-181a			
	Bcl-2 mRNA	0.41 (P=0.08)	0.40 (P=0.09)
	Bcl-2 Protein	-0.11 (P=0.65)	-0.22 (P=0.37)
Mir-125b			
	Bak mRNA	0.35 (P=0.14)	-0.12 (P=0.64)
	Bak Protein	0.16 (P=0.50)	0.05 (P=0.83)

Table 7.2: Pearson's correlational co-efficients of cytosolic associated miRNA fold changes in expression against mRNA/Protein fold changes in expression for the corresponding gene of interest in placebo and rhGH treated groups.

7.4.4 – Mitochondrial miR-125b / miR-181a miRNA Expression

The change in fold expression of miR-125b in mitochondrial fractions from baseline values was found to be significantly decreased in rhGH treated samples compared to placebo treated controls (-2.86±0.74, -4.61 – -1.11, P \leq 0.05) at 8 days post-treatment (Figure 7.4 A). However no significant differences were observed between the treatment groups at 1, 15 or 22 days post-treatment. No significant differences were observed in the level of expression of miR-181a from mitochondrial fractions at any time point compared to baseline measurements between rhGH and placebo treated groups (Figure 7.4 B).

No significant correlation was found for the fold expression changes from baseline levels between mitochondrial associated miR-125b and either Bak mRNA or protein (Table 7.3). Additionally, mitochondrial associated miR-181a fold expression changes did not significantly correlate with changes in the expression of either mRNA or protein Bcl-2 levels (Table 7.3).

Mitochondrial_miRNA		Placebo	GH
Mir-181a			
	Bcl-2 mRNA	-0.20 (P=0.48)	0.37 (P=0.13)
	Bcl-2 Protein	-0.16 (P=0.57)	-0.31 (P=0.21)
Mir-125b			
	Bak mRNA	-0.08 (P=0.74)	0.37 (P=0.13)
	Bak Protein	-0.22 (P=0.35)	-0.17 (P=0.51)

Table 7.3: Pearson's correlational co-efficients of mitochondrial associated miRNA fold changes in expression against mRNA/Protein fold changes in expression for the corresponding gene of interest in placebo and rhGH treated groups.



B)



Figure 7.3: A) Changes from baseline measurements (mean \pm SEM) in the expression of miR-125b miRNA levels from the cytosol of PBMCs' in rhGH treated compared to placebo treated samples. B) Changes from baseline measurements (mean \pm SEM) in the expression of miR-181a miRNA levels from the cytosol of PBMCs' in rhGH treated compared to placebo treated samples.







Figure 7.4: A) Changes from baseline measurements (mean \pm SEM) in the expression of miR-125b miRNA levels from isolated PBMC mitochondria in rhGH treated compared to placebo treated samples. **B**) Changes from baseline measurements (mean \pm SEM) in the expression of miR-181a miRNA levels from isolated PBMC mitochondria in rhGH treated compared to placebo treated samples. (* P<0.05 compared to placebo).

7.5 – Discussion

Here we report, for the first time, significantly decreased protein concentrations of the pro-apoptotic Bcl-2 family member Bak in isolated PBMC mitochondria following rhGH administration. These decreases were observed at one and eight days posttreatment compared to placebo treated controls. While the level of expression of Bcl-2 plays an important role in determining whether or not a cell will undergo apoptosis, Zong et al. [367] demonstrated that the suppression of anti-apoptotic Bcl-2 family proteins was not sufficient to induce apoptosis in the absence of Bak and another proapoptotic Bcl-2 family member, Bax. Indeed, considerable evidence exists to indicate that mitochondrial initiation of apoptosis is mediated via either a Bax or a Bak dependent pathway [58, 349, 367, 368]. In addition to this, the Bak protein has been found to be a more potent inducer of cell death than Bax and it has been demonstrated that over expression of the Bak gene induces high levels of apoptosis regardless of the level of the anti-apoptotic Bcl-2 proteins (Bcl-2 and Bcl-xL) [59, 354]. While it is well established that both GH and IGF-1 exert a significant down-regulation of the level of expression of the Bax gene [17, 210, 298, 352], to our knowledge the effect of GH on Bak gene expression has not been demonstrated previously. In addition, conclusive evidence linking the effects of IGF-1 to Bak regulation has not been reported. Although Khan et al. [369] found that Bak protein expression was significantly down-regulated in mesenchymal stem cells (MSC's) following only one hour incubation in the presence of IGF-1 (50 µg/L), this treatment was administered in combination with the survival factor, fibroblast growth factor-2 (FGF-2) (50µg/L). Thus, any effect witnessed on the regulation of Bak protein levels in that study could not solely be attributed to the actions of IGF-1. In addition, while Cui et al. [370] observed that in-vitro administration of IGF-1 (100µg/L) significantly decreased the ratio of Bak to Bcl-xL mRNA expression in porcine parthenotes, they failed to report the level of expression of these genes. Therefore, considering that IGF-1 is a proven upregulator of Bcl-xl expression [17, 340, 371, 372], to what extent the hormone exerted an effect on Bak expression is unclear from their observations.

While rhGH was found to significantly decrease Bak protein levels at one and eight days post-treatment, no significant differences were observed in protein concentrations after either 15 or 22 days following the cessation of treatment. These results could have important implications in light of the considerable evidence for the association between

chronic excesses in GH concentrations and apoptosis. In addition to the observed metabolic alterations resulting from chronic elevations in GH and IGF-1 concentrations which can lead to the development pro-apoptotic conditions in-vivo [21-23, 124, 343], case studies have reported that a prolonged history of rhGH abuse can lead to the development of cardiomyopathy and heart failure [373-375], conditions which are associated with deregulated apoptosis [376, 377]. Significant increases in cardiomyocyte apoptosis has been reported in myocardial biopsies from acromegalic patients, apparently contributing to cell loss and functional abnormalities in acromegalic cardiomyopahy [377-379]. Indeed, the degree of cardiac apoptosis was found to exhibit a significant positive relationship with both serum IGF-1 concentrations and the reported duration of acromegalic disease [378, 379]. Cardiomyocyte apoptosis is also found to be abnormally stimulated in the hearts of animals and humans with arterial hypertension, with apoptosis preceding both the impairment of ventricular function and the development of heart failure in hypertensive patients [377]. Interestingly, high levels of circulating IGF-1 have also been reported in patients with essential hypertension and hypertensive cardiomyopathy [377]. In light of these observations, the finding that the anti-apoptotic effects exerted by rhGH on Bak protein levels does not persist, at least up until 15 days following the cessation of treatment could have significant health consequences for individuals who are administering rhGH at supra-physiological concentrations.

Bak mRNA was observed to be down-regulated following rhGH administration at all time points compared to placebo treated controls. However unlike the decrease in Bak protein concentrations these results were not found to be significant. Our results at the transcriptomic and proteomic level for Bak therefore show a "mismatch" of expression. However, it has been experimentally validated that multiple miRNAs can target the same mRNA target, suggesting it is likely that there are a number of miRNAs expressed within a cell at any given time that will determine the overall changes in the expression and function of specific genes [94, 360, 380, 381]. For instance, in addition to miR-125b, Bak mRNA is also known to be directly targeted by miR-125a, miR-26a and miR-29b [100, 101, 382]. MiR-125b has been shown to significantly decrease Bak mRNA inducing mRNA degradation [228], however not all miRNAs function in this manner. Indeed, certain miRNAs simply act to repress gene translation, leaving the mRNA molecule intact [93, 102]. Several studies have observed protein silencing by

miRNA with or without a change of mRNA levels [383-387]. Thus, it is possible that the dual effect of mRNA degradation and translational inhibition, resulting from a combinational targeting of multiple miRNAs on Bak mRNA, may be responsible for the significant changes observed in Bak protein concentrations following rhGH administration.

Considering the effect of rhGH on Bak protein concentrations, it is surprising that the level of expression of miR-125b in cellular lysate was not found to be significantly affected by rhGH treatment compared to placebo treated controls. In addition, miR-125b expression levels were not correlated with either Bak mRNA or protein expressions in both treatment groups. These results would suggest that GH does not exert its effects on the level of expression of Bak through the regulation of miR-125b, possibly indicating that other miRNAs known to target Bak mRNA may play a role [100, 101, 382].

However, evidence strongly suggests that the control of mRNA expression by miRNA is combinatorial, meaning that it is not a one miRNA : one mRNA interaction but rather a combination of multiple miRNAs targeting the same mRNA that determines the level of translational regulation [94, 360, 380, 381]. The combinational effect of multiple miRNAs targeting a specific mRNA could have a considerably higher biological significance than an individual miRNA targeting the same mRNA [94]. Thus, the expression of miR-125b may not, on its own, be considered sufficient to induce the observed decreases in Bak protein concentrations. However, miR-125b acting in combination with other miRNAs may contribute to a significant down-regulation of Bak protein expression.

An alternative explanation is that rhGH induced significant increases in miR-125b levels early in the treatment program and that these elevations in expression had subsided at the post-treatment time points analysed. It has been reported that the concentration of an individual miRNA relative to its mRNA targets is likely to be more important to mediating its biological effects than its absolute copy number [227]. In addition, although miRNAs are generally assumed to have a very long half-life, corresponding to many hours or even days, such a slow turnover may not be a universal feature of miRNAs since they often play a role in rapid developmental transitions which require a more active miRNA metabolism [388, 389]. While miR-125b was reported to

have a half-life of 225 hours (~ 9 days) in mouse embryonic fibroblasts, the same miRNA was reported to exhibit rapid turnover (4-6 hours) following lipopolysaccaride (LPS) stimulation in murine phagocytes and human dendritic cell lines [390-392]. Thus, it is plausible that rhGH induced significant increases in the expression of miR-125b early in the treatment program which, as a result of increased turnover, were subsequently down-regulated relative to decreases in Bak protein concentrations. Indeed, it has been suggested that a reduction in the availability of respective complementary mRNA targets may result in the enhanced destabilization of miRNA [388, 393]. In order to carry out their function, upon maturation miRNAs are incorporated with proteins into miRISC where their association with target mRNAs prevent their degradation [388, 393]. XRN-1, a 5' - 3' exoribonuclease, has been shown to mediate the degradation of mature miRNA, but requires that it is released from the miRISC in order for its 5' end to become accessible to the enzyme [388, 393]. Hence, it may be that in the absence of complementary targets, miRNA could be specifically released from miRISC and degraded, making the proteins available for loading with new miRNA [388].

In the present study, expression levels of Bcl-2 mRNA and protein in PBMCs were not found to be significantly affected by rhGH administration. While this finding was unexpected, Bcl-2 protein concentrations have previously been found to remain unaffected in animal based trials following GH administration in-vivo [210, 211]. Cuesta et al. [210] examined the effects of four weeks rhGH treatment, administered subcutaneously at a dosage of 2mg/kg/day, on the regulation of Bcl-2 family proteins in senescence-accelerated mice (SAM), a murine model of accelerated aging. While they reported that the ratio between anti-apoptotic (Bcl-2) and pro-apoptotic (Bax and Bad) proteins was significantly enhanced following GH administration, this was attributed to a decrease in the expression of pro-apoptotic protein levels as the expression of Bcl-2 was found to be unaffected. Liang et al. [211] investigated, in-vivo, the effect of six days rhGH treatment on a human gastric cancer cell line (BGC823 cells) following their inoculation to induce tumor xenografts in nude mice. They reported that the level of Bcl-2 protein expression in xenograft tumors was not significantly different between mice treated with rhGH (2IU/kg/day) and saline administered controls. In contrast, invitro studies conducted on human lymphoid and monocytic cell lines have consistently demonstrated that GH elicited a positive effect on the expression of the Bcl-2 protein [18, 19, 208, 339]. Treatment of peripheral blood lymphocytes including both CEM/C7 and IM-9 cells (lymphoid cell lines of T and B lineage respectively) with rhGH at concentrations of up to $500\mu g/L$ for 12 to 48 hours resulted in significant enhancement of the expression of both Bcl-2 mRNA and protein levels [18, 208]. In addition, U937 cells (a cultured human monocytic cell line), previously treated with either 50 or $500\mu g/L$ of rhGH for up to 24 hours were shown to have significantly up-regulated Bcl-2 protein concentrations [19].

Variations in the duration of rhGH treatment could possibly account for the conflicting results observed by experiments in-vitro and in-vivo. It has been shown that GH signalling associated with short term survival, following cytokine deprivation and cell cycle arrest in a lymphoid cell line (Ba/F3 cells) is correlated with an up-regulated expression of Bcl-2 [339]. However, the expression of another anti-apoptotic Bcl-2 family member, Bcl-xL, which is reportedly crucial to the mediation of cytokine induced cell survival processes, appears to be involved in GH induced long term cellular survival coupled to cellular proliferation [394]. Of relevance to these observations, evidence of a reciprocal regulation between these two proteins has been reported [51, 395, 396]. Endogenous Bcl-xL protein levels were found to be repressed in Bcl-2 transgenic mice, while endogenous Bcl-2 protein expression was found to be down-regulated in T lymphocytes from a transgenic mouse model exhibiting overexpression of Bcl-xL [396]. That over-expression of either Bcl-2 or Bcl-xL results in a reciprocal down-regulation is indicative of the existence of a shared regulatory feedback pathway [395]. In addition, while the expression of Bcl-2 has been reported to be largely dependent on the activation of NF-kB, the regulation of Bcl-xl expression is reported to be regulated via both NF-kB dependent and independent signalling pathways [339]. Thus, although NF-kB appears crucial to mediating the effects of GH on the expression of at least Bcl-2, it is likely that other signalling molecules regulated by GH in-vivo are involved in regulating Bcl-xL expression. Interestingly, in-vivo administration of IGF-1 (1mg) in rats was found to significantly increase Bcl-xL protein concentrations from isolated heart mitochondria after 24 hours [17]. In addition, in-vitro IGF-1 administration significantly enhanced the expression of Bcl-xl mRNA and protein levels in PC12 cells following 24 hours incubation [340]. Thus, it is possible to assume that, in the present study, the effects of rhGH on Bcl-2 might have been counteracted by additional hormonal regulations exerted on other anti-apoptotic proteins, such as Bcl-xL.

MiR-181a is one of several miRNAs that directly target and repress translation of Bcl-2 [99, 360, 397]. Considering no rhGH mediated effect on the Bcl-2 level of expression was observed in the current study, it is not surprising that no significant difference was observed in miR-181a expression in cellular lysate in both rhGH and placebo treated subjects. In addition, miR-181a expression levels were not correlated with either Bcl-2 mRNA or protein expressions in both treatment groups.

The presence of four miRNAs have been identified in isolated mitochondria in the present study, adding PBMCs to the short list of cell types that have been found to contain mito-miRNA [104-107]. To date, miRNAs have been specifically associated with mitochondria isolated from rat liver [105], mouse liver [104], human HeLa epithelial cells [106] and human skeletal muscle cells [107]. In addition to miR-181a and miR-125b, the two miRNAs tested as endogenous references, miR-24 and miR-92 were found to be mitochondrially located. While miR-181a, miR-125b and miR-24 have all previously been documented in isolated mitochondria from human skeletal muscular cells [107], to our knowledge this is the first time that miR-92 has been shown to be localised to in mitochondria.

A MACS technique was utilized to isolate mitochondria in the present study, which has been experimentally validated to provide a better quality, quantity and purity of mitochondrial fraction (89% enrichment) than the traditional differential centrifugation method of mitochondrial isolation (59% enrichment) [233]. Indeed, the performance of the MACS method was even found to compare favourably with the more sophisticated and expensive ultracentrifugation method of isolation (88% enrichment) [233]. Several peer reviewed publications [107, 233, 238] have validated the MACS mitochondrial isolation method at the protein level, showing specific cytosolic proteins (Glycogen Synthase, GAPDH, Beta-actin) to be poorly detected in mitochondrial fractions while specific mitochondrial proteins (Prohibitin, TOM-22, ATP synthase) were found to be highly enriched. The purity of mitochondrial fractions isolated via MACS has also been confirmed with the assessment of compartmental mRNA and DNA levels [107]. Barrey *et al.* [107] undertook the analysis of two nuclear encoded genes (HIST2AA3 and GAPDH) and observed very low cytosolic mRNA levels and an absence of nuclear

DNA contamination in isolated mitochondria. Using transmission electron microscopy, this group also showed that the membrane and ultra-structural integrity of mitochondria remained intact following MACS isolation [107]. In the present study, mRNA from three nuclear encoded genes (18s, GAPDH and HRPT1) were not found to be expressed in the mitochondrial RNA extract, while protein concentrations of the mitochondrially located Bcl-2 and Bak fell below detection parameters in cellular lysate (data not shown), lending further support to our claim that the mitochondrial isolation procedure used yielded a highly enriched mitochondrial fraction. Additionally, in keeping with all mito-miRNA studies undertaken to date [104-107], RNase treatment of isolated impermeable mitochondrial organelles was carried out to remove any externally bound cytosolic RNA molecules prior to mitochondrial RNA extraction.

No significant correlations were found to exist between miR-181a or miR-125b expression levels in mitochondrial fractions and their respective mRNA targets in either treatment group (Table 7.3). As miR-181a and miR-125b have been experimentally validated to target Bcl-2 [99, 227, 360] and Bak [102, 226, 228] respectively, that no association was detected between their expression profiles is likely a reflection of the small sample size utilized in the present study. Expression levels of miR-181a from mitochondrial fractions were not found to be significantly affected in rhGH treated subjects compared to placebo treated controls. However miR-125b was observed to be down-regulated in isolated mitochondria following rhGH administration at all time-points measured. Furthermore, this down-regulation was found to be significant at eight days post-treatment in comparison with the placebo group. Thus, the results from this study suggest that rhGH may exert a regulatory effect on the expression levels of at least some mito-miRNAs. However, identifying what the significance of this rhGH induced effect may be and how its regulation is mediated remain important issues which must be addressed.

It has been hypothesized that post-transcriptional regulation via mito-miRNAs might provide a sensitive and rapid mechanism to fine tune level of protein expression from the mitochondrial genome in response to changes in cellular metabolic demands [106]. Indeed, miRNAs identified in isolated mitochondria from HeLa cells have been predicted *in-silico* to target mitochondrial RNA [106]. In addition, argonaute 2 (AGO2), the principal active protein of the cytoplasmic miRISC, has been found in several studies [104, 106, 107] to be present in isolated mitochondrial fractions. GH mediated

signalling pathways have been demonstrated to directly target mitochondria, exerting regulatory control over the activity of ETC complexes and the rate of ATP synthesis [187, 192, 193, 296]. Thus, it is possible that rhGH induces changes in mitochondrial function through mito-miRNA regulated changes in the expression of mitochondrial proteins.

Another hypothesis is that mitochondria serve as a storage site for miRNAs [103, 105, 361]. Both Kren *et al.* [105] and Barrey *et al.* [107] identified mito-miRNAs which exhibited no obvious mitochondrial mRNA targets, nor were they found to be complementary to nuclear mRNAs encoding mitochondrial proteins. In addition, all mito-miRNAs identified to date have predicted cytoplasmic protein targets with many of these found to be involved in the regulation of cellular processes such as apoptosis, proliferation and differentiation [103-105]. Thus, it is conceivable that a select group of miRNAs are sequestered into mitochondria for storage and that their signal mediated release into the cytoplasm mediates an additional level of control over cellular processes [105].

It has been proposed that mito-miRNAs released into the cytoplasm are trafficked into P-bodies, cytoplasmic granules involved in mRNA degradation that also contain the necessary machinery for translational inhibition [103, 398]. In fact, it has been reported that P-bodies establish frequent and prolonged contacts with mitochondria [103, 398]. Huang et al. [398] observed that 50 - 70% of P-bodies were found to associate with mitochondria in established human cell lines, indicating that their interaction is not the result of a random association. In addition, the association between mitochondria and Pbodies is reported to be dynamic, with fluorescence microscopy imaging showing that more than 80% of P-bodies establish a link with mitochondria at least once within a three minute interval, with a median duration of eighteen seconds [103, 398]. Whether this transient association involves the binding of P-body proteins to proteins on the surface of the mitochondrial outer membrane remains to be determined, however the proximity between the two organelles does make possible the exchange of metabolites, RNAs and proteins [103]. Furthermore, experimental evidence has arisen to suggest that cytosolic ribosomes bound to mitochondria may control a co-translational import process for at least some nuclear encoded mitochondrial transcripts and it is plausible that mitochondrial P-body interactions allows for regulation of these membrane bound mRNAs [398-400]. Of note, while it is well established that AGO2 associates with miRNAs and is responsible for anchoring these molecules to miRISCs, evidence supporting the mitochondrial localization of complete and fully functional miRISCs is currently lacking [90]. Interestingly, it has been indicated that AGO2 carries out specific functions which cannot be replicated by other argonaute proteins [90]. Knockdown of AGO2 in human HEK293 cells was found to significantly impair miRNA mediated repression compared to the knockdown of other AGO proteins [401]. In addition, knockout of AGO2 is embryonically lethal in mice, while knockout of AGO1 or AGO3 is not [402]. Thus, it is possible that AGO2 plays a crucial role in trafficking miRNA between mitochondria and OMM associated p-bodies, where translational repression occurs subsequent to the formation of miRISCs. In the present study, the pre-treatment of isolated mitochondria with RNase indicates that all identified mito-miRNAs are located within the organelle as those associated with membrane bound P-bodies and ribosomes would have been destroyed. Thus, in order to determine whether mitomiRNAs are released from mitochondrial storage and subsequently associate with membrane bound granules, further studies incorporating the analysis of both RNase treated and untreated mitochondrial fractions are warranted.

If GH does indeed mediate the release of mito-miRNA from the organelle, the mechanism by which the hormone exerts this effect remains to be determined. Kren et al. [105] suggest that an increase in mitochondrial membrane permeability may represent a mechanism by which miRNAs sequestered into mitochondria are released into the cell. Indeed, the disruption of $\Delta \psi_m$ is known to result in an increased permeability to small molecules, including miRNAs [403]. $\Delta \psi_m$ is a dynamic entity, subject to transient depolarizations termed "flickers", a phenomenon which has been attributed to occur in response to intracellular Ca²⁺ signals mediating an increase in intra-mitochondrial Ca²⁺ concentrations [404-407]. Since GH has been reported to stimulate an increase in intracellular free Ca²⁺ concentrations in cells expressing wild type GH receptors [170], it is possible that GH induced changes in mitochondrial permeability mediates the release of mito-miRNAs. However, differences in the expression levels of mitochondrially located miR-181a and miR-125b observed following rhGH administration in the present study would seem to indicate that rhGH mediates a preferential and selective active release of mito-miRNAs. While the mechanism for such a mode of action remains to be determined, it most likely involves the activity of P-bodies targeted to the mitochondrial outer membrane.

In summary, significant decreases in Bak protein concentrations following rhGH administration could not be solely attributed to changes in the expression of miR-125b. That difference in Bak levels did not persist past eight days post-treatment and therefore could have important health implications for any individual taking rhGH at supraphysiological concentrations for performance enhancing purposes. In addition, our results suggest that rhGH may exert an effect on the expression of at least one of the mitochondrial-miRNAs investigated, although how this effect is mediated remains to be determined. It should be noted that subjects recruited for this study were resistance trained, which of itself influences rates of endogenous GH secretion. Whether the same effects would be induced following rhGH administration in untrained individuals has yet to be elucidated. The data presented here points towards the existence of GH induced anti-apoptotic effects which may in part be mediated via changes in the expression of mitochondrial-miRNAs. These preliminary and interesting observations warrant further investigation.
8. Final Discussion and Conclusion

8.1 GH and IGF-1 mediated effects on Mitochondrial Function.

Healthy mitochondrial function is characterized by both the production of ATP at rates sufficient to meet physiological demand and avoidance of the generation of ROS at rates that would lead to elevated levels of oxidative damage [6]. As cellular rates of ATP production have previously been shown to be increased in the presence of GH [192], attention was focused on the consequences of this putative up-regulation on rates of oxidative phosphorylation. The influence of rhGH and IGF-1 on the efficiency of oxidative phosphorylation was addressed in Study One, through examination of relative changes in $\Delta \psi_m$ and mitochondrial O_2^- levels from PBMCs *in-vitro*. The magnitude of $\Delta \psi_m$ is a major determinant of the efficiency of oxidative phosphorylation as the proton permeability of biological membranes increases exponentially at high values, leading to a waste of energy and an increase in the rate of ROS production [14, 408].

At the physiological concentrations utilised we proposed that neither rhGH nor IGF-1 would exert any significant effect on $\Delta \psi_m$ and that mitochondrial O_2^- levels would be either maintained or decreased compared to untreated control samples. At least for rhGH treated samples this was found to be the case, with mitochondrial O_2^- levels found to be significantly decreased at concentrations of $5\mu g/L$ and $10\mu g/L$ in lymphocytes and $10\mu g/L$ in monocytes, while $\Delta \psi_m$ values remained unaffected. These results suggest that at these concentrations rhGH elicited an increase in the efficiency of electron transfer along the ETC, lowering the rate of superoxide production while a tight coupling between O_2 consumption and the rates of ATP synthesis maintained $\Delta \psi_m$ within physiologically optimum values of 100 - 120mV [14].

With supra-physiological concentrations of rhGH and IGF-1 it is proposed that both $\Delta \psi_m$ and mitochondrial O_2^- levels would be significantly increased compared to untreated samples. Our hypothesis was that at these concentrations both hormones would impose a level of cellular stress at which the synthesis of ATP would become rate-limiting, leading to increases in $\Delta \psi_m$ to values that impede the efficiency of oxidative phosphorylation. Contrary to our proposals, neither $\Delta \psi_m$ nor mitochondrial O_2^- levels were found to be significantly increased by rhGH at supra-physiological concentrations, which could possibly be due to the existence of an as yet unidentified negative feedback pathway initiated by the hormone at these concentrations. IGF-1 was found to induce no significant effect on either of these variables that would seem to

indicate that this hormone does not impact mitochondrial function in PBMCs under the concentrations analysed.

Although analysis of $\Delta \psi_m$ together with mitochondrial O_2^- levels did provide evidence favouring GH mediated regulation of the efficiency of mitochondrial energy production, it should be noted that the efficiency of oxidative phosphorylation was not determined directly in this study. Traditionally, determination of the efficiency of energy production by oxidative phosphorylation is based on calculation of the P/O ratio, which is the number of ATP molecules produced per oxygen atoms reduced by the respiratory chain [64]. However, this method of analysis requires that measurements be carried out on isolated mitochondria for which both structural and functional interactions with other cellular components are absent [409]. This includes possible interactions with signalling pathways that could mediate effects on oxidative phosphorylation. In contrast, the method of analysis utilised in Study One allowed for the interpretation of mitochondrial function in live cells without compromising cellular function. It should also be noted that as samples in this study underwent treatment *in-vitro*, any factors exclusively present in-vivo which exert an influence over the hormonal effects mediated on mitochondrial function have not been accounted for. For example, the presence of binding proteins in-vivo, such as growth hormone binding protein (GHBP) which controls the distribution of circulating GH and insulin-like growth factor binding protein-3 (IGFBP-3), which together with acid-labile subunit (ALS) is known to modulate the activity of IGF-1 [410, 411], likely influence any mitochondrial effects mediated by these hormones at specific concentrations. In addition, administration of rhGH *in-vivo* is known to increase the rate of lipolysis leading to elevated plasma FFA concentrations, an effect which in and of itself has been shown to negatively impact mitochondrial function, down-regulating genes required for oxidative phosphorylation [21, 293].

Mitochondrial responses to rhGH and IGF-1 administration were further addressed in Study Two, again through analysis of relative changes in $\Delta \psi_m$, but also for the first time through examination of how these hormones affect levels of the hROS, 'OH and ONOO- within mitochondria. The examination of these variables was carried out under several respiratory conditions, which to varying degrees allowed for control over the sites of electron entry into the ETC at complexes I and II, in an effort to identify the role played by individual complexes in mediating hormonal effects on the efficiency of mitochondrial function. It was found that respiration mediated by both complex I and fatty acids produced significantly higher hROS levels compared with respiration mediated by complex II, leading to an impairment of mitochondrial function as indicated by significant decreases in $\Delta \psi_m$. The mitochondrial responses to physiological and supra-physiological concentrations of both rhGH and IGF-1 were assessed under saturated substrate concentrations in Study Two in contrast to the endogenous intracellular substrate conditions utilized in Study One.

Variability in the efficiency of oxidative phosphorylation has previously been shown under different physiological conditions within several tissue types when using complex I respiratory substrates [64]. However, the same variability was not found under conditions of complex II mediated respiration [64]. In addition, several studies have identified complex I to be the principal site of electron leak contributing to the production of ROS along the respiratory chain [303, 307, 308]. Thus, in line with expectations from Study One, it was hypothesized that under conditions of respiration initiated at complex I, no significant effects would be exerted on $\Delta \psi_m$ but that hROS values would be attenuated at physiological concentrations in the case of both hormones. Additionally, values for both variables were predicted to be significantly elevated at supra-physiological concentrations of rhGH and IGF-1. In contrast, it was proposed that neither hormone would mediate any significant effect under conditions of complex II mediated respiration. Such findings would provide support for the notion that variability in the level of activity of complex I plays an important role in mediating mitochondrial responses to the actions of these hormones.

However, it was found that neither rhGH nor IGF-1 exerted any significant effect on $\Delta \psi_m$ or hROS values, at any concentration compared to untreated samples under the respiratory conditions analysed. That rhGH treatment at concentrations of 5µg/L was not found to induce a mitochondrial response under the respiratory conditions of Study Two contrasts with the findings of Study One where the same treatment was found to mediate a change in the rates of mitochondrial ROS production. As we concluded, it is likely that subtle effects induced by rhGH, which at physiological concentrations manifested as an increase in mitochondrial efficiency in Study One, were negated in Study Two owing to the effects exerted on $\Delta \psi_m$ by saturating concentrations of respiratory substrates. Unfortunately as a result, whether or not the activity of complex I plays any role in mediating mitochondrial responses to the actions of GH could not be

determined from the observations in Study Two. However, it is worth noting that high hROS values were recorded under saturated conditions of both complex I mediated and lipid derived respiration which GH was not capable of attenuating at the concentrations tested. Despite the findings from Study One that showed significant decreases in mitochondrial O_2^- levels with physiological concentrations of GH *in-vitro*, this could have implications for the administration of rhGH *in-vivo*, which has previously been associated with substantial increases in both circulating concentrations and musculoskeletal uptake of NEFA's [412]. Such elevated supplies of lipid-derived substrates to mitochondrial matrix, leading to oxidative damage which can negatively impact mitochondrial function [113].

Evaluation of mitochondrial metabolism in the presence of respiratory linked substrates, as outlined in Study Two, is not possible in whole cells as some of the respiratory substrates used are incapable of permeating intact cellular membranes [219]. Digitonin induced permeabilization of the cell surface membrane was advantageous over more traditional methodologies carried out on isolated mitochondria as it allowed for the analysis of mitochondrial function under these respiratory conditions while keeping the organelle's relationship with its surrounding cellular structure intact [219]. However, a limitation of this technique is that cellular permeabilization inevitably leads to a loss of endogenous substrates and proteins from the cytosol [219]. Additionally, the supply of exogenous respiratory substrates to the ETC creates experimental conditions that do not accurately reflect the physiological conditions within whole cells [62]. Hence, although rhGH and IGF-1 treatment was conducted on whole cells over a period of four hours, prior to analysis under conditions of cellular permeabilization, we acknowledge that this process could have altered GH mediated mitochondrial responses compared to those seen in Study One.

Moving forward, there is much scope for further research into the effects these hormones exert on mitochondrial efficiency. Notably, there is a need for these hormone mediated effects to be examined under conditions that more closely resemble those present *in-vivo*. Controlled trials administering rhGH to healthy subjects have been reported to induce up to three fold elevations in serum lipid concentrations with free oleic acid, triglyceride palmitate and cholesterol oleate in particular exhibiting significant increases [332]. Thus, analysis of GH and IGF-1 mediated mitochondrial

responses in the presence of these lipids, at concentrations that would mimic the extracellular environment in-vivo, would account for any interaction effect between the hormonal and substrate impact on the efficiency of mitochondrial function. Additionally, it should be noted that in practice, rhGH and IGF-1 are often abused in combination with anabolic-androgenic steroids, most commonly synthetic testosterone, in order to enhance the potency of their anabolic effects [413-416]. In light of this, it is evident that any combinatorial effects elicited by either rhGH or IGF-1 when used in conjunction with other anabolic agents could have important implications for mitochondrial metabolism and should be investigated.

Key to the understanding of how GH and IGF-1 mediate changes in mitochondrial responses could be the elucidation of associated signalling pathways directed at the organelle in addition to the identification of specific protein targets. Although phosphorylation sites have been identified on all of the respiratory chain complexes, to date the signalling pathways involved in their regulation remain largely unknown [6, 61]. Hence, further investigations could ascertain whether GH or IGF-1 affect the phosphorylation status of any of these identified sites on respiratory chain complexes. Additionally, examination of the dynamics associated with any interactions that are identified is warranted, in terms of the percentage of proteins that are either phosphorylated or dephosphorylated at specific hormonal concentrations. It should be noted that only in a few cases have the physiological responses to specific phosphorylation of mitochondrial respiratory complex proteins been resolved [14]. It is therefore important that the functional consequences concerning $\Delta \psi_m$, ATP synthesis and ROS production for any individual site whose phosphorylation status is found to be affected by GH or IGF-1 be subsequently addressed.

8.2 Implications for Cellular Viability.

Both GH and IGF-1 are potent "survival factors" whose anti-apoptotic effects are well documented [17, 18, 212, 297]. However, elevations in GH and IGF-1 levels to supraphysiological concentrations are characterized by the development of pro-apoptotic conditions *in-vivo* [373-375]. Additionally, the development of acromegaly has negative health implications associated with increased levels of apoptosis [377-379]. It is therefore relevant to consider the implications for cellular viability imposed by GH and IGF-1 mediated effects on mitochondrial function at both physiological and supraphysiological concentrations. This was addressed in Study One through analysis of relative changes in the level of activity of the mtPTP. The activity of this intermembrane channel represents a key step in the execution of mitochondrial mediated apoptotic pathways [54]. Its opening occurs in response to physiological stresses, most notably the augmentation of intra-mitochondrial free ROS concentrations [54]. Hormonal impacts on cellular viability were also assessed directly, as changes in the percentages of viable, apoptotic and necrotic cells following treatment were determined.

Considering the expectation to observe augmented rates of mitochondrial ROS production at supra-physiological concentrations of rhGH and IGF-1, we hypothesized that both hormones would impose significant increases in the level of mtPTP opening at these concentrations. It was proposed that this would have negative implications for cellular viability which would be reflected by significant elevations in the percentages of cells found to be undergoing apoptosis.

Contrary to this, neither hormone was found to have a significant effect on either mtPTP activity or the percentage of cells undergoing apoptosis at any concentration administered. This was not surprising given that neither hormone was found to induce an increase in the level of mitochondrial oxidative stress, even at supra-physiological concentrations in Study One. Thus, we concluded that the mitochondrial responses mediated by these hormones do not of themselves adversely affect cellular viability, even at supra-physiological concentrations. As previously stated, it must be noted that the *in-vitro* nature of this study precludes the involvement of factors exclusively present *in-vivo* which could mediate pro-apoptotic effects. Indeed, noted limitations of *in-vitro* analysis include the lack of a homeostatic environment and an impairment of intracellular signalling [417]. Consequently, how these factors interact with hormone

mediated mitochondrial responses to impact cellular viability were not addressed by Study One.

In consideration of the noted limitations of *in-vitro* analysis, Study Three assessed hormonal effects impacting PBMCs *in-vivo* following one week of rhGH administration, at a dose of 1mg per day, in healthy male subjects compared to placebo treated controls. The potential implications of rhGH administration at this dosage for cellular viability were addressed through the analysis of the hormone mediated effects on the expression of apoptotic regulatory proteins, namely the anti-apopotic Bcl-2 and the pro-apoptotic Bak. Both proteins play a key role in determining whether a cell will activate central apoptotic pathways in response to mitochondrial mediated stimuli [349, 351]. Furthermore, changes in the expression of these proteins was assessed over a period of 22 days post-treatment in order to investigate whether the temporal extent of the hormone mediated effects on their regulation would persist. Through this Study Three aimed to determine whether the putative protection provided by rhGH's noted anti-apoptotic effects remains sufficient, in the long term, to counter the development of pro-apoptotic stimuli that are associated with prolonged elevations in GH concentrations *in-vivo*.

In line with its role as a potent survival factor, it was hypothesized that rhGH administration would result in an up-regulation of Bcl-2 levels while Bak levels would be down-regulated. Additionally, it was proposed that the significance of these initial anti-apoptotic effects would become attenuated by 22 days post-treatment. Indeed, one week rhGH administration (1mg per day) was found to significantly decrease Bak protein levels at 1 and 8 days post-treatment, while no significant differences were observed in protein concentrations after 15 or 22 days following the cessation of treatment. However, contrary to the presented hypothesis, the level of expression of Bcl-2 was not found to be significantly affected by rhGH which would indicate, at least in the case of PBMCs, that this hormone does not mediate its anti-apoptotic effects via the regulation of this protein *in-vivo*. That the reported anti-apoptotic benefits elicited by rhGH administration via a reduction in Bak protein concentrations for cellular viability considering the noted pro-apoptotic risk factors associated with prolonged supra-physiological GH concentrations *in-vivo* [33, 416]. Such factors include the

development of type II diabetes mellitus, hypertension and cardiomyopathy as well as the alteration of lipid and hormonal profiles [33, 416].

It is worth noting that only two candidates from the Bcl-2 family of proteins that regulate the mitochondrial execution phase of apoptosis were assessed in the study. The limitation of this becomes apparent in consideration of the fact that, to date, a total of 25 members of the Bcl-2 group have been identified, each of which plays a role in the regulation of mitochondrial mediated apoptotic pathways [51]. In addition, both GH and IGF-1 have been shown to mediate short-term changes in the expression of a number of these proteins, including the pro-apoptotic Bax [17, 298, 418] and Bad [418] as well as the anti-apoptotic Bcl-xL [17, 209, 418]. Thus, further research assessing how *in-vivo* rhGH administration affects long-term changes in the expression of treatment is warranted. In addition, it is worth examining whether rhGH administration induces similar effects on the expression of these proteins in other cell types. In particular, the prevalence of cardiomyopathy and heart failure in acromegalic patients and the association of these conditions with deregulated apoptosis in cardiac tissue [377-379] make cardiomyocytes a prime target for investigation in this type of study.

8.3 Regulation of Mitochondrial Mediated Apoptosis by rhGH: The Role of miRNA

Determination of the effects exerted by the administration of rhGH on the expression of Bcl-2 family proteins provides key insight into the regulatory mechanisms behind GH's anti-apoptotic effects. We further aimed to build upon these findings through the investigation of factors which may have contributed to these rhGH induced changes. With regard to this, it is likely that post-transcriptional regulation of gene expression by miRNA molecules play an important role in mediating any effects induced by rhGH on these apoptotic regulatory proteins [355]. While miRNA molecules have traditionally been thought to exert their post-transcriptional effects in the cytosol, the recent discovery of the mitochondrial compartmentalisation of these molecules has given rise to speculation that control over their storage and release from the organelle may act as a mechanism for intracellular signalling and play a role in regulating mitochondrial processes [105]. Furthermore, a portion of the mito-miRNA molecules identified in previous studies have predicted gene targets *in-silico* which are consistent with those

involved in apoptotic signalling pathways [105]. Hence, it was our intention to examine whether both cytosolic and mitochondrial associated miRNA signalling could be responsible for the rhGH induced changes observed in the expression of Bcl-2 associated regulatory proteins. To this end, changes in the expression of miR-181a and miR-125b, which are known translational inhibitors of Bcl-2 [99, 227, 360] and Bak [102, 226, 228] respectively, were analysed from the cytosolic and mitochondrial fractions of PBMCs' following rhGH administration in Study Three.

In accordance with the rhGH induced changes predicted to occur in the gene and protein expression levels of Bcl-2 and Bak respectively, we hypothesized that cytosolic levels of miR-181a would be significantly decreased while the expression of miR-125b would be significantly elevated following rhGH administration. A reversal of these effects was proposed in the case of miRNA residing within mitochondria. Significant increases in miR-181a levels and significant decreases in miR-125b levels may indicate a respective storage and release of these miRNA in response to rhGH. Pursuant to our hypothesis regarding the long-term effects induced by rhGH administration on apoptotic regulatory protein levels, observed changes in the expression of either cytosolic or mitochondrial derived miRNA molecules were not expected to persist up to 22 days post-treatment.

In support of the presented hypothesis, it was indeed found that mitochondrial associated miR-125b was down-regulated following rhGH administration, although this was only significant at eight days post-treatment. Cytosolic levels of miR-125b were not observed to be significantly up-regulated or down-regulated compared to pre-treatment values, likely indicating that rhGH does not exclusively exert its effects on Bak protein expression via the regulation of miR-125b. However, the observed trend in GH induced Bak protein expression changes, together with those of cytosolic and mitochondrial derived miR-125b levels, suggests that miR-125b may play at least a partial role in regulating the expression of Bak and that the mediation of this role likely involves both cytosolic and mitochondrial signalling pathways. Indeed, it has previously been demonstrated that multiple miRNA's, working in combination with each other, are capable of impacting the regulation of a single gene [94, 380, 381]. Thus, it was concluded that it is possible miR-125b, in conjunction with the combinatorial effects of other Bak targeted miRNA's, may have contributed to the significant effects induced by rhGH on Bak protein concentrations. Contrary to observations made with miR-125b, neither cytosolic nor mitochondrial levels of miR-181a were significantly affected following rhGH administration which was not surprising considering that there was also a failure to observe any rhGH mediated effect on the level of Bcl-2 expression.

It is a limitation of this study that only one individual miRNA which has been validated to target Bcl-2, together with a single miRNA validated to target Bak, were the subject of investigation. As recent evidence points towards a combinatorial effect elicited by miRNA's on the regulation of mRNA, it has become evident that the combination of multiple miRNA's targeting the same gene ultimately determine whether the expression of that gene is up-regulated or down-regulated in response to a stimulus [94]. Thus, future studies not only need to focus on whether rhGH induced changes in the expression of individual miRNA's subsequently impacts the expression of their predicted apoptotic regulatory protein targets, but must also pay attention to the interaction effects resulting from changes in the expression of multiple miRNA's predicted to target the same gene.

While the results presented in Study Three provide evidence supporting the existence of mitochondrial associated miRNA signalling pathways, questions remain regarding the role that such pathways play in mediating hormonally induced anti-apoptotic effects, in addition to the functional processes behind their regulation, which must be addressed in future studies. Considering the differences observed in the expression levels of mitochondrial located miR-181a and miR-125b following rhGH administration, it was concluded that rhGH affects the release of mito-miRNA in a selective manner. However, based on current observations alone, the mechanism by which such a mode of action is mediated remains to be elucidated.

To date, a limitation of studies which have attempted to identify mito-miRNA's, including Study Three presented in this thesis, is that in order to ensure all identified miRNA's were located within the organelle, researchers utilized methodologies involving the pre-treatment of isolated mitochondria with RNase [104-107]. Consequently, any miRNA which may have been associated with the OMM was destroyed along with the subsequent knowledge of any role it may have played in mito-miRNA mediated signalling. Indeed, considering recent evidence associating both P-bodies and cytosolic ribosomes with the OMM [103, 398-400], it is plausible that miRNA residing on this membrane represents an important "missing link" to the

association between cytosolic and mitochondrial miRNA signalling pathways and incorporation of their analysis into future mito-miRNA studies is warranted.

Finally, it is worth investigating whether the effects elicited by rhGH on mitochondrial efficiency, as outlined in the first two studies in this thesis, exert any impact on the regulation of mito-miRNA signalling. Indeed, it has previously been demonstrated that the activation of RNA interference (RNAi) pathways is dependent on mitochondrial activity, while the induction of mitochondrial dysfunction has been shown to significantly alter mito-miRNA profiles [103], pointing towards the possibility that the efficiency of mitochondrial energy production may play a role in mediating such effects.

8.4 Conclusions

In closing, the evidence presented in this thesis points towards the mitochondrial effects elicited by rhGH being beneficial at physiological concentrations that are within the range seen following the stimulation of peak endogenous GH responses in normal subjects. However, it appears that these benefits are negated following rhGH treatment within the supra-physiological range, the concentrations of which are only observed, *in-vivo*, in the presence of exogenous rhGH taken at excessive doses for performance enhancement purposes. In addition, rhGH was found to be incapable of countering mitochondrial dysfunction induced in the presence of saturating substrate conditions at any administered concentration. Taken together with observations that the anti-apoptotic effects elicited on the regulation of Bak protein concentrations following rhGH administration *in-vivo* were not seen to persist past eight days post-treatment, these findings could have important health implications for any individual intent on improving their athletic capabilities through the administration of rhGH at supra-physiological dosages.

While the data presented in Study Three of this thesis points towards the existence of mito-miRNA signalling pathways under the regulation of rhGH, these findings must be validated and expanded upon in future studies. Moving forward, advancement of knowledge within this field of study necessitates the elucidation of signalling pathways which underlie the hormonal regulation of both mitochondrial efficiency and mitochondrial mediated apoptosis. Indeed, interpretation of how these two vital physiological processes are regulated could potentially have important clinical applications and warrants further study.

9. References

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