Evaluation of the Role of Paracrine/Autocrine IGF-1 System In Skeletal Muscle Adaptation

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Abstract

Muscle is highly responsive to changes in functional demand, with overload and increased activity leading to hypertrophy, while decreased load, denervation and joint immobilization can cause muscle atrophy. It has also been long appreciated that the mechanism of load-dependent muscle hypertrophy and atrophy are locally regulated and now increasing evidence suggests a role for locally expressed insulin-like growth factor I (IGF-1). Although it is accepted that overload and stretch are likely to mediate skeletal hypertrophy by an increase in local IGF-1, it is not clear to what extent changes in local IGF-1 gene expression are regulated in response to ageing and neuronal injury, which lead to muscle atrophy. There is even less information on how the two local IGF-1 isoforms, IGF-1Ea and MGF are differentially regulated. This thesis presents the hypothesis that muscle response to mechanical and electrical stimuli can be determined by changes in local IGF-1 expression based on the premise that insulin-like growth factor (IGF-1), functioning in an autocrine/paracrine mode, is an important mediator of skeletal muscle adaptation. The experiments focused on the central role of local isoforms of muscle IGF-1, IGF-1Ea and MGF, in muscle hypertrophy, atrophy and loss of skeletal muscle strength during senescence and in response to spinal injury. In summary, the experiments suggested that local modulation of IGF-1, particularly transcription of autocrine splice variant MGF, is stimulated by mechanical loading as well as some component of neuromuscular activity. A recurring observation was that the two IGF-1 isoforms were differentially regulated in response to loading, senescence and neuronal injury both in muscle and spinal cord tissue. In addition, it appeared that absence of neuromuscular activity affects the expression of IGF-1 gene, as passive cycling of spinal transected muscle did not appear to activate MGF expression in muscle. The results of this thesis supports evidence that IGF-1 is a positive regulator of muscle growth, but its downregulation may not directly determine muscle atrophy. However, as it is likely that the signalling pathways that control proteolysis and protein synthesis converge downstream, there is a possibility that indirect modulation of IGF-1 signalling may enhance the process of muscle atrophy.
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Abbreviations
ALS: Acid labile subunit
BHLH: basic Helix-loop-helix
DNA: Deoxyribonucleic acid
EGF: Epidermal Growth factor
GH: Growth hormone
HGF: Hepatocyte growth factor
IGF: Insulin-like growth factor
IGFBP: Insulin-like growth factor binding protein
IGF-1R: Insulin-like growth factor 1 receptor
IRS: Insulin receptor substrate
IRE: Insulin regulatory element
MAP-kinase: Mitogen activated protein kinase
MyHC: Myosine heavy chain
MRNA: messenger ribonucleic acid
PCR: Polymerase chain reaction
PI3-kinase: Phosphatidylinositol-3-kinase
UTR: Untranslated region
Chapter I
General Introduction
1.1 **Introduction**

It has been four decades since Salmon and Daughday (1956) made the observation that the action of growth hormone (GH) on cartilage is mediated through a factor absent in hypophysectomized animals. Insulin-like growth activity in serum fractions was attributed to factors originally termed as non-suppressible insulin-like activity somatomedins, as their effect could not be suppressed by the addition of anti-insulin serum (Froesch et al 1966). In 1972, Pierson and Temin identified factors from calf serum, which stimulated the proliferation of fibroblasts in tissue culture and hence termed this as multiplication-stimulating activity.

The amino acid structure (Rinderknecht and Humbel 1978, Blundell and Humbell, 1980) and nucleotide sequence analysis (Jansen et al 1985) of the somatomedins revealed conclusively that they were members of the insulin gene family. In 1987, the terminology Insulin-like growth factors 1 and II (IGF-1 and IGF-II) was adopted universally to avoid confusion in describing the family of somatomedins.

Insulin and Insulin-like growth peptides comprise of a large superfamily of ancient anabolic peptides represented across the animal phyla. It is thought that duplication of the ancestral insulin-like' gene gave rise to the insulin and IGF predecessors, with subsequent duplication events, resulting in IGF-I and IGF-II (Duguay 1999). Since then insulin and IGF-1 have evolved into distinct roles with insulin transducing a large number of metabolic functions in cells, whereas IGF-I specializes in growth and differentiative function (reviewed by LeRoith et al 1993).

1.1.2 **IGF-1 Structure**

1.2.1 **Gene structure:**

A single gene spanning more than 70 kilobases and consisting of six exons and at least five introns encodes IGF-1 in both humans and rats (Rotwein et al 1986, Shimatsu and Rotwein 1987). Two promoters, one adjacent to Exon 1 and the
other to Exon 2, govern gene transcription. Exons 1 and 2, each contain multiple
transcription start sites, and are also alternatively spliced (Gilmour 1994). The
resulting variant mRNA transcripts with different 5' UTR have been classified as
Type 1 (exon 1-3) and Type 2 (exon 2-3). These 5'UTR mRNA variants are
differentially regulated during development in a tissue specific manner (Lowe et

In addition to transcription from two promoters, IGF-1 is regulated by post-
transcriptional events, which yield several mature mRNA transcripts. The mature
70-amino acid peptide translated from all these mRNA variants remains
unchanged and is encoded by exons 3 and 4 (Bell et al 1986, Gilmour 1994).
Therefore, in addition to exons 1 and 2, alternative splicing occurs between exon
5 and 6 in rats. This yields four possible pre-pro IGF-1 peptides encoded by
either: Class 1 IGF-1Ea (exons 1, 3, 4, 6), Class 2 IGF-1Ea (exons 2, 3, 4, 6),
Class 1 IGF-1Eb (exons 1, 3, 4, 5, 6) and Class 2 IGF-1Eb (exons 2, 3, 4, 5, 6)
(reviewed by Gilmour 1994).

In humans the alternative splicing pattern is different as exon 5 can be a terminal
exon. There are therefore in each Class 1 and 2 transcripts, three different
possibilities of: Ea (exons 3, 4, 5, and 6), Eb (exons 3, 4, 5) and Ec (exons 3, 4,
fourty two bases of exon 5 and exon 6). Hence the human IGF-1Ec isoform
corresponds to rat IGF-1Eb (Chew et al 1995).

1.2.2 Primary structure:
The primary structure of IGF-1 was first described as similar to that of pro-
insulin as it has an amino terminal B region and an A region which is separated
by a short connecting C domain. However unlike pro-insulin, it has a D region
extension peptide and an E peptide at its carboxyl terminus (Lowe 1988).

The variant mRNA isoforms IGF-1Ea and IGF-1Eb are so named because they
encode different E peptides. These different E peptides are cleaved from the pre-
pro-IGF-1 to yield a mature peptide consisting of the B, C, A, and D domains.
The function of the E peptide remains unknown although Eb can be glycosylated in vitro and a small part of it can be immunologically detected in human serum (Siegfried et al 1992).

Although only two different complementary DNA (cDNA) isoforms have been identified in rat hepatic and non-hepatic tissue, a number of different names have emerged. This has been in order to try and distinguish between endocrine IGF-1 that is produced largely by the liver and autocrine IGF-1 produced locally in tissue. In muscle IGF-1Ea has been termed as Muscle liver-type IGF-1 (muscle L.IGF-1) (Goldspink 1998), and also muscle IGF-1 (mIGF-1) (Musaro et al 2001). IGF-1Eb, similar in structure to IGF-1Ec in humans, is upregulated in response to stretch and exercise and has therefore been renamed Mechano-Growth factor (MGF) (Yang et al 1996).

![Fig 1.1](Image) Schematic of rat IGF-1 gene and derived IGF-1 mRNAs from gene splicing. Class 1 and 2 derive from the use of different transcription start sites. Unlike its human counterpart, Exon 5 of the rat gene is a cassette exon rather than a terminal exon. Exons 3 and 4 encode the mature IGF-1 peptide and first 16 amino acids of the E domain.
1.3 IGF-1 Receptors

The cellular effects of Insulin/IGF-1 family of ligands are mediated by cell-surface receptors, which include the Insulin receptor (IR), IGF-1 receptor (IGF-1R), and IGF-II receptor, otherwise known as the Mannose-6-Phosphate receptor. The effects of insulin are mediated by the insulin receptor, and a related subset of alternatively spliced receptors, which also stimulate proliferation in response to IGF-I. The IGF-II/mannose-6-phosphate receptor does not appear to have a role in IGF signal transduction, but is instead thought to act as a clearing vehicle, reducing the extracellular levels of IGF-II by endocytosis (Nissley et al 1991). The type I receptor therefore mediates most of the biological actions of IGF-I and IGF-II, with its binding affinity (kd) for IGF-I approximately 1nM, 2-to 10-fold lower for IGF-II and 100- to 500-fold lower for insulin.

Both the Insulin and the IGF-1 receptor are structurally similar, being alpha2beta2 (α2β2) transmembrane glycoproteins with tyrosine protein kinase domains in the cytoplasmic portion of the beta subunits (Siddle 1992). The alpha subunits are located on the extracellular face of the plasma membrane and contain the ligand-binding site, while the beta subunits span the membrane and are responsible for the tyrosine kinase activity required for signal transduction (Kato et al 1993).

Ligand binding to the receptor results in a conformational effect in the interaction of the alpha and beta subunits so that they draw together. This interaction triggers off an autophosphorylation event in the tyrosine kinase domain of the receptor. The intrinsic tyrosine kinase activity of the IG-1 receptor then mediates the different biological actions of IGF-1 by phosphorylating endogenous cellular substrates. Several of the substrates that have been identified are also phosphoproteins, the most well characterized being the designated Insulin receptor substrate-1 (IRS-1). IRS-1 is proposed to be a multi-site ‘docking’ protein, which upon phosphorylation associates with other cellular proteins such as the phosphatidylinositol 3’ kinase (PI-3-K). This frequently occurs through src-homology 2 (SH2) domains. Other proteins activated by phosphorylation
include Extracellular signal-related kinases 1 and 2 (ERK-1 and ERK-2) (reviewed by Cohick and Clemmons 1993).

1.4 IGFBPS

IGFs in biological fluids are found bound to Insulin-like Growth Factor Binding Proteins (IGFBPs). These proteins serve to increase the IGF-1 half-life from ten minutes to almost twelve hours in some cases and to transport IGF-1 both in circulation and in peripheral tissues. All of the six IGFBPs, (IGFBP1 to 6) that have been characterized to date, have much higher binding affinities to IGF-1 and II than to the IGF-1R, and therefore determine the bioavailability of the ligands (Jones and Clemmon 1995). In addition to stabilizing IGF-1, IGFBPs confer tissue specificity through 1) their different expressions within different tissues, 2) different posttranslational modifications that can alter cell biological activity and 3) direct receptor mediated, IGF-1-independent activities. IGFBPs can play either a stimulatory or inhibitory role, in increasing the effects of IGF-1.

IGFBPs are highly conserved cysteine-rich proteins that have about 50% amino acid homology between them. Structurally, IGFBPs have a highly conserved N-terminal domain that is thought to determine the IGF binding, and a carboxyl-terminal, that is important for protein-to-protein or protein-to-extracellular matrix (ECM) interactions (Hwa et al 1999). The central domain site is of little similarity between IGFBPs and is commonly subject to proteolysis/cleavage. Although in all the binding proteins, IGF-1 binding is built around the highly conserved cysteine residues in the N-terminal, the carboxyl terminal does play a role in ligand recognition as cleavage of the domain greatly reduces the binding affinity for IGF-1 (reviewed by Rosenfield et al 2000).

The most abundant binding protein, IGFBP-3 binds 90% of circulatory IGF-1 in a complex consisting of IGF-1, an Acid labile subunit (ALS) and IGFBP-3. IGFBP-1, IGFBP-2, and IGFBP-4 are also present in serum, but at much lower concentrations (Hwa et al 1999). The ALS-IGFBP-3 complex appears to be responsible for keeping IGF-1 from crossing the capillary barrier and it is only upon the cleavage of ALS from the complex that coupled IGFBP-3 and IGF-1
can reach peripheral tissues (Jones and Clemmons 1995). IGFBP-1 is highly expressed in liver and kidney, where its regulation is associated with changes in body metabolism and also during reproduction. Its promoter region contains an Insulin Regulatory element and hence it is negatively regulated by insulin. Accordingly, it is strongly expressed during catabolic circumstances, and its gene expression is also strongly stimulated by glucorticoids (Cohick and Clemmons 1993). IGFBP-4, 5 and 6 are expressed in many muscle cell lines and also have been observed in vivo in muscle tissue (Ewton and Florini 1995, Bayol et al 2000).

Most of the time, IGFBPs inhibit IGF-1 action by decreasing its binding to the receptor, however IGFBP-3 is able to modulate IGF-1 receptor activation independently of IGF-1 binding. Phosphorylated forms of IGFBP-1, 3 and 5 also affect IGF-1 binding affinity and hence presentation to the receptor (Kelley et al 1996). Recent evidence indicates that most binding proteins are subject to cleavage by specific proteases. For example, IGFBP degrading proteases secreted by breast and prostate cancer cells act as growth stimulators as they increase IGF-1 availability (Cohick and Clemmons 1993). These proteases are also under physiological regulation and their timed release during the cell cycle could control differentiation, as seen during ovarian follicular development. IGFBP5 and IGFBP-3 are able to bind the ECM and directly stimulate the cells through their receptor. It is in this way that IGFBP5 exerts its anti-apoptotic effects on muscle cells, thereby acting synergistically with IGF-1 to increase cell survival during differentiation (James et al 1993, Hwa et al 1999).

1.5 Role and Regulation of IGF-1

IGF-1 is an important mediator of growth hormone action in the body. The Somatomedin hypothesis was conceived on the basis of Salmon and Daughday's (1956) finding that GH did not induce growth in the cartilage of hypophysectomized rats. This proposed that GH actions were all mediated by hepatic IGF-1, which was circulated round the body and exerted an anabolic effect. This theory was modified upon the discovery that almost every tissue in
the body expressed IGF-1 and IGF-1 receptors locally. Nevertheless, it was still accepted that GH would regulate the effects of local IGF-1 (reviewed by Van den Brande 1999).

Recent advances have cast doubts upon this hypothesis. In particular, it is not clear as to whether GH regulates local IGF-1 levels in all tissues. In addition, GH is able to exert direct effects on certain tissues such as adipocytes. The modulation of IGF-1 activity therefore seems to be broadly sub-divided by its function either as an endocrine hormone or as a local autocrine/paracrine growth factor.

1.5.1 Serum IGF-1: The GH/IGF-1 Axis
GH produced from the pituitary somatotrophs exerts its effects by binding to the growth hormone receptor (GHR). It is then circulated in a complex with its binding protein growth hormone binding protein (GHBP). Upon binding to hepatic GHR, GH induces the expression of IGF-1. As the liver has the highest expression of GHR, it accordingly expresses a large fraction of circulatory/serum IGF-1. However, it is to be noted that other tissues including bone, muscle, and kidney all express GHR and may therefore contribute to the levels of serum IGF-1. Liver/serum IGF-1 has a negative effect on pituitary GH expression, thereby establishing a negative feedback loop (reviewed by Arvat 2000).

Loss of GH in the mature organism leads to retardation in body growth otherwise known as dwarfism. GH deficiency, a well-characterized condition, results in a reduction in muscoskeletal mass, impaired motor, vascular and cardiovascular function (Reviewed by Meling and Nylen 1996). These GH-deficient adults have both reduced GH levels and serum IGF-1 levels. Upon treatment using recombinant GH there is a restoration of vascular integrity, increased bone mass density and skeletal muscle mass (Bross et al 1999). Similarly, when GH is increased in acromegalics and gigantism, circulating IGF-1 levels are raised. Therefore as a general rule, GH will have a direct impact on serum IGF-1 levels.
However it is also clear that GH has direct effects, independent of IGF-1 mediated action. These effects have been observed in igf-1 knockout mice, which have normal GH levels. GH has a direct effect on adipocyte tissue, where it increases lipolysis and serum free fatty acids (Vernon et al 2000). This explains the reduction in body fat and increases in protein-to-fat ratio observed after GH treatment in normal adults. GH levels are, in turn regulated by levels of free fatty acid, and the adipostat Leptin. In contrast, the lipolytic effect is not observed in adults receiving IGF-1 treatment. To a lesser extent, GH stimulates the gene expression of other mitogenic factors such as Hepatocyte growth factor, (HGF), in liver cells (Ekberg et al 1992) and Epidermal growth factor, (EGF), in cartilage (Tajima et al 1994).

It is not clear how GH regulates IGF-1 gene expression and whether the alternative promoters P1 and P2 are differentially activated. The data on GH control of IGF-1 promoters has been generated by studies on hypophysectomised rats and was investigated only in relation to GH control of hepatic tissue (Adamo et al 1991). It was determined that GH activates both P1 and P2 equally (Mittanack et al 1997). However, in addition there was evidence of constitutive activation of the P2, the minor promoter in non-hepatic tissue, which was unaffected by GH activity (Le Stunff et al 1995). Therefore, there may be alternative regulatory elements within the promoter regions of IGF-1 gene, which allow for GH-independent, tissue-specific activation.

1.5.2 Regulation of Circulatory IGF-1

IGF-1 Binding Proteins

As IGFBPs play a key role in determining IGF-1 bioavailability and also in potentiating or inhibiting IGF-1 actions, the effect of serum IGF-1 on tissues does depend to a large extent on the levels of binding proteins. IGFBPs are shown to modulate the effects of IGFs in various ways including an inhibitory model whereby they can sequester IGFs from their receptors, or an enhancing model in which the can present IGFs at their site of action and a receptor-independent model that may involve IGFBP receptors (Rajaram et al 1997).
More than 95% of circulating IGF-1 is bound in a trimeric complex with IGFBP3, and an acid labile subunit. It was originally thought that GH had a direct impact on the IGFBP3 levels in the liver by regulating hepatic expression. However, in the recent Cre/Lox P model where \textit{igf-1} gene in the liver has been deleted (Liver IGF-1 Deficient or LID), BP3 levels are also 75% reduced while GH levels are almost fourfold that in normal mice (Yakar et al 1999). The IGFBP3 levels are in direct correlation with serum IGF-1 levels suggesting that GH control of IGFBP3 levels is only via IGF-1 (Binoux 1997).

As a general rule, serum IGF-1 levels correlate well with IGFBP3 levels. This is thought to be an in-built safety control that is disturbed in acromegalics, who have high serum IGF-1 levels and low IGFBP3 levels. This may in turn explain their increased risk to colonic neoplasms (Rosen 1999). It is indeed becoming increasingly clear that the measurement of serum IGF-1 alone does not give a clear indication of its function and the measurement of ‘free’ and ‘bound’ IGF-1 may give a better indication of its effect on cellular mechanisms.

**Nutrition**

During moments of severe nutritional changes such as undernutrition, malnutrition and obesity, GH regulation of circulatory IGF-1 is overridden (Thissen et al 1994, Gautsch et al 1998). At these times, calorie-intake has a direct impact on serum IGF-1 levels due to its effect on hepatic tissue. The importance of nutrition in the regulation of animal growth explains why it plays a dominant role in regulating IGF-1 action. Poor nutritional status impairs the hepatic induction of IGF-1 while in obese individuals GH levels are decreased but serum IGF-1 levels remain normal. As malnutrition leads to catabolism, it directly impacts on the function of IGF-1 and GH, which are anabolic hormones. Hence during fasting, tissues become GH resistant and serum IGF-1 levels are low (Clemmons and Underwood 1991). The regulation of hepatic IGF-1 by the protein/calorie intake is also post-transcriptional. This is partially due to the regulation of binding proteins such as IGFBP1. In addition, other catabolic stresses such as illness, sepsis, and trauma, play a role in determining the levels of circulating IGF-1 (Rosen et al 1999).
Genetic factors
Recent population studies including two of twins, have suggested that the levels of serum IGF-1 may be a heritable phenotype. A study of healthy inbred mice of the same body weight, length and similar GH levels revealed that serum IGF-1 levels differed between them by almost 30%. This study reflects the broad range of inter-individual variation amongst human populations (reviewed by Rosen et al 1999). This variation has been one of the factors that has cast doubts on the link between the decline of GH/IGF-1 axis and the onset of sarcopenia (skeletal muscle loss) with increasing age. This is due to the observation that there is a lack of conformity of low serum IGF-1 levels in the elderly (Arvat 2000). It has also been confirmed that there exists polymorphic variations within the IGF-1 gene that may cause differences in serum IGF-1 levels (Rosen et al 1998). These variations would be particularly relevant if they occurred within the promoter or enhancer regions of the gene, thereby affecting normal regulatory control. Similarly this phenomena would be applicable to the other modulatory or synergistic genes for example, IGFBP3.

1.5.3 The Role of Circulatory IGF-1
The role of IGFs in development is emphasized in igf-1 receptor gene knockout mice, which die of respiratory failure due to poor development of respiratory muscles. In contrast, igf-1 knockout mice are born normal yet show a severe retardation of postnatal growth and are infertile (Baker et al 1993, Liu et al 1993). However it has been difficult to determine whether circulatory and/or autocrine/paracrine IGF-1 determine postnatal tissue growth.

Factors affecting GH production including mutations in the anterior pituitary, result in retarded postnatal growth. Initially, these individuals and animals have almost normal birth weights, strongly supporting the GH-independent effects of IGF-1 on foetal growth. However, by puberty, growth appears to be stunted, suggesting that GH is responsible for the pubertal growth spurt (reviewed by Butler and LeRoith 2001). It was also assumed that GH was responsible for the
pubertal growth spurt, due to the observation of a corresponding peak in GH and thus serum IGF-1 levels during adolescence. The establishment of the Cre/Lox P model whereby hepatic igf-1 was knocked-out (LID) raised a number of questions regarding the basic tenets of IGF-1 physiology (Yaker et al 2000). Unexpectedly, these hepatic IGF-1 knockouts pups, were born with normal body weights, grew at a normal rate and were fertile. The mice had a normal appearance despite the reduction in 75% of serum IGF-1 and IGFBP3 levels. This has in turn raised the question as to whether either ‘free’ or unbound serum levels area is a better indicator of IGF-1 effects on body growth. Given the coincidental increase in serum IGF-1 levels, and strong correlation between GH and serum IGF-1 levels, it is clear that GH does control hepatic liver IGF-1 production. It is also clear from these models that serum IGF-1 levels in turn regulate GH production through a negative feedback mechanism. In the case of the LID mice and Laron’s dwarfism (exhibits GH receptor defects), serum IGF-1 is low and GH levels are abnormally high. Although the LID models may rule out the importance of the role played by hepatic IGF-1 in growth, the GH deficient models seem to still indicate that GH has a role to play and may coordinate the growth spurt through its direct effects on tissue IGF-1s (Woods et al 1995). However, tissue IGF-1 levels did not differ between control and transgenic mice.

The number of different organs that have been shown to respond directly to GH may lend support to the theory that postnatal growth spurt is regulated by local IGF-1. Recombinant GH supplementation in GH deficient individuals leads to an increase in IGF-1 mRNA in lung, kidney, heart, bone, skeletal muscle and adipose tissue (Fielder 1996). GH has also been observed to directly stimulate the proliferation of chondrocytes and growth plate germinal cells. In addition there seems to a synergistic effect with IGF-1, which implies that both GH and IGF-1 can act directly on bone to stimulate growth. The effects of GH in skeletal muscle are also thought to be through the upregulation of local IGF-1. Administration of recombinant IGF-1 (rhIGF-1) in Snell’s dwarf mice model shows a tendency to increase muscle, weight, but this effect is lower than that seen with GH administration. Both GH and IGF-1 supplementation increase protein synthesis in
the muscle. However, in the liver, only GH increases protein synthesis (reviewed by Butler and LeRoith 2001).

The emergence of these transgenic models has cast confusion about what the function of the large amount of IGF-1 derived from the liver can be. One of the conclusive findings was that serum IGF-1 regulates GH secretion in an endocrine manner (Yakar et al 1999). Circulatory IGF-1 may also regulate insulin metabolism. In the Laron dwarf, LID mice and following chronic administration of GH, insulin resistance develops specifically at the level of the insulin receptor in skeletal muscle tissue (Yakar et al 2000). This is accompanied by hyperinsulinamia and decreased IGFBP-1 levels. While administration of rhIGF-1, improved insulin sensitivity, it is still unclear whether IGF-1 directly corrects insulin resistance by direct effect or merely through its reduction of GH levels (Arvat 2000). IGF-1 is known to have an insulin-like effect and chronic elevations tend to increase insulin sensitivity, suggesting that it may also additional action on skeletal muscle gluconeogenesis independent of the GH effect. Intravenous bolus injection of both IGF-1 and IGF-II induced hypoglycaemia and enhanced glucose uptake from serum and incorporation into glycogen (Zapf et al 1986). The insulin-like effects of IGF-1 in both hypophysectomized and normal rats appear to be mediated by free IGF-1 rather than IGFBP-bound IGF-1 (Giacca et al 1990). This also supports the hypothesis that the main site of action of IGF-1 on glucose metabolism is the muscle rather than liver or adipose tissue, the target tissue of insulin. Hence IGF-1 has been proposed to be of therapeutic value in conditions involving insulin resistance such as Type 2 diabetes, and obesity.

1.5.4 Autocrine/Paracrine IGF-1

Almost all tissues express IGF-1 as well as the IGF-1 receptor. It is also clear that GH induces IGF-1 expression in many tissues. Hypophysectomised animals treated with GH show an increase in Igf-1 gene expression in the pancreas, muscle, intestine, kidney, brain, adipose tissue as well as the liver. However, it is now clear that IGF-1 can also have GH-independent actions. For example GH
apparently does not affect prenatal development, as GH and GHR null mice are no different from their wild-type littermates at birth. In contrast, Igf-1 null mice are born smaller than the wild type, and most die in the early neonatal stages (Liu et al 1993). Those that survive are severely retarded and also infertile (Liu et al 1993).

Postnatally, tissue type appears to largely determine the role and regulation of autocrine/paracrine IGF-1. The heterogeneity of tissue/cellular response depends on: the type of tissue, the state of differentiation of the cell, the number and activity of cell surface receptors, the cellular microenviroment and the method of delivery of IGF-1. IGF-1 is demonstrated to stimulate the differentiation of developing progenitor cells to adult myelin-forming oligodendrocytes (McMorris et al 1986). It also stimulates the proliferation and differentiation of myoblasts (Florini et al 1992) osteoblasts and adipocytes (Smith et al 1988).

Local IGF-1 production is thought to be important in the regulation of growth and differentiation in response to local environmental changes. In these circumstances tissue response appears to be under direct control of autocrine/paracrine IGF-1, independent of GH regulation. For example, IGF-1 directly regulates ovarian function and uterine growth. In hyposectomised rats, oestrogen treatment of increases the level of ovarian IGF-1 mRNA, while decreasing the level of hepatic IGF-1 ( ). In addition, animals featuring GH resistance or deletion are fertile in comparison to LID mice.

IGF-1 appears to have an important role in tumouriogenesis, as a number of tumour cell lines have been shown to synthesize both IGFs and IGF-1 receptors in larger concentrations than seen in normal cells. Some of these cell lines grow in absence of exogenous growth factors and their proliferation in serum-free media can be inhibited by antibodies that bind either IGF-1 or IGF-1 receptors (reviewed by Le Roith et al 1999). The tumour suppressor protein, p53, has been shown to suppress proliferation by increasing IGFBP-3 transcription and down regulating IGF-1 receptors in haemopoietic cells.
Local IGF-1 synthesis is also stimulated in response to local injury. For example IGF-1 immunoreactivity is elevated after injury of peripheral nerves, skeletal muscles, and endothelial cells of the arteries (Hansson et al 1987, Jennische et al 1987). Given the widespread actions and variety of response stimulated by autocrine IGF-1, its role in muscle will be evaluated in light of different stages in development and in response to specific stimuli.

1.6 Skeletal Muscle:
1.6.1 Structure and Development
Skeletal muscle tissue serves not only to allow for body movement, but is also the largest protein store in the body. It is also an important site for thermoregulation and glucose metabolism. Striated muscle consists of giant long cylindrical multinucleated cells known as a myofibres. Each muscle fibre is made up of rod-like structures known as myofibrils. In the embryo, myogenic precursor cells are mainly derived from the lateral plate and paraxial somatic mesoderm. The development of skeletal muscle is complex requiring external cues that regulate the migration of these committed myoblasts to their correct target sites (reviewed by Monti et al 2001). Proliferation and differentiation of myoblasts at their target is driven by the expression of muscle-specific transcription factors, the MyoD and MEF2 family. These basic-Helix-Loop-Helix proteins (i.e. MyoD, Myf-5, myogenin and MRF) act in concert to coordinate a multistep process where myoblasts withdraw from the cell cycle, elongate and fuse into multinucleated myotubes (Lassar et al. 1994). These Myogenic Regulatory Factors (MRF), regulate the expression of muscle-specific genes such as myosin, actin, creatine kinase, troponin.

Early in development, trophic factors such as Hepatocyte Growth factor (HGF), and IGF-1, act on both the mesoderm and neural tissue at each segmental level to induce matched muscle precursor cells and motorneurons. Motor axons from the spinal chord navigate to their target muscles as soon as they are formed. It has been known from decades that soluble neurotrophic activity is present in nerve and nerve targets (Hollyday et al 1977). Sensory neural input is clearly required
for the development of specialized muscle fibres, and it is also thought that target fibres may promote sensory neuron maturation via retrograde neurotrophic factors in order to ensure correct central connectivity. Based on the original observations of Hollyday and Hamburger (1976), who found that the sizes of the lateral motor columns and dorsal root ganglia were influenced by the size of target that was innervated, it is thought that these growth factors also serve simultaneously as neurotrophic factors. Thus IGF-1 may act during development to coordinate growth between the size of the nervous system and the target field that requires innervation (Recio-Pinto and Ishii 1988). IGF-1 gene expression is correlated with synapse formation during developmental and regeneration.

Although innervation is not needed required for the basic muscle patterning, nerve-dependent signals later regulate the phenotype and properties of the muscle fibres. Ultimately the correct mixture of fibre types, are specified in each muscle with particular tendon attachments and become innervated by the appropriate motor neuron. The formation of perfectly aligned myofibrils is a dramatic example of supramolecular assembly of eukaryotic cells.

Unlike most other organs, muscle growth in cell number is limited to the prenatal period, with mammals being born with or soon reaching the full complement of cells. Fiber number becomes fixed either before birth or shortly after birth. Therefore, during post-natal growth, growth of skeletal muscle occurs only by an increase in fibre size, termed as hypertrophy, as opposed to prenatal hyperplasia. The muscle fibres increase in size by either an increase in length and/or increase in circumference (reviewed by Allen et al 1999).

1.6.2 Skeletal Muscle Diversity

There exits great diversity between sub-types of skeletal muscle fibres in physiological regulation, excitation-contraction, energy metabolism and ion homeostasis. Separate groups of muscles also differ in the heterogeneity of their fibre types, with each specific makeup reflecting their biological adaptation to specific functional demands. Based on immunohistochemical evaluations, histochemical assays which exploit the pH stability of the myofibril proteins,
several different fibres can be identified. Molecular biochemical techniques have since correlated each histochemical identifiable muscle fibre with a highly specific expression profile of myofibril proteins. The sarcomere is the contractile unit of striated muscle and consists of one set of thick (myosin molecules) filaments and thin (actin) filaments. Myosin molecules, consists of two heavy chains (MyHC) and four light chains (MLC). The MyHC genes play a predominant role in specifying the muscle fibre contractile properties. There are at least nine different MyHC isoforms identified in adult mammalian skeletal muscles (Reviewed by Hamalainen and Pette 1995). The main ones found in the hind limbs are summarized in the table below:

<table>
<thead>
<tr>
<th>MyHC isoforms</th>
<th>Nomenclature</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic</td>
<td>HCemb</td>
<td>Myotubes, regenerating fibres</td>
</tr>
<tr>
<td>Neonatal</td>
<td>HCneo</td>
<td>Neonatal muscle, regenerating fibres</td>
</tr>
<tr>
<td>Fast-twitch</td>
<td>HClIib</td>
<td>IIB, IIAB fibres</td>
</tr>
<tr>
<td>Fast-twitch</td>
<td>HClIia</td>
<td>IIA, IIBD, IDA, IIC, IC</td>
</tr>
<tr>
<td>Fast-twitch</td>
<td>HC2x</td>
<td>2X, IIBD, IDA fibres</td>
</tr>
<tr>
<td>Slow-twitch</td>
<td>HCI (BHCcard)</td>
<td>Type I, IC, IIC fibres</td>
</tr>
</tbody>
</table>

The diversity observed within skeletal muscles reflects their high degree of functional specialization, which is the key feature which forms the core basis of muscle plasticity. During maturation, changes in environmental conditions act to modulate muscle phenotype. Furthermore, when changes in hormonal environment or in the functional demand outside of the normal range of muscle activity occur, a fully differentiated skeletal muscle is able to adapt itself to the new conditions by modifying its mass as well as changing its fibre type composition.
1.6.3 Skeletal muscle adaptation

Skeletal myofibres are unique in that they are not only postmitotic cells, but also truly multinucleated. This poses unique questions as to how the cells are modulated during muscle adaptation. The multinucleated nature of muscle fibres has led to the concept of a DNA unit or myonuclear domain, which is defined as the theoretical amount of cytoplasm supported by a single myonucleus (Cheek 1985). It is thought that the changes in skeletal fibre size and mass could conceivably be regulated by an increase in the myonuclear domains (by increasing myonuclear number) or by an increase in the size of existing domains (through increased protein synthesis). More recent studies indicate that the relative nuclear volume is unchanged in hypertrophied muscle as compared to the control muscle. This lends support to the hypothesis that myonuclear number increases during muscle hypertrophy in an attempt to maintain the nuclear to cytoplasmic volume ratio (Hall et al 1989). However, it is important to keep in mind that the concept of a nuclear domain is theoretical and does not sufficiently account for the regulation and distribution of individual proteins within the muscle fibre (Allen et al 1999).

Cardiac and skeletal muscles of humans and other vertebrates respond to changes in physiological demands by remodeling the architecture of individual myocytes and of the tissues in which they reside. The responses can take several forms: changes in the overall mass of the tissue (hypertrophy versus atrophy); changes in the spatial relationship of myofibres and other cellular and extracellular components of muscle tissue; and reprogramming of gene expression to alter specialized metabolic and contractile properties of myofibres (Olson et al 2000). Chronic changes in a number of physiological factors, including neuromuscular activity, hormones levels, and mechanical loading result in both quantitative and qualitative changes in protein metabolism and gene expression in muscle. These adaptations in turn, alter the size and/or metabolic properties of the muscle fibres, resulting in physiological capabilities that meet the new functional demands.
Changes in MyHC expression.

Motor neurons are organized in a continuous column along the anterior posterior axis in the ventral horns of the spinal chord. Each motor neuron column is further divided into pools that innervate a specific muscle. The significance of neural control in determining the phenotypic expression of muscle has been illustrated by the use of cross-innervation experiments chronic stimulation and denervation studies (reviewed by Monti et al 2001, Wigmore and Evans, 2002). The character of motoneurons within each pool tend to be matched to the contractile properties of the target muscles. Generally, all fibres composing a specific motor unit appear to contain immunohistochemically identical myosins, suggesting that the phenotypic expression of muscle is dictated by neuromuscular activity. The motor neuron pools are further divided into a series of motoneuron classes that synapse with and drive contraction of specific muscle fibres. Therefore gentle muscle contractions are driven by slow motoneurons and hence recruit the slow-contracting oxidative fibres. On the other hand, for fast phasic movements faster motor neurons are recruited, giving a more forceful contraction.

Different models of muscular activity and inactivity play a role in altering gene expression of MyHC family of proteins in skeletal muscle. The marked differentiation in myofibres that occurs after birth and is characteristic of adult MyHC is thought to be coordinated in a thyroid hormone (T(3), 3,5,3'-triiodothyronine) level-dependent fashion. Hypothyroidism, as well as interventions that unload or reduce weight-bearing activity of muscle, causes slow to fast MyHC isoform conversions. On the other hand, chronic low frequency stimulation, hypothyroidism as well muscle overload induces pronounced fast-to-slow transitions in both MyHC isoforms and fibre types (reviewed by Adams et al 1999). Both stretch alone and electrical stimulation alone are capable of activation of the slow type gene and repression of the fast-type genes (Goldspink 1998). However, a more rapid and complete switch MyHC gene expression is seen when both electrical activity and mechanical stimuli are combined as observed during resistance or endurance training (Goldspink et al 1999).
The regulation of MyHC expression appears to be strongly regulated by transcriptional events. Recent findings using promoter-reporter constructs have clearly demonstrated that an increase in mechanical stress leads to the increase in B-MHC transcription by activating its promoter (McCarthy et al. 1999). On the other hand, in non-weight bearing muscle, there is a decrease in B-MHC protein synthesis. The decrease in translation appears to be regulated within the 3'UTR of its mRNA (Ashley and Russel 2000).

It is well established that changes in neural stimulation patterns result in changes in the levels of fibre-type specific isoforms of most muscle proteins, such changes in protein composition take weeks to occur. This is due to the relatively slow turnover of most muscle proteins (Hamalainen and Pette 1995). Individual genes are thought to be differentially sensitive to distinct patterns of electrical activities, mechanical stress and hormones. Recent studies focusing on defining the signaling pathways and underlying mechanisms that govern changes in MHC isoforms have uncovered a complex network which may more accurately reflect the large functional diversity and hence heterogeneity among individual fibres.

Changes in Muscle mass

The phenotype of adult-stage muscle can be profoundly altered in response to specific mechanical perturbations, for example mechanical overload or non-weight bearing. These changes were thought to reflect changes in neuromuscular activity, however it is now clear that mechanical stress alone has an effect on changes in muscle mass.

The idea that an increase in mechanical input to muscle is the primary determinant of muscle hypertrophy has long been established (Folkman et al. 1978). The basic underpinning for this idea is provided by the observation that cellular growth is critically dependent on cellular stress, which is internally coupled to extracellular signaling pathway through the cytoskeleton (Ingber 1997, Huang et al. 2000). A number of different ways of overloading the muscle to produce a hypertrophic response have devised, including sectioning the
gastrocnemius portion of the Achilles tendon leaving the remaining plantaris and soleus muscle to support the body weight (Goldberg 1969). Using this method, there is a rapid increase in the muscle mass of the synergistic muscles as they undergo compensatory growth. In addition, stretch and stimulation of the tibialis anterior muscle has also been shown to induce rapid hypertrophy (Goldspink et al 1999).

In contrast to muscle postnatal growth, the muscle hypertrophy does not appear to require the presence of pituitary GH. The same manner of muscle compensatory growth occurs in hypophysectomized animals, and starving animals as that seen in normal animals (Goldberg 1967). These experimental results suggested that muscle hypertrophy was independent of the GH/IGF-1 axis and therefore may be mediated by paracrine and autocrine factors.

Further experiments appear to suggest that there is a direct correlation between increased load and the hypertrophic response implying that mechanical input itself is a sufficient stimulus for anabolic mechanisms intrinsic to the postnatal muscle cell. However the concept that paracrine or autocrine factors either play an intermediary role or they themselves cause myogenic growth have arisen from the observation that load induction is accompanied by an increase in peptide growth factors such as IGFs.

1.7 IGF-1 and Muscle Growth

IGF-1 expression is relatively high in neonatal muscle and decreases rapidly after birth, with adult muscle fibres expressing very low levels of IGF-1 (Alexandrides et al 1989). In addition, IGF-1 content in muscle seems to be partially influenced by the GH axis as hypophysectomy results in a decrease in muscle IGF-1 expression and this is restored after GH treatment (Underwood et al 1986 and Isgaard et al 1988).

As mentioned previously, compensatory growth in muscle appears to be a localized process, which is induced by the recruitment of local factors. IGF-1
expression increases in muscle in response to overload induced by tendon ablation, weightlifting, stretch and stimulation and eccentric exercise. IGF-1 response in muscle has therefore been linked to muscle response to mechanical stimulus as well as to repair and regeneration mechanisms. It is also evident that IGF-1 induced in muscle is locally produced and therefore not from systemic source or related to the GH/IGF-1 axis.

Several approaches have been used to define more accurately the role of local IGF-1 in the induction of muscle hypertrophy in response to loading. Myoblasts cultured in vitro, can hypertrophy in response to exogenous IGF-1 as well as a result of IGF-1 overexpression (Semsarian et al 1999, Vandenburgh et al 1991). In addition transgenic mice overexpressing IGF-1 only in skeletal muscle (Barton-Davis et al 1998) as well as infusion of IGF-1 directly into the tibialis anterior of the adult rat is characterized by muscle hypertrophy (Adams and McCue 1998). Since overexpression of IGF-1 induces muscle hypertrophy and increased mechanical stress induces muscle IGF-1, it appears that functional demand therefore induces muscle hypertrophy by stimulating IGF-1 expression.

However, there are still doubts raised as to whether hypertrophy induced by mechanical stress is directly coupled to the induction of autocrine/paracrine IGF-1. Overload induced in dennervated or previously unweighted muscle does not respond with an increase in muscle IGF-1, although there appears to be a hypertrophic or growth response. Infusion of IGF-1 in unloaded muscle does not appear to reverse the atrophic response. In addition there is very little data regarding the regulation of different IGF-1 transcripts in muscle.

With renewed interest in the role of muscle IGF-1 in postnatal growth, further work is required to distinguish between the response of muscle to local stimuli versus systemic hormones. In particular, it is important to establish how different IGF-1 transcripts are regulated in response to local stimuli as it may reveal the distinction between local and systemic regulation of IGF-1, and therefore shed some light on the different roles of the same IGF-1 protein under different circumstances.
1.8 Aim of These Studies

The regulation, biological actions and potential clinical use of IGF-1, particularly in reversing muscle frailty during ageing, is under active investigation. This review has focused on the current state of knowledge about the gene structure of mammalian IGF-1 and its role in development and compensatory muscle growth. It has emphasized the growing distinction between the role of IGF-1 as a systemic hormone versus its role as an autocrine/paracrine growth factor. Although it is accepted that overload or stretch is linked to an increase in the local production of IGF-1, leading to skeletal hypertrophy, it is not clear to what extent local IGF-1 changes can be linked to muscle adaptive response. The overall purpose of this dissertation is to determine how ageing, mechanical loading and neuronal injury influence changes in local IGF-1 expression and whether different IGF-1 transcripts are produced in response to different stimuli. This will also shed some light as to whether IGF-1 is a mediator of changes in muscle fibre size in response to positive or negative stimuli.
Chapter II

Development And Validation Of A Quantitative RT-PCR Method To Measure Changes In Gene Expression Of IGF-1 Receptor
2.1 Introduction

The adaptation of muscle to overload is thought to be reflected in altered patterns of gene expression of the IGF-1 system. An accurate, reproducible and sensitive method was required to quantify local expression of IGF-1 system in muscle. Four methods have commonly been used to quantify gene expression; Northern blotting, In situ hybridisation, RNase protection assays and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The main limitation of the first three methods has been their comparatively low sensitivity. RT-PCR in comparison has a detection limit of 10-100 -fold lower than the other methods.

RT-PCR is an in vitro method for enzymatically amplifying defined sequences of RNA. As RNA cannot be a direct template for PCR the first step requires the reverse transcription of RNA into complementary DNA, cDNA using dedicated RNA-dependent DNA polymerases (Reverse transcriptase). The cDNA is then exponentially amplified by DNA polymerases. The RT step is primed by using specific primers, random hexamers or oligo-dT. Different RNA transcripts exhibit varying secondary structure and this can affect the activity of the reverse transcriptase, hence the PCR quantification. Taq polymerase is less sensitive to such effects but with exponential amplification, errors are compounded. Therefore, to establish accurate PCR quantification it is important to optimise the RT step as well as the amplification step.

For the accurate quantification of low abundant gene expression two RT-PCR methods were compared: Quantitative Competitive RT-PCR and Real-time RT-PCR.

2.2 Quantitative Competitive RT-PCR (QC-RT-PCR).

The technique involves the amplification of the sequence of interest and a second control sequence, which is reverse-transcribed and co-amplified in the same reaction as the native mRNA sequence. The internal standard acts as a reference point and controls for the tube-to-tube variation during reverse transcription and subsequently, cDNA amplification. The internal standard consists of synthesised...
RNA, with the same primer sites as the native sequence. Normally, quantitative competitive RT-PCR (QC-RT-PCR) methods use serial dilutions of the internal control with a constant amount of unknown mRNA in the RT-PCR (Wang et al 1989). A titration assay is then used to determine the amount for each unknown sample. The method is based on the assumption that the target and internal control molecule (ICM) are amplified at the same PCR efficiency. Therefore, if the initial ICM and target molecule have identical PCR efficiencies, at equimolar concentrations, the ratio of target to ICM PCR products should be one to one (reviewed by Ferre 1994). Comparison of a competitor that differs in any way from the native mRNA will produce non-quantitative results (McCulloch et al 1995). For precision and accuracy this procedure requires 3-to-5 reactions for each sample, rendering the technique labour-intensive and subject to error. Recently, a modification of this method has been developed, which still uses a competitor to account for internal variation, but the final quantitative results are obtained by a comparison to a standard curve that utilises a native RNA with sequence identity to the mRNA of interest (Tsai and Wiltbank 1996). This standard-curve method allows the direct quantification of each sample by comparison to a standard curve.

In the standard curve method, two standards are constructed. One consists of the internal control molecule, and the other is identical to the native sequence to be amplified. The former will be known as the Internal Control and the latter as the Target. Different amounts of target RNA are then co-amplified with a constant amount of the competitor RNA. The two different species are distinguished by size using high-resolution gel analysis. The bands were quantified by densitometric analysis using a phosphoimager (Molecular Dynamics). Regression analysis was used to produce a ‘line-of-best fit’, that would determine whether there was a linear relationship between the initial quantities of competitor and target cDNA, after PCR amplification.
2.2.1 Materials and Methods

Preparation of Oligonucleotides

Primers used in the study were derived from rat IGF-1 receptor cDNA sequence [EMBL Ac no. NM052807]. The reverse transcriptase primer, RT803 (5' CTACTACTACAAAATCGCCCT 3') was tagged with a T7 promoter sequence to allow for straightforward in vitro RNA synthesis of the internal control and target molecules. All primers were synthesized by Genosys Europe, Cambridge, UK. The primers were dissolved in double-distilled water to a concentration of 100mM.

Conditions for cDNA Synthesis

All cDNA synthesis was undertaken with a Superscript II Rnase H- (GibCo BRL). RNA were denatured at 65°C for 5 min and chilled in ice before added to the reverse transcription solution containing 0.5 mM dNTPs, 20μM of specific primer, 10 units/μl Superscript II RT, 4μl of 5x Superscript buffer (250mM Tris-HCl, 375 mM KCl, 15 mM MgCl₂), 1 unit/μl Rnase inhibitor (GibCo BRL), and 10mM dithiothreitol (DTT) in a final volume of 20μl. The cDNA synthesis reaction was performed at 37°C for 60 mins.

Preparation of Standard RNA

0.5g of fresh adult rat muscle tissue was homogenised in 4M guanidinium thiocyanate buffer as described by Chomczynski and Saachi (1987). The extracted RNA was dissolved in diethyl pyrocarbonate water (DEPC H₂O) and quantified spectrophotometrically with the Gene Spec I (Naka Instruments). The RNA was run on ethidium bromide stained gels and its integrity confirmed by visual inspection of the 18S and 28S ribosomal RNA under ultra violet light.

Using the cDNA transcribed from total RNA, the Target DNA product was amplified with the PCR primers RT803 and RIGFRF (5' CTCGCTGTGGGGCTCG 3') using Bioline Taq (Bioline UK). PCR amplification was performed in a total volume of 50μl per reaction using Bioline Taq (Bioline) as before. 2μl of cDNA (1 in five dilution of total cDNA) was added to each reaction, which contained 1 unit of Taq DNA polymerase, Bioline
Buffer, 0.2mM dNTPs, 0.4μM sense and antisense primers. Following a 5 min hot start at 94°C, amplification was undertaken by denaturation at 92°C for 30s, annealing at 62°C for 30s and extension at 72°C for 40s in a PCR reaction of a final volume of 50μl.

In order to keep the RT and PCR efficiency of the control and target molecules as close as possible, the control molecule was amplified from the insulin receptor sequence (EMBL Ac. No. NM017071) with PCR primers with the RIGFRF and RT803 sequences tagged to each end. This yielded a PCR product of 627 bases. This region of the insulin receptor has relatively high homology to the IGF-1R, and yet is sufficiently different to avoid the formation of heterogeneous PCR products during the simultaneous amplification of the target and control molecules. The desired DNA bands were excised and purified by QIA Gel Extraction kit (Qiagen, Crawley, U.K.). The purified products were cloned into a pGEM T-easy vector (Promega, UK). The inserts were fully sequenced by MWG Biotech (Cambridge UK). The control molecule was confirmed to have the appropriate primer sequences tagged to both ends, but different internal sequences.

For RNA transcription, the inserts were amplified by PCR using Platinum Taq as before and run on a gel to confirm the correct size. The purified DNA was dissolved in diethyl pyrocarbonate water (DEPC H₂O), and in vitro RNA transcription carried out using a T7 polymerase RNA kit (Promega). The reaction was carried out at 37°C for 1 h and the DNA digested by incubation with RNase-free DNase (1unit/μl) at 37°C. The synthesised RNA was extracted with chloroform/3 methylbutan-1-ol (49:1, volume for volume), precipitated with 100% ethanol and washed with 70% ethanol. The RNA pellets were then air-dried and quantified by spectrophotometry.

**PCR Amplification and Quantification of PCR Products.**

PCR amplification was performed in a total volume of 50μl per reaction using Bioline Taq (Bioline) as before. For negative controls the same procedure was applied to a reaction mix containing no cDNA.
Each RNA sample was analysed using Competitive RT-PCR as described by Zhang et al (1999). Briefly, five separate cDNA synthesis reactions were undertaken with increasing quantities ($10^4$ to $10^5$) of molecules of target RNA and fixed amounts of $10^6$ molecules of Control cRNA in successive cDNA reactions. From each of the five completed cDNA reactions 3 aliquots were taken, added into a PCR mix and individual aliquots amplified at either 21, 25 and 30 cycles. The PCR amplification efficiency for each the Target (TM) and Control Molecules (CM) was also determined.

RT-PCR products were analysed in 5% polyacrylamide gel stained with a 1 in 10000 dilution of Vistra Green dye (Amersham Life Sciences). The DNA bands were then quantified directly on the FluorImage™ system of a Phosphoimager (Molecular Dynamics). The quantification value of the bands in the gel was designated the optical density (pixels). In each case the optical density from target template (Dt) and Control template (Dc) were obtained and used to calculate the RNA number. A schematic illustrating the process of constructing a standard curve is shown in Fig 2.1.

**Design of primers for Competitor and Target standards**

*Target Standard: IGF-1R – 740 bp,*

*Internal Competitor: IR- 627 bp*

---

**Fig 2.1**: A schematic diagram illustrating the steps taken in construction of the target and internal control molecules required for the quantification of PCR products based on the standard curve method.
Assay of unknown samples

1. The mean value of ratios (n=5) of target DNA band density (Dt) to the control band density (Ds) was calculated from each of the original cDNA’s amplified from the three consecutive cycles.

2. Log values of the mean values was plotted against log values of the amounts of the total RNA in the original cDNA synthesis reaction, creating a standard curve.

3. From the curve unknown quantities in the native sample could be determined by spiking the cDNA reaction with the pre-determined amount of Control cRNA.

2.2.3 RESULTS

Fig 2.2A shows the amplification native /target IGF-1 receptor cDNA sequence and the internal competitor. The sequence of the competitor is different except at the primer sites where the sequences are identical. The PCR products were separated on a 5% polyacrylamide gel and showed that there was both an increase in amounts of native product (upper band) and a decreased amount of competitor product (lower band) with increasing amounts of initial target RNA used, as expected.

Each of the cDNA reactions was subdivided into aliquots and each aliquot amplified for either 21, 25 or 30 cycles. The measured ratios of target DNA band intensity (Dt) to competitor DNA band intensity (Ds) between the different cycles is plotted in Fig 2.2B. As indicated on the graph by the standard deviation bars, the measured ratios between the different numbers of cycles were similar indicating parallel amplification of both the competitor and target templates.
**Key**
(moles x $10^6$)
1. 8
2. 4
3. 1
4. 0.8
5. 0.4
6. 0.1
7. 0.08
8. 0.04
9. 0.01
10. 0.0008

**Fig 2.2A:** Shows PCR products of $8 \times 10^6$ to $8 \times 10^8$ molecules of native /target IGF-1 receptor cDNA each amplified with $10^6$ molecules of the internal competitor for 25 cycles. The PCR products separated on a 5% acrylamide gel showed that there was both an increase in amounts of native product (upper band) and a decreased amount of competitor product (lower band) with increasing amounts of initial target RNA used. **B:** The measured ratios of target DNA band intensity ($D_t$) to competitor DNA band intensity ($D_c$) between the different cycles is plotted. As indicated on the graph by the standard deviation bars, the measured ratios between the different numbers of cycles were similar indicating parallel amplification of both the competitor and target templates.

In order to construct a standard curve, the relationship between the target input and the ratio of band intensities of $D_t$ to $D_c$ was determined using linear
regression analysis. A straight line can be constructed by plotting the logarithm of the ratio of input target (or native cDNA) and competitor RNAs against the logarithm of the initial amounts of the target RNA (Fig 2.3).

\[ y = 0.057x - 0.1293 \]
\[ R^2 = 0.9662 \]

Fig 2.3: Shows the mean ratios of target DNA band intensities (Dt) to competitor DNA band intensities (Dc) amplified from each 2 separate experiments run at 21, 25, and 30 cycles analysed with a log-log plot. The straight line is the regression line, which is used as the standard curve from which unknown values are calculated.

As can be seen from the Fig 2.2b, and Fig 2.3, fairly consistent results can be reproduced from this method. This is partly dependent on the detection method used, as a more sensitive method will yield more accurate results. From Fig 2.2a it can be clearly seen that the points with the highest deviation are likely to be values that correspond with the points furthest from the equimolar ratio of the Target to Competitor RNA. The variation can also be measured by the standard deviation as the ratios at the lower concentrations of input target DNA varied much more widely than those closer to the concentration of the input competitor DNA (see Fig 2.2b). This implies that in the determination of unknown samples it may be important to determine a rough estimate of the equimolar ratio of the target to competitor DNA so as to be able to set parameters as to the limits of the assay for unknown samples. Bearing this in mind, it may be necessary to have a rough estimate of the concentration of unknown samples if a more accurate
quantification is to be carried out. As can be seen at the much higher concentrations of input target DNA, the competitor DNA band intensity is not quantifiable (Fig 2.2a) and therefore it is likely that the amplification efficiencies are no longer linear. Therefore, even using an extrapolated standard curve (see Fig 2.3), the results beyond the set parameters are likely to be inaccurate.

Although it is claimed that the Standard curve QC-RT-PCR is linear over more than 2 orders of magnitude, it can be clearly seen from Fig 2.2a, that the accuracy and sensitivity of the assay diminishes at the higher and lower ends of the Target molecules. Using a more sensitive method of detection and increasing the number of amplification cycles may decrease this limitation to the assay. However, increasing the number of PCR cycles seemed to increase the standard error, as expected (results not shown).

The greatest advantage of the standard curve QC-RT-PCR method over the 'original' is that the amplification efficiency of the Target and Competitor do not have to be equal. This is because the absolute amount of competitor RNA is not used to calculate the values of the native or unknown samples (Tsai et al 1996). It only requires that the amplification efficiency of the native and Target RNA be similar. A direct comparison was made by determining the ratio of density of target and competitor RNAs at different amplification cycles. It was found that the ratios were similar (Fig 2.2b). This is likely as they are of similar sequence length, same primer sequence and even the base content between the sequences is not widely different. From the values of the regression line, which is close to 1 (Fig 2.3) it can be seen that this assay produces reproducible results.

2.3 Real Time PCR
Recent advances have facilitated the application of fluorescence techniques to RT-PCR leading to suitable instrumentation that can monitor the PCR reaction in real-time. The PCR was carried out in a Lightcycler (Roche Molecular Biochemicals, Mannheim, Germany), which combines amplification, detection and quantification. The Lightcycler performs RT-PCR in small volume glass
capillary tubes, contained in a rotor-like carousel, which are heated and cooled by an airstream. The carousel is rotated past a blue-light emitting diode, which stimulates fluorescence and a photon multiplier detects the changes in fluorescence.

In this method, absolute quantification of the unknown sample was determined by constructing a standard curve for each individual amplicon. The standard curve was generated by running a serial dilution of calibrated external standards corresponding to the gene of interest along with unknown samples. The PCR products were detected by the binding of a fluorescent dye, SYBR Green. The dye binds to double-stranded DNA and the bonding increases the fluorescence by energy transfer hence exhibits very little fluorescence at the beginning of the experiment. At the end of each elongation step there is an increase in the amount of newly synthesised DNA, and therefore an increase in the fluorescent signal. The Lightcycler records fluorescence readings after every PCR cycle.

It therefore follows that the more the template present at the beginning of the reaction, the fewer cycles it takes to reach a point at which the fluorescent signal is significantly above the background fluorescence. This semi-arbitrary set point is defined as the threshold cycle (Ct) and is the key to accurate and reproducible quantification using fluorescence-based RT-PCR. The Ct value is inversely proportional to the log of the initial copy number and therefore a standard curve can be generated by plotting the Ct values against the logarithm of the initial copy numbers of the standards. Unknown values are then calculated from the linear regression of that standard curve (Bustin et al 2000).

2.3.1 Methods and Materials

Total RNA Extraction

Total RNA was extracted and quantified as for use in QC-RT-PCR.

Primer Design

The primers used for production of both the Standards and for real time PCR were derived from rat IGF-1 receptor sequence. Two sets of primers were used:
1) RT803 and RIGF1RF: This produced a product of 740bp spanning exons 3 and 4 of the IGF-1 receptor transmembrane region;

2) LCIGF-1RF and LCIGF-1RR were designed using the Omega Version 2.0 (Oxford Molecular) and synthesised by Sigma Genosys. They were designed to produce a 350bp amplification product, across introns within the region RT-primed by RT803. Primer and product melting temperatures (Tm in °C) were calculated with the %GC method.

Optimisation of Reverse Transcription

As there is a fundamental assumption that cDNA synthesis efficiencies are identical for both standards and target RNA, ensuring efficient reverse transcription was an important step in determining precise quantification of target mRNA copy number.

For all cDNA reactions the amount of total RNA, 2µg, and 0.5 dNTPs and 1unit/µl of RNase inhibitor all in a 20µl reaction mix was kept constant. The effect of the following factors were considered:

1. Choice of RT- the different RTs tested were Superscript™ II (GibCOBRL, UK), Expand RT (Promega, UK) and Omniscript RT (Qiagen, Crawley, UK). The reactions were set up according to stated manufacturers protocols:

2. Choice of RT primers- three different primers were also tested; 20µM specific primer RT803, 0.5µl of 50ng of random hexamers and 0.5µl of 250ng of a modified oligo-dT primer (RoRi oligo-dT).

Construction of IGF-1 receptor Standards.

The Standards used consisted of in vitro transcribed Target RNA as described for Competitive RT-PCR. This cRNA was quantified and the copy number determined. The purified RNA was then mixed with 2µg of liver mRNA (does not have IGF-1 receptor) extracted in the same way as the samples. The Standard RNA was then reversed transcribed as described above.
Lightcycler PCR Optimisation

The conditions for LC PCR were optimised by determining the optimal MgCl₂, cDNA and primer concentration, as well as the annealing temperature. PCR master mixes were assembled carefully, with recently calibrated Gilsons to minimise pipetting errors. In addition, different amounts of tRNA were added to the PCR master mix to minimise primer artefact formation. Each master mix of the IGF-1 receptor reactions consisted of:

<table>
<thead>
<tr>
<th></th>
<th>Vol [μl]</th>
<th>[Final]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast start Sybr Green</td>
<td>2.0</td>
<td>1x</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.0</td>
<td>2.25 mM</td>
</tr>
<tr>
<td>Forward primer</td>
<td>0.5</td>
<td>5pM</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.5</td>
<td>5pM</td>
</tr>
<tr>
<td>H₂O (PCR grade)</td>
<td>15</td>
<td>----</td>
</tr>
<tr>
<td>Total volume</td>
<td>18.0</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1

To 18μl of the master mix in the glass capillaries, 2μl of cDNA was added as PCR template. The capillaries were sealed and briefly centrifuged at 3000rpm and placed into the Lightcycler rotor.

The following lightcycler protocol was used for IGF-1 receptor online detection using Faststart Sybr Green (a hot start protocol), and including a melt curve program. The hot start was carried out 95°C, for 10 minutes to activate the Taq polymerase, as well as to reduce non-specific amplification. The amplification and quantification phase was carried out for 55 cycles with denaturation at 95°C for 15s, annealing at 61°C for 4s, product extension at 72°C for 18s, and fluorescence acquisition (quantification of double-stranded DNA) at 87°C for 2s. The melting curve programme used to distinguish between different products amplified was initiated at 72°C with a stepwise increase every 0.1°C/s until 95°C.
Normalisation

RT-PCR specific errors are easily compounded by any variation in the starting material between samples, especially when the samples are obtained from different individuals. The accepted method of minimising these errors and correcting for sample-to-sample error is to amplify simultaneously with the target gene, a cellular RNA template that serves as an internal reference (Suzuki et al 2000). In this study the mRNA for housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used to normalise sample-sample variation. In addition GAPDH mRNA levels were compared to total RNA concentration.

2.3.2 Real Time PCR Results

Primer Design

The specificity of the amplification was determined by melting curve analysis and also by visualisation of PCR products separated by gel electrophoresis. Optimal primer design was important. In addition to ensuring the resultant PCR product was less that 400bp, the primers have to be very specific with any mismatch or wobble resulting in double products. Non-specific priming was decreased by selecting primers with a only one or two G/Cs within the last five nucleotides at the 3’end.

RT optimisation

The use of RoRi Oligo(dT) and random hexamers to prime the reverse transcription reaction was determined as inefficient for the expression analysis of low abundance mRNAs. Using the two methods IGF-1 receptor mRNA could not be detected from the control muscle samples. The use of short sequence specific RT primers (RT803) about 100bp from the PCR reverse primer was the most efficient way of reverse transcription as it resulted in the amplification of a clear PCR product, without non-specific products. The RT primers were determined not to interfere with the subsequent PCR amplification due to their low melting temperatures (42°C). This method of maximised the sensitivity of the PCR, as well as increased the likelihood of product specificity (see Fig 2.3).
The choice of reverse transcriptase was also important. Despite all RTs used being modified or engineered derivatives from Moloney murine leukemia virus Reverse transcriptase (MMLV-RT), Superscript II (Rnase H-) was found to significantly inhibit the PCR reaction, resulting in no product formation. It was only after incubation of the cDNA at room temperature for 48 hours or at 4°C for two weeks that any amplified product could be detected. The most compatible and efficient RT was determined to be Omniscript from Qiagen.

Fig 2.4 Illustrates how differential priming of RNA templates during cDNA synthesis markedly affects the amplification of PCR product. The red peak represents the sample primed with specific primer RT803, while the black represents the same sample primed using oligo(dT) RoRi. The PCR amplification conditions are otherwise identical. The peak at 82°C represents primer dimerisation

**Accurate Quantification**

Non-specific products, such as primer dimers are easily determined by their lower melt curve profile (See Fig 2.4, where the amplification of RoRi oligo(dT)-primed O10 Sol C yields no PCR product). Accurate quantification by SYBR Green depends on ensuring that there is no primer dimer and or any other
contaminants that would otherwise give a false result. Primer artefacts were particularly common when starting cDNA concentrations were low and two methods were used to minimise this: 1) Reduction of the concentration of PCR primer in the master mix. This was altered according to the starting template, however the optimal concentration was determined to be 5-2.5 pM in each 20μl reaction; 2) The addition of picogram amounts of tRNA in certain templates decreased the primer dimer peak at low template concentrations (results not shown). This like the optimal Magnesium concentration had to be determined empirically.

The melting temperature of IGF-1 receptor products was determined as 89°C (see Fig2.3). To ensure the elimination of non-specific fluorescence signal, quantification of the fluorescent signal was acquired at 87°C. High temperature acquisitions also kept the fluorescence of the negative control around 1 unit, hence reduced the background fluorescence.

**Amplification Efficiencies of IGF-1 Receptor RNA Standards and Native mRNA.**

For reliable quantification, the amplification efficiency during PCR must be equal for the standards used in the calibration curve and the native mRNA present in the unknown samples. The relationship between the initial amount of target present $T_0$ and the amount of DNA produced after $n$ PCR cycles, $T_n$, can be expressed as $T_n = T_0 (1 + E)^n$ where $E$ is PCR efficiency, or the fraction of template replicated per cycle. The efficiency can be easily calculated, at the exponential phase from the standard curve as $E = 10^{\frac{1}{\text{slope}}}$ (See Fig 2.5 below).
Fig 2.5: Illustrates an optimised run, which would be typically used for quantification. The run was carried out using faststart Sybr green. PCR conditions are denaturation at denaturation at 95°C for 15s, annealing at 61°C for 4s, product extension at 72°C for 18s, and fluorescence acquisition at 87°C. The graph in the lower right hand corner is the linear regression analysis of the standards, which forms the standard curve, from which the unknown samples are calculated.

The y intercept of the standard curve defines the C, gives an indication of the sensitivity of the assay. It is an indication of the point at which the cycle at which the minimum input cDNA can be consistently detected. The correlation of input standard DNA to the output fluorescence as emitted by the amplification product is determined by r of the standard curve analysis. The closer r is to -1, the greater the accuracy of the standards. The sensitivity of the lightcycler PCR was evaluated using different starting amounts of IGF-1 receptor recombinant RNA from 10^3 to 10^7 molecules. To confirm the reproducibility of the lightcycler PCR at low template copies, inter-assay variation was determined by 3 experiments on 3 different days using different pre-mixes. The minimal detection limit was 10^4 /capillary with a satisfactory linearity of r^2 =0.97. The inter assay variability was approximately 5.4%, but increased to 19% when the PCR efficiency decreased for example at very low starting amounts of cDNA. At very low levels of starting
cDNA amounts, (below $10^4$ molecules), the PCR efficiency decreased as indicated by the low PCR product peaks, and high primer dimer peaks.

**Normalisation**

Although, GAPDH mRNA levels in the muscle did not change after tendon ablation, there was a significant decline with increased age. Therefore GAPDH could only be used to normalise the values between control and experimental samples, but not between samples of different ages. In addition, it was found that there was a wide range of individual variation between rats of the same age group (see Fig 2.5).

**Fig 2.6A:** shows that levels of GAPDH expression do not change significantly 5 days after tendon ablation. The controls unloaded contralateral leg, while the experimental refer to the overloaded hindlimb. There is a wide range of individual variation between samples, which is unlikely to be caused by RNA quantification errors. **B** illustrates the changes of GAPDH expression level with age emphasising that it is not an appropriate control gene to normalise samples across all ages. Young refers to 3 month, Mature to 13 months and Old to 24 months.

**2.4 Discussion**

Optimisation and consistency were as critical for obtaining reproducible results for real time RT-PCR as for QC-RT-PCR. However, there is no doubt that real-time PCR was significantly less variable than conventional PCR. This is partially due to the fact that there are less steps taken in obtaining the final data for real-
time PCR compared to QC-RT-PCR such as gel loading and densitometric detection. It was also clear that the amplification efficiency of the PCR influenced the reproducibility of the assay; therefore optimisation of the PCR was a critical step. One of the largest sources of variation was due to the huge variation in the efficiency of the enzymes in the supplied in the PCR kits (Roche, UK).

Due to the fewer steps required for the quantification of samples using the real-time PCR, there is less requirement for a normalisation gene. It was determined that GAPDH was not suitable as a normalisation gene for all the samples as the values varied between individuals. However, as the GAPDH levels did not change significantly between the control muscle and overloaded muscles of the same animal, this variation was assumed to be real and not simply as a result of quantitative errors. Overwhelming evidence supports the unsuitability of GAPDH as an internal control as it varies significantly between individuals and also decreases with age (Bustin et al 2000). Ribosomal RNA (rRNA) has been used as an alternative to housekeeping genes, however it also has a few drawbacks. One of the drawbacks is that rRNA is expressed at much higher levels than any other genes as it consists of 85% of total cellular RNA. Therefore it would essentially have the similar drawbacks as normalising to total RNA. In addition highly proliferating cells may have increased rRNA levels (Barbu et al 1989).

Standard curve competitive PCR (QC-RT-PCR) relatively simplifies the process of quantifying gene expression in comparison to the ‘original’ competitive PCR method. The latter would require a titration and precision assay for each sample. In comparison, the standard curve C-PCR only requires an assay to establish a standard curve thereby rendering it less labour-intensive. In addition, while comparing the Target and unknown samples using real-time PCR it was found that the two amplicons had similar PCR efficiencies. However the attempt to use the assay to quantify unknown samples (results not shown), there was inconsistent duplication of results. The same sample amplified with a set amount of competitor produced widely varying results. It was concluded that the inconsistency was most likely produced during the RT step, whereby the
unknown samples were not reverse transcribed at the same rate as the competitor. This would immediately change the ratio of the unknown to competitor, and therefore not conform to the calculated relationship of the standard curve. It is hypothesised that inhibitors that are introduced during RNA extraction may act to inhibit reverse transcription or else the secondary structure of the mRNA may also interfere with RT. This emphasises that in all quantitative PCR, the RT step carries much weight in determining how accurate the quantification is.

In summary, real-time PCR was considered significantly more accurate, sensitive, as well as less labour-intensive than QC-RT-PCR. It is also more adaptable for use with a wide number of genes; therefore for all the other genes quantified it was used as the method of choice.
CHAPTER III

Effect Of Ageing And Mechanical Loading On Local IGF-1 Gene Expression In Muscle
3.0 Introduction

Ageing is difficult to define as it occurs at many different levels including social, psychological, physiological, cellular and molecular. A generally acceptable description is that there is a progressive decline or impairment of function of the cell, tissue or organ that leads to a loss in an adaptive response to stress (Holliday 1998). This is postulated to lead to a growing risk of succumbing to age-related diseases, which in turn increase the probability of death. Senescence of skeletal muscle is associated with a marked decrease in skeletal muscle mass and strength and increased muscle fatigability, otherwise known as Sarcopenia. As well as muscle loss, ageing is characterised by a decrease in bone mass and density. These changes though gradual from adulthood, seem to rapidly accelerate after the sixth decade of life (Bross 1999). The decline in maximal aerobic capacity, and bone strength, often leads to increased risk of falls, and substantially reduces the amount of physical activity that can be performed by elderly men and women.

At a histological level, muscle loss results from the selective loss and atrophy of the type II, fast twitch fibres (Carlson 1998). This preferential loss has been attributed to their reduced innervation compared with the type I fibres. The fast fibres are gradually deinnervated (Carlson 1998). The remodelling of motor units and loss of type II fibres partially explain the changes in relative fibre type composition of aged muscles (Alnaqbeeb 1987). The changes in the myosin isoform composition result in a reduction in the peak force that can be generated by muscles at fast contraction speeds. There also appears to be a decrease in the fractional synthesis rates of individual muscle proteins. Compared to young subjects, middle-aged and older men and women, have a 31% and 44% reductions, respectively, in synthetic rates of myosin heavy chain (Balagopal et al 1997). Given the key role of myosin heavy chain as a contractile protein, these changes would further contribute to the alterations in muscular endurance, fatiguability and contractility.

Ageing is also associated with an increase in total body fat. Hence the decline in fat-free mass due to loss of skeletal muscle loss is countered by an increase in central adiposity, which in turn is linked to increased insulin resistance (Bross
A decrease in the daily activities of the elderly was initially thought to account for the age-related decline in muscle function. This is because in models of muscle disuse, there is observed muscle atrophy. However, it has since become clear that even highly trained and active older athletes do not maintain the muscle function or body composition at levels seen in active young subjects (Klitgaard 1990). So while there is evidence that physically active older people maintain higher levels of muscle mass, it is clear that there are some irreversible physiological changes linked to ageing per se. This is emphasized by the fact that disuse atrophy can be reversed by physical exercise whilst the intrinsic changes to ageing seem largely irreversible.

It is well established that the neuro-endocrine system regulates much of development, as well as the maturation of the ovarian and testicular functions. Normal ageing has been attributed to the observed decrease in anabolic hormone levels (reviewed by Bross 1999). Therefore the decline in the function of the hypothalamic-pituitary-adrenal axis with increased age has long been proposed to lead ultimately to the frail phenotype. In particular, ageing has been characterised by changes that closely resemble those in growth hormone deficient adults, such as reduction in muscle mass and strength, bone strength, an increase in total and abdominal fat, hypertension and cardiovascular mortality. The decline in the GH/IGF-1 axis, the so called ‘Somatopause’ is thought to be due to the impairment of the somatotrophs in the pituitary resulting in a decrease in the mean pulse amplitude and fraction of GH secreted. This is accompanied by a parallel decrease in circulatory IGF-1 levels.

Other hormones, which modulate circulatory IGF-1 levels, also show declining levels. Of most clinical importance is the effect of ageing on the pancreatic system, which controls insulin production. 40% of individuals aged 65 to 74 have impaired glucose tolerance or diabetes mellitus and it is estimated that nearly half of the elderly individuals with diabetes are undiagnosed (Harris 1990). Changes in numbers of pancreatic insulin-receptors and other post-receptor changes result in decreased relative insulin secretion from the B cells, as well as increased peripheral insulin resistance. Insulin replacement restores IGF-1 levels in
individuals with type I (insulin-dependent) diabetes mellitus and improves GH receptor status as well as increase GH sensitivity. There is also decrease in gonadonal sex steroids, testosterone and oestrogen, secretion. The dramatic decrease in circulatory oestrodial in women who reach menopause about the age of 50 is linked to age-related changes in the CNS and hypothalamus-pituitary unit (reviewed in Hortobagyi et al 1995). The change in men is subtler, with a gradual decline in the availability of total free testosterone. Menopause is associated with age-related osteoporosis and sarcopenia. Oestrogen hormone replacement is clearly shown to reduce the decline in bone mass density and abdominal fat accumulation, and this action is thought to be partially through its stimulation of non-hepatic IGF-1. Oestrogen also stimulates the upregulation of hepatic GH receptors thereby activating the negative feedback mechanism to increase GH sensitivity and ultimately decreases circulatory IGF-1 levels (reviewed in Arvat 2000 and Ravaglia et al 2000). Testosterone levels also seem to have a positive correlation with circulatory IGF-1 levels (Bross et al 1999).

Despite all the evidence that correlates the increase in fraility during ageing with decreased anabolic hormone levels, hormone replacement seems to have only a moderate impact on increasing muscle strength in patients. The strongest evidence linking the decrease in GH/IGF-1 axis to ageing has been the age-related appearance of certain physiological changes resembling symptoms observed in GH deficient individuals. While, a certain level of GH is crucial for the maintenance of musculoskeletal mass and vascular integrity, the extent to which it depends on serum IGF-1 during senescence seems to be modest. GH supplementation in the elderly increases fat-free mass, muscle mass and whole body protein synthesis, but fails to improve on muscle strength or increase bone density (Welle et al 1996, Bross 1999). There seems little argument for the costly programme of GH supplementation when these modest improvements are matched or even surpassed by exercise regimes. Serious side effects of GH supplementation including oedema, and carpal tunnel syndrome have further deterred the continuation of clinical trials (reviews Hortobagyi et al 1995, Bross 1999).
The role of the GH/IGF-1 axis in ageing has been questioned by recent evidence. The first doubts cast on what role circulatory IGF-1 plays in determining skeletal muscle integrity were initiated by the establishment of knockout mice deficient of liver IGF-1 (liver IGF-1 deficient, LID). The LID model suggests that serum IGF-1 is relatively unimportant for muscle growth (Yakar et al 1999). Increasing GH in the elderly does not result in increased skeletal muscle IGF-1 mRNA. However, the high levels of GH induce insulin resistance, suggesting that circulatory IGF-1 may regulate/modulate insulin metabolism. In a bizarre overturn of established thinking, it is now postulated that increased GH/IGF-1 secretion may increase ageing and shorten lifespan (Gems and Patridge 2001).

Of five ways known to extend longevity in mammals, three affect GH/IGF-1 levels. The Ames dwarf (Prop-1\textsuperscript{df}), the Snell dwarf (Pit1\textsuperscript{sw}) both result from mutations in transcription factors controlling pituitary development resulting in GH deficiency and an increase in lifespan. A mouse model of Laron’s dwarfism (GH receptor resistance) also has increased longevity (reviewed by Gems et al 2001)). These phenotypes appear to directly contradict the hypothesis that a reduction in anabolic hormones leads to increased ageing. In addition caloric restriction in genetically normal mice not only extends lifespan, but also decelerates age-dependent changes in multiple organs, cell types, intracellular and extracellular processes. This seems to suggest, that contrary to conventional wisdom, the very wide range of age-dependent changes could be controlled by a ‘single clock’ (Flurkey et al 2001).

Age-related loss of muscle mass and functional properties is a result of a complex hierarchical system of basic ageing processes and cell adaptive responses. This loss of function is thought to result from either increased susceptibility of muscle fibres to injury and/or decreased ability to repair muscle fibres. The mechanism of injury in all muscle is primarily mechanical in nature. Due to the postmitotic nature of muscle cells, satellite cells are required for muscle repair following mechanical damage. In turn, damage seems to be the signal for regeneration and repair. Therefore the normal maintenance of skeletal muscle function involves repeated cycles of injury and satellite cell-mediated
repair. Three critical changes in old muscle are that: muscle fibres are injured more easily, the fibres regenerate less successfully and structural and functional repair appears incomplete (Goldspink 1998).

The regenerative capacity of skeletal muscle depends on the stimulation of satellite cells, their ability to proliferate and fuse to form new myofibres. Carlson et al (1989) clearly established that older muscle transplanted into younger recipients regenerate better than younger muscles into older recipients. An earlier study by Vandenburgh (1984) had determined an age-dependent difference between the ability of extracts of young and old crushed regenerating muscle to stimulate myogenic cell proliferation and myotube formation. These studies suggested that age-related defects in the microenvironment of older muscle were largely responsible for their declined abilities.

IGF-1 is a critical mediator of development, regeneration, hypertrophy and survival in the neuro-muscular system. Following the recent evidence based on transgenic models, there is increasing emphasis that autocrine/paracrine IGF-1 plays a larger role in local tissue adaptation. Muscle has an intrinsic ability to adapt to increased load by undergoing hypertrophy. The two local muscle isoforms, IGF-1Ea and MGF appear to be positive regulators of muscle growth as they are upregulated in regenerating and hypertrophied muscles subject to stretch and stimulation (Goldspink 1998). Mature IGF-1 is known to induce growth, not only by satellite cell activation, but also through increasing protein synthesis in muscle tissue (Adams 1998).

Numerous studies have confirmed that the decline in the proliferative capacity of satellite cells during senescence contributes to the pathology of muscle ageing (Charkravarthy 2000). The decreased ability of senescent muscle to induce satellite cell proliferation and protein synthesis, led investigators to hypothesize that IGF-1 response would be diminished. However, studies measuring the expression levels of IGF-1 have failed to show any differences in both resting muscle and in both mRNA and peptide levels (Hamilton et al 1995). This work has not involved exercise studies or distinguished between the expression of
different isoforms. Paradoxically, the overexpression of IGF-1 locally in the muscles of transgenic mice, or using adeno-viral vectors, reverses muscle atrophy and restores the proliferation capacity of satellite cells in old mice (Musaro et al 2001, Barton-Davis et al 1999 and Charkravarthy et al 2000). The aim of this study was to determine the expression of IGF-1 isoforms in relation to the ability to adapt to mechanical overload at different ages. We hypothesized that the early response of muscle to overload would be an increase in both IGF-1Ea and MGF isoforms and that IGF-1 mRNA expression in old animals would be diminished to reflect the blunted response of ageing skeletal muscle to overload. Activated satellite cells express MyoD, a member of the muscle regulatory transcription factors (Seale et al 2000). Therefore, it was anticipated that measuring changes in the expression of this myogenic factor, would shed some light on the functional implications on the age-related ability of muscle to express MGF and IGF-1Ea.

As the effects of IGF-1 are modulated by IGFBPs a more detailed picture of changes in expression during ageing and hypertrophy is presented by considering their role. Both IGFBP-4 and 5 are expressed in muscle and appear to change with muscle function (Ewton and Florini, 1995). IGFBP-5 in particular is thought to be regulated by IGF-1 and is highly upregulated during differentiation. There have been no studies investigating changes of local IGFBPs in muscle with age. As IGFBP expression appears to change with hypertrophy, comparing the expression in the young and old animals would add an additional parameter relating to muscle plasticity.

As muscle mass is known to increase in response to overload and stretch, it was decided to use the method of synergistic muscle tenotomy, which stretches as well as increasing the load on the intact muscle. The resulting hypertrophy, as show by Goldberg (1969) in hypophysectomized rats, is independent of the GH/IGF-1 axis. Therefore, this system was considered appropriate for studying the early response of autocrine IGF-1 splice variants to overload. Caloric restriction is a well-known intervention known to retard ageing in mammals. Studies in C. elegans, suggest that the IGF-1/Insulin system may integrate systems that define an organisms rate of ageing. Dietary intervention has also
been shown to delay the atrophic changes related to senescent muscle (Boreham et al 1988). The effect of caloric restriction on the expression of local IGF-1s was therefore also investigated.

3.1 Material and Methods.

3.1.1 Animal Experiments

Male Sprague Dawley rats were weaned at 21 days and at 3 months (mths), they were randomly allocated into ad libitum-fed (AD) or diet restricted (DR) groups, which were individually caged. AD animals had unlimited access to food whilst DR rats were fed an identical diet to 70% of the AD food group intake. All rats were kept in conditions of constant temperature and light controlled environment (12:12 hr light dark cycles). Three different sub-groups of 18 rats each, were classified in each of the AD and DR groups as 3mth (young), 13mth (mature), and 24mth (old). All procedures were performed while the animals were anaesthetized.

Experiments were initiated by unilateral excision of the distal gastrocnemius tendon. Rats were anaesthetized with Halothane and a 2cm incision was made over the distal portion of the muscle. Connective tissue was then separated from the tendon, which was then transected at the tendon-muscle junction. The skin was closed with sutures. Sham operations were carried out on the contralateral leg, which would then act as the control. After the animals recovered from anesthesia and they were observed to use both legs almost within 1 hour. They all survived until they were killed, after 1, 2, 3 and 5 days (a minimum of 4 animals per group), when the animals were again anaesthetized with CO$_2$ and decapitated. The plantaris and soleus muscles were immediately removed, weighed, and one part snap-frozen in liquid nitrogen and stored at $-70 \degree C$. The other part was submerged in cold denaturing solution and stored at $-70 \degree C$ for RNA extraction.
3.1.2 RNA isolation and Analysis.
Total RNA was isolated by the guanidine thiocyanate method of Chomczynski and Saachi (1987), as described in detail in Chapter 2. The extracted RNA was dissolved in diethylpyrocarbonate-treated water and quantified spectrophotometrically with the Gene spec I (Naka Instruments). The integrity of the RNA was confirmed by visual inspection of ethidium bromide-stained 18s and 28s ribosomal RNA under ultra violet light.

3.1.3 Reverse Transcription
The RNA was transcribed as described in Chapter 2. To facilitate the efficiency of reverse transcription (RT) in transcripts expressed at low levels such as MGF and IGFBP-4, short specific primers 50-100 base pairs downstream of the PCR reverse primers were used (Pfeffer et al 1995). Hence a mixture of random hexamers and specific decamers were used in the same reaction. The RT primer for MGF (5' TTGCAGGTTGCT 3') is taken from exon 6.

3.1.4 Real-time quantitative PCR.
IGF-1Ea is transcribed from exons, 3, 4 and 6, while MGF consists of exons 3, 4, 5 and 6 of the rat igf-1 gene. The same forward primer was designed from the conserved exon 3. The reverse primer for IGF-1Ea spans the exon 4 and 6 boundary, while MGF reverse primer crosses the boundary between the exon 4 and 5. The locations of all the primers for IGF-1 isoforms are shown in Fig 1 below.

The primers and conditions that were used for PCR are presented in Table 3.1. All other primers for IGFBP-4 and 5 were designed with the Omega software package and synthesized by Sigma Genosys. Real-time PCR was carried out on the Roche lightcycler. Each primer set was optimized as described in chapter 2. For negative controls the same procedure was applied to a reaction mix containing no cDNA.
Rat IGF-1Ea mRNA (Embl Accession No. D00698)

ggacgtacctaaatgagcgcacctcaataaagatacacatcatgtcttcacctctttatctgtgactgtgctttcatcgt
tgctcactttaaccagctgcgcacacagccgaccagagacttttgccgggtgagctggtggacgctcttcagt
tctgtgtgtgaccacaggggttttacttcaacaagcccaaggtcatgtgctccagcatcgggacaccacacacgagggcattgtgatgagttgctctcggagctgtgatctgaggaggctggagatgtactgtgctccgctgaagctta
cuaagttgcagctgtgctccagtccggccggcggccagggcactgacatgcccagctcgttccatccgggcccagcgccacactgacatgccacacagacagcgattgtggatgagtgttgcttccggagctgtgatctgaggaggctggagatgtactgtgctccgctgaagctta
tcttttaggaggtggtt
gtttaaaaaaaaaaacaaaaaaca

Rat MGF, also IGF-1Eb mRNA (Embl Accession No. X06107)

ggacgtacctaaatgagcgcacctcaataaagatacacatcatgtcttcacctctttatctgtgactgtgctttcatcgt
tgctcactttaaccagctgcgcacacagccgaccagagacttttgccgggtgagctggtggacgctcttcagt
tctgtgtgtgaccacaggggttttacttcaacaagcccaaggtcatgtgctccagcatcgggacaccacacgagggcattgtgatgagttgctctcggagctgtgatctgaggaggctggagatgtactgtgctccgctgaagctta
cuaagttgcagctgtgctccagtccggccggcggccagggcactgacatgcccagctcgttccatccgggcccagcgccacactgacatgccacacagacagcgattgtggatgagtgttgcttccggagctgtgatctgaggaggctggagatgtactgtgctccgctgaagctta
tcttttaggaggtggtt
gtttaaaaaaaaaaacaaaaaaca

Figure 3.1 Sequences of the two IGF-1 isoforms indicating differential splicing of the IGF-1 gene as well as showing the location of the forward and reverse primers used during RT-PCR. The different colours represent different exons, with Red as Exon 3, Black as Exon 4, Blue as Exon 6 and Pink as Exon 5.
Table 3.1.
Primers used in the PCR in 5’ to 3’ direction.

<table>
<thead>
<tr>
<th>Gene</th>
<th>F Primer</th>
<th>R Primer</th>
<th>T\textsubscript{A} °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1Ea</td>
<td>GCTTGCTCACTTTACCAGC</td>
<td>AAGTACTTCTTCTGGGTC</td>
<td>62</td>
</tr>
<tr>
<td>MGF</td>
<td>GCTTGCTCACTTTACCAGC</td>
<td>AAGTACTTCTTCTGGGTC</td>
<td>61</td>
</tr>
<tr>
<td>IGFBP4</td>
<td>GCATTGACATCCGCAACG</td>
<td>ACCTGTGAGGTCGGAAG</td>
<td>57</td>
</tr>
<tr>
<td>IGFBP5</td>
<td>GGAGACAATCCCTCAACGGATG</td>
<td>AGCACCCTTGTAATCGGATTGG</td>
<td>60</td>
</tr>
</tbody>
</table>

3.1.5 Statistical analysis.
The student’s t-test was used to test the differences in the means of the overloaded and control muscles at the various age groups. One-way analysis of variance was used to determine significant differences between the means, with the level of significance set at p<0.05. Values are expressed as means +/- standard error mean (SEM).

3.2 Results
In Chapter 2, it was observed that the absolute values measured in different lightcycler runs could vary significantly and therefore to control for this variation the relative values (to controls) of each gene were used to test the hypothesis.

3.2.1 Effect of Loading.
After 5 days of gastrocnemius tendon ablation, there was an increase in the wet weight of synergistically overloaded plantaris muscle of 60%, 35% and 20% in the muscles of young, mature and old rats, respectively. See Table 3.2

Table 3.2.

<table>
<thead>
<tr>
<th>Samples</th>
<th>YC</th>
<th>YE</th>
<th>MC</th>
<th>ME</th>
<th>OC</th>
<th>OE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>0.128</td>
<td>0.188</td>
<td>0.325</td>
<td>0.414</td>
<td>0.351</td>
<td>0.411</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.026</td>
<td>0.033</td>
<td>0.065</td>
<td>0.047</td>
<td>0.074</td>
<td>0.067</td>
</tr>
</tbody>
</table>

Y=Young, C= Control limb and E=Overloaded leg. n=6
3.2.2  Effect of loading and age on IGF-1Ea, MGF, IGFBP-4, and IGFBP-5 gene expression.

There was no change in IGF-1Ea mRNA both at the baseline and overloaded muscles in the young, mature and old muscles after 5 days of overload (Fig. 2A).

There was a significant increase in MGF mRNA at all ages in overloaded muscle relative to control muscle, (Fig. 2B). For the young muscle, \( p < 0.001 \) and in both mature and old muscles \( p < 0.05 \). \( n = \) minimum of 6.

**Fig. 3.2.** Quantification of gene expression using RT-PCR 5 days after tendon ablation in young (Y), mature (M) and old (O) samples. Control (C) refers to the control leg, while Experimental (E) is in reference to the overloaded leg. A: There is no change in in IGF-1Ea mRNA levels during ageing or after tenotomy. B: There is a significant increase in MGF levels in the overloaded muscles (E) compared to the contralateral control legs. (**\( p < 0.001 \) and *\( p < 0.05 \) ) \( n = \) a minimum of 6 in each group.
As shown in Fig 3.4A, there are no significant changes in mRNA expression of IGFBP-5, 5 days after tendon ablation at all ages. However in contrast, after 5 days of overload, IGFBP-4 levels increase significantly across all age-groups (*p<0.05 see Fig 3.4B).
Activated satellite cells express MyoD, a member of the muscle regulatory transcription factors (Seale et al 2000). By measuring the expression of this myogenic factor some light may be shed on the functional implications of the age-related ability of muscle to express MGF and IGF-1Ea. MyoD expression is known to be fibre-specific, and therefore changes in expression were only measured in the soleus muscle. Changes in gene expression in MyoD over 1, 2 and 3 days were also measured in order to correlate it to changes in MGF (see Fig 3.3). MyoD baseline mRNA levels were higher in old muscles in comparison to
those in young muscle, \( P<0.05 \) (Fig 3.5). Although there was a significant increase in the expression of MyoD in both young and old overloaded muscles, the degree of increase in the old muscle was attenuated in comparison to that in young muscle.

![Changes in MyoD levels after tenotomy](image)

Fig 3.5: Quantification of MyoD mRNA message in the soleus muscle after tenotomy. The average levels in the contralateral leg, which also served as the control, represent Day 0. \( \ddagger \) indicates that baseline MyoD levels are significantly higher in the muscles of old rats compared to those in the young \( P<0.05 \). **\( P<0.01 \) and *\( P<0.05 \).

**Effect of Caloric Restriction**

Caloric restriction had no significant effect on the changes in gene expression in either the control or overloaded muscles at all ages. This lack of effect may be due to the fact that restricting food intake to 70% of the ad lib-fed rats is likely to be still well within the range of normal food intake. Therefore, in order to observe the effects of diet restriction it is hypothesised that a much more severe restriction of the food intake would have to be undertaken. The samples taken from the DR animals were therefore grouped with those from the AD thereby \( n=6 \) was found in each group.
3.3 Discussion

Original observations by Goldberg (1969), in which he showed that muscles of hypophysectomised rats were still able to undergo hypertrophy in response to work-induced overload, suggested that this was a localized process. As, illustrated in Table 3.2, there is an increase in the wet weight of the overloaded muscles, implying that hypertrophy is confined to the experimental leg. As shown here this simple reproducible way of inducing hypertrophy showed that muscle compensatory growth could take precedence over hormonal-induced growth. There was a much larger increase in the wet weight of overloaded muscles of young animals compared to the old and mature suggesting that with increasing age, there was a decreased ability to respond to muscle overload. The increase in the wet weight of muscles in the mature and old muscle compares favourably with previous experiments (Adams 1996, DeVol 1990). There were no signs of inflammatory response, so the weight increases cannot be attributed to oedema.

This study showed for the first time that in muscle, IGF-1 mRNA isoforms are differentially regulated in response to mechanical overload. Increases in muscle IGF-1 have been observed during work-induced compensatory overload, in regenerating fibres after acute and chronic exercise and during passive stretch in mammalian muscle (Yang et al 1997, DeVol 1990, Czerwinski et al 1994, Marsh et al 1997, and Adams et al 1996). However, in previous work, no distinction was made between the different transcripts. Indeed, the previously reported early increase in IGF-1 gene expression may be a reflection of the increase in MGF rather than IGF-1Ea gene expression. Further support of the latter hypothesis may lie in the observation of Eliakim et al (1997), who detected no increase in predominant IGF-1 mRNA species in muscle following overload. He clearly observed no increase in Exon 1 start site transcripts which constitute almost 90% of IGF-1 mRNA in muscle. These transcripts are likely to be IGF-1Ea, levels of which are approximately ten-fold of those of MGF (see Fig 3.2). It can also be argued that due to the use of far less sensitive assays to quantify changes in gene expression, changes in MGF would have gone undetected. Local IGF-1 expression is likely to be dissociated from hepatic/circulatory IGF-1 due to the
localized effects of hypertrophy observed in only the overloaded muscle (Adams 1998). This suggests the presence of a transient and activity-sensitive local trophic factor, which accumulating evidence indicates is MGF, the mRNA of which increases markedly in response to stretch and stimulation (Yang et al 1997 and McKoy et al 1999).

While the stimulus responsible for increased MGF expression is likely to be stretch, the mechanism by which mechanical induction stimulates gene expression in muscle is still unknown. Goldspink and co-workers have suggested that the dystrophin cytoskeleton complex may provide a link between mechanical stimuli and MGF expression (Yang et al 1997, McKoy et al 1999, Goldspink 1999). They observed that in contrast to normal muscle, MGF was not detectable in dystrophic muscle, even after stretch (Goldspink 1999). Recent evidence supports the observation that the cytoskeleton plays a role in transducing signals that cause the upregulation of growth factors. Certain membrane associated receptors; the integrins have been identified, which are activated upon mechanical stress (Ingber 1997).

MGF and IGF-1Ea splice variants, apparently yield the same mature peptide, which is derived from the highly conserved exons 3 and 4 of the IGF-1 gene. A mechanism of extracellular endoproteolysis of the IGF-1 prohormone, results in the same mature peptide, but different E peptides (Gilmour 1994), which may function as independent growth factors (Siegfried et al 1992). It has been suggested that IGF-1 precursors could be pluripotential, in a form analogous to that of prohormone proopiomelanocortin and proglucagon (Siegfried et al 1992). Of particular interest is the observation that a synthetic peptide derived from the rat Eb (equivalent to Ec in humans) domain induces proliferation in epithelial cells (Siegfried et al 1992). The role of the growth promoting properties of the E peptide in MGF, acting as a separate growth factor is supported by observation that muscle cell lines stably transfected with MGF show greater proliferative rates of mononucleated myocytes than in the control or in those cells transfected with IGF-1Ea (Yang et al 2002). Furthermore, an antibody raised against the synthetic IGF-Eb related precursor peptide can detect a protein product of

69
immunological similarity in human and mammalian tissues extracts (Siegfried et al 1992).

The early induction of MGF, following mechanical overload indicates that this may play an important role in the increase in protein synthesis, proliferation and differentiation of satellite cells in response to stretch and damage. IGF-1 expressed by muscle is believed to increase protein synthesis and to activate satellite cells, which proliferate, differentiate and fuse with the pre-existing muscle fibres (Adams 1996). In gamma-irradiated mouse skeletal muscle, where the proliferative capacity of satellite cells has been destroyed, vector-delivered IGF-1 is only able to induce partial hypertrophy (Phelan and Gonyae 1997). Muscle response to overload occurs in two phases (Snow 1990). At early time points, the increase in muscle DNA and RNA content has been related to the proliferation and differentiation of satellite cells (Adams 1999).

The stages of satellite cell proliferation, and differentiation have also been shown to be marked by the production of IGFBP-4 and IGFBP-5 (Awede 2000, Ewton and Florini 1995). IGFBP-4 is the main binding protein produced during the proliferation of myoblasts. This is reflected in Fig 3.3, where IGFBP-4 mRNA levels are significantly up regulated at all ages after five days of overload. This suggests that compensatory growth by the overloaded the muscle involved satellite cell activation. Despite the lower baseline levels of IGFBP-4 the relative increase between the control and overloaded legs are similar at all stages. IGFBP-4 is reportedly produced by the connective tissue, but its role during muscle proliferation is not clear (Ewton et al 1995).

IGFBP-5 increases dramatically upon differentiation, but is also thought to inhibit the earlier phase of myoblast proliferation. Five days after tendon ablation it was expected that some of the satellite cells should have differentiated and fused with the existing myotubes. Therefore it was expected that there would be an accompanying increase in IGFBP-5 levels following overload. However, there is only a modest increase in the young muscle and none in the mature and old muscle. IGFBP-5 is reported to be upregulated by IGF-1 and muscle enriched
transcription factors, with its promoter activated by the induction of these factors upon differentiation (Jennische and Hall 2000). Alternatively, the very low levels of IGFBP-5 may also correspond to the lack of IGF-1Ea upregulation. However due to the direct correlation of IGFBP-5 to muscle transcription factors it is more possible that an increase of IGFBP-5 in overloaded muscle would be detected at a later time point.

The attenuated increase of MGF mRNA levels in old muscle after tenotomy correlates well with the decreased expression of myogenic factors in senescent muscle subjected to exercise. In agreement with previous observations there was a marked increase in MyoD mRNA levels in both the young and old rats 24 hours after tendon ablation. Concurrent with data from Musaro et al, (1995) and Marsh et al (1997), old muscle expresses higher baseline levels of myogenic factors and after exercise do not achieve the same levels of increase as young muscle. Although it is not known why resting levels of MyoD should be higher, it has been proposed that this may be as a result of increased dennervation and impaired reinnervation, a phenomena that is associated with ageing in muscle (Marsh et al 1997).

It is proposed that age-related loss in muscle mass is due to the failure to activate satellite to repair cumulative damage incurred by cycles of regeneration/degeneration (Barton-Davis et al 1998). Restoration of muscle strength using virally mediated over-expression of IGF-1 supports the hypothesis that a deficit in local trophic factors may be linked to age-related muscle atrophy. Exogenous locally infused IGF-1 has been shown to restore the satellite cell proliferative potential of immobilized old skeletal muscle (Charkravarthy et al 2000). The significant decrease in baseline levels of MGF with increasing age may partially explain the decrease in capacity of satellite cells to proliferate in the elderly (Carlson et al 1998, Schultz 1994). The down-regulation of the IGF-1 system particularly the decrease in MGF mRNA levels, in response to overload is likely to play an important role in the impaired regenerative potential of old skeletal muscle.
CHAPTER IV

Age-Related Alterations In The Regulation Of The IGF-1 Receptor Expression.
4.0 Introduction

In the last chapter it has been clearly shown that muscle adaptation to increased load as determined by IGF-1 responsiveness varies with age. In particular, evidence by various researchers tended to suggest that the effects of IGF-1 effect on cell growth is most prominent during foetal and early postnatal life, and decreases significantly with senescence (Alexandrides 1989, Quiroga et al 1995, Baker et al 1993). High affinity receptors for IGF-1 are reported in skeletal muscles at all ages but manifest a distinct pattern of expression during development. IGF-1 receptor number is very high in fetal muscle, remains constant until the fourth week of life, and then decreases to 4-5 fold lower in adult muscle (Alexandrides et al 1989). In relation to this, basal IGF-1 stimulated receptor phosphorylation and tyrosine kinase activity is several-fold higher in partially purified receptor preparations from fetal muscle compared to an equal number of receptors from adult muscle (Yee et al 1989).

The main function of IGF-1 is to regulate cellular growth and metabolism. Cellular consequences of IGF-1R activation in muscle include; myoblast proliferation and differentiation and subsequent growth and hypertrophy of myofibres, glucose transport and glycogen synthesis, modulation of calcium release in the sarcoplasmic reticulum (Renganathan et al 1997) {McCarthy, Vyas, et al. 1999 292 /id}, and also activation of cellular protective mechanisms in response to oxidative stress (reviewed by Allen 1999). IGF-1 also stimulates protein synthesis and decreases the rate of protein degradation in muscle tissue (Bark 1998). It is therefore an important mediator of net positive protein balance. With advancing age, there is a decrease in the rate of protein synthesis as well as a loss in the ability of IGF-1 to increase protein synthesis in skeletal muscle (Welle et al 1996, Balagapol et al 1997). In addition, it has been shown that externally applied IGF-1 was unable to stimulate protein synthesis and amino acid transport in aged rat and mouse muscles (Dardevet et al 1994, Willis et al 1997). This observation mirrors the increased resistance of aged skeletal muscle to insulin (Ryan 2000 for review, Dardevet et al 1994). These studies have indicated that loss of muscle loss cannot be simply attributed to the declining levels of anabolic hormones.
Age-associated muscle resistance to IGF-1 has been partially attributed to the large decreases in the IGF-1 receptor number, after the first few weeks of birth. Although there is general consensus that there is a sharp decrease in the IGF-1 receptor protein and mRNA in rats after the fourth week of birth, conflicting observations have been made as to whether this decline continues into senescence. Dardavet et al (1994) estimated that there was 60-80% decrease in receptor levels in adults compared to those at birth and also concluded that the decline in receptor number continued into senility. On the other hand, other groups have maintained that the decline in receptor number stops 6-8 months after birth (Alexandrides et al 1989, Werner et al 1989, Willis et al 1997). Furthermore, conflicting data has been published regarding the expected parallel decrease in receptor mRNA. Some groups have either observed no parallel decrease in receptor mRNA or even recorded an increase in IGF-1 receptor mRNA compared to younger controls (Marsh et al 1997). This has led to the hypothesis that increased age may be accompanied with an impairment in the translational mechanism for the IGF-1 receptor, decreased stability of IGF-1 receptor mRNA and/or increased degradation or internalization of the receptor protein.

Reports of increased or enhanced protein synthesis in fetal and neonatal muscle have also been attributed to the possibility of different receptor isoforms. Alexandrides and co-workers (1993) first proposed the possibility of an alternative receptor due to their observations of an immunologically distinct fetal receptor, which disappeared after a few weeks of birth. This foetal receptor seemed to share the same alpha subunit but possess a different beta subunit to the 'normal' adult IGF-1 receptor (Alexandrides et al 1993). This receptor was associated with accelerated growth as it was fully stimulated by concentrations of IGF-1 that are submaximal for the adult receptor. The beta subunit of this fetal receptor differed in molecular weight from that of the adult with the latter determined as 105 kD and the former 95 kd. Since then, various reports support the existence of a distinct fetal beta subunit (Quiroga et al 1995, Mascotti et al 1997 Moss and Livingston 1993). In the muscle cell line C2C12, this 'foetal'
receptor is highly expressed in proliferating myoblasts and downregulated upon differentiation (Barenton et al 1993). It is possible, that alternative gene splicing maybe the mechanism used to developmentally regulate the expression of IGF-1R.

Although there is considerable evidence for multiple receptor species, there is only one receptor gene published to date (Abbot et al 1992). In addition, there has been no indication of multiple mRNA species, as a single mRNA species of 11kb has been detected in humans, chicken and rodent species, by Northern blotting (Ullrich 1991, Abbot et al 1992, Pedrini 1994). However, using RNase protection, two different IGF-1 receptor transcripts have been identified in several tissues. One transcript is identical with the published cloned IGF-1R and the other lacks 3 nucleotides in the extracellular portion of the beta subunit-encoding region (Yee et al 1989). Although these two transcripts have been shown to differ slightly in the molecular weight of their B-subunits, they do not appear to account for the developmentally different receptor. This suggests that other developmentally regulated transcripts may exist, but have not been detected due to the relative insensitivity of the Northern blotting technique and low expression levels of the receptor mRNA.

As with previous measurements of local IGF-1, it is not known whether the decreased action of IGF-1 with age is an inevitable result of ageing or as a consequence of decreased physical activity. Increased physical activity, as well as stretch has been shown to reverse the resistance of aged skeletal muscle to IGF-1 and Insulin (Willis et al 1997). While the enhancement of IGF-1 action has been largely attributed to post-binding receptor mechanisms, an increase in IGF-1 receptor mRNA and protein has also been reported (Marsh et al 1997, Willis et al 1997). Recent evidence suggests that skeletal muscle regeneration in postnatal life recapitulates the events that occur in the normal course of embryonic development. Therefore in order to determine the plasticity of old muscle, it is important not only to determine changes in growth factor levels, but also establish changes in receptor activity.
Using the previously described model of tenotomy induced work overload, this study proposed to investigate the contribution of changes in IGF-1 receptor expression and number in the altered action of IGF-1 with ageing. I hypothesized that the old animals would have decreased baseline levels of IGF-1 receptor mRNA to reflect protein levels. The slower regenerative capacity of older animals compared to the young and mature, would also be reflected by an attenuated upregulation of receptor mRNA levels after 5 days overload. In addition I set out to determine whether the basis for the heterogeneity of the IGF-1 receptor was due to alternative RNA splicing of the beta subunit. The latter experiment would also shed some light on possible post-transcriptional regulation of the receptor mRNA.

4.1 Methods

4.1.1 Animal Experiments

Samples collected from the same animals used in Chapter 3 were used to carry out this investigation. In summary, Male Sprague Dawley rats were weaned at 21 days and at 3 months (mths) randomly allocated into ad libitum-fed (AD) and diet restricted (DR) groups, which were individually caged. AD animals had unlimited access to food whilst DR rats were fed an identical diet to 70% of the AD food group intake. Three different sub-groups of the rats were classified in each of the AD and DR groups as 3mth (young), 13mth (mature), and 24mth (old). Experiments were initiated by unilateral excision of the distal gastrocnemius tendon as described by DeVol et al (1990). Sham operations were carried out on the contralateral leg, which would then act as the control. After five days the animals were sacrificed. The plantaris and soleus were immediately removed, weighed, snap-frozen in liquid nitrogen and stored at −70 °C.

Muscle and brain tissue was also collected from 1-day Sprague Dawley neonate rats. The animals were decapitated 24 hours after birth.
4.1.2 RNA isolation and Analysis.
Total RNA was isolated by the guanidine thiocynate method of Chomczynski and Saachi. The extracted RNA was dissolved in diethylpyrocarbonate-treated water and quantified spectrophometrically with the Genespec I (Naka Instruments). The integrity of the RNA was confirmed by visual inspection of ethidium bromide stained 18s and 28s rRNA under ultra violet light.

4.1.3 Quantitative RT-PCR
The protocols by which the receptor mRNA levels were determined using real-time PCR are described and discussed in Chapter 2. Changes in levels of mRNA following tenotomy were determined in the young (3month), mature (13month) and old (24month) old rats.

4.1.4 3'RACE PCR

cDNA synthesis
In eukaryotic nuclei, every pre-mRNA undergoes post-transcriptional processing to generate mature mRNA, which is exported to the cytoplasm for translation. Post-transcriptional modifications include, capping at the 5' end, polyadenylation at the 3' end and splicing or removal of intervening sequences (introns) (reviewed by Manley 1995). The technique, Rapid Amplification of cDNA ends (RACE-PCR), is a PCR-based technique which was developed to facilitate the cloning of full-length cDNA 5 prime and 3 prime-ends after a partial cDNA sequence has been obtained by other methods (Schaefer 1995). The total cellular RNA is reverse-transcribed with an oligo(dT)$_{17}$ primer linked to a G/C-rich anchor sequence. The oligo(dT) portion of the primer hybridizes to mRNA poly(A) tails, thereby generating a heterogenous pool of cDNAs primed at all possible positions along the poly(A) tail. The anchor sequence on the oligo(dT) anchor served to prevent progressive shortening of the homopolymeric (dA) stretches representing the poly(A) tail during PCR amplification (Salles et al 1999). Once the cDNA transcripts are synthesized they are stable and can be used to determine the 3' end, including the poly(A) status of any mRNA within the pool by simple PCR. Subsequent PCR amplification of this pool was done with a message-specific primer and a primer with the same sequence as the anchor.
The oligo(dT)$_{17}$ primer used in this study, RoRi (Harvey et al 1991) had been established as effective for isolating 3'UTR of various myosin heavy chain myosins (Kiri et al 2002). 5 ug of total RNA was reverse transcribed in a 20ul volume reaction using Superscript II RNase H- (GibCoBRL, UK) according to the instructions outlined by the manufacturer. Each reaction contained 5ug of RNA, 0.5ug of oligo(dT) anchor in DEPC-distilled H$_2$O, 5X Buffer (250mM Tris-HCl, 375 mM KCl, 15mM MgCl$_2$), 0.1 M DTT, 10 units RNase Inhibitor (GibCoBRL) and 200units of the Reverse transcriptase.

Oligonucleotide design

Due to the fact that most of the antibodies that were used to determine IGF-1 receptor variants were derived from peptide sequences, downstream from the tyrosine kinase domain, it was necessary to pick a primer upstream from these sites. Two primers (RIGFR-F and RIFNE) were chosen in the tyrosine kinase domain of the IGF-1R according to published sequences, which show high identity with both the insulin and insulin-related receptors (IRR) and therefore are likely to be conserved in any insulin-receptor family members (Ullrich 1991, Shier and Watt 1990). The upstream primer, RIGFR-F was degenerate, to accommodate any nucleotide differences between the three receptors. The reverse primer comprised of the Ro sequence that was tagged onto the oligo-dT primer. The authenticity of the PCR product observed was verified by running a nested PCR. This involved using an optimised dilution of the first PCR product to run a second PCR using a forward primer just downstream of the first primer and the inner Ri sequence as a reverse primer. (Fig4.1A below).
PCR Optimisation

cDNA cloning in this study was based on using insulin receptor family specific degenerate primers and a general primer contained within the RT primer RoRi. To optimize the use of the degenerate specific primers RIGFR-F (Fig 2B), PCR amplification temperature was dropped from the predicted 62°C to 55°C. At these cycling conditions (94°C-1min, 55°C-1min and 72°C-1min), only a smear was amplified. Increasing the annealing temperature resulted in the amplification of only short products that were due to mispriming and not the desired products. This highlighted a dangerous pitfall of using RACE-PCR to determine new members of a gene family, or to determine 5’ or 3’ cDNA sequences. It was clear
that PCR seemed to favour the shortest products the oligonucleotides allow to 
amplify. It was therefore important to ensure that the starting RNA for cDNA 
synthesis was full-length size. In addition there was a tendency to co-amplify 
non-specific ubiquitously expressed sequences. By reducing the concentration of 
the general primer (Ro and Ri) relative to the specific primer (RIGFR-F), the 
formation of non-specific products was reduced (Bespalova 1998). When an 
equimolar concentration of 0.5uM was used no specific product was amplified. 
With decreasing amounts of the general primer, the level of unspecific 
background decreased, with the best result achieved with a ratio of 10:1 of the 
general primer Ro to the specific primer RIGFR-F. (Results not shown). The 
specificity of the products was confirmed by nesting the PCR products using 
specific primer RIFNE and general primer Ri.

Cloning and sequencing
PCR products were separated by electrophoresis on 1.5% agarose gels. The 
products were viewed under UV light and cut out using fresh razors. The gel 
products were then melted down and the PCR products purified using Qiagen 
DNA clean up kits (Qiagen, UK) according to the manufacture’s protocol. 
Purified PCR products were cloned into PGem T-Easy vector (Promega, UK) 
according to the manufacturer’s protocol. Three clones of each PCR product were 
partially sequenced by the dideoxy chain termination procedure (Sanger 1977) 
using the Sequenase Version 2.0 DNA sequencing kits (Amersham UK). On 
verifying that the clones contained some homology to the IGF-1 receptor 
sequence, the clones were sent off to MWG-Biotech (Cambridge, UK) for full 
sequencing.

4.2 Results
4.2.1 Characterization of the IGF-1R mRNA by 3’RACE
The experiments were carried out to determine whether alternative splicing 
within the IGF-1R gene could account for the immunological heterogeneity 
observed.
Different annealing temperatures and primers led to the detection of slightly different PCR products. In the first PCR run (before nesting), using RIGFR-F and RO, 3 bands of approximately 1.3 Kb, 1Kb and 800bp were identified in, brain and muscle at different age-groups (See Fig 2: Lanes 1 to 8). These PCR products were fully sequenced and were determined to correspond to the carboxyl terminal domains of the IGF-1R, insulin receptor (Ullrich 1991) and Quaking protein (Ebersole et al 1996) respectively. Following nesting the PCR using primers RIFNE and RI, the specificity of two bands corresponding to the IGF-1R and insulin receptor were also confirmed by sequencing.

**KEY**
1. OC Soleus
2. Fetal Brain
3. Young Brain
4. Fetal Muscle
5. Neonate Muscle
6 YE Muscle
7. Fetal muscle
8. YE muscle
9. OC Muscle
10. Liver
11. Purified 1.5kb IGF-1R transcript
12. Gut

![Fig 4.2](image.png)

Fig 4.2. A representative picture showing the different PCR products amplified in various tissues using 3'RACE. Lanes 1-8 were amplified using primers RIGFR-F and RO. Lanes 7-13 were nested reactions using RIFNE and RI primers. The PCR conditions were as follows, Hotstart of 92°C-5 mins, 35 amplification cycle of: 92°C-45s, 58°C-1 min, 72°C-1min.

In the fetal brain and neonate muscle a 1.5kb band was also detected. This higher MW band was also faintly detected in young and old muscle following overload. The major difference between the two IGF-1 receptor bands lay in the lengths of the 3’ untranslated (3’ UTR) region of the gene sequence. The 1.3kb bad corresponded to the published rat IGF-1R cDNA. The sequence below represents the 3'UTR of the IGF-1R of the 1.5kb fragment (Fig 3). The highlighted region represents a previously unpublished sequence.
Fig 4.3. 3’UTR of the 1.5Kb transcript of the IGF-1 Receptor. The highlighted region represents the addition to the published sequence. The underlined sequence ATT A are the destabilization motifs thought to reduce RNA stability. The AAGAAA sequence may serve as a polyadenylation signal.

The published sequence ends with two cytosine bases instead of continuous adenosine bases. This suggests that either the published data may have be an error in the published sequence which has resulted from priming of an internal poly-A tract or that IGF-1R is regulated developmentally by its adénylation status. In summary no novel IGF-1R-related cDNA could be detected using degenerate primers.

The reproducibility of the results was different according to the tissue tested and PCR amplification conditions. It proved very difficult to amplify IGF-1 receptor in control muscle. In these samples, only the insulin receptor was amplified with regularity. The intensity of the other bands was very faint and failed to be reproducible.

4.2.2 Effect of loading and age on IGF-1R gene expression.

In order to quantify the changes in IGF-1R gene expression with increased age and after tendon ablation, real-time quantitative RT-PCR was carried out on RNA samples obtained from the previously described model of tenotomy induced work overload. Real-time QC-RT-PCR with non-degenerate primers has been proven to be reliably reproducible and also to be a sensitive method ensuring good detectability of low abundance mRNAs (described in Chapter 2). There was no significant change in baseline IGF-1R mRNA levels with increased age. The increase in the young and mature animals after tenotomy was approximately two-fold (50% increase) compared to the controls, whilst there was less than a 25% increase in the overloaded old muscles. Hence, a significant increase in IGF-1
receptor mRNA levels (p<0.05) was found in overloaded muscles in the young and mature, but not old overloaded muscles compared to their controls. Although OE was not significantly different from OC; if the IGF-1R expression was induced in the old, it is possible that the magnitude of the induction was too small to be determined as significant from this small sample size. (Fig 4.4)

**IGF-1 receptor mRNA changes**

![IGF-1 receptor mRNA changes](image)

Fig 4.4. IGF-1 receptor mRNA (quantification 5 days after tenotomy. Values are ± S.E.M: n=a minimum of 6 rats/group. Samples within the group were tested using the paired t-test. ANOVA was used to determine whether there was any significance between the means *Significantly different

4.3 Discussion

Muscle responds to mechanical overload by activating the local IGF-1 system. The effects of IGF-1 are likely to be enhanced by the increase in IGF-1R activity. My results demonstrate that one of the ways receptor activity could be increased is by an increase in gene expression. An increase in IGF-1 receptor mRNA and number has been observed following overload, eccentric exercise and during regeneration of young muscles (Marsh et al 1997, Willis et al 1998, Dardavet et al 1994 and Singh et al 1999). This increase in receptor activity has been measured in terms of both receptor number and downstream consequences, such as protein synthesis, and glucose uptake.
In contrast to the young and mature rats, there was an attenuated increase in mRNA levels in the old rats 5 days after work-induced overload. In the previous chapter there was some indication that regeneration in the elderly was likely to be delayed. In this study the attenuated growth of old muscles in comparison to that in the young rats, is also reflected by the lack of increase in IGF-1 receptor mRNA in old overloaded muscles. Willis et al (1997) noted that young muscles responded to a bout of exercise with enhanced muscle IGF-1 activity 24hours before elderly rats. The results of this study support the hypothesis that old muscle does respond to exercise, however not as readily as the young. In addition, the results of this study show that muscle activity is also linked to an induction of IGF-1 receptor gene expression. Previously, results from Chapter 3 have shown that MyoD, a muscle-specific transcription factor is upregulated with overload. Other studies have established that MyoD gene expression is activated through IGF-1R signaling. This suggests that muscle activity increases IGF-1 activity through an increase both in IGF-1R signaling and also IGF-1R expression.

With maturation, and into senescence, there is a dramatic decrease in receptor activity. Evidence from measuring the changes in receptor activation and immuonoreactivity has suggested the existence of a developmentally regulated beta subunit (Mascotti et al 1997, Quiroga et al 1995, Alexandrides et al 1989). The molecular nature of this receptor had not been elucidated. This investigation confirmed that there are unlikely to be any other existing IGF-1R mRNA isoforms that could account for amino acid changes in the beta subunit of IGF-1R. Although the decrease in IGF-1 receptor activity may be still due to the existence of a developmentally regulated receptor variant, the immunological differences observed may therefore be a result of post-translational modifications such as glycosylation or even phosphorylation. Certain oncogenes can also alter the phosphorylation status of the receptor beta subunit. An example of this class of oncogenes is pp60src. This receptor variant, is however likely only to contribute to the decrease in activity after birth and not to the age-related muscle resistance to IGF-1.
Receptor number is regulated through gene transcription and also possibly at the pre-translation stage of mature mRNA. The age-related decrease in mammals, has been attributed to a decrease in receptor protein shortly after puberty. Although, receptor protein was not determined in this study, it would be logical to assume that the decrease in receptor protein would be reflected by a decrease in baseline mRNA level. However this was not the case, as baseline receptor mRNA levels did not change significantly between the young and old rats. This is consistent with either a decrease in mRNA translation and/or increase in protein degradation with increased age.

Degenerate primers were used in this study to look for novel receptors. However no novel IGF-1R-related cDNA was found. Instead, the results of this investigation raised the possibility that receptor mRNA stability and translation could be developmentally regulated through poly(A) tail shortening. Polyadenylated IGF-1R mRNA in control mature and old rats, was not detectable by RT-PCR using oligo(dT) primed cDNA. However it was detectable in neonate muscle and also at all ages following exercise or overload. Initially, it was assumed that this was the case due to low levels of receptor mRNA in control adult and old muscle. However, gene quantification by RT-PCR using specifically primed cDNA showed that there was no significant difference in the mRNA levels between the control and overloaded leg. The only detectable difference was in the poly(A) tail of the neonatal IGF-1R mRNA and adult control muscle.

The polyadenylation signal of the IGF-1 receptor has not been previously identified although it is known to be represented in the poly(A)+ pool of mRNA (Ullrich et al 1986). The 1.5kb transcript represents a mRNA with a previously undetermined poly(A) stretch. However the highly conserved polyadenylation signal, AAUAAA motif or other frequent variants could not be located. There is a possibility that the motifs AAGAAA, or AAATCA can serve as a polyadenylation signal (Beaudoing et al 2000). The former motif has been previously recognized in a mammalian gene to act as a poly(A) signal (Anand et al 1997). It was expected that the poly(A) signal would be found 11-30
nucleotides (nt) upstream of the poly(A) tail, however in this case the two motifs lie more than 200nt upstream. Non-canonical poly(A) signals are thought to function less efficiently than the commonly found AAUAAA or AUUAAA variants (Wickens et al 1984, Beudoing 2000). The possession of variant AAUAAA sequences in naturally occurring genes may reflect the intentional possession of weak poly(A) site as a simple approach to reduce the amount of mRNA produced by a particular gene (reviewed in Proudfoot and Whitelaw 1990). It is not clear in this study why previous investigations have not been able to identify different transcripts of IGF-1 receptor mRNA. The implication has been that there is a single poly(A) site, although it was not identified. The shorter IGF-1R transcript is likely to be more abundant than the longer, or alternatively, is reverse-transcribed more efficiently using oligo(dT) and therefore amplified more efficiently during PCR. Another reason why polyadenylation sites may not be identified is because oligo(dT) priming may bind to internal poly(A) tracts, and therefore not allow for the characterization of the full-length 3'UTR.

A simple approach to modulate output from a gene is by controlling the stability of the gene transcript. Eukaryotic genes associated with transient expression such as interferon, c-myc, and various growth factor mRNAs all have AU rich sequences in 3'UTR region as well as the sequence AUUU in tandem (Chen et al 1995). This characteristic was displayed by the IGF-1R mRNA as well as two AUUU sequences were identified. These sequences serve as destabilizing sequences. The insertion of this sequence into the otherwise highly stable B-globin mRNA made it extremely unstable. The function of the poly(A) tail is clearly to stabilize RNA. Deadenylated RNA is relatively unstable, degrading after a few hours instead of a few weeks (Shaw et al 1986).

The identification of the two different transcripts may suggest that IGF-1R is also regulated developmentally by its adénylation status. Although the IGF-1R transcript is always detectable using by RT-PCR using hexamers, the lack of amplification of polyadenylated transcripts using oligo(dT)-primer in the adult and old muscle control tissue seems to indicate that deadenylation of the mRNA may occur. In both yeast (Decker and Parker 1993) and eukaryotes (Decker et al
1994), it appears that shortening of the poly(A) tail can be the first step in mRNA degradation. Such regulation has also been observed in the IGF-1R genes in Xenopus and trout (Groigno 1996, Ellies et al 1998). Therefore, the increase in IGF-1R mRNA after exercise and also in foetal tissue may be due both to an increase in gene transcription, as well as more stable mRNA species, as indicated by the presence of the 1.5kb transcript.

The decrease in IGF-1R activity from maturity to senescence involves the complex regulation of transcription, translation and post-binding events. A decrease in baseline protein levels may be accounted for by a decrease in translation due to increased mRNA degradation. However, this hypothesis would not explain the observed muscle resistance and post-receptor uncoupling of downstream signaling events. The increase of IGF-1R mRNA during hypertrophy is likely to be due to the activation of IGF-1R promoter by growth factors such as bFGF and PDGF (Hernandez-Sanchez et al 1996). These growth factors are also upregulated in response to muscle overload. The lack of increase in IGF-1R in the senescent muscle after exercise may also be attributed to the deficit of these growth factors (reviewed by Grounds 1998). Like MGF, generalized decrease in the production and response of muscles of old rats to bFGF and PDGF has been observed (Mezzogiorno et al 1993). This once again emphasizes the point that differences in the local environment in vivo probably play an important role in the response of young compared to old muscles.
CHAPTER V:
Role Of IGF-1Ea And MGF In Spinal Transection-Induced Muscle Atrophy
5.0 Introduction

Adult skeletal muscle is a dynamic tissue capable of changing phenotype depending on the functional demands placed on it. As discussed in the previous chapters, stimuli such as resistance exercise and overload to muscle give rise to hypertrophic responses. Other conditions such as disuse, malnutrition, and microgravity lead to atrophy of the muscle. After spinal cord injury, significant changes occur in muscles that receive innervation from the site of injury. Severe atrophy characterised by a decrease in cross-sectional area of the muscle fibre and altered expression of myosin heavy chain proteins is observed in both human patients (Round et al 1993) and experimental animals (Dupont-Verseteegden et al 1999).

Although, with spinal transection, there is no direct damage to the muscle, innervation to the muscle is disrupted and spinal motor neurons caudal to the lesion lose neural input from the descending brainstem pathways. This influences the tonic and phasic firing patterns and consequently affects muscle contractile and metabolic activity (Salmons and Henrikkson 1981, Houle et al 1999). These changes in turn are thought to induce a rapid transformation from slow to fast phenotype in hind limb muscles after injury, which leads to a loss of strength and endurance of the muscle (Dupont-Verseteegden et al 1999, Lieber 1986). Changes in myosin heavy chain (MyHC) gene expression in atrophying muscle have also been reported under a number of experimental conditions including microgravity, hind limb suspension and chronic denervation (reviewed by Allen et al 1995). These studies support the hypothesis that appropriate neural activity is an important feature of maintaining the expression of slow MyHCs (Houle et al 1999).

However, it is not clear what molecular mechanisms lead to the decrease in muscle fibre size and hence atrophy. Muscle atrophy could be as a result of either an increase in proteolysis and/or a decrease in myonuclear number. The upregulation of calcium-dependent proteases (Calpains) observed 6-12 hours following hind-limb immobilization and after spinal injury is thought to cause increased proteolysis (Tang et al 2000). This phase is followed by an increase in
protein degradation due to the release of cytokines such as TNF-alpha, which are associated with muscle wasting (Zarzhevsky 2001).

In the same analogy as hypertrophy, muscle atrophy would also be expected to be characterised by myonuclear modulation. In support of this hypothesis, recent studies have demonstrated that muscle atrophy as a result of reduced neuromuscular activity is also associated with a decrease in myonuclear number, which at least may be partially responsible for the decrease in fibre size (Allen et al 1997, Dupont-Verseteeegden et al 1999). There is some controversy as to whether myonuclear loss occurs in all fibre types and also contradictory reports on whether a greater decrease is observed in type I fibres compared to type II in response to spinal cord injury (Dupont-Verseteeegden et al 1999, Allen et al 1995). In soleus muscle, the decrease in myonuclei number is observed to be directly correlated to the decrease in myofibre size (Dupont-Verseteeegden et al 2000).

The mechanism by which these nuclei disappear is not clear although there is a possibility that it is by programmed cell death or apoptosis (Allen et al 1997). Apoptosis is characterised by a stereotyped sequence of structural changes shared by a wide variety of cells dying in different circumstances (reviewed by Wyllie 1997). The morphological features of apoptosis include cell shrinkage, membrane blebbing, cytoplasmic condensation, condensation of the nuclear chromatin and endonuclease-catalysed degradation of DNA (Kerr et al 1972). A particular feature of apoptosis is that the plasma membrane remains intact until very late in the process and specific mechanisms are induced to ensure phagocytosis of apoptotic bodies. In contrast, disruption of the plasma membrane appears early in necrosis with consequent inflammation and tissue damage. A family of a dozen related genes encoding for cysteine proteases (cysteine-containing asp-ases or caspases) have been identified as a common endpoint for signalling pathways to programmed cell death (Thornberry 1998). Mammalian skeletal muscle myoblasts undergo programmed cell death during normal differentiation and development (Wang and Walsh 1996, Smith et al 1995), but following fusion, myotubes are post-mitotic and it has been claimed that they
acquire a resistance to apoptosis (Wang and Walsh 1996). However, observations based on the latter model raise doubts as to whether apoptosis does occur in mature myofibres in vivo.

On the other hand, several workers have recently examined the possibility that apoptosis may play a role in some pathologies affecting mature muscle. Apoptotic nuclei have been observed in muscles undergoing atrophy due to hind limb unweighting (Allen et al 1997), denervation (Tews et al 1997) and also in dystrophic mice (Tidball et al 1995). The most comprehensive study confirming that apoptotic cell death can occur in postmitotic multinucleated skeletal muscle was based on a treating C2C12 myotubes with a cytotoxic agent, staurosporine (McCardle et al 1999). In contrast to other studies, they used various methods to identify several classical features of apoptosis. In addition to identifying DNA degradation using terminal deoxynucleotidyl transferase (TDT)-mediated dUTP nick end labelling (TUNEL), they also determined that procaspase 3 was activated by cleavage and caspase 3 could be identified in apoptotic myonuclei.

Various interventions have been recently shown to prevent the decrease in myonuclear number and/or fiber size during muscle atrophy. Muscle atrophy induced by spinal cord injury is reduced by two interventions: motor-assisted cycling exercise (to be known as passive exercise) and foetal spinal cord transplantations into the lesion cavity (to be called transplantation) (Dupont-Versteegden et al 1999, and Houle et al 1999). Complete restoration of the muscle to its normal size was achieved by a strategy combining both transplantation and passive exercise (Dupont-Versteegden et al 2000). It has also been shown that step training on the treadmill reduced atrophy in spinal cord-injured cats (Roy et al 1996). The beneficial effects of exercise are thought to be mediated partially through reflex activity, as blocking neural activity with a conductance-blocking agent cancelled the previously observed benefits (Dupont-Versteegden et al 2000). Foetal tissue transplantation is thought to exert its beneficial effect by providing a substratum supportive of axonal re-growth (Houle et al 1999). In both scenarios it is likely that the attenuation of atrophy and neuron re-growth is partially mediated by growth factors. It is possible that
the transplants release some trophic factors that require muscle activity to exert their action, or alternatively there could be factors released from exercising muscles that are involved in neuronal outgrowth. However, it is not clear where the growth factors would be expressed and how they would act to increase spinal nerve regeneration or reduce/counter muscle atrophy.

McCall et al (1998) demonstrated that administration of short daily bouts of exercise combined with GH/IGF-1 treatment significantly reduced the decrease in both myonuclear number and fiber size during hind-limb unloading, whereas exercise or hormone treatment alone had a minimal effect. This raises the possibility that regulation of myonuclear number is a dependent upon the interactive effects of IGF-1, and/or some other growth factors, as well as some component of neuromuscular activity (Allen et al 1997, Allen et al 1999). The expression levels of IGF-1 in muscle and the nerve are known to transiently increase in partially denervated muscle (Caroni et al 1993). It is thought that IGF-1 participates in the restoration of the neuromuscular system and assists in avoiding degenerative events. Pu et al (1999) also showed that endogenous IGF-1 activity in or near nerves was required for motor neuron survival following sciatic nerve crush. In addition, muscle IGF-1s have been shown to be involved in regulation of satellite cell proliferation and hypertrophy. However, the involvement of IGF-1 in the attenuation of atrophy still remains controversial. Criswell et al (1998) has showed that atrophy induced by unloading was not prevented by the overexpression of IGF-1.

The anti-apoptotic role of IGF-1 in various cells and also its involvement in hypertrophy makes it an attractive candidate for the attenuation of muscle atrophy. A previous study had shown that muscular atrophy induced by spinal injury was ameliorated by passive exercise (Dupont-Versteegden et al 2000). However, the exercise regime had to be initiated shortly after the injury, thereby suggesting that there was a time window in which it was most beneficial to start the exercise. The goal of this present study was to explore the mechanism by which passive exercise prevents skeletal muscle atrophy following spinal cord transection and to determine the time window at which it was most beneficial to
start the exercise regime. I hypothesised that MGF and IGF-1 expression would increase shortly after injury in the muscle and nerve, thereby preventing muscle degradation and initiating nerve regrowth. I also proposed that anti-apoptotic action of IGF-1 would reduce the loss of myonuclei and therefore serve to attenuate a decrease in fibre size.

(This work was carried out in collaboration with C. Petersen’s group. All animal experiments, optimisation and validation of the immunohistochemistry procedures were carried out in her laboratory. Data generated in her lab is clearly indicated).

5.1 Methods

5.1.1 Spinal transection protocol

Complete transection of the thoracic (T10) spinal chord was done by creating an aspiration lesion 2-3 mm in length while the animals where under anaesthesia with ketamine (60mg/kg) and xylazine (10mg/kg). Following surgery, rats received Penicillin Procaine G and a dextrose saline injection. Manual expression of the urinary bladder was carried out twice daily. Following transection rats were assigned into different groups. Those receiving no further manipulation (Tx), rats exercised on a motor-driven cycling apparatus (Tx Ex) and control rats. Exercise was performed using a custom built motor-driven bicycle. Rats were suspended in full body slings and their feet secured to the pedals. Cycling speed was maintained at 45 revolutions per min and each exercise bout consisted of two 30-min exercise periods with a 10-min rest in between. To detect changes in myonuclei number, an exercise regime of 5 days was carried out starting 10 days after spinal transection.

Following completion of the exercise regime, animals were deeply anesthetized with sodium pentobarbital (180mg/kg) and the hindlimbs were removed at the hip joint and chilled on ice. Soleus muscles were dissected and weiged. Muscles were embedded in freezing medium and snap frozen at resting length in liquid nitrogen cooled isopentane and stored at $-70^\circ$C for immunocytochemistry.
5.1.2 Immunohistochemistry procedures: Counting myofiber nuclei

Dystrophin staining was performed to visualize the sarcolemma. Cross sections of soleus muscles were cut on a cryostat (8um), air dried and stored at -20OC. For dystrophin detection, sections were rehydrated in 2% PBS for 15 minutes. The sections were then reacted with 0.25% \( \text{H}_2\text{O}_2 \) to block endogenous peroxidase activity. Sections were then incubated with Dystrophin antibody (mouse anti-human dystrophin, NCL-DYS2, Vector Labs) diluted 1:4 in PBS for 2 hours at room temperature. The sections were then briefly rinsed in PBS twice, and a rat anti-mouse alkaline phosphate-conjugated IgG secondary antibody (Pharmigen, San Diego, CA) was applied. The alkaline phosphatase substrate kit (Vector Labs, Burlingame, CA) was used to yield red colour for dystrophin staining. To count myofiber nuclei, a Hoechst dye was applied after with a dystrophin staining. The sections were then fixed in 2% paraformaldehyde and Hoechst-33258 nuclear dye (Molecular Probes, Eugene, OR) was applied at 1.2ng/ml for 30 min. Sections were viewed with a fluorescent microscope with the use of an ultraviolet filter package. Nuclei within the dystrophin-positive sarcolemma were counted in 70-100 fibers and the number of nuclei expressed per 100 fibers. Numbers were not expressed per unit fibre cross-sectional area because fiber area changes with experimental manipulations (Dupont-Versteegden et al 1998).
5.1.3 Detection of Apoptotic Nuclei

Apoptotic nuclei were identified using a TdT-mediated dUTP nick end-labelling (TUNEL) assay (Boehringer Mannheim). This assay is based on the fact that apoptotic nuclei display DNA strand breaks. TdT can then be used to label these DNA strand breaks with flourescein-conjugated dNTP. Incorporated flourescein was then detected by anti-flourescein antibody conjugated with horseradish peroxidase. TUNEL detection was performed according to the protocol supplied with the assay. Specifically, sections were fixed in 4% paraformaldehyde at room temperature, for 15 minutes. Endogenous peroxide activity was then blocked using 0.3% peroxidase in 70% methanol. The sections were permeabilized in 0.1% Triton-X and 0.1% sodium citrate at 4°C. TUNEL mix was added to the sections at a 1:5 dilution with 30mM Tris, 140mM sodium cacodylate, and 1mM CoCl₂. Labelling mix was incubated at 37°C for 1 hour. Sections were rinsed and the flourescein antibody was applied for 30 min at 37°C. Sections were rinsed and DAB substrate (Vector Labs) added for colour development. The sections were then dehydrated and cover slipped. All positive nuclei of in each muscle section were counted.
Fig 5.2 Increased frequency of apoptotic nuclei with spinal cord transection. Cross section of soleus muscles from control (A), Tx10 (B) and Tx10Ex5 (C) rats were assayed for TdT-mediated dUTP nick end labelling (TUNEL) reactivity (brown). Arrows indicate examples of TUNEL-positive nuclei. Bar for A-C, 25 μm.

All animal experiments and immunohistochemistry was carried out in C. Peterson’s lab and the data was analysed and reproduced with their permission

5.1.4 Changes in the local IGF-1 system following spinal cord injury

Short-term effects of cycling on muscle: Time window experiment

Adult Sprague Dawley rats were divided into 5 groups of 4 rats each. In all but one (the Control group SC-) of the 5 groups the spinal cord was completed transected at T_{10}. Two groups underwent transection and were killed 2 and 5 days later (Tx2 and Tx5, respectively). The other two groups were transected, left for 2 and 5 days, had one bout of exercise and were sacrificed 24 hours later (Tx2Ex and Tx5Ex respectively) using an overdose Sodium Pentobarbital. In each of these groups the Soleus muscles were harvested by snap freezing in liquid nitrogen and stored at -70°C before RNA extraction. These samples were then analysed for changes in IGF-1, MGF and IGF-1 receptor expression.

Effects on neuronal tissue

Adult Sprague Dawley rats were divided into three groups. In two of the groups, of four rats each, the spinal chord was completed transected at T_{10}. One group was transected and killed three days later (Tx3) and the other transected, after
three days exercised and sacrificed four hours later (Tx3 Ex). The third group consisting of two animals served as the controls (Con). In each of these groups, samples of the soleus muscles, (Sol), thoracic spinal chords (Thr) and lumbar spinal chords (Lum) were harvested for RNA extraction. These samples as the above were analysed for changes in IGF-1, MGF and IGF-1 receptor expression.

Quantitative PCR:
All the total RNA samples were quantified using the Genespec I (Naka instruments Japan). In each study 2ug of total RNA was reverse transcribed using Omniscript (Qiagen, UK) according to the manufacturer's instructions. To facilitate the reverse transcription of rare mRNA species, specific primers were used during the reaction. The Quantitative PCR was carried out using the Lightcycler (Roche instruments, UK). The optimization of the PCR conditions quantification of IGF-1Ea, IGF-1 receptor and MGF expression are described in detail in Chapters II and III.

5.2 Results
5.2.1 Changes in myofiber size and nuclei after spinal cord transection
10 days after spinal cord transection there was a decrease of 25% in the number of myofiber nuclei in soleus muscle (see Fig 5.4A). In control soleus muscle, nuclei were found in almost every myofiber on any given cross section, however in transected soleus muscle, many small fibers did not exhibit nuclei on the cross section (results not shown). The myofibre nuclear number did not increase with exercise compared to transection alone. To investigate the possible mechanism of myofibre nuclear loss, soleus muscle cross sections were assayed for apoptotic nuclei by TUNEL, as well as for the presence of caspase 3. Control soleus muscle showed little TUNEL positively, but the number of TUNEL-positive nuclei was increased >35-fold with spinal cord transection p<0.05. However most of the TUNEL positive nuclei were not muscle nuclei (see Fig 5.3). No samples stained positive for the presence of Caspase 3. The number of TUNEL-positive nuclei decreased by significantly (p<0.05) with exercise compared with transection alone (See Fig 5.3).
5.2.2 Changes in the local IGF-1 system following spinal cord injury

Short-term effects of cycling on muscle: Time window experiment

This study was carried out to determine whether there was a time window at which the effects of the exercise would be most beneficial. There were no significant changes in the levels of IGF-1Ea in the soleus at either 5 days or 2 days following transection or with exercise. MGF mRNA in the soleus muscle did not significantly increase after spinal transection and following exercise. As with IGF-1Ea there was no significant difference in the change in mRNA levels between the times chosen to exercise the rats following transection.

Although IGF-1Ea levels are still approximately 10-fold higher than MGF expression levels, the absolute values of the two isoforms are significantly lower than those observed in Chapter 3. This anomaly was attributed to the effect of a PCR inhibitory factor in the spinal transaction samples. Different methods were used to dilute this effect, such as diluting the cDNA as well as decreasing the starting RNA amount. However, the optimal PCR efficiency was never fully achieved, hence the low fluorescence readings, relative to that of the standards.
Fig 5.5: Quantitation of changes in expression of IGF-1 isoforms in muscle after spinal transection. Comparisons were made of the differences between the levels of A: IGF-1Ea and B: MGF when the exercise regime was started 2 or 3 days after transection. In all the cases only the Soleus muscle was analysed. n=4
Effects on neuronal tissue.

In the muscle there were no significant changes in IGF-1Ea levels following spinal transection (see Fig 5.4A). The levels of IGF-1EA mRNA in the thoracic spinal chord did not change following spinal transection (Thr Tx3). However after exercise there is an upregulation of IGF-1Ea mRNA levels. There appears to be no evidence of IGF-1Ea mRNA in the lumbar spinal cord (Lum Con and Lum Tx3) (see Fig 5.5A). This was verified also using standard block PCR. However these levels may be very low and hence escape detection when run on an agarose gel and viewed under UV light. After exercise IGF-1Ea mRNA could be detected in the thoracic spinal cord (Thr Tx3 Ex).

MGF mRNA was present in the soleus muscle but did not significantly change after spinal transection, with or without passive exercise (see Fig 5.4B). Following transection there is a significant increase in MGF mRNA levels in the thoracic spinal cord. This increase was significantly higher following exercise (see Fig 5.5B). Although there was clear evidence of MGF expression in the lumbar region, there were no significant changes in mRNA levels after transection alone, or transection and exercise.
Fig 5.6: Analysis of the effects of a single burst of exercise on the expression of IGF-1 isoforms in neuronal and muscle tissue after spinal transection. A: IGF-1Ea expression does not change except around the lesion and also in the lumbar region following exercise. B: MGF levels increase around the lesion following spinal transection and are further upregulated by passive exercise. Sol, Thr, and Lum represent the tissues Soleus, Thoracic and Lumbar spine respectively. * Significantly different from control (p<0.05).

5.3 Discussion

A dramatic loss of muscle mass is associated with spinal cord injury. This study confirms that the rapid decrease in myofibre size is associated with the loss of myonuclei from the myofibres. The loss of myonuclei is thought to be due to an attempt to maintain the myonuclei domain (the ratio of cytoplasm supported by a single myonucleus) (Allen et al 1999, Cheek et al 1985). The increase in apoptotic nuclei detected by TUNEL staining suggested that myonuclei were lost by programmed cell death. However, further investigations revealed that most of the apoptotic nuclei were not muscle cells. This was verified by cross comparison of myonuclei identified by Hoechst and dystrophin staining and TUNEL staining. From Fig 5.3, apoptotic nuclei can be seen to increase after exercise and yet most of the nuclei stained appears to be between the fibres. When compared to Fig 5.2, it is clearly that myonuclei can be clearly distinguished from other nuclei and it
was determined that the majority of stained nuclei (blue stain) is not myonuclei. This widespread TUNEL staining suggested that spinal transection had a wider effect than just on mature myofibres. Borisov et al (2001) noted that the vascular bed in dennervated muscle underwent dramatic remodelling during postdennervation period that included rapid loss of capillaries. Therefore some of the dead cells may be represented by vascular cells. The increase in the number of TDT-positive non-myogenic cells suggests that elimination of other cell types may be a necessary component of the regulated remodelling of skeletal muscle as a multicellular tissue to conditions of altered usage (Allen et al 1999).

When using TUNEL to identify apoptotic cells, another alternate method should be used for confirmation as the reliability and specificity of TUNEL labelling has been questioned (Merritt et al 1995,). The second method used in this case was based on identification of Caspase 3 positive cells. However, failure to detect any Caspase 3 staining suggested that these cells may either be not apoptotic, or else cell death is activated via a different pathway. Previous studies on muscle have noted that apoptosis within muscle can be attributed to various pathways such as p53 – caspase 3 pathway (McArdle et al 1999, Mukasa et al 1999), as well as proteosome pathway linked to ubiquitin-dependent proteases (Van Royen et al 2000). Although a number of studies have claimed to observe apoptosis in mature muscle, some of the features described do not fit the stereotypical characteristics of apoptosis. In particular the later stages of the pathological appearance of dystrophic muscle, muscle after exercise have appeared necrotic (McCardle et al 1999). It is therefore still unclear by what mechanism myonuclei would be lost.

However Tidball and coworkers (1995) have also pointed out the difficulties of the precise location of apoptotic features to muscle fibres in degenerating tissue. Because apoptosis is a rapid process, the number of TUNEL-positive myonuclei at any given time is likely an underestimate of the nuclei that have undergone cell death 10 days after transection. TUNEL-positive myonuclei may also be already in the final stage of apoptosis after caspase-3 detection has decreased because
caspase-3 is transiently activated at the initial stage of apoptosis and then the active form rapidly degraded (Mukasa et al 1999).

As the number of myonuclei decreased with atrophy due to spinal injury it was determined whether exercise-related reduction of atrophy was associated with a restoration of myonuclear number. Surprisingly, this was not the case, as myonuclei number did not increase in response to the cycling regime. This observation is not in accord with the theory of the myonuclear domain as it suggests that there may be greater flexibility than initially proposed as to what the ration of cytoplasm to myonucleus may be. However, in an analogy to the hypertrophic response observed after muscle overload, it is possible that the short-term effect of cycling involves an increase in protein synthesis and a decrease in proteolysis thereby increasing the net positive protein balance. Therefore a long-term regime of passive exercise would therefore result in both an increase in protein synthesis and also the restoration of myonuclei number to control levels. In support of this hypothesis, both McCall et al (1998) and Dupont-Versteegden et al (2000) have reported that in response to a longer –term regime of exercise, myonuclear number is increased.

Given that in the previous chapters I found that MGF expression increased in response to mechanical stress, it was expected that in response to passive exercise there would be a similar upregulation in muscle tissue after spinal transection. The short bout of exercise did not result in a significant increase in any of the IGF-1 isoforms, or receptor in the muscle. This may be due to the fact that unlike previous studies, this exercise regime is a simple circular movement of low force generation as opposed to the movement against resistance that is required to attain muscle hypertrophy. The cycling movement may simply increase the sensitivity of muscle tissue to the effects of IGF-1 and MGF, thereby preventing the otherwise observed muscular atrophy. Upon dennervation, muscle tissue has been shown to develop a resistance to the effects of IGF-1 (Czerwinski et al 1993). Stretching the muscle reverses this resistance and the resulting IGF-1 activity in turn would lead to increased protein synthesis thereby attenuating muscle atrophy. In addition, protein translation increases shortly after exercise
therefore it is possible that IGF-1 peptide increased and prevented further cell death as shown by the decrease in apoptotic nuclei after exercise (Fig 5.4B).

The lack of increase in IGF-1Ea and MGF in muscle was surprising as a number of other investigations have noted a transient increase in IGF-1 levels in muscle upon nerve injury induced by sciatic nerve crush. Based on this observation, it was proposed that IGF-1 was the elusive trophic factor that provided a link between muscle activity and nerve survival. Ishii and coworkers (Reviewed by Ishii, Glazner and Pu 1994) proposed that due to IGF-1’s dual role as a neurotrophic factor and a mitogen in muscle tissue, IGFs could coordinate growth between the nerve and its target field during development and also after injury. Therefore following nerve injury, the muscle could provide trophic support by retrograde transport.

The finding that MGF is upregulated in the damaged spinal cord and further upregulated by the passive exercise without any detectable increase in the muscles indicates that it is not likely to be retrogradely transported from the muscle. It suggests that MGF is expressed in neuronal tissue per se. Although IGF-1 has been previously detected in neuronal tissue, no distinction was made between the different isoforms. After nerve transection, IGF-1 was upregulated in the sciatic nerve distal to the injury site (Ishii et al 1994, Caroni et al 1990 and Caroni et al 1993). Using in situ hybridisation, IGF-1 transcripts were localised to Schwann cells and invading macrophages. It is therefore likely that the significant increases in IGF-1Ea and MGF around the lesion following exercise are expressed by the glial cells and macrophages and play a beneficial role in the survival of the injured spinal cord. IGF-1 in spinal cord tissue enhances motoneurone sprouting, reduces apoptosis in motoneurones during normal development and after spinal transection, promotes survival of Schwann cell division maturation and growth and enhances faster recovery after crush or freeze injury of the nerve (reviewed Borst et al 1997).

However, it is clear that the effect of exercise is being transmitted to the thoracic spinal cord by a signalling mechanism as yet unidentified. It is likely that the
electrical activity from muscle can transmit signals to the neuronal tissue adjacent to the lesioned area. Therefore in these experiments the possibility of trophic factors such as MGF acting in a paracrine manner cannot be excluded. It is therefore possible that either IGF-1 isoforms or other signals can still have a retrograde effect.

As with the muscle response to hypertrophy, it was found that IGF-1Ea and MGF were not upregulated in the same pattern in neuronal tissue following spinal injury and also passive exercise. Although both IGF-1Ea and MGF seem to be expressed in a kind of gradient which declined further away from the lesioned area, there was a different pattern of expression for the two local isoforms. While MGF increased significantly in the thoracic spinal cord after transection and was upregulated further by passive exercise, IGF-1Ea mRNA levels were only increased following exercise. In the lumbar region, MGF was not upregulated at all, while IGF-1Ea was only detected after exercise. These results support the hypothesis that MGF expression may be closely linked to the cellular stress response. Therefore in response to the first cellular insult, MGF mRNA increases and then is further upregulated as the tissue is exposed to further stress in the form of passive exercise.

Although there was no increase in IGF-1 gene expression in muscle, given that both IGF-1Ea and MGF peptides are positive regulators of muscle growth, they are likely to be involved in the passive exercise-induced reversal of muscle atrophy following spinal injury. It is not also not clear how, a signalling molecule produced by the muscle is likely to activate the IGF-1 gene resulting in the upregulation of IGF-1 transcripts in the spinal cord. This data supports the hypothesis that the different IGF-1 isoforms may have different roles within the same tissue. Due to MGF’s early expression pattern, it may play protective role an early mediator of cellular response to stress. From the pilot study it had been established that the changes in IGF-1 system were most pronounced around the site of the lesion. Therefore the benefit of the exercise would have been better assessed by changes in IGF-1 variants in spinal cord samples.
CHAPTER VI

General Discussion And Conclusions
6.1 Summary

Skeletal muscle is composed of multinucleated fibres formed after differentiation and fusion of muscle precursors. These myofibres adapt to changes in their workload by regulating fibre size by mechanisms that are only now, starting to be clarified. Atrophy and hypertrophy refer to changes in the diameter of these pre-existing muscle fibres. This thesis presents the hypothesis that muscle response to positive and negative stimuli can be determined by changes in local IGF-1 expression based on the premise that insulin-like growth factor (IGF-1), functioning in an autocrine/paracrine mode, is an important mediator of skeletal muscle adaptation. The experiments focus on the central role of local isoforms of muscle IGF-1, IGF-1Ea and MGF, in muscle hypertrophy, atrophy and loss of skeletal muscle mass and strength during senescence.

To better understand the regulatory mechanisms that control the interrelations of hypertrophy and atrophy processes during skeletal muscle adaptation to changes in workload I addressed the following questions:

1. Whether changes in gene expression of muscle IGF-1 isoforms, receptors and binding proteins are modulated by muscle adaptation to overload by synergist muscle ablation;
2. Whether muscle IGF-1 is downregulated during spinal transection induced atrophy;
3. Whether muscle IGF-1 mediates the attenuation and reversal of spinal transection-induced atrophy after exercise;
4. Whether the impairment of muscle regeneration during ageing is reflected by changes in either muscle IGF-1 and IGF-1 receptor gene expression and/or reduced IGF-1 signalling.

6.1 IGF-1 and Muscle hypertrophy

The earliest studies on muscle growth determined that muscle mass increased in response to increased loading by an increase in mass, a process that was later confirmed to be hypertrophy rather than hyperplasia (reviewed by Allen et al 1999). In this study the model of gastrocnemius tendon ablation, was used to induce hypertrophy in the synergistic muscles of the soleus and plantaris.
Although it is fairly established that local IGF-1 gene expression increases in response to exercise and overload, there have been no previous studies distinguishing the regulation of different IGF-1 isoforms. Surprisingly, MGF mRNA expression increased in response to overload while IGF-1Ea mRNA levels remained steady.

Yang et al noted that rabbit muscle subjected to stretch and stimulation responded with an increase in muscle mass that was also associated with an increase in both IGF-1 mRNA isoforms (Yang et al 1997). Other studies have also observed a large increase in IGF-1 peptide and mRNA in response to eccentric exercise (reviewed by Hameed et al 2002). Therefore the differentiating factor in this model may be that there was no sign of muscle damage, suggesting that transcriptional regulation of MGF may be directly regulated by mechanical stress. In contrast, IGF-1Ea may be induced by cytokines that are released during muscle damage. This would suggest that different promoters regulate IGF-1Ea and MGF and hence different signaling molecules may coordinate the transcription of the different isoforms.

The finding that local IGF-1 mRNA isoforms were differentially regulated in response to mechanical overload raised the question as to what the role of the different peptides was especially as they differ only in the E peptide. As the E peptide is truncated in mature IGF-1 peptide, there has been an assumption that it has no function. This finding suggests that MGF may have a distinctive role from that played by IGF-1Ea and that this distinction may lay in the truncated peptide. In the model of tendon ablation, it was not clear what role the early increase in MGF gene expression could play other than that has been previously allocated to mature IGF-1 protein.

IGF-1 signaling has been shown to increase protein synthesis as well as induce cell proliferation and differentiation in muscle cells. In addition various studies have shown conclusively that IGF-1 can promote hypertrophy both in vitro and in vivo (Marsh et al 1997, Musaro et al 1999, and Allen 1999). The results of this study support the hypothesis that local IGF-1 acts as a positive regulator of
muscle growth and may do so by inducing satellite proliferation and differentiation as indicated by the correlated increased expression of MyoD and MGF in response to overload.

However, the study does not comprehensively address the question as to whether muscle adaptation to overload is directly coupled and mediated by an increase in IGF-1. IGF-1 as well as other trophic growth factors have been shown to act as initiators of load-independent hypertrophy muscle hypertrophy in vitro. In addition an increase in mechanical input to muscle in vivo is accompanied by an increase in IGF-1 as well as muscle mass (reviewed by Booth 1999). The two separate observations have led to the suggestion that mechanical stimulus is coupled to muscle growth via a mechanism transmitted through the actions of growth factors such as IGF-1. However, in view of the conservation of signaling pathways activated by the different growth factors as well as mechanical input, it is more likely that there are conserved pathways of positive regulation of muscle growth, which act synergistically to increase muscle mass in response to overload. In support of this hypothesis, recent evidence has shown that the PI (3)K/Akt pathway promotes hypertrophy by activating protein synthesis (Rommel et al 2001 Bodine et al 2000). This pathway is also activated in response to integrin signaling as a result of muscle stretch and also conserved among the activation of other tyrosine kinase receptors.

6.2 IGF-1 and Muscle Atrophy
Muscle atrophy occurs as a consequence of denervation, injury, joint immobilization, bed rest, glucorticoid treatment, cancer and aging (Jagoe and Goldberg 2001). The maintenance of muscle mass is thought to be controlled by a balance between protein synthesis and protein degradation pathways which shift towards protein degradation during atrophy. The decline of endocrine or circulatory IGF-1 was initially proposed to be a factor leading to muscle frailty during ageing. Recently the attention has turned towards the central role of muscle local IGF-1 in hypertrophy. IGF-1 as a positive regulator of hypertrophy has therefore emerged as an attractive candidate to mediate both hypertrophy and atrophy in muscle. Various reports have proposed that reduced IGF-1 signaling is
involved in muscle atrophy and results from decreased muscle exercise, reduced GH and insulin levels as well as treatment with drugs like corticosteroids (Bross 1999).

In my thesis, the model of spinal transection was used to stimulate muscle atrophy in adult rats. The rapid decrease in muscle mass accompanied by a loss in myonuclei was attributed to the reduced electrical activity to the muscle as a result of direct damage to nerves in the spinal cord. As with other studies a reduction in IGF-1Ea or MGF mRNA levels in muscle tissue did not accompany atrophy. However it is possible that the loss of innervation may lead to IGF-1 resistance or inhibition of IGF-1 downstream signaling pathways, which may therefore the direct effect of reducing cellular effects of IGF-1 and therefore may tip the balance in favor of protein degradation.

The recent discovery of conserved molecular markers for multiple models of skeletal muscle atrophy have suggested that there may be common atrophy-inducing pathways. Muscle atrophy, is linked to protein degradation systems which appear to be regulated by ubiquitin ligases (Bodine et al 2001) These pathways appear to be distinct from the pathways promoting muscle hypertrophy and therefore may indicate that there are both defined positive and negative regulators of muscle growth.

Passive cycling of the transected rats attenuated spinal transection-induced atrophy and it was hypothesized that in a similar pattern to that of overload-induced hypertrophy model, local IG-1 isoforms would be upregulated. However, Both IGF-1Ea and MGF mRNA levels within muscle did not change in response to either spinal transection or passive cycling after spinal transection. The attenuation in muscle atrophy and rescue of apoptotic nuclei suggested that a physiological change had taken place in response to the stretch.

It therefore appears that another signalling pathway can act to increase protein synthesis and prevent apoptosis in response to stretch. Clearly, the regulation of IGF-1 gene transcription and translation in muscle is complex and even
comparing the two positive stimuli (mechanical overload as opposed to passive stretch), there appears to be a different response. These combined results may suggest that there exist thresholds at which different signal are triggered resulting in graded responses. Hence there would be increased recruitment of local growth factors with increased mechanical stress as defined by the extent of stretch and/or overload. Cellular damage to the muscle tissue during severe exercise regimes would also enhance the release of inflammatory cytokines, which would in turn coordinate the expression of other synergistic trophic growth factors such as EGF and HGF.

6.3 IGF-1 and Neuronal Injury

Neurotrophic factors regulate the growth and survival of selected populations of neurons in the CNS and PNS. An essential role for neurotrophic factors has been established in neuronal development and maintenance of normal activities in adulthood, with IGF-1 exerting potent effects on motor neurone survival and nerve sprouting in targeted muscles (Caroni et al 1990). Evidence from animal studies has indicated that IGF-1 receptor mRNA and protein are widely expressed in the spinal cord including the motor neurone (Ishii et al 1994) Furthermore, IGF-1-like immunoreactivity has been localized in both spinal motoneurones and their innervated muscles and is known to be transported rapidly in both an anterograde and retrograde manner in the sciatic nerve (Pu et al 1999).

It was therefore expected in the spinal transection model used in my thesis that IGF-1 expression would increase in the lesioned area after injury. Although an increase in IGF-1 expression has been detected in other spinal transection models after injury, no other studies have differentiated between the expression of the two IGF-1 transcripts. Once again, the two local isoforms were differentially regulated with MGF increased after exercise while IGF-1Ea gene expression remained level.
There is growing evidence that exercise of muscles after spinal transection or injury, appears to increase neuronal sprouting and regeneration around the lesioned area, as well as attenuate muscle atrophy. Given the well-established protective effects of IGF-1 in response to a variety of cellular insults, I hypothesized that local IGF-1 alone, or in combination with other trophic factors would play a role in the rescue of neuronal tissue in the injury zone, in response to exercise. Both IGF-1 isoforms IGF-1Ea and MGF were increased in the lesioned area, but only IGF-1Ea increased in the lumbar region further removed from the area of injury. The findings in this study support the numerous reports that show that IGFs, their receptors and binding proteins are induced following traumatic injury to the nervous system and have a neuroprotective effect.

As discussed in the section above, passive exercise was accompanied by the attenuation of muscle atrophy. This effect is likely to be linked to the regenerative effect seen in the transected nerve. As there was no increase in IGF-1 expression in the muscle, it is not clear how the effects of exercise are transmitted to the thoracic spinal cord. To date, there is no clear description of the mechanism by which molecules could transmit signals from the nerve to the muscle or vice versa.

The early increase in MGF suggests that this isoform may play a role in reducing the incidence of infarction and neuronal loss soon after spinal transection. As observed in the muscle response to mechanical overload, there was differential expression of the two local IGF-1 isoforms after spinal transection. It is possible that MGF acts in an early protective role in response to various cellular stresses to mimic that of stress proteins such as heat shock proteins.

Alternatively the different E peptides may determine the half-life of the two isoforms either through a proteolysis or through regulating IGFBP binding. It is however not clear what the significance of having the same mature peptide as opposed to different E peptides would be. Indeed the answer to the different roles of the isoforms will lie in the fate of the E peptides. Further studies are required to determine what role the E peptide plays in regulation of IGF-1 activity.
6.4 IGF-1 and Ageing

The striking loss of skeletal muscle mass and strength that occurs in very old animals and humans has been attributed to a number of different effects including impaired muscle regeneration, under-nutrition and lack of vitamin D, decreased hormone levels (e.g. GH/IGF-1, testosterone, oestrogen), reduced physical activity and a loss of nerves that innervate the muscles (Bross 1999). Different organs in the whole organism appear to age at different rates such as the function of reproductive organs in human females, declining (menopause) long before the age-related weight loss in muscle, spleen and liver. In addition there appear to be a complex pattern of individual variation that is partially genetically-biased (Kirkwood 2001). In reflection of the variation and complexity of ageing in the whole organism, senescence in muscle appears to have a more detrimental effect on the fast type 2B myofibres, than on the slow type. However, there is an overall decline in the function of muscle during senescence, which is conserved even in healthy and active old people (Carlson and Faulkner 1998). Over the last decade, reduced GH/IGF-1 has been explored as a probable cause for ageing in muscle. However, no conclusive evidence has been found linking the rate of ageing directly to the decreased levels of GH/IGF-1 signalling. With the recent evidence, showing that postnatal growth depends on autocrine/paracrine IGF-1 rather than hepatic IGF-1, the focus has turned on the central role of local forms of IGF-1 particularly in muscle.

In particular, my thesis sought to define whether there is a decline in local IGF-1 that either causes the loss in muscle strength or reflects impaired plasticity of muscle during senescence. There was no difference between baseline levels of IGF-1 isoforms between the young and old animals. However following muscle overload, which can be defined as a challenge to muscle adaptive capacity, MGF levels in old muscle did not increase to the levels of the young muscle. This seems to suggest that ageing in muscle results in a reduction in muscle plasticity, which is reflected by the attenuated rise in MGF, IGF-1 receptor and MyoD mRNA levels after synergistic overload in the old versus young muscles.
The focus on how to clinically prevent age-related muscle wasting and motor neurone loss has recently swung from of patients with systemic GH/IGF-1 supplementation to examining the possibilities of boosting local muscle forms of IGF-1. Encouraging results have been recently obtained from the overexpression of muscle IGF-1Ea and transgenic mice overexpressing a localized muscle form of IGF-1 (Musaro et al 2001, Charkravarthy et al 2000). These results have observed a reversal of age-related muscle wasting with remarkable conservation of muscle strength in senescent mice. In conjunction with the results obtained in my thesis there appears to be possibility that an impairment or reduction in local muscle IGF-1 signalling may be correlated with ageing in muscle. What is not clear is how these results fit into the theories that are now seeking to reveal how cellular ageing is related to the general senescent phenotype, in this case how a reduction in local MGF release can be linked to the wider observation of a decline in muscle wasting and ultimately death.

In one of the current view of ageing (the disposable soma theory) it is proposed that longevity requires investments in somatic maintenance and repair that must compete against investments in growth, reproduction and activities that enhance fitness (Kirkwood and Austad 2000). This theory suggests that there is a finite amount of metabolic resources upon which all survival mechanisms required by an organism to combat intrinsic deterioration (DNA damage, protein oxidation, etc) can be drawn from. Therefore the competition for metabolic resources between reproduction and bodily maintenance has been settled in such a way that somatic cells are repaired at a level that is high enough to keep the body in good shape through the all important years, but not high enough to keep it going forever (Kirkwood et al 2000). In this hypothesis, senescence can be attributed to the myriad of tiny faults that accumulate throughout the lifetime of an organism. Ageing in tissues is the sum of the cellular and molecular damage imposed on the body by living. Therefore genes of particular importance for ageing and longevity are likely to be those governing the durability and maintenance of the soma.
Almost all cells in the body express IGF-1 receptor ubiquitously. Therefore the effects of circulatory IGF-1 as well as any locally-produced IGF-1 is able to exert its effect on various parts of the body. GH/IGF-1 therefore would present itself as an attractive candidate as a gene determining the rate of senescence in different tissues (Winograd et al 1991). The somatomedin hypothesis accordingly was extended to propose that age-related muscle wasting was attributed to a decline in GH/IGF-1 in elderly people. However, recent evidence may be now pointing a finger towards the role of local IGF-1 isoforms in somatic maintenance rather than circulatory IGF-1. In many somatic and reproductive cells, local IGF-1 coordinates growth, differentiation and maintenance. Clearly in muscle, IGF-1 is a positive regulator of growth and also enhances healing and regeneration in response to cellular damage in both myogenic and neuronal cells. Therefore on a fundamental level, IGF-1 functions to encourage somatic repair and maintain myofibre size. The reduced capacity of ageing muscle to produce MGF after exercise may either indicate a reduced capacity for repair therefore reflect the impaired function of or be a cause for the reduced plasticity of senescent muscle. Although in my thesis I was unable to address the question of why muscle would age, the reduced ability of senescent muscle to upregulate MGF and therefore maintain somatic maintenance, would be likely to accelerate the deterioration of muscle tissue.

This hypothesis may appear contradictory in the light that exercise, in particular weightlifting is encouraged to combat muscle wasting in elderly people (Tseng et al 1995). However weightlifting not only results in an increase in MGF levels (albeit an attenuated increase compared to that in young animals) it also results in an increase in IGF-1 activity, including an upregulation of IGF-1 receptor signaling, muscle sensitivity to IGF-1 and also IGFBPs with stimulatory action such as IGFBP-5. This would have the effect of not only decreasing muscle atrophy, but also encouraging neuronal survival as shown in my thesis. Therefore, despite the danger of deleterious cellular damage by exercise, the likely net effect of a moderate exercise regime would be to combat muscle wasting (Lacour 2002). It is likely that in elderly individuals muscle weakness after to illness, due to under-nutrition, or reduced physical activity would benefit
from supplementation of local muscle IGF-1 combined with simple stretches, similar to passive cycling. This regime could be used to kick-start their recovery process. Given that muscle wasting has been shown as a strong predictor of mortality, local muscle supplementation should be considered as a serious contender to enhance the quality of later life.

6.5 IGF Signalling and Regulation: Seeing The Bigger Picture

Local modulation IGF-1, particularly transcription of autocrine splice variant MGF, is stimulated by mechanical loading as well as some component of neuromuscular activity. During hypertrophy increased levels of muscle activation appear to result in greater MGF, IGF-1R and IGFBP-5 expression. On the other hand, it appears that absence of neuromuscular activity affects the expression of IGF-1 gene, as passive cycling of spinal transected muscle does not appear to
activate MGF expression in muscle. My thesis supports evidence that IGF-1 is a positive regulator of muscle growth, but may not determine muscle atrophy. However, it is likely that the signalling pathways that control proteolysis and protein synthesis are likely to converge downstream. There is now increasing evidence that this convergence occurs downstream the IGF-1R signalling pathway. The questions that still go unanswered revolve around the exact nature of signals resulting from varying levels of neuromuscular activity. Whether the signals be mechanical, electrical and/or biochemical, it appears to regulate local IGF-1 system in a very specific way.

If local muscle response is partially mediated either by modulation of autocrine/paracrine IGF-1, or its downstream signalling pathways, it raises the question as to what role is played by the circulating GH and/or IGF-1. GH is likely to have some direct impact on muscle tissue through its GH receptors and also to modulate muscle insulin response. Systemically, in adult muscle tissue it appears that hepatic IGF-1 regulates the insulin signaling (Yakar et al 2001) while on a local level, IGF-1 controls cellular growth, protein synthesis and apoptosis (Butler and LeRoith 2001). In this light, IGF-1 signalling is centrally involved in the deployment of metabolic resources both on a systemic and local level.

The genes identified from several studies in model organisms as affecting ageing are rate also control the energy metabolism, e.g. via the insulin/IGF-1 signalling pathway (Gems and Patridge 2001). The ageing studies may start to shed light on why two systems on a local and systemic level may have evolved to regulate the same gene on a systemic and local level. Therefore as shown on this thesis local IGF-1 is necessary for the maintenance and repair of the somatic cells such as muscle tissue. On the other hand, GH control of circulatory IGF-1 (even as a sum of muscle and hepatic IGF-1) serves to coordinate both nutrition through insulin metabolism and pubertal growth spurt including the maturity of reproductive organs.
Studies in drosophila and C. Elegans have shown that IGF-1/Insulin signaling pathway appear to predict the lifespan (Gems and Partridge 2001). These observations have suggested controversially that increased IGF-1 levels may shorten the lifespan of organisms. Observations of mutant mice with GH-deficiency hormones also appear to support this theory (Flurkey et al 2001). Perhaps there is a suggestion that in early adulthood, selection supports the a trait that promotes survival and reproductive success even if that trait leads directly to loss of fitness and even death of the organism at later ages (Kirwood 2002).

In this case, systemic IGF-1 would promote fertility and strength in young adults, but with increased age, the anti-apoptotic effects of IGF-1 signalling would lead to the escape of damaged cells and therefore increase the risk of developing cancer. Therefore circulatory IGF-1 levels may decline to protect proliferating cells. The paradox is that post-mitotic cells such as muscle and neurons require a greater level of maintenance and repair and would be exposed to greater damage with the decline in systemic IGF-1. Therefore in particular, muscle tissue may have been programmed to regulate local IGF-1 isoforms so as to provide a mechanism to maintain muscle function in response to local growth, while preserving the overall survival of the organism. Therefore IGF-1 isoforms such as MGF may prove to have a short-lived effect that would only promote local growth and not contribute to an increase in systemic IGF-1. Such short-lived action would protect other proliferating cells from possibly deleterious effects such as malignant transformation.

A long-held theory was that there was a gene/mechanism that measured lifespan and then activated the destructive process (Kirkwood and Austad, 2000). However, recent research into ageing as well as evolutionary theories reject the proposal that organisms are programmed to die, as the ageing gene concept suggests and instead supports the hypothesis that organisms are programmed to survive. The problem is that in spite of the formidable array of mechanisms most species appear not to be programmed well enough to last indefinitely. Thus at a fundamental level there is a positive relation between IGF-1’s basic cell maintenance and repair systems and protection against the damage that causes
ageing. However the tradeoff is that as the organism ages, there is an accumulation of damage to cells. These cells are either deleted by apoptosis or their replication is blocked by stress-induced senescence. But this either decreases the number of cells that are available for maintenance or requires another cell to divide an extra time. Cell division increases the risk of somatic mutation, and so the grim reaper gathers his harvest in the end (Kirkwood 2002).

Future work is needed to determine what signals allow muscle tissue to activate different isoforms as well as to distinguish between the systemic and local effects. Monitoring the effects of translation of local IGF-1 peptides MGF and IGF-1Ea should also provide a fascinating insight into the role of the local growth factors, particularly during senescence.
References


hormone (GH), and IGF-I plus GH on body growth and IGF binding proteins in hypophysectomized rats. Endocrinology 137(5):1913-20.


186. Siegfried JM, Kasprzyk PG, Treston AM, Mulshine JL, Quinn KA, Cuttitta F. 1992. A mitogenic peptide amide encoded within the E peptide domain of


The tables below summarise the raw data from various lightcycler QC-RT-PCR runs. Each table has a graph drawn from the corresponding data as indicated in each legend.

Gene expression was determined in various rat age groups after tendon ablation in young (Y, 3month), mature (M, 13month) and old (O, 24month) samples. Control (C) refers to the control leg, while Experimental (E) is in reference to the overloaded leg. The samples had to be run in batches in order to minimise inter-assay variation and the limitations of space within the PCR machine. Only the values of the samples which were run twice were included.

GAPDH Gene Expression
The table below corresponds to Fig 2.5, which shows changes in GAPDH gene expression 5 days after tendon ablation.

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<th>Mean</th>
<th>SD</th>
<th>SE</th>
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IGF-1Ea Gene expression
Corresponds to Figure 3.2A, showing the effects of overload on both the soleus and plantaris muscle 5 days after tenotomy.

<table>
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<tr>
<td>OE</td>
<td>6</td>
<td>258.900</td>
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</tr>
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MGF Gene Expression

The data below corresponds to Figure 3.2B, measuring changes in MGF mRNA levels five days after tenotomy.

<table>
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</table>

MGF Timeline

The data below is plotted on Fig 3.3, which shows changes in MGF gene expression over days 1-to-3 after tendon ablation. All the samples were taken from the soleus muscle. In each group n= 4.

<table>
<thead>
<tr>
<th></th>
<th>day 0</th>
<th>day 1</th>
<th>day 2</th>
<th>day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>11.230</td>
<td>20.658</td>
<td>25.870</td>
<td>32.562</td>
</tr>
<tr>
<td>SD</td>
<td>2.706</td>
<td>0.838</td>
<td>0.279</td>
<td>0.379</td>
</tr>
<tr>
<td>SD</td>
<td>0.695</td>
<td>0.850</td>
<td>0.557</td>
<td>0.726</td>
</tr>
</tbody>
</table>

MyoD timeline

This data corresponds to the plotted graph on Fig 3.5, which is meant to correlate changes in MyoD over 3 days which changes in MGF expression in Fig 3.3. In each group, n= 4 (each animal had control and experimental values and therefore day 0 was set at the mean of all control values). All the samples were taken from the soleus muscle as above.

<table>
<thead>
<tr>
<th>Gene expression</th>
<th>day 0</th>
<th>day 1</th>
<th>day 2</th>
<th>day3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>2.618</td>
<td>11.168</td>
<td>5.403</td>
<td>2.948</td>
</tr>
<tr>
<td>SD</td>
<td>0.969</td>
<td>1.446</td>
<td>0.211</td>
<td>0.771</td>
</tr>
<tr>
<td>SE</td>
<td>0.280</td>
<td>0.723</td>
<td>0.105</td>
<td>0.385</td>
</tr>
<tr>
<td>Old</td>
<td>9.668</td>
<td>12.253</td>
<td>7.071</td>
<td>7.647</td>
</tr>
<tr>
<td>SD</td>
<td>1.527</td>
<td>0.014</td>
<td>3.250</td>
<td>3.227</td>
</tr>
<tr>
<td>SE</td>
<td>0.441</td>
<td>0.007</td>
<td>1.625</td>
<td>1.6135</td>
</tr>
</tbody>
</table>
IGF-1 Receptor Gene Expression

The table below consists of raw data used to plot Fig 4.4, showing changes in IGF-1 receptor gene expression 5 days after tenotomy. Samples were taken from both the soleus and plantaris muscles.

<table>
<thead>
<tr>
<th>Gene Expression</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>YC</td>
<td>7</td>
<td>3.101</td>
<td>1.431</td>
<td>0.5410</td>
</tr>
<tr>
<td>YE</td>
<td>7</td>
<td>6.199</td>
<td>3.101</td>
<td>1.1720</td>
</tr>
<tr>
<td>MC</td>
<td>12</td>
<td>4.607</td>
<td>2.979</td>
<td>0.8598</td>
</tr>
<tr>
<td>ME</td>
<td>12</td>
<td>7.705</td>
<td>4.577</td>
<td>1.3214</td>
</tr>
<tr>
<td>OC</td>
<td>10</td>
<td>4.182</td>
<td>2.067</td>
<td>0.6536</td>
</tr>
<tr>
<td>OE</td>
<td>10</td>
<td>5.314</td>
<td>3.049</td>
<td>0.9641</td>
</tr>
</tbody>
</table>

Changes in IGFBP-5 Gene Expression

The table below shows the raw data used to plot Fig 3.4, which illustrates the changes in IGFBPs 4 and 5, five days after tenotomy. n=6 in each group.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>YC</td>
<td>20.8206</td>
<td>7.083</td>
<td>22.158</td>
<td>3.245</td>
</tr>
<tr>
<td>YE</td>
<td>43.615</td>
<td>10.878</td>
<td>31.920</td>
<td>4.036</td>
</tr>
<tr>
<td>MC</td>
<td>4.697</td>
<td>2.151</td>
<td>4.445</td>
<td>1.524</td>
</tr>
<tr>
<td>ME</td>
<td>24.594</td>
<td>9.568</td>
<td>5.615</td>
<td>1.490</td>
</tr>
<tr>
<td>OC</td>
<td>3.533</td>
<td>1.623</td>
<td>1.120</td>
<td>0.189</td>
</tr>
<tr>
<td>OE</td>
<td>12.974</td>
<td>2.059</td>
<td>2.781</td>
<td>0.318</td>
</tr>
</tbody>
</table>

Spinal Transection Experiments

In all the spinal transaction experiments all samples were taken from the soleus. In each group n=4. Both the tables below correspond to Fig 5.5A and Fig 5.5B respectively.

IGF-1 Expression
Sol Con | 17.17 | 0.289 | 0.145
Sol Tx Ex | 20.52 | 11.818 | 5.909
Thr Con | 20.36 | 11.384 | 5.692
Thr Tx | 20.54 | 6.948 | 3.474
Thr Tx Ex | 41.74 | 20.204 | 10.102
Lum Con | 0 | 0 | 0
Lum Tx | 0 | 0 | 0
Lum Tx Ex | 5.23 | 0.625 | 0.313

MGF Expression

<table>
<thead>
<tr>
<th>Samples</th>
<th>Gene expression</th>
<th>SD</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sol Con</td>
<td>3.26</td>
<td>0.043</td>
<td>0.021</td>
</tr>
<tr>
<td>Sol Tx</td>
<td>3.41</td>
<td>0.047</td>
<td>0.024</td>
</tr>
<tr>
<td>Sol Tx Ex</td>
<td>3.43</td>
<td>0.059</td>
<td>0.030</td>
</tr>
<tr>
<td>Thr Con</td>
<td>0.11</td>
<td>0.153</td>
<td>0.077</td>
</tr>
<tr>
<td>Thr Tx</td>
<td>1.38</td>
<td>0.823</td>
<td>0.412</td>
</tr>
<tr>
<td>Thr Tx Ex</td>
<td>3.94</td>
<td>1.398</td>
<td>0.699</td>
</tr>
<tr>
<td>Lum Con</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lum Tx</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lum Tx Ex</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>