An investigation of IGF-I isoforms in human skeletal muscle. The effects of age and exercise

Mahjabeen Hameed BSc (Hons)
Royal Free and University College Medical School

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This thesis is dedicated to the memory of my parents
Abstract

This thesis has focused on the expression of the different insulin like growth factor-I (IGF-I) isoforms (IGF-IEa, IGF-IEb and MGF) in human skeletal muscle, with specific reference to exercise and the ageing process. To enable this, a method of real time quantitative PCR was developed for the accurate detection and quantification of these splice variants. Studies were performed both in our laboratory and at the Copenhagen Muscle Research Centre, Denmark.

The acute response of IGF-IEa and MGF to a single bout of high resistance exercise was studied in young (n=8, age 29.5±1.5 years) and old (n=7, age 74.4±1.8 years) subjects. At rest there were no significant differences between resting levels of either isoform between the young and old subjects. The study showed that when measured 2½ hours after the end of the exercise bout, the mechano-sensitive isoform, MGF, was significantly upregulated in the young but not the old subjects (P<0.05). No change was observed after exercise in the IGF-IEa isoform in either age group suggesting that the isoforms were differentially regulated. Furthermore, in situ hybridisation using oligo probes specific for MGF mRNA confirmed its localisation within the muscle fibres. No significant correlation was observed between the change in MGF expression and the muscle myosin heavy chain isoform composition was observed.

A second study in young (n=10, age 20-27 years) and old subjects (n=10, age 67-75 years) evaluated the effects of a single bout of prolonged (1 hour) eccentric muscle damaging cycling exercise. In this study, and in contrary to the weightlifting study, a significant increase was observed in MGF mRNA levels in both the young and old subjects after the end of the exercise challenge (P<0.05), whereas levels of IGF-IEa again showed no significant increase. Thus suggesting that older muscle might require some myofibrillar disruption and/or sarcolemmal damage caused by eccentric exercise as a prerequisite for stimulating MGF expression.

The effects of a period of resistance training in older people (n=19, age 70-82 years) performed over 12 weeks revealed a significant increase in the mRNA of both MGF and IGF-IEa and also the third isoform detected, IGF-IEb (P<0.05). In addition, the role that growth hormone (GH) might play in regulating the IGF-I splice variants in muscle with training was also investigated in this study. Recombinant GH administered to subjects without exercise, favoured expression of the IGF-IEa isoform. When resistance training was combined with GH administration, enhanced levels of MGF were observed but not IGF-IEa. This suggests that GH administration may lead to an overall upregulation of the primary IGF-I transcript, which is spliced towards MGF when mechanical activity in the form of resistance training is imposed.

The results of the work in this thesis have shown that it possible to detect and quantify IGF-I mRNA transcripts in human skeletal muscle, even those expressed at low levels. It has also shown that there may be differential regulation of the different isoforms in young and old muscle in response to exercise, which would support the idea that these isoforms of IGF-I have different physiological actions.
Acknowledgements

I would like to thank Professor Geoff Goldspink for giving me the opportunity to study for a PhD. His academic guidance and direction of the project has made this experience an invaluable one.

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<tr>
<td>ALS</td>
<td>Acid labile subunit</td>
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<tr>
<td>BHLH</td>
<td>Basic helix-loop-helix</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<td>GH</td>
<td>Growth hormone</td>
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<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<td>IGF</td>
<td>Insulin-like growth factor</td>
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<td>IGFBP</td>
<td>Insulin-like growth factor binding protein</td>
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<td>IGF-IR</td>
<td>Insulin-like growth factor I receptor</td>
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<td>IRS</td>
<td>Insulin receptor substrate</td>
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<td>IRE</td>
<td>Insulin regulatory element</td>
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<tr>
<td>MAP-kinase</td>
<td>Mitogen activated protein kinase</td>
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<td>MyHC</td>
<td>Myosin heavy chain</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PI3-kinase</td>
<td>Phosphatidylinositol 3 kinase</td>
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General Introduction

Muscle is a remarkably plastic tissue, altering its size and phenotype in response to challenges that may be placed upon it. It is able to respond to functional overload by increasing its size (hypertrophy). In contrast, removal of load caused by microgravity or joint immobilisation causes it to decrease its size (atrophy). Whilst the mechanisms which govern these different responses are still not fully understood, a number of key factors in the regulation of muscle mass have been identified. In addition, one of the most striking features of the ageing process is the loss of muscle mass, which has more recently been termed 'sarcopenia'. This loss of muscle mass determines to a large extent the loss of muscle strength and power that occurs with age. Such functional changes have important implications on the quality of life of the older person, in that it decreases their ability to perform simple everyday tasks as well as impairing its role as a dynamic metabolic store, endogenous store of heat and a form of protective padding (reviewed by Harridge and Young, 1998).

There is some evidence to suggest an age-related decline in the rates of muscle protein synthesis (Welle et al. 1993; Yarasheski et al. 1993; Welle et al. 1995; Balagopal et al. 1997; Hasten et al. 2000). Amino acids and proteins are the primary substrates for skeletal muscle mass maintenance. An increase in muscle mass will inevitably lead to a net gain in protein, whereas a decrease in muscle mass, a net loss. There is a fine balance between the factors that regulate protein synthesis and those that regulate protein breakdown. Hormones and growth factors that encode for proteins such as testosterone and IGF-I act to stimulate, while myostatin works as a negative regulator of protein synthesis. On the other side of the equation, calpain and proteosomes appear to function by regulating protein degradation. The complex
nature of these interactions are not yet fully understood but the work in this thesis has focussed on the expression of genes that regulate muscle protein synthesis.

It has long been accepted that strength and progressive resistance training leads to gains in muscle mass and strength in young individuals. Moreover, the responsiveness of aged muscle to strength training has also been confirmed by a considerable number of longitudinal studies (Frontera et al. 1988; Brown et al. 1990; Skelton et al. 1995; Welle et al. 1996; Harridge et al. 1998) in which elderly people have performed similar progressive resistance regimes as young people. Training studies in older people have also shown an increase in the rates of muscle protein synthesis (Yarasheski et al. 1993). However, the precise underlying mechanisms which trigger this increase have yet to be determined.

It is becoming increasingly more apparent that local growth factors produced by the muscle may play an important role in the adaptation of muscle to overload. In particular, the role of IGF-I as a positive regulator of muscle mass has been highlighted by a number of animal and cell culture studies. For example, genetic manipulation of IGF-I in animal muscle has shown that it has considerable anabolic effects in both young and old muscles (Barton Davis et al. 1998; Musaro et al. 2001). However, recent work has shown that the involvement of IGF-I is more complex than initially envisaged, with the individual splice variants of the IGF-I gene having been shown to have quite independent roles. In vivo animal studies (Yang et al. 1996; McKoy et al. 1999; Hill and Goldspink, 2003) and in vitro (Yang and Goldspink, 2003) experiments have suggested that the main isoform produced by the liver, IGF-IEa, and the locally produced activity sensitive, mechano growth factor
(MGF), are both positive regulators of muscle growth. In contrast to these animal and cell culture studies, there is little information concerning the role of these IGF-I splice variants in human skeletal muscle. This thesis is therefore concerned with the role that these different isoforms may play in the regulation of muscle mass with exercise, with specific relevance to old age.

**Aims**

The specific aims of this thesis are:

1) To develop a quantitative PCR technique to measure IGF-I isoforms in human skeletal muscle.

2) To study the acute effects of a single bout of high resistance exercise on the expression of different isoforms of IGF-I in the muscle of young and old individuals.

3) To investigate the acute effects of a prolonged bout of eccentric exercise on the different isoforms of IGF-I in the muscle of young and old individuals.

4) To determine the effects of a period of resistance training on IGF-I mRNA expression in the muscles of elderly individuals and the role that GH may play in modifying this response.
Chapter 1

Introduction and Review of the Literature
Muscle loss with age

The ageing process is associated with a number of physiological changes. One of the most striking of these is the loss of muscle mass (sarcopenia) (reviewed by Harridge et al. 1998). This loss of muscle mass is in part responsible for the decline in muscle function as we get older. This includes its mechanical function (i.e. generating force, power, or braking movement), its role as a dynamic metabolic store and generator of heat (Griffiths et al 2001). In addition, the decline in muscle mass also accounts for a significant proportion of the decline observed in maximal aerobic power with increasing age (Flegg and Lakatta, 1988). As physical function declines with age, simple daily tasks such as rising from a chair unaided or climbing stairs become increasingly more difficult, and eventually impossible to perform. This has important consequences for older people whose quality of life is reduced through a loss of independence and increased reliance upon others and also for the strain put upon health and social services charged with the care of an increasingly ageing population. The mechanisms by which muscle is lost with age and what possibilities there might be for restoring muscle mass and function in later life, are still not fully understood. However, interventions that augment muscle mass can help older men and women maintain or regain their ability to perform daily functional activities and live independently (reviewed by Harridge and Young, 1998).

Why is muscle lost with increasing age?

Sarcopenia is likely to be caused by a combination of several inter-related factors. At an anatomical level, it is clear that the loss of muscle with age reflects a reduction in the actual number of muscle fibres (Lexell et al. 1988). There is also electromyographical evidence to suggest that there is a reduction in the number of
functioning motor units as a consequence of the ageing process. Remaining / surviving motor units are larger in size, due to the re-innervation of abandoned and dying muscle fibres through a process of collateral nerve sprouting (Brown, 1972; Campbell et al. 1973; Sica et al. 1976). Thereby implying that part of the fibre population undergoes a denervation and re-innervation process with increasing age (Lexell et al. 1998). There are also a number of structural and compositional differences that are characteristic of ageing muscle such as an increase in the amount of connective tissue and intra-muscular fat (Frontera et al. 1991). These compositional changes can, in part, be explained by some fibres losing their capacity to be reinnervated. Having become permanently denervated, these fibres are eventually lost and replaced by fat and fibrous tissue. These changes in aged muscle reduce the contractile tissue volume available for locomotive and metabolic functions and act, presumably, as a friction brake to slow contractile velocity (Bross et al. 1999).

Histological analysis of aged muscle has shown a change from the traditional mosaic arrangement of type 1 and type 2 fibres into one in which fibres of the same type are ‘grouped’ together (Nygaard and Sanchez, 1982; Lexell et al. 1986; Lexell and Downham, 1991). It is thought that this grouping together in clusters of certain fibre types can be largely attributed to the age-related process of denervation and partial reinnervation mentioned earlier. Cross sectional analysis of muscle has shown that the characteristic angular shape of individual muscle fibres also changes with age. In the very elderly, many fibres appear flattened, crushed or even banana-shaped, with this shape change being more pronounced in the type 2 fibres (reviewed by Andersen, 2003). Lower proportions of the fast contracting myosin heavy chain
(MyHC) isoforms have also been reported in the vastus lateralis of aged muscle, which is in part due to an atrophy of the faster type 2 fibres (Klitgaard et al. 1990). This preferential atrophy of the fast twitch, type 2 fibres could be in part explained by their reduced reinnervation capacity compared with type I fibres (Tseng et al. 1995). Furthermore, an increase in the number of muscle fibres that express more than one MyHC isoform appears to be a characteristic of the ageing process (reviewed by Andersen, 2003).

Approximately 20% of muscle weight is protein (Tzankoff and Norris, 1977) therefore, changes in muscle mass will probably be associated with alterations in muscle protein turnover (which includes muscle protein synthesis and breakdown) (Brozek, 1952; Proctor et al. 1998). With age, there is suggested to be a general decline in whole body protein synthesis. However, it is now generally agreed that when normalised to fat free mass (FFM), there is very little difference, if any at all, in whole body protein turnover rates between young and old individuals (Fukagawa et al. 1988; 1989; Welle et al. 1993; Yarasheski et al. 1993; Welle et al. 1994; Benedek et al. 1995; Millward et al. 1997; Hasten et al. 2000; Volpi et al. 2001). Contrary to this, Balagopal et al (1997) reported an age-related decline of 20% in whole body protein turnover rates in elderly subjects, even after correcting for FFM. One limitation of whole body measurements is that they are susceptible to influence from dietary intake. The results of the aforementioned study could therefore, in part, be explained by the 5-day weight maintaining diet that was given to the elderly subjects prior to measurements being made. The protein requirements for elderly people have been suggested to be higher than those for young individuals (Pannemans et al. 1998). However, the question of protein requirements for elderly
people remains a difficult one. Work by Millward (in press) and co workers have shown that elderly people probably have no greater absolute need for protein, but that food with a high protein:energy ratio, rather than an increase in absolute protein requirements per day would be beneficial. Limiting the protein intake in these elderly subjects may have had the effect of further reducing whole body protein synthesis, particularly in the study by Balagopal et al. However, this was not the case in other studies of this kind where no effect of age on whole body protein synthesis was reported, even where controlled diet plans were used, albeit that they were for 3 and not 5 days (Welle et al. 1993; Yarasheski et al. 1993; Welle et al. 1994; Hasten et al. 2000). Muscle contributes less than 30% to whole body protein turnover. Therefore small changes in muscle protein synthesis or breakdown are difficult to detect using measurements of whole body protein turnover (Bross et al. 1999). Many studies have therefore measured muscle protein rates directly to determine the effects of aging on skeletal muscle and the results of these studies have been summarised in Table 1.1. Indeed, a number of authors have suggested that the basal rates of mixed muscle protein synthesis are lower in elderly people when compared with younger individuals (Welle et al. 1993; Yarasheski et al. 1993; Welle et al. 1995; Balagopal et al. 1997; Hasten et al. 2000). However, there is also conflicting evidence in the literature to suggest that basal rates of muscle protein synthesis are unaffected by age (Volpi et al. 1999; 2001). These discrepancies in basal muscle protein synthesis rates with age are further highlighted in some studies that report different rates of synthesis in the different muscle fractions. For example, although Balagopal et al. (1997) report similar rates of synthesis in the sarcoplasmic muscle fraction between young and old subjects, they also report lower rates of MyHC and mixed muscle protein synthesis in elderly when compared with young individuals. This was also
the case in the study by Hasten et al (2000), where rates of MyHC synthesis were lower in the elderly subjects but rates of mixed muscle protein synthesis were no different to the young. It is also possible that some workers controlling the diet and activity of the subjects whereas others (Volpi et al. 1999; 2001) allowing subjects to continue with their usual routine prior to making the measurements, may have caused some of these discrepancies in the results. As mentioned earlier on in this section, protein turnover includes both muscle protein synthesis and its breakdown. In order to determine protein turnover requires the measurement of both. Different groups using different methods to measure these parameters may have also caused the discrepancies in results. In the main, the studies reported above (Welle et al. 1993; Yarasheski et al. 1993; Welle et al. 1995; Hasten et al. 2000) used a non specific and relatively insensitive technique to measure protein breakdown, which involved measuring whole body 3-methylhistidine excretion. Work by Volpi and co workers however used a more direct approach, the AV balance method. This method relies on the measurement of phenylalanine enrichments and concentrations in the femoral artery and vein to estimate muscle protein synthesis, breakdown and net balance. Whether muscle protein synthesis rates are reduced with age is still the matter of much debate and much needs to be done to improve and standardise methods for reliable and consistent results.

The reasons why muscle fibres are lost, fast fibres atrophy and protein synthesis rates may be reduced in old age are not fully understood and remain the important questions concerning the aetiology of sarcopenia. Much work has focussed on the relationship between the age-related decline in levels of the growth hormone/insulin
like growth factor-I (GH/IGF-I) axis and muscle loss with age. This work has been evaluated in the next section.
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<td>Welle et al (1993)</td>
<td>Myofibrillar</td>
<td>480 min L-[1-1^3C] leucine</td>
<td>3 days controlled meat free diet + activity</td>
<td>All male</td>
<td>Lower in elderly</td>
</tr>
<tr>
<td>Welle et al (1995)</td>
<td>Myofibrillar</td>
<td>480 min L-[1-1^3C] leucine</td>
<td>3 days controlled meat free diet + activity</td>
<td>5.4; 5.4</td>
<td>Lower in elderly</td>
</tr>
<tr>
<td>Hasten et al (2000)</td>
<td>Myosin heavy chain</td>
<td>840 min L-[1-1^3C] leucine</td>
<td>3 days meat free controlled protein</td>
<td>4.3; 3.4</td>
<td>Lower in elderly</td>
</tr>
<tr>
<td>Balagopal et al (1997)</td>
<td>Myosin heavy chain</td>
<td>600 min L-[1-1^3C] leucine</td>
<td>5 days weight maintaining diet</td>
<td>4.4, 4.4; 4.4</td>
<td>Lower in middle aged and elderly</td>
</tr>
<tr>
<td>Balagopal et al (1997)</td>
<td>Mixed</td>
<td>600 min L-[1-1^3C] leucine</td>
<td>5 days weight maintaining diet</td>
<td>4.4; 4.4; 4.4</td>
<td>Lower in middle aged and elderly</td>
</tr>
<tr>
<td>Rooyackers et al (1996)</td>
<td>Mixed</td>
<td>240 min L-[1-1^3C] leucine</td>
<td>5 days weight maintaining diet</td>
<td>6.6; 7.7; 7.9</td>
<td>Lower in middle aged and elderly</td>
</tr>
</tbody>
</table>

Table 1.1 Basal muscle protein synthesis in young and elderly people. (Adapted from Dorrens and Rennie, 2003).
Growth hormone/IGF-I axis

Growth hormone (GH) is secreted from somatotroph cells located within the anterior pituitary gland in a pulsatile manner, with one of its main actions being synthesis of the anabolic peptide insulin like growth factor-I by the liver. The GH/IGF-I axis plays an essential role in postnatal growth and development. Levels of these hormones reach their peak during adolescence and are maintained at somewhat lower levels during adulthood. However, with increasing age there is a further decline in the circulating levels of GH and IGF-I. (Rudman et al. 1981; Corpas et al. 1993; Lamberts et al. 1997; Morley, 1995). The relationship between the age-related decline in the GH/IGF-I axis and loss of muscle mass and strength has been extensively studied. In young adults who were growth hormone deficient, the administration of recombinant GH (rhGH) was shown to have positive effects on muscle mass and function (Cuneo et al. 1991). Another study where GH deficient adults were treated with GH for an extended period of time concluded that there was not only increased muscle strength but also decreased body fat (Beshyah et al. 1995). This lead to the belief that older individuals with decreased levels of circulating GH and IGF-I would also benefit from rhGH therapy. However, studies which have combined GH administration and resistance training in both young (Yarasheski et al. 1992) and older men (Yarasheski et al. 1995) have shown that the rates of protein synthesis are no greater when resistance training is combined with GH than when resistance training is performed alone. Furthermore, in older people the changes in muscle mass and function have also been reported to be similar between both groups (Lange et al. 2002). The roles of circulating GH and IGF-I, particularly with regards to muscle adaptation in later life are still unclear. In support of an argument that systemic growth factors may be of relatively minor importance in muscle hypertrophy, is one study which showed that the overloaded muscles in hypophysectomized rats were
still able to hypertrophy despite significantly reduced systemic IGF-I levels (Adams and Haddad, 1996). These findings, coupled together with the simple observation that it is only challenged muscles which hypertrophy and not all the muscles of the body, highlights the importance of a 'local' system of muscle adaptation.

The somatomedin hypothesis

The original somatomedin hypothesis originated in the 1950's in early efforts to understand how somatic growth was regulated by factors secreted by the pituitary. It was determined that pituitary-derived GH did not act directly on its target tissues to promote growth, and that there were intermediary substances involved (Daughday and Reeder, 1966). The term 'somatomedin' was later adopted to reflect the growth promoting actions of these substances (Daughday et al. 1972), and some two decades later, IGF-I (and IGF-II) were characterised, with IGF-I being the somatomedin regulated by GH (Rinderknecht and Humble, 1978; Klapper et al. 1983). Both substances were termed 'insulin-like' due to their ability to stimulate glucose uptake into fat cells and muscle. The original hypothesis became the widely accepted model of IGF action: namely, that the effect of GH on longitudinal body growth was mediated in an endocrine fashion, solely by liver-derived IGF-I. This hypothesis was later revised in the 1980's when D'Ercole and co workers discovered that extra-hepatic tissues also expressed IGF-I, thus suggesting that IGF-I also had an autocrine/paracrine effect (D'Ercole et al. 1984). In 1985, an alternative view was proposed by Green and co-workers known as the 'dual effector hypothesis' (Green et al. 1985). This hypothesis suggested that GH had direct effects on peripheral tissues not mediated by IGF-I and that GH stimulated local IGF-I production for autocrine/paracrine action. However, current evidence suggests that the situation is
more complex than any of these early hypotheses envisioned. It is now clear that GH, in addition to stimulating hepatic IGF-I synthesis, stimulates the formation of a ternary IGF binding complex, including IGFBP-3 and the acid labile subunit (ALS), which stabilises IGF-I in the serum. Furthermore, the results of recent gene deletion experiments using the Cre-loxP model of gene deletion have questioned the role of liver IGF-I and the bound form of circulating IGF-I in controlling postnatal growth and development (Sjogren et al. 1999; Yakar et al. 1999). This homologous recombination system creates a liver specific deletion of the IGF-I gene and allows normal expression of this gene in other non-hepatic tissues, such as heart, muscle, fat, spleen and kidney. When testing the effect of this liver specific gene deletion of IGF-I on growth and development in these animals, it was found that circulating IGF-I levels were markedly reduced at six weeks of age compared with wild type animals. However measurements of body weight made from 3 to 6 weeks, body length and individual organ weights at 6 weeks were no different between the knockout animals and their wild type littermates. Thus, postnatal growth and development was considered normal without the contribution of liver derived IGF-I (Sjogren et al. 1999) and emphasising the role of the local IGF-I system in this process. A schematic representation of the somatomedin hypothesis is shown in Figure 1.1.
Figure 1.1 Schematic representation of the somatomedin hypothesis. (A) The original hypothesis proposed that the effect of GH on longitudinal body growth was mediated exclusively by IGF-I (originally termed somatomedin). (B) The revised hypothesis proposed that IGF-I was also expressed by most extra-hepatic tissues, thus suggesting a possible autocrine/paracrine role for IGF-I as well as endocrine. (C) Current evidence has shown that liver-derived IGF-I and the bound form are not essential for postnatal growth and development. Abbreviations: ALS (acid labile subunit), GH (growth hormone), IGF-I (insulin-like growth factor-I), IGFBP-3 (insulin-like growth factor binding protein-3). (Adapted from LeRoith et al. 2001).
**GH and the GH receptor**

GH belongs to a large family of peptides (Miller and Eberhardt, 1983) and is known to exert its effects by binding to the growth hormone receptor (GHR) (Bazan, 1989; 1990; Cosman et al. 1990). It circulates in a complex with its binding protein, growth hormone binding protein (GHBP). Upon binding to the hepatic GHR, GH induces the expression of IGF-I. As the liver has the highest expression of the GHR, it accordingly expresses a large fraction of circulatory/serum IGF-I. However, it is also known that other tissues including bone, muscle and the kidney also express the GHR and may therefore contribute to the levels of serum IGF-I. Circulating IGF-I inhibits GH secretion, providing a negative feedback loop on the actions of GH in peripheral tissues. Two hypothalamic factors, GH releasing hormone (GHRH) and the inhibitory hormone, somatostatin (SS), are known to act together to regulate GH secretion from pituitary somatotrophs. More recently, another GH-secretory factor that is derived from a peripheral organ has been isolated. Ghrelin, a 28-residue peptide, is expressed at high levels in endocrine cells of the stomach (Kojima et al. 1999). GH secretion is therefore likely to be controlled by a combination of hypothalamic and peripheral signals.

**GHR signal transduction pathways**

The actions of GH are mediated by its binding to the transmembrane GHR. As with most cytokine receptors, the GHR utilises the JAK-STAT signal transduction pathway. The activated GHR associates with JAK2 (Janus kinase 2). JAK2 is a tyrosine kinase, which upon activation by GH phosphorylates STATs 1, 5a, and 5b (signal transducers and activators of transcription) on tyrosines. Upon phosphorylation by GHR and JAK2, the STAT proteins translocate to the nucleus
where they bind to specific DNA sequences and activate gene transcription. Activation of the GHR also induces tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins IRS-1 and IRS-2. These behave as docking proteins to direct signals from the IGF and insulin receptors to various downstream signalling pathways. Activation of the insulin like growth factor-I receptor (IGF-IR) also stimulates the phosphorylation of these IRS proteins, thus providing for cross talk between GH and IGF-I at the level of signal transduction (Reviewed by Le Roith et al. 2001). IGF-IR signal transduction pathways are discussed in detail later on in this chapter.

GH stimulation has been shown to regulate the gene expression of IGF-I and many of the IGF binding proteins (IGFBPs). However, it is still not clear precisely how GH regulates IGF-I gene expression and whether the alternative promoters P1 and P2 on the IGF-I gene are differentially activated. The data on GH control of IGF-I promoters has only been generated by studies on hypophysectomized rats and was investigated in relation to GH control of hepatic tissue (Adamo et al. 1991). Mittanak et al. (1997) showed that GH activates both P1 and P2 equally. However, there is also evidence from studies on the ovine IGF-I gene to suggest that only transcripts initiating at P2 are GH sensitive (Lyall, 1996). In addition, there is evidence of constitutive activation of the P2 (the minor promoter) in non-hepatic tissue, which was unaffected by GH activity (Le Stunff et al. 1994), suggesting that there may be alternative regulatory elements within the promoter regions of the IGF-I gene, which allow for GH-independent, tissue specific expression.
The insulin like growth factor system

The insulin like growth factors (IGF-I and IGF-II) belong to a family of polypeptides with marked structural similarities to insulin and possess both anabolic and mitogenic properties both in vivo and in vitro (Blundel et al. 1983; Rinderknecht and Humble, 1978a; b). The IGF system is comprised of the ligands: IGF-I, IGF-II and insulin, six well characterised binding proteins, and cell surface receptors that mediate the actions of these ligands (IGF-IR, insulin receptor and the IGF-II mannose-6-phosphate [M-6-P] receptor). The work in this thesis focuses on IGF-I. The following sections will therefore describe this particular system in detail.

IGF-I gene structure

In both human beings and rats, the IGF-I gene spans more than 70 kilobases and consists of six exons and at least five introns (Rotwein et al. 1986; Shimatsu and Rotwein, 1987; Rotwein, 1991). Two promoters, one adjacent to exon 1 and the other to exon 2 govern gene transcription. Both promoters lack a “TATAA” box, a “CCAAT” box and other typical proximal control elements, yet neither resemble a GC-rich “housekeeping” promoter (Adamo et al. 1991; Jansen et al. 1991; Hall et al. 1992). Exons 1 and 2 are alternative leader exons derived from different transcription start sites, and encode part of the signal peptide (Jansen et al. 1983; Tobin et al. 1990). The resulting variant mRNA transcripts with different 5’ untranslated regions (UTR) have been classified as Type/Class 1 (exon 1-3) and Type/Class 2 (exon 2-3) (for schematic representation of the IGF-I gene see Figure 1.2). These 5’UTR mRNA variants are differentially regulated during development in a tissue specific manner (Lowe et al. 1988). Class 1 mRNA transcripts have been shown to be expressed in rat liver and a wide range of non-hepatic tissues both in prenatal and
postnatal animals (Hoyt et al. 1988; Adamo et al. 1989). In contrast, Class 2 mRNA transcripts were undetectable or barely detectable in non-hepatic tissues (Hoyt et al., 1988; Adamo et al, 1991), suggesting that the basal promoter associated with exon 1 is active in a wide range of tissues.

In addition to transcription from two promoters, IGF-I is regulated by post-transcriptional events, which yield several mature mRNA transcripts. In all of these variants exons 3 and 4, which encode the mature 70 amino acid peptide are constant (Bell et al. 1986; Gilmour, 1994) whereas, exons 5 and 6 are subject to a complex alternative splicing pattern. This is a complex mechanism by which exons are arranged in different combinations from pre-mRNA. It is an important and common process for generating protein diversity and regulating gene expression in higher eukaryotes (Chew, 1997).

**IGF-I primary structure**

The primary structure of IGF-I is similar to that of pro-insulin as it comprises an amino terminal B region and an A region that is separated by a short connecting C domain. Unlike pro-insulin however it also has a D region extension peptide and an E peptide at its carboxyl terminus (Lowe, 1985). The different E peptides are cleaved from the pre-pro-IGF-I to yield a single mature IGF-I peptide consisting of the B, C, A, and D domains (Kim et al. 1991; Jansen et al. 1991).
<table>
<thead>
<tr>
<th>IGF-I isoform</th>
<th>Names used in the literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-IEa</td>
<td>L.IGF-I&lt;sup&gt;1&lt;/sup&gt;, m-IGF-I&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>IGF-IEb</td>
<td>IGF-IEb (rats)&lt;sup&gt;3&lt;/sup&gt;, MGF&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>IGF-IEc</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2 The different names used to describe the IGF-I isoforms in the literature.

Figure 1.2. Schematic representations of the human IGF-I gene and its splice variants. Exons are shown as ‘boxes’, introns as ‘lines’. The arrows under exons 1 and 2 indicate multiple transcription start sites. Promoter elements 5’ to exons 1 and 2 are shown as P1 and P2 respectively. Polyadenylation signals are shown by the letter ‘A’. Splicing patterns are shown by ‘broken lines’. The protein-coding potential of the exons is indicated underneath the IGF-I gene. Exon 1 or 2 is spliced to exon 3 to give rise to either class 1 or class 2 mRNA transcripts.
Figure 1.3. Human exon 4-5 (Eb) and exon 4-5-6 (Ec) cDNA sequences aligned with rat exon 4-5-6 (Eb) cDNA. Homology to human Ec is denoted by the ‘asterisks’ under the sequence, exon boundaries are marked by the ‘broken lines’ and in-frame stop codons are underlined in human Ec and rat Eb. The numbering refers to the human Eb sequence cloned by Rotwein (1986). Taken from Chew et al (1995).
IGF-I mRNAs that encode different E peptides

Alternative splicing of exons 5 and 6 of the IGF-I gene results in IGF-I mRNAs encoding different E peptides. Exon 4 usually splices to exon 6 (Jansen et al. 1983), but can also splices to exon 5, representing 1-10% of IGF-I transcripts (Rotwein, 1986). A minor isoform results from splicing of exons 4 and 5, and from an alternative 5' splice site in exon 5 to exon 6 (Chew et al. 1995). A schematic representation of the human IGF-I gene and the splicing pattern of its splice variants is shown in Figure 1.2. 3' alternative splicing of the primary IGF-I gene generates six different IGF-I mRNAs in humans: Class 1 or 2 IGF-IEa, IGF-IEb and IGF-IEc, and these are indicated on the figure.

In the rat, the splicing pattern of exon 5 is different from that in the human as exon 4-6 or exon 4-5-6 splicing occurs (Roberts et al. 1987; Shimatsu and Rotwein, 1987), and exon 5 is a cassette exon of 52 base pairs (bp), rather than a terminal exon as it is in humans. This therefore generates 4 different IGF-I mRNAs: either Class 1 or Class 2 IGF-IEa and IGF-IEb. The nomenclature is highly confusing and it should be noted that the human IGF-IEc isoform corresponds to the rat IGF-IEb isoform. This confusion in the nomenclature has been further compounded by different groups further re-naming the isoforms. Table 1.2 shows the different names used for each of the IGF-I isoforms in the literature. Comparison between the human and rat cDNA and deduced peptide sequences suggests that the exon 4-5-6 splice variants in both species are equivalent (Roberts et al. 1987; Shimatsu and Rotwein, 1987). The deduced E-peptide sequences of the rat and human 4-5-6 mRNAs have been shown to share 73% homology and this has been shown in Figure 1.3. It has been suggested that the rat hepatic exon 4-5-6 mRNA variant may generate circulating and GH-
responsive IGF-I (Lowe et al. 1988; Lund, 1994). It is therefore likely that the human exon 4-5-6 splice may also play a role in generating circulating IGF-I (Chew et al. 1995).

The role of E peptides
As previously mentioned, IGF-I cDNAs predict at least four different mRNA species (six in humans) and at least two different IGF-I translational products (Daughaday and Rotwein, 1989). These different IGF-I precursors or pro hormones: proIGF-IA and proIGF-IB have identical initial sequences, which are encoded by exon 4 but different carboxyl-terminal extensions. Therefore IGF-I is synthesised as precursor molecules that are post-translationally processed to a biologically active mature peptide. It is believed that transcripts encoding proIGF-IA are more abundantly expressed than those encoding proIGF-IB (Nagaoka et al. 1991). The physiological roles of these precursors and those of the cleaved E peptides (Ea and Eb) are essentially unknown. However, the significant sequence homology for IGF-I during evolution would suggest that these E peptides or pro hormones may have important biological functions independent of that of IGF-I (Wilson et al. 2001). An amino acid sequence within the E domain of proIGF-IB was shown to have mitogenic activity in normal and malignant bronchial epithelial cells (Siegfried et al. 1992). More recently, work by Tian et al (1999) demonstrated that recombinant E-peptides, corresponding to sequences in the carboxy terminal of proIGF-IA from rainbow trout, possessed mitogenic activity in heterologous systems. In addition, Yang and Goldspink (2002) reported that the IGF-IEc peptide had a different role to that of mature IGF-I in myoblast proliferation and differentiation. This work and later work by Hill and Goldspink (2003), suggests that the IGF-IEc peptide is involved in the activation of satellite cells. Studies of this kind would therefore suggest that the
different E peptides of IGF-I have distinct biological roles, independent to that of IGF-I.

**IGF-I receptor**

The cellular effects of IGF-I are mediated by its cell surface receptor, the IGF-IR (Figure 1.4). The IGF-IR is a heterotetrameric glycoprotein composed of two extracellular α-subunits and two transmembrane β-subunits. The IGF-IR is similar in structure to the insulin receptor and both receptors share >50% amino acid identity. The α-subunits contain the ligand binding region of the receptor, while the β-subunits are composed of a short extracellular domain, a membrane spanning segment and a large cytoplasmic region containing a tyrosine kinase domain and sites of tyrosine and serine phosphorylation (Reviewed by Stewart and Rotwein, 1996).

![Diagram of IGF-IR](adapted_from_stewart_and_rotwein,1996)

**Figure 1.4.** IGF-IR. Functional domains within the α and β chains are labelled and disulfide bonds responsible for maintaining receptor heterotetramer are indicated by the 's-s'. (Adapted from Stewart and Rotwein, 1996).
Ligand binding to the receptor results in a conformational change whereby the α and β-subunits are drawn together. This then leads to receptor autophosphorylation in the tyrosine kinase domain. This tyrosine phosphorylation is an essential activating step for receptor function.

It is known that the biological activity of a hormone or growth factor does not simply reflect the level of hormone or growth factor, but also depends on the concentration of the receptor and the affinity of its interaction. There is evidence that there is an impairment of both IGF-IR density and signalling in aged skeletal muscle (Renganathan et al. 1997). However, IGF-IR number, binding capacity and affinity were shown to increase in the soleus muscle of old mice after physical exercise (Willis et al. 1998), suggesting that old animals retain plasticity for IGF-1R.

**IGF-IR signal transduction pathways**

This intrinsic tyrosine kinase activity of the IGF-IR mediates the different biological actions of IGF-I by phosphorylating endogenous cellular substrates. Many of the substrates that have been identified are also phosphoproteins, with insulin receptor substrate-I (IRS-I) being the most well characterised. IRS-I behaves as a multi-site ‘docking’ protein, which upon phosphorylation associates with other intracellular proteins with src homology 2 (SH2) domains (LeRoith et al. 1995). Different proteins containing SH2 binding domains bind to IR-S such as Grb2 Nck (adaptor proteins), c-Crk (a Grb2 and Nck homolog), Syp a protein tyrosine phosphatase (SH-PTP2) and the p85 subunit of phosphatidyl-inositol-3 kinase (PI3-kinase). The recruitment of Grb-2 through IRS-1 leads to the activation of the ras mitogen-activated protein (MAP) kinase pathway. Activation of PI3-kinase leads to activation
of the enzyme p70 S6 kinase, which may be involved in mitogenesis (LeRoith et al. 1995). Other proteins activated by phosphorylation include extracellular signal related kinases 1 and 2 (ERK-1 and ERK-2).

**IGF binding proteins**

Over 97% of IGFs are bound to members of a family of six proteins, the IGF binding proteins (IGFBPs 1-6), which bind IGF-I and IGF-II with an affinity equal to or greater than that of the IGF-I receptors (Jones and Clemmons, 1995). These IGFBPs are modulators of IGF action and essentially serve to: transport IGFs from the circulation to peripheral tissues, maintain a reservoir of IGFs in the circulation, potentiate or inhibit IGF action and mediate IGF-independent biological effects (Reviewed by Stewart and Rotwein, 1996). The actions of the individual binding proteins is summarised in Table 1.3.

IGFBPs are highly conserved cysteine proteins that vary in length from 216 to 289 amino acids. Structurally, IGFBPs have a conserved N-terminal domain involved in IGF binding, and a carboxyl terminal, that is important for protein-protein or protein-extracellular matrix (ECM) interactions (Hwa et al. 1999). The central portion of each binding protein is unique and commonly subject to proteolysis. Although IGF-I is hought to bind largely around the highly conserved cysteine residues, the carboxyl terminal does play a role in ligand recognition, as cleavage of the domain greatly reduces the binding affinity for IGF-I (Reviewed by Rosenfield et al. 2000).

In the circulation, over 90% of IGF-I is found as heterotrimeric complex bound to the most abundant binding protein, IGFBP-3, and the acid labile subunit (ALS). ALS, as
the name suggests, loses its ability to form ternary complexes and is irreversibly destroyed under acidic conditions (pH<4.5). Its main physiological role is thought to be to extend the half lives of IGFs from 10 minutes, when in the free form, and 30-90 minutes when in binary complexes, to more than 12 hours when bound in ternary complexes. Another role of ALS is the prevention of the non-specific metabolic effects of the IGFs, such as causing severe hypoglycaemia (reviewed by Biosclair et al. 2001). Other IGFBPs found in the bloodstream include IGFBP-1, IGFBP-2 and IGFBP-4, but are present at much lower concentrations. IGFBP-4, IGFBP-5 and IGFBP-6 are expressed in many muscle cell lines and have also been reported to be present in muscle tissue in vivo (Ewton and Florini, 1995; Bayol et al. 2000). There is some evidence to suggest that alterations in IGFBP expression, namely IGFBP-4 and IGFBP-5 occur in unloaded or overloaded soleus muscle, and that this may play a role in skeletal muscle adaptation to changes in loading (Bonaventure et al. 1999).
<table>
<thead>
<tr>
<th>IGFBP</th>
<th>Potential functions</th>
<th>Cell surface/matrix binding</th>
<th>Modification by proteolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP-1</td>
<td>Minor serum carrier; potentiates/inhibits IGF action</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>IGFBP-2</td>
<td>Minor serum carrier; potentiates/inhibits IGF action</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>Major serum carrier; potentiates/inhibits IGF action: IGF-independent modulator of cell proliferation</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>IGFBP-4</td>
<td>Inhibits IGF action</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>IGFBP-5</td>
<td>Potentiates/inhibits IGF action</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>IGFBP-6</td>
<td>Potentiates/inhibits IGF action</td>
<td>No</td>
<td>Possibly</td>
</tr>
</tbody>
</table>

Table 1.3. Actions of the individual IGFBPs. (Adapted from Stewart and Rotwein, 1996).
Autocrine/paracrine IGF-I

As previously mentioned, almost all tissues express IGF-I, including skeletal muscle. It is also clear that GH induces IGF-I expression in many of these tissues and that IGF-I can have GH independent actions. For example, GH has not been shown to affect prenatal development, as GH and GHR null mice appear no different from their wild type littermates at birth. Contrastingly, *Igf-1* null mice are smaller compared with their wild type when they are born, and most die early in the neonatal stages. Those that survive are severely retarded and also infertile (Liu *et al.* 1993).

Postnatally, the role and regulation of autocrine/paracrine IGF-I is largely determined by tissue type. A number of factors influence the heterogeneity of the tissue/cellular response: the tissue type, the state of differentiation of the cell, the number and activity of cell surface receptors, the cellular microenvironment and the method of delivery of IGF-I. IGF-I has been demonstrated to stimulate the proliferation and differentiation of myoblasts (Florini *et al.* 1986), osteoblasts and adipocytes (Smith *et al.* 1988).

Local IGF-I production is believed to be important in the regulation of growth and differentiation following changes in the local environmental. The stimulation of IGF-I production in muscle in response to overload has been the topic of much recent research. Given the widespread action of local IGF-I in a number of tissues, it is beyond the scope of this literature review to detail all of these. The following sections will therefore focus on evaluating its role in skeletal muscle growth, with specific reference to age.
Muscle hypertrophy

Hypertrophy is the increase in muscle size. It has long been accepted that mechanical stress can have an effect on muscle mass. There are a number of ways of overloading animal muscles to produce a hypertrophic response, some of which are discussed briefly in the next section of this chapter. In man however, this is most easily achieved through high resistance or strength training. By definition, strength training involves progressively overloading the neuromuscular system using near maximal muscle contractions against high resistance, where loads near to their 1 repetition maximum (1-RM) are lifted (Porter et al. 1995). Body builders in contrast tend to lift less heavy loads to achieve definition, so that muscles can be visually separated from each other. This is achieved using 'split' system exercise programmes, i.e. a few selected muscles or muscle groups are exercised in each exercise session (Tesch, 1994). It is clear that as hypertrophy is limited to the muscle exercised and not to all muscles, there are 'local' factors regulating this process. It is now believed that IGF-I plays a key role in the hypertrophic adaptation of muscle to exercise.

Muscle IGF-I and hypertrophy

The anabolic effects of IGF-I have been clearly demonstrated by numerous in vitro studies, where it has been shown that IGF-I acts to increase the diameter of myotubes, suppress protein degradation, increase amino acid uptake and stimulate protein synthesis (Florini, 1987; Vandenburgh et al. 1991; Florini et al. 1996; Semsarian et al. 1999; Rommel et al. 2001; Bodine et al. 2001).

In vivo studies, employing models known to result in muscle hypertrophy have reported that IGF-I expression in the muscle increases early on in the process of
hypertrophy. Studies utilising the model of stretch-induced hypertrophy of the muscle have reported an increased expression of muscle IGF-I mRNA (Schlechter et al. 1986; Czerwinski et al. 1994). A study by DeVol et al (1990) demonstrated that there was a three-fold increase in IGF-I mRNA levels in the soleus and plantaris muscles in 11-12 week old female rats following tenotomy-induced hypertrophy. This particular study employed hypophysectomized rats, which further suggests that the observed increase in IGF-I mRNA expression was GH independent. Later studies utilising a similar model of functional overload in both normal and hypophysectomized rats found that both mRNA and protein levels of IGF-I were increased in muscle, prior to the attainment of significant hypertrophy, and remained elevated for up to 28 days during the hypertrophy process (Adams and Haddad, 1996). Furthermore, when investigating the time course of changes in markers of myogenesis following increased loading, the myogenic processes were activated early on, and it was suggested that IGF-I might be modulating this response (Adams et al. 1999). In another study which utilised treadmill training of GH-suppressed rats, levels of IGF-I mRNA and protein increased by 55% and 250% respectively (Zanconato et al. 1994).

Few studies to date have investigated the IGF-I response to exercise and overload in human skeletal muscle. In a study of young military recruits, Hellsten et al (1996) reported an increase in IGF-I immunoreactivity after seven days of strenuous exercise. Later, in a study investigating IGF-I mRNA levels, Bamman et al (2001) reported a 62% increase in IGF-I mRNA concentration in the muscle, 48 hours after an acute bout of eccentric, but not concentric contractions. More recently, a study by Psilander et al measured mRNA levels of some of the isoforms of IGF-I after heavy resistance training.
in young subjects. In this study levels of total IGF-I (IGF-I_Eabc) and IGF-I_Ebc were measured before, immediately after, and 1, 2, 6, 24 and 48 hours post exercise. The study reported a decrease in levels of total IGF-I and no change in levels of IGF-I_Ebc mRNA after the exercise. It is difficult to determine the individual isoform response to training from this study, as measurements were made of the isoforms grouped together.

**IGF-I stimulated hypertrophy**

There is also evidence that experimental manipulations of the levels of IGF-I in muscle can induce muscle hypertrophy both *in vitro* (Vandenburgh *et al.* 1991) and *in vivo* (Coleman *et al.* 1995; Adams and McCue, 1998). For example, overexpression (Coleman *et al.* 1995) or direct infusion (Adams and McCue, 1998) of IGF-I in muscle results in hypertrophy, whereas inhibition of intracellular signalling components associated with IGF-IR activation can prevent this response (Bodine *et al.* 2001). In another study, examining the association between local IGF-I overexpression and atrophy induced by hind limb unloading it was concluded that overexpression of IGF-I in the muscles of transgenic mice, was not shown to prevent unloading-induced atrophy (Criswell *et al.* 1998).

**IGF-I signalling pathways involved in skeletal muscle hypertrophy**

The signalling pathways by which IGF-I promotes skeletal muscle hypertrophy remain unclear, with roles suggested for both the calcineurin/NFAT (nuclear factor of activated T cells) pathway (Musaro *et al.* 1999; Semisarian *et al.* 1999) and the PI3-kinase/Akt pathway (Rommel *et al.* 1999). These pathways have been summarised in Figure 1.5 below. More recently, studies investigating the hypertrophic response both *in vitro* and *in vivo* have reported that it is the Akt/mTOR
pathway and not the calcineurin pathway, which is involved in promoting hypertrophy, by activating downstream targets such as p70 S6 kinase (Rommel et al. 2001; Bodine et al. 2001). In addition, it was also reported that IGF-I might in fact act via Akt to inhibit the calcineurin/NFAT signalling pathway during this process (Rommel et al. 2001).
Figure 1.5. Schematic overview of the downstream signalling pathways of IGF-I, emphasising the role of the PI(3)K/Akt/mTOR pathway in skeletal muscle hypertrophy. Adapted from Rommel et al. 2001.
Expression of different muscle IGF-I isoforms in response to overload

Most of the studies conducted to date have looked at the expression of total IGF-I in response to muscle overload. As mentioned earlier on in this chapter, IGF-I exists as different isoforms, which are derived from the IGF-I gene by a process of alternative splicing. In human beings there are three isoforms: IGF-IEa, IGF-IEb and IGF-IEc (see Figure 1.2). The first isoform is similar to the main isoform produced by the liver. Several different abbreviations exist in the literature for this isoform as shown in Table 1.2: IGF-IEa (Rotwein et al. 1986), L.IGF-1 (Yang et al. 1996) and m.IGF-1 (Musaro et al. 2001). The second isoform, IGF-IEb is thought to be predominately expressed in the liver and its role in muscle is as yet unknown. In people, exons 5 and 6 were thought to be mutually exclusive however, the third isoform, IGF-IEc in human beings (IGF-IEb in the rat) is a splice variant resulting from a novel splice acceptor site in the intron preceding exon 6. This isoform contains the first 49 base pairs of exon 5 (52 base pairs in the rat) and exon 6 (Chew et al. 1995).

Yang et al (1996) demonstrated that following acute stretch by hind limb immobilisation in the extended position, rabbit EDL muscles expressed both IGF-IEa and IGF-IEb mRNAs. However, very little, if any IGF-IEb mRNA could be detected in the control muscles not subjected to stretch. This lead to the conclusion that this isoform was sensitive to mechanical signals or micro cellular damage. In this light, the isoform was re-named mechano-growth factor (MGF). To save any further confusion in the nomenclature of this isoform, this splice variant will subsequently be referred to as MGF. The notion that MGF was mechano-sensitive was supported by two further studies. For example, when stretch was combined with electrical stimulation of the tibialis anterior muscle, MGF mRNA was upregulated more so than by stretch alone.
(McKoy et al. 1999). Secondly, following stretch and immobilisation of the muscles of MDX mice (mice lacking the gene for dystrophin, an important cytoskeletal structural protein), MGF mRNA could not be detected. IGF-IEa mRNA was however present in detectable amounts in these animals (Goldspink et al. 1996). Furthermore, recent studies by Haddad and Adams (2002) have shown that MGF mRNA was significantly increased by at least 2-fold after a single bout of resistance type exercise in rat muscles.

**MGF-stimulated hypertrophy**

It has been shown that MGF is an inducer of muscle hypertrophy in experiments where a plasmid gene construct containing MGF cDNA (under the control of muscle regulatory elements) was introduced into the tibialis anterior muscle of mice by intramuscular injection. This resulted in 20% increase in muscle mass within two weeks, and the analysis showed that this was due to an increase in the size of the muscle fibres. (Goldspink, 2001).

**IGF-I stimulated hypertrophy in aged muscle**

In older animals, overexpression of the different IGF-I isoforms has been associated with the prevention of some of the age-related effects on skeletal muscle, namely, the decline in muscle mass (Barton-Davis et al. 1998; Musaro et al. 2001). For example, Barton-Davis and co-workers injected a recombinant adeno-associated virus (AAV) directing overexpression of IGF-I (Ea isoform), into the extensor digitorum longus (EDL) muscles of young and old mice. The construct, containing a myosin light chain MLC1/3 promoter resulted in an overexpression of IGF-I in the muscles, but did not increase circulating IGF-I levels. Four months post injection, the injected muscles of the younger animals were on average 15% larger and 14% stronger than
the non-injected muscles. In the older animals, age-related changes in muscle were therefore prevented and the injected muscles were 27% stronger than the non-injected older animals. A second, more recent study also demonstrated an age-related improvement in muscle mass and function in transgenic animals bred to over-express the IGF-I_Ea isoform (which was termed m.IGF-I) (Musaro et al. 2001). At 6 months of age, the mean diameter of fibres in the transgenic animals was 32 μm compared with 18 μm in the wild type. There was however, preferential hypertrophy of the faster fibre types (46 μm in transgenic animals compared with 32 μm in wild type) with little effect on the slow fibres (16 μm compared with 18 μm). This was attributed to the low expression of the MLC regulatory cassette (1/3 locus) in the slow muscle fibres. Examination of the older transgenic animals revealed that they were protected against the normal age-related muscle loss, whilst there was significantly atrophy in their wild type littermates.

Is aged muscle as sensitive to damage and overload?

The above studies give good reason to suggest that maintaining local IGF-I synthesis could play an important part in maintaining muscle mass in old age. Studies have shown that upregulation of IGF-I in response to muscle injury or mechanical damage is altered in older animals (Marsh et al. 1997; Owino et al. 2001). The regenerative capacity of the tibialis anterior muscle in young, mature and old rats were determined in the study by Marsh et al (1997) following an injection of the myotoxic anaesthetic, bupivacaine. In young animals, there appeared to be a transient surge in IGF-I mRNA levels early on in the regeneration process, which diminished by the 15th day of recovery. In contrast, levels of IGF-I mRNA in the mature and older animals remained elevated between 5-15 days of recovery. Thus IGF-I mRNA expression changed from a transient surge in
young regenerating muscles to sustained elevated levels, associated with a failure to complete the regeneration process in the older animals. However, this study did not determine the type of IGF-I involved. The recent study by Owino et al., (2001) used synergistic muscle tendon ablation to overload the soleus and plantaris muscles of young, mature and old rats, to determine if there were any age-related differences in the response of the different IGF-I isoforms in muscle. This study showed that as a result of overload MGF mRNA was 3 times higher in the young muscle as compared with mature muscle and 5 times higher than in the older muscles. IGF-IEa mRNA levels were also upregulated by the challenge, but showed no clear age-related effect. The study therefore suggested an age-related reduction in the ability to upregulate the MGF isoform. Furthermore, it was suggested that the two IGF-I isoforms were differentially regulated. This was based on the observation that that MGF mRNA levels significantly increased 5 days after surgery, but no significant changes in levels of IGF-IEa mRNA were seen.

If IGF-I, more specifically the MGF isoform, were an important local repair factor, then these data would be in general agreement with other work that has shown an impairment in the response of aged skeletal muscle following overload. Three critical changes occur in aged muscle: muscle fibres are injured more easily (Zerba et al. 1990), muscle fibres regenerate less successfully (Carlson and Faulkner, 1989) and structural and functional recovery is incomplete (Brooks and Faulkner, 1990). Contraction-induced injury provides a possible mechanism for the atrophic changes with increasing age (Faulkner et al. 1995). In two studies, isometric force was used to characterise force deficit after a number of high force pliometric (eccentric) contractions in young and old mice. Greater force deficits were observed in older animals when compared with young, after 10 minutes (Zerba et al. 1990) and 28
days (Brooks and Faulkner, 1990) following contractions. A more recent study by Vignaud et al (2003) used a very different approach to damage the soleus muscle in both adult and senescent rats. The study reported incomplete regeneration of these muscles, 3 weeks following myotoxic injury (using snake venom) in the senescent rats when compared with adult.

Adaptability of aged human muscle

The adaptability of aged muscles, in particular the possibility of improving muscle strength and increasing muscle mass in older individuals, has been the focus of much work. Many studies have related low muscular strength with other conditions, such as increased susceptibility to falls and fractures and increasing dependence on others (reviewed by Porter et al. 1995). Thus, emphasising the importance of improving muscle mass and strength in the elderly population.

With animal studies having shown that there are age-related impairments in the responsiveness of muscle to damage and overload, this might suggest that older people may be less adaptable to strength training exercise than younger individuals. This seems not to be the case, as a large number of studies conducted to date have shown the positive effects of strength training in older individuals (Frontera et al. 1988; Brown et al. 1990; Charette et al. 1991; Hicks et al. 1991; Grimby et al. 1992; Nichols et al. 1993; Rice et al. 1993; Roman et al. 1993; Pyka et al.1994; Skelton et al. 1995; Welle et al. 1996; Harridge et al. 1998). With a number of studies also showing that even the muscles of very frail, very elderly individuals (>85 years) are able to hypertrophy in response to muscle strengthening exercise. (Fiatarone et al. 1990; 1994; 1999; Harridge et al. 1999). In general, these studies suggest that in terms of changes in the amount of
weight that can be lifted (1-repetition maximum [1-RM]), isometric strength and muscle cross sectional area (CSA), older people respond to training in a similar way to young people.

The local IGF-I response to strength training exercise in human skeletal muscle has not been widely studied. In one such study investigating IGF-I peptide levels in muscle following 10 weeks of strength training in old men and women (aged 72 – 98 years), it was shown that there was a ~500% increase in the levels of IGF-I immunoreactivity within the muscle fibres of these subjects after the training period (Fiatarone-Singh et al. 1999). This indicates that IGF-I peptide levels in older muscles may adapt over the longer term to exercise training. However, the effects of exercise training on IGF-I peptide levels in younger individuals were not investigated in this study, to determine if there was an age-related difference in the muscle IGF-I response to exercise. It was also reported that the increase in amount of weight that could be lifted after training was proportional to the significant increase in IGF-I immunoreactivity in muscle after training. In addition, they observed increased ultrastructural damage and an increase in the number of fibres expressing embryonic MyHC isoforms in the post-training biopsy samples. This study therefore suggested that muscle damage provoked the ‘initial’ step in the remodelling process, which ultimately leads to muscle regeneration. Other studies however, have shown that what we commonly interpret as damage are in fact not the result of damage provoking regeneration but regeneration itself, associated with that invasion of myofibres by satellite cells (Yu et al. 2004).
Mechanisms of local action

There are two likely mechanisms through which IGF-I is believed to stimulate muscle hypertrophy. The first is through stimulating protein synthesis and the second is through the activation of satellite cells (discussed in the next section). For a muscle to hypertrophy there must inevitably be a change in the net rate of protein turnover. Changes in muscle protein synthesis will particularly be effected by the type, duration and nature of contractile activity, but also by dietary intake (Rennie and Tipton, 2000). It is generally agreed that the rate of muscle protein synthesis is increased after resistance exercise in both young and elderly individuals. In the first ever reported human study, Chesley et al (1992) found a 50% increase in muscle protein synthesis immediately after resistance exercise. A later study by Yarasheski et al (1993) reported a 36% and 60% increase in the basal fractional rate of protein synthesis in young and old subjects, respectively, after 2 weeks of resistance training. This idea was further supported by more recent studies in which an increase in MyHC and mixed protein synthesis rates was reported following resistance exercise (Yarasheski et al. 1999; Hasten et al. 2000; Balagopal et al. 2001). Contrary to this, one such study by Welle et al (1995) reported no significant increase in myofibrillar protein synthesis rates in young and old subjects after 3 months of resistance training. There is now a large body of literature to suggest that either oral amino acid ingestion (Tipton et al. 1999) or intravenous infusion (Biolo et al. 1997) can alter the net protein balance after exercise. As detailed earlier in this chapter, binding of IGF-I to its cell surface receptor activates the PI(3)K/Akt/mTOR signalling pathway. This then triggers a cascade of downstream events attributed to PI3-kinase activity, including the activation of p70 S6 kinase, which phosphorylates the S6 protein component of ribosomes, possibly modulating the rate of translation and thereby
protein synthesis (reviewed by Adams, 1998). Indeed, Baar and Esser (1999) showed a correlation between p70/S6 kinase activity and the long-term increase in muscle mass following training. Thus suggesting that p70 6 kinase phosphorylation may be a good marker for the phenotypic changes that characterise muscle hypertrophy and may play a role in the load induced skeletal muscle growth.

The role of satellite cells in muscle growth and repair

Satellite cells are small mononucleate precursor cells, which are typically located between the basal lamina and sarcolema of muscle fibres (Figure 1.6). These cells are believed to remain mitotically inactive but are mobilised by increased mechanical loading or damage, playing a role in both adaptation and repair of muscle. Satellite cells only account for approximately 5% of the nuclei present within muscle fibres. However, when activated, they have the capacity to divide extensively in order to produce sufficient mononuclear myoblasts to replace the damaged muscle fibres. They do this by fusing, either with one another or with existing but damaged muscle fibres, in order to repair and repopulate the traumatised muscle (reviewed by Goldring et al. 2002). These cells are important for postnatal growth of muscle and are an important means by which adult muscle mass is formed and maintained. Their number decreases gradually after birth and there is recent evidence from studies of human satellite cells in culture that the replicative potential of these cells is reduced in later life (Decary et al. 1997).

Recent studies using immunohistochemical techniques and N-CAM/Leu-19 staining have shown that satellite cell number (the ratio between the number of satellite cell nuclei and the total number of nuclei minus the myonuclei and nuclei of satellite cells) is increased in power-lifters when compared with untrained subjects (Kadi et al. 1999),
and that the number of satellite cells increased in the trapezius muscle after 10 weeks of strength training (Kadi and Thornell, 2000). This study also showed a significant relationship between the CSA of the muscle after training and the increase in number of myonuclei in the individual muscle fibres. These data suggest that an increase in the number of nuclei provided by satellite cells is required to maintain the nuclear domain during hypertrophy.

DeVol et al (1990) suggested that during muscle growth, locally produced IGF-I had two distinct functions with respect to satellite cell involvement, those being stimulation of both proliferation and differentiation of satellite cells. This was further confirmed by in vitro studies demonstrating that IGF-I stimulated proliferation, differentiation and fusion of satellite cells into growing myotubes (Florini et al. 1991; Delany et al. 1994). A more recent cell culture study by Yang et al (2002) in which C2C12 cells were transfected with either MGF or IGF-I peptides, suggested that the MGF E-peptide was specifically involved in the proliferation of satellite cells, whereas mature IGF-I stimulated both proliferation and differentiation of these cells. The role of IGF-I in satellite cell activation was further supported by experiments in which direct infusion of IGF-I into the tibialis anterior muscle of adult rats resulted in an increased total muscle protein and DNA content (Rosenblatt et al. 1994; Adams and Haddad, 1996; Adams and McCue, 1998). Further evidence for the role of these cells in hypertrophy is provided by an experimental approach whereby satellite cells were prevented from proliferating following treatment with γ-radiation. The subsequent overloading of the adult rat soleus muscle failed to evoke hypertrophy of the overloaded irradiated muscles (Phelan and Gonyae, 1997).
Interestingly, recent work has shown that acute overexpression of IGF-I can increase the number of times that satellite cells can replicate, possibly explaining some of the positive effects this treatment has had on the age-related changes in skeletal muscle, which were discussed earlier in this chapter. (Chakravarthy et al. 2000a; Chakravarthy et al. 2000b; Chakravarthy et al. 2001). As these studies involved overexpression of the IGF-I gene, they give no indication about which IGF-I splice variants may be involved. The work of Yang and Goldspink (2002) and Hill and Goldspink (2003), suggests that MGF is involved with the initial activation of satellite cells. Therefore, in the present study it was important to study the expression of the different isoforms of IGF-I in human muscle in response to exercise.
Figure 1.6 A summary of some of the molecular events involved in satellite cell activation during skeletal muscle regeneration. Satellite cells are activated in response to a mechanical signal e.g. injury, denervation, exercise or stretching. HGF is also thought to activate satellite cells through its receptor, c-Met, expressed in quiescent satellite cells. Activation of quiescent satellite cells results in the expression of the myogenic factors MyoD and/or Myf5 and the generation of mpc. IGF-I is thought to play a role in both mpc proliferation and in the differentiation of mpc into myotubes. Abbreviations: mpc: myogenic precursor cells, HGF: hepatocyte growth factor. (Taken from Hameed et al. 2002)
Summary

Mechanically overloading a muscle exercise causes changes in muscle gene expression. This leads the muscle to hypertrophy, which involves a complex process of repair and remodelling. IGF-I is believed to play an important role in these adaptive processes. Many studies in animal muscle have focussed on the role of this growth factor in the hypertrophic response of muscle to exercise. However, there was little knowledge about the expression of IGF-I and its splice variants in human skeletal muscle. The above review of the literature has focused on the current state of our understanding of the structure of the mammalian IGF-I gene and its role and regulation in muscle growth and repair. The overall aim of the work in this thesis was to study the expression of IGF-I isoforms in human skeletal muscle, and how they respond to different types of exercise. A specific focus is placed on differences in their expression that might result from the ageing process.
Chapter 2

Development of a quantitative polymerase chain reaction method to measure IGF-I isoforms in human skeletal muscle
2.1 Introduction

Much of the initial work for this thesis has focused on the development of a quantitative polymerase chain reaction (PCR) method, which allows the accurate measurement of the different isoforms of IGF-I: IGF-IEa, IGF-IEb and MGF (IGF-IEc) in human skeletal muscle. This chapter outlines the developmental work and begins with an overview of the different methods available for measuring gene expression. Due to the limited tissue size available in the studies detailed in Chapters 3-5, the most suitable and sensitive method for measuring the changes in gene expression was believed to be real time quantitative PCR. This is further expanded upon later in this chapter. The second part of this chapter (Technique Development) focuses on optimisation strategies employed in developing a sensitive, reliable and reproducible quantitative PCR assay using LightCycler technology. Many of the initial experiments were concerned with characterising the different isoforms of IGF-I in human skeletal muscle and these were carried out prior to the availability of a real-time quantitative PCR set-up in our laboratory. Pilot studies using conventional PCR methods were used to identify and characterise the different isoforms of IGF-I in human muscle and these are detailed in Appendix 1. These early experiments also investigated the option of using a ‘less invasive’ syringe needle aspiration technique for muscle sampling, instead of the more conventional needle biopsy technique. Whether this technique could be used reliably to obtain adequate muscle from the vastus lateralis muscle and whether the samples obtained could then subsequently be used for quantitative PCR was also investigated in a later pilot study, details of which are given in Appendix 2.
2.2 Background

The Polymerase Chain Reaction

Emerging technology now enables the measurement of genes that are expressed at low copy numbers including autocrine growth factors, which may be produced by only a small percentage of cells within a given tissue. The polymerase chain reaction (PCR) was first developed in the mid-1980s as a novel method for the study and analysis of low abundant gene sequences from small amounts of starting material (Mullis and Faloona, 1987). Techniques available for detecting specific DNA sequences before the advent of PCR required considerable amounts of tissue, were time consuming and involved lengthy cloning methods. PCR offers a unique tool, which enables the production of large numbers of copies of specified sequences of DNA for analysis, without the need for cloning. The starting material for a PCR is DNA that contains the sequence of interest to be amplified. It is not necessary to isolate the sequence to be amplified, as this is defined by the specific primers used in the reaction. Since the introduction of PCR, the basic protocol has been further modified to allow for the study of messenger RNA (mRNA). Here, the starting material is RNA and the PCR is used in combination with reverse transcription (RT-PCR) to amplify specific regions of mRNA. Whether DNA or RNA, the amount of starting material needed to perform a PCR is very small. For RT-PCR, the mRNA is first converted into single stranded complementary DNA (first strand cDNA) by primer extension, which can be achieved by three methods: Since virtually all eukaryotic mRNA molecules have a run of adenine nucleotide residues called a poly(A) tail at their 3’ end, the first method involves using oligonucleotides consisting only of deoxythymidine [oligo(dT)] to prime the mRNA from the poly(A) tail. An alternative to this is to use random hexamers (dN)6. These are short primer
sequences which bind at non-specific points along the RNA template. If the mRNA sequence to be amplified is known, short, sequence specific primers (often called dodecamers) can also be used for first strand cDNA synthesis. The use of dodecamers in this process is discussed further later on in this chapter.

Quantification of nucleic acids

There are four main methods available for quantification of nucleic acids: Northern blotting and in situ hybridisation (Parker and Barnes, 1999), RNase protection assays (RPA) (Hod, 1992; Saccomanno et al. 1992) and quantitative RT-PCR (Weiss et al. 1992; Ferre, 1992). cDNA micro-arrays (Bucher, 1999), a fifth method, is still very much in its infancy due to the high cost considerations. Northern analysis provides information about the integrity of the RNA samples, mRNA size and the presence of splice variants. RPA offers the most useful tool for mapping transcript initiation and termination sites and intron/exon boundaries. Unlike Northern blot analysis, it is also able to distinguish between mRNAs of similar size and can give information about the primary transcript of the gene. The main advantage of these two methods are that their reaction kinetics are relatively easy to determine. In situ hybridisation is the only one of these methods to allow localisation of the mRNAs to specific cell types within a tissue. However, the main disadvantage of all three of these methods is that the sensitivity achieved is not sufficient for many practical applications. These procedures are also lengthy and labour intensive to perform, with in situ hybridisation being the most complex and time-consuming of the three. A further drawback of the Northern blotting and RPA techniques is that they usually require the use of radioactive substances for labelling. When autoradiography is combined with the used of densitometry there are problems of over saturation unless extensive
calibration is carried out to ensure that all the measurements are made at the lower linear part of the curve. This reduces its sensitivity further and makes it unsuitable for experiments in which there may be large changes. The problems encountered when using optical densitometry can be overcome by using a phospho-imager, but these are expensive instruments. Blotting and autoradiographic methods also require relatively large amount of starting material, which can rule out studies where the sample size is limited, such as work involving human biopsy material. In contrast, PCR based methods for the quantification of nucleic acids are highly sensitive (Wang and Brown, 1999) and therefore require very tiny amounts of starting material (even as little as one cell). Quantitative PCR methods are highly flexible and can be used to compare the levels of mRNAs in different sample populations, to characterise patterns of mRNA expression, to differentiate between closely related mRNAs (e.g. splice variants of the same gene) and to analyse RNA structure. One drawback is that the kinetics of the PCR reaction are comparatively more complex than the more conventional hybridisation techniques. The conditions when using PCR as a quantitative method must therefore be well established for each gene of interest. Factors such as specificity, sensitivity and reproducibility of the PCR must therefore be carefully optimised in order to establish a reliable and successful quantitative assay. Therefore, quantitative PCR can initially be laborious to perform, but after careful optimisation of the PCR reaction conditions, results can be obtained in less than one hour.

Quantitative PCR methods

mRNA can be quantified using either semi-quantitative or quantitative methods. Montgomery and Dallman (1997) first described a rather laborious semi-quantitative
RT-PCR method, which involved regular sampling of the RT-PCR reaction followed by dot-blot analysis. Quantitative RT-PCR methods can be categorised into non-competitive or competitive. Non-competitive PCR involves the co-amplification of the target with a second RNA molecule with which it shares neither the primer recognition sites nor any internal sequence (standard). The PCR is run under conditions that allow for no competition between target and standard (Reischl and Kochanowski, 1999). The major disadvantage of this technique is that it compensates for the possibility that both the RT and PCR efficiencies are likely to be different, even when investigating relative changes of transcription within a sample (Ferre, 1992). In contrast, competitive RT-PCR involves using an internal standard that is almost identical to the primary target. This competitor molecule shares the same primer recognition and internal sequences as the target and only differs from it in size by the inclusion or exclusion of a small number of base pairs (Gilliland et al. 1992). Because they are virtually identical, both should be amplified with the same efficiency and can be distinguished by the size difference when visualising the products on a gel. As the name suggests, this type of PCR generates competition between target and competitor for reagents. A series of PCR tubes containing the target are spiked with serial dilutions of known copy numbers of the internal standard. The greater the concentration of the internal standard, the more likely it is that the primers will bind and amplify it, rather than the target. After gel electrophoresis, a comparison of the intensities of ethidium bromide stained standard and target amplicons allows target quantification (reviewed by Bustin 2001).
mRNA quantification using real-time quantitative PCR

When the term quantitative PCR is used today it is highly likely that the method being referred to is real-time quantitative PCR. Higuchi et al (1993) documented the first real-time PCR method, yet this technology has only recently become mainstream. Real-time quantitative PCR is the detection and measurement of products generated during each cycle of the PCR process, which are directly proportional to the amount of template prior to the start of the PCR process (Ginzinger, 2002). To achieve this two things are necessary: a method of detecting the accumulation of PCR product and an instrument in which to perform the thermocycling that is adapted to record the results during each PCR cycle in real time. Real-time PCR potentially offers a highly sensitive technique for the quantification of mRNA. With this method only a very small number of the cycles in a PCR reaction contain useful information. The early cycles have undetectable amounts of the DNA product and late cycles (the plateau phase) are almost as uninformative. The quantitative information in a PCR reaction comes from those few cycles where the amount of DNA grows logarithmically from barely above background to the plateau. This is known as the log linear or exponential phase of the PCR and is considered the condition of constant amplification efficiency (see Figure 2.1). Often only 4-5 cycles out of 40 will fall in this log-linear portion of the curve (Rasmussen et al. 1998; Rasmussen et al. 2001). With real-time PCR it is possible to measure the number of cycles necessary to detect a signal (threshold cycle) and use this to determine the starting levels of mRNA present in the sample.
Figure 2.1. A typical example of a PCR amplification profile illustrating the nomenclature typically used in real time Q-PCR experiments. The amplification profile is the plot of fluorescence signal vs PCR cycle number. The baseline is defined as the PCR cycles in which a signal is accumulating but is beneath the limits of detection of the instrument. The signal measured during these PCR cycles is used to plot the threshold. The threshold is calculated as 10 times the standard deviation of the average signal of the baseline fluorescent signal. A fluorescent signal that is detected above the threshold is considered a real signal that can be used to define the threshold cycle (Ct) for a sample. The Ct is defined as the fractional PCR cycle number at which the fluorescent signal is greater than the minimal detection level. The Ct values of different samples are then used to calculate the relative abundance of template for each sample. Adapted from Ginzinger (2002)
Real-time quantitative PCR vs conventional PCR

The introduction of real-time PCR technology has markedly improved and simplified the quantification of nucleic acids and this technology has more recently become an invaluable tool for many scientists working in different disciplines. How it compares to conventional PCR methods is summarised in Table 2.1.

<table>
<thead>
<tr>
<th></th>
<th>Conventional PCR</th>
<th>Real-Time PCR</th>
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<tbody>
<tr>
<td><strong>Run Time (35 cycles approximately)</strong></td>
<td>Up to 6 hours (including running on gel)</td>
<td>25-40 minutes</td>
</tr>
<tr>
<td><strong>Set-up Time</strong></td>
<td>Lengthy as reagents added individually</td>
<td>Reaction-mix contains (Taq DNA polymerase, reaction buffer, dNTPs and SYBRGreen I dye) therefore saves time during set-up</td>
</tr>
<tr>
<td><strong>Gel Electrophoresis</strong></td>
<td>Necessary to visualize PCR products</td>
<td>Not necessary Reaction specificity visible online</td>
</tr>
<tr>
<td><strong>Quantitative Analysis</strong></td>
<td>Semi-quantitative analysis by measuring band intensity following gel electrophoresis. Problems with over saturation unless a phospho-imager is used</td>
<td>Accurate quantification of PCR products relative to a set of standards of known concentration</td>
</tr>
<tr>
<td><strong>Contamination Risk</strong></td>
<td>Each reagent is added individually, increasing contamination risk</td>
<td>Ready to use reaction mix minimises risk of contamination during set up and handling</td>
</tr>
<tr>
<td><strong>Safety Risk</strong></td>
<td>DNA intercalating reagent ethidium bromide used during gel electrophoresis (carcinogen)</td>
<td>None</td>
</tr>
</tbody>
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Table 2.1 Comparison of conventional PCR with real-time PCR using LightCycler technology.
Real-time PCR using LightCycler technology

The LightCycler (Roche Molecular Biochemicals, Manheim, Germany) is one of the several commercially available real-time PCR systems. It performs the PCR in small-volume glass capillaries that can hold a volume of up to 20µl (Figure 2.2 inset). These are contained within a rotor like carousel, which is heated and cooled by temperature-controlled airflow. The capillaries are positioned within a rotation symmetric chamber to ensure homogeneous temperature distribution within all samples (see Figure 2.2).

![Figure 2.2. LightCycler real-time PCR instrument (Roche Molecular Biochemicals). The picture shows the rotor like carousel containing 32 glass capillaries. The inset shows the individual glass capillaries.](image)

The carousel is rotated past a blue light-emitting diode and fluorescence is read by three photodetection diodes with different wavelength filters that allow the use of spectrally distinct fluorescent probes. The LightCycler system is compatible with
assays based on DNA binding dyes such as SYBR green, hydrolysis and hybridisation probes. Details of quantification using SYBR Green I are given below. The probe-based assays use two sequence specific oligonucleotides labelled with two different fluorescent dyes. The detection is based on the generation of a fluorescent signal by fluorescent resonance energy transfer (FRET) when the two probes bind to the target sequence. The LightCycler system can carry out up to 32 reactions at one time and one run takes between 20 and 55 minutes to complete, depending on the type of chemistry employed. Fluorescent readings are taken at the end of every cycle of the PCR and are displayed on-screen in real-time immediately after each measurement, giving the user the option of extending or terminating the run if need be.

**Quantitative PCR using a double strand DNA binding dye (SYBR green I)**

SYBR green I binds in the minor groove of double stranded DNA (dsDNA) in a sequence independent way (Morrison et al. 1998). In its unbound state SYBR green I dye has relatively low fluorescence, but when it binds to DNA during elongation its fluorescence increases over 100-fold. As the amount of DNA increases during the different phases of PCR, the amount of fluorescence from the dye increases proportionally.
At the beginning of amplification, the reaction mixture contains the denatured DNA, the primers and the dye. The unbound dye molecules weakly fluoresce, producing a minimal background fluorescence signal which is subtracted during computer analysis.

After annealing of the primers, a few dye molecules can bind to the double strand. DNA binding results in a dramatic increase of the SYBR Green I molecules to emit light upon excitation.

During elongation, more and more dye molecules bind to the newly synthesized DNA. If the reaction is monitored continuously, an increase in fluorescence is viewed in real time. Upon denaturation of the DNA for the next heating cycle, the dye molecules are released and the fluorescence signal falls.

**Figure 2.3.** DNA detection using SYBR Green I dye. (Taken from LightCycler Online Resource Site – Roche Diagnostics).
Figure 2.3 summarises the events that occur during a real-time PCR reaction using SYBR Green I. During the denaturation step of the PCR SYBR Green I does not bind. During annealing, the specific primers hybridise to the target sequence forming small regions of dsDNA to which the SYBR Green I binds and emits fluorescence. In the final step of the PCR, elongation, the PCR primers are extended and the entire length of the DNA is now double stranded. This leads to a maximum amount of SYBR Green I dye being bound. When monitored in real time, the increase in fluorescent signal can be observed at the end of the elongation phase, which then falls off once the DNA is denatured. The excitation and emission spectra of SYBR Green I are shown in Figure 2.4.

SYBR Green I is an easy to use and highly reliable detection method, which obviates the need for target-specific fluorescent probes, but the primers used for PCR solely determine its specificity. The main drawback in using SYBR Green I is that it binds to any dsDNA molecule formed during the reaction, whether this is the target of interest or a non-specific product like a primer dimer. This non-specific binding of SYBR Green I dye makes the quantification of low copy numbers more difficult. Staining with SYBR Green I on the LightCycler system is thought to detect from approximately 1 to $10^9$
copies of a target sequence. However, there are ways of overcoming this problem when using this system. The products of a PCR reaction can be melted down by increasing the temperature of the sample. A melting curve is then achieved by plotting the fluorescence as a function of the temperature (Ririe et al. 1997). At the melting temperature ($T_m$) of the product, a sharp drop in the level of fluorescence is seen (Figure 2.5). Non-specific products such as primer dimer tend to have much lower $T_m$ and broader melting peaks than the longer specific products, which have characteristic sharp, narrow melting peaks (see Figure 2.5). Melting curve analysis allows the user to correct for the contribution of non-specific products, thereby extending the sensitivity of the technique to a single copy per reaction (Morrison et al. 1998).

**Figure 2.5.** Figures A and B show typical melting curve profiles of a specific product and a no-template negative control. The LightCycler software presents the user with two graphs. Figure A shows melting curve data as Fluorescence vs Temperature and Figure B shows the calculated negative derivative plot of the same data. Figure B is commonly used to determine the specificity of the reaction. Specific products (shown in red) characteristically have sharp, narrow peaks whereas, non-specific amplifications such as primer dimer (shown in black), melt at lower temperatures and over a broader range.
Quantification strategies

Absolute quantification

Quantification by external standards is often referred to as ‘absolute’ quantification, because an actual number or concentration of the transcript can be obtained per cell, total RNA or unit mass of tissue. The absolute amount of target nucleic acid of interest is determined using external standards. These standards usually contain sequences that are the same as, or differ only slightly from the target sequence, and their primer binding sites are always identical to the target sequence. This ensures equivalent amplification efficiencies between standards and target molecules, which is essential for absolute quantification. This method requires the construction of an absolute standard curve (plot of $C_T$ – crossing points of different standard dilutions against log of amount of standard) generated using a dilution series of the standards. Amplification of the standard dilution series and of the target sequence is carried out in separate tubes. Comparing the $C_T$ of the unknown samples with the standard curve allows calculation of the initial amount of target used in real-time PCR. A standard curve is generated for each of the individual targets being measured to ensure accurate reverse transcription and PCR amplification profiles (Bustin, 2000). The accuracy of this method depends largely on the accuracy of the standards and it is important to select an appropriate standard for the type of target to be quantified. Both of these points are discussed later on in this chapter.

Relative quantification

This type of quantification of mRNA determines the changes in steady state transcription of a gene (Bustin, 2000). In this approach, the ratio between the amount of target molecule and a reference molecule within the same sample is calculated. It
is essential that the expression level of the reference molecule, such as a control gene (examples of common control genes are given in the next section), do not vary under experimental conditions or in different states of the same tissue (e.g. diseased vs normal). Their level is therefore used as a reference value for quantification. The relative quantification procedure varies depending on whether the target molecule and reference molecule are amplified with comparable or different efficiencies.

**Different amplification efficiencies**

Amplification efficiencies will differ between the target and reference molecules if primer binding sites, PCR product sequences and PCR product sizes differ in each (i.e. the reference molecule is different to the target). In this case two standard curves are generated, one for each. Given that the expression of the reference does not change in different samples, the ratio of the two PCR products varies according to the expression level of the target gene.

**Comparable amplification efficiencies**

If amplification efficiencies between target and reference molecules are the same then one standard curve is sufficient. The standard curve is produced as described in the previous section but with a dilution series of only the reference. Unknown amounts of target and reference in the same sample are calculated by comparing the \( C_T \) values with the standard curve of the reference sample.
The use of 'control genes' for normalisation in quantitative PCR

'Control genes' or 'housekeeping genes', as they are sometimes called, are those genes that are constitutively expressed to maintain cellular function (Yamamoto et al. 1989). When using quantitative PCR technology, it is essential to take into account that there will be considerable variation of starting material between samples, especially if the samples have been obtained from different individuals. Ignoring this issue can lead to misinterpretation of the expression profile of any target genes being studied. This is where the concept of 'normalisation' against a 'reference' becomes relevant. As mentioned in the previous section, expression of mRNA can be normalised to total RNA concentration. Here, equal amounts of total RNA (e.g. 1μg) are taken from each of the samples and used to make the cDNA. When using this method it is essential to reverse transcribe all of the samples at one the time, using the same reagents and reaction conditions to minimise any operator error. When normalising to a control gene, the first thing that needs to be determined is whether the chosen gene is affected in any way by the experimental treatment and also if its expression levels are similar to those of the mRNA being studied (Schjerling, 2001). It is common to screen many genes until a suitable gene is found and the most common control genes used are listed in Table 2.2. Using control genes for correcting sample-to-sample variation seems to be an accepted method for minimising these errors. However, the problem when studying muscle arises when the intervention being studied is something like exercise, as a result of which many control genes commonly used for this purpose are affected.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>Beta actin</td>
<td>Cytoskeletal structural protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Oxidoreductase in glycolysis and gluconeogenesis</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
<td>Constitutes 85-95% of total cellular RNA</td>
</tr>
<tr>
<td>β2M</td>
<td>Beta-2-microglobin</td>
<td>Beta chain of major histocompatibility complex class I molecules</td>
</tr>
<tr>
<td>α-actin</td>
<td>Alpha actin</td>
<td>Cytoskeletal structural protein</td>
</tr>
<tr>
<td>RPL13a</td>
<td>Ribosomal protein L13a</td>
<td>Structural component of the large 60S ribosomal subunit</td>
</tr>
<tr>
<td>UBC</td>
<td>Ubiquitin C</td>
<td>Protein degradation</td>
</tr>
<tr>
<td>HMBS</td>
<td>Hydroxymethyl-bilane synthase</td>
<td>Heme synthesis, porphyrin metabolism</td>
</tr>
<tr>
<td>HPRTI</td>
<td>Hypoxanthine phosphoribosyl-transferase I</td>
<td>Purine synthesis in salvage pathway</td>
</tr>
<tr>
<td>SDHA</td>
<td>Succinate dehydrogenase complex, subunit A</td>
<td>Electron transporter in the TCA cycle and respiratory chain</td>
</tr>
<tr>
<td>YWHAZ</td>
<td>Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide</td>
<td>Signal transduction by binding to phosphorylated serine residues on a variety of signalling molecules</td>
</tr>
</tbody>
</table>

Table 2.2 11 commonly used control genes for normalisation in quantitative PCR. (Adapted from Vandesompele et al. 2002)
2.3 General Methods and Materials

Vastus lateralis muscle samples from 2 healthy volunteers were used for method optimisation purposes. Details of the needle biopsy technique are described in the relevant sections in Chapters 3-5.

Total RNA isolation

Total RNA was extracted from muscle samples using Tri Reagent (Sigma, UK) according to the manufacturers recommendations. The frozen weight of the tissue samples was determined prior to homogenisation. Tissue samples were then homogenised in 0.5 ml Tri Reagent (1ml/50-100 mg tissue) using a hand held Polytron homogeniser for 20-50 seconds. After homogenisation, the homogenate was spun at 12,000 x g for 10 minutes at 4°C in a bench top centrifuge to remove any insoluble material. The supernatant, which contained RNA and protein, was then transferred to a fresh tube. To ensure complete dissociation of nucleoprotein complexes the samples were left to stand for 5 minutes at room temperature. 100µl chloroform was added to the tube (0.2ml/1ml Tri Reagent). The samples were then shaken vigorously for 15 seconds and then allowed to stand for 2-15 minutes at room temperature. The resulting mixture was centrifuged at 12,000 x g for 15 minutes at 4°C. After centrifugation, the mixture separates into 3 phases: a red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA). The aqueous phase was transferred into a fresh tube and mixed with 250µl of isopropanol. The sample was then allowed to stand at room temperature for 5-10 minutes, after which it was centrifuged at 12,000 x g for 10 minutes at 4°C. The RNA precipitate formed a pellet on the side and bottom of the tube. The extracted RNA was dissolved in diethylpyrocarbonate (DEPC) treated water and stored at -70°C until required. Total
RNA was then treated with 10 units of RQ1 RNase-free DNase (Promega, Madison, WI) at 37°C for 30 min followed by inactivation at 65°C for 10 min in order to avoid amplification of contaminating genomic DNA. The entire extraction procedure was carried out in a fume hood due to the toxic nature of the chemicals used for RNA isolation.

**Measurement of total RNA concentration**

Total RNA concentration was determined by spectroscopy at 260 nm using the Gene Spec I (Naka Instruments). The quality of the RNA was then assessed by visual inspection of ethidium bromide stained 18s and 28s rRNA under ultra violet light.

**Electrophoresis of RNA**

To ensure the integrity of the RNA samples, total RNA was separated under denaturing conditions using formaldehyde agarose gel electrophoresis.

**Preparation of 1.2% formaldehyde gel**

3.6g of agarose was added to 231ml of DEPC-treated water and boiled for 2 mins to allow the agarose to dissolve. After cooling to 55°C, 60 ml of 5 x MOPS buffer (0.1M MOPS, pH 7.0, 40mM sodium acetate, 5mM EDTA, pH 8.0) and 9 ml of 37% formaldehyde were added. The gel was poured and allowed to set.

**Preparation of RNA samples**

5μg of total RNA was mixed with 4μl of 5 x MOPS buffer, 7μl of formaldehyde and 20μl of formamide. Samples were incubated at 65°C for 15 minutes and then chilled on ice. After brief centrifugation, 4μl of gel loading buffer (50% glycerol, 1mM
EDTA pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol FF) was added to each of the samples. Samples were then loaded onto the gel, which had been allowed to pre-run for 5 minutes at 5 V/cm and electrophoresed in 1x MOPS buffer for 2-2.5 hours at 80-100V. To confirm the integrity of RNA samples following electrophoresis, visual inspection of ethidium bromide stained 18s and 28s ribosomal RNA bands was performed under ultra violet light. All reagents were supplied by Sigma.

Handling RNA
Ribonucleases (RNases) are very stable and active enzymes capable of destroying RNA, even when present at very low concentrations. All plastic and glassware was therefore treated with diethyl pyrocarbonate (0.1% DEPC in water) to avoid any possible RNase contamination.

Primers for cDNA synthesis and PCR
Primer sequences were specifically designed from human mRNA sequences and accession numbers for these sequences are given in Table 2.3 below. All primers used for cDNA synthesis and real time PCR were designed using Omiga version 2.0 software (Oxford Molecular, UK) and these were synthesised and purified by high-pressure liquid chromatography (HPLC) (Sigma Genosys, Cambridge, UK). Primers used for conventional RT-PCR in the study described in Appendix 1 were taken from Chew et al, 1995. Sequence analyses were performed using BLAST searches on the EMBL database. Nucleotide sequence alignments and pair-wise alignments for sequence comparisons and primer positioning were performed using the CLUSTAL W programme, part of the EMBL genome analysis facilities. Table 2.4 lists the
sequences of all the primers (forward and reverse) used in the studies described in Chapters 3-5, given in the 5’ to 3’ direction.

<table>
<thead>
<tr>
<th>Target</th>
<th>Accession No</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-IEa</td>
<td>X57025</td>
</tr>
<tr>
<td>IGF-IEb</td>
<td>X57025/U40870</td>
</tr>
<tr>
<td>MGF</td>
<td>U40870</td>
</tr>
<tr>
<td>α-actin</td>
<td>AF182035</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM002046</td>
</tr>
<tr>
<td>MyoD</td>
<td>X56677</td>
</tr>
</tbody>
</table>

Table 2.3 The different mRNA targets investigated using real time quantitative PCR and their corresponding accession numbers.
Table 2.4 Primers used for real time quantitative PCR and conventional PCR given in the 5' to 3' direction with their corresponding annealing temperatures and the expected product size of the PCR products. Primes used for conventional RT-PCR, taken from Chew et al. 1995 are named MGF-2 and are shown in ‘blue’. The sequence of the specific RT primer used for cDNA synthesis is given at the end.

First strand cDNA synthesis

Following RNA extraction, total RNA was then reverse transcribed into cDNA using Omniscript reverse transcriptase (Qiagen, UK). 0.5μg of total RNA was then mixed with DEPC treated water in a total volume of 10μl and heated to 65°C for 5 min before transfer to ice. The samples were then mixed with 2μl First Strand Buffer (10x), 2μl dNTPs (5mM each), 50 pmol random primers or 15 pmol of sequence specific primer, 1μl RNase inhibitor (10 units/μl) and 1μl Omniscript Reverse Transcriptase (4 units/μl). The reaction volume was made up to 20μl using DEPC.
treated water. The samples were then incubated at 37°C for 1 hour followed by 5 min at 93°C to inactivate the reverse transcriptase.

**RT-PCR**

For initial PCR work (see Appendix I) conventional RT-PCR was used to determine the presence of the different IGF-I isoforms in human muscle. PCR reactions were set up in a volume of 50μl and each reaction mix contained 5μl of 10x PCR buffer (100 mM Tris-HCl, 500 mM KCl pH 8.3), 2μl of dNTPs (10mM of each dATP, dCTP, dTTP, dGTP) (Bioline, UK), 1μl forward primer (25pmoles), 1μl reverse primer (25pmoles) and 1μl of first strand cDNA. MgCl₂ concentration was optimised for each PCR reaction and ranged between 1.0mM to 3mM (Bioline, UK). The reaction components were gently mixed and briefly centrifuged. The samples were heat denatured at 94°C for 5 minutes and then 2.5units of Taq polymerase (Bioline, UK) were added. Amplification was then carried out for 35 cycles of consisting of a denaturation step at 94°C for 1 minute, an annealing step at the specific T°C of the target being amplified for 1 minute and a final elongation step at 72°C for 1 minute. An additional elongation step at 72°C for 10 minutes was included at the end to ensure all PCR products had 3’ A overhangs to facilitate cloning of the PCR products. RT-PCR was performed on a conventional PCR machine (Hybaid PCR Express).

**Real Time Quantitative PCR using SYBR Green I**

Quantification of mRNA was carried out using LightCycler technology (Roche Diagnostics, UK). Reactions were performed in a volume of 20μl per capillary for the LightCycler format. The 20μl reaction mix contained 10μl of a SYBR green mix
(QuantiTect, Qiagen, UK), 0.5-10pmol of each forward and reverse primer, 2μl cDNA (made from 0.5μg RNA) and nuclease free water to make up the reaction volume.

SYBR green I was used as the method of detection. A dilution series of standard DNA (which contained the target sequence of interest) of 'known' concentrations was included in each run from which a standard curve was created (see Figure 2.6). It was relative to this standard curve that samples of unknown concentration were quantified.

On completion of the PCR, all PCR products formed were melted to attain a melting curve profile, which enabled the specificity of the reaction to be determined. All runs were performed in duplicate and mean values were subsequently used for analysis.

Figure 2.6. A typical standard curve generated from a dilution series of standard DNA of known concentration.
Typical programme

A typical LightCycler programme protocol when using the QuantiTect SYBR Green PCR kits (Qiagen, UK) consists of four main steps:

**Activation Step (1 cycle)**

HotStarTaq DNA polymerase is activated by this heating step. HotStarTaq has no enzymatic activity at ambient temperatures and this prevents the formation of misprimed products and primer-dimers during reaction set-up and the first denaturation step. The enzyme is activated by a 15 minute incubation step at 95°C.

**Amplification Step (35-55 cycles)**

This step most commonly consists of three segments: (i) Denaturation at 94°C for 15 sec (ii) Annealing at a temperature approximately 5-8°C below the Tₘ of the specific primers being used for an optimised incubation time of between 20-30 sec, so as to increase the specificity of primer binding to target and (iii) Extension at 72°C for a time that is calculated depending on the length of the amplicon (approximately 5 sec for every 100bp of product) An additional fourth step can be included in the amplification step, where fluorescence acquisition is recorded at a temperature just below that of the specific product, so as to avoid any fluorescent signal from non specific products such as primer-dimers and to ensure accurate quantification of only the desired product. This step of either 3-4 segments is then repeated for 35-55 cycles depending on the amount of template DNA present.

**Melting curve (1 cycle)**

This step also consist of three segments: (i) a rapid denaturation at 95°C (ii) a short hold at a temperature approximately 10°C higher than the primer annealing
temperature during amplification step and (iii) a slow denaturation at 95°C. During the slow denaturation step, fluorescence is measured continuously to generate a high-resolution melting curve for every sample.

**Cooling**

Following the melting curve step, the LightCycler instrument is cooled down to 40°C for 30 sec. This is simply to allow the carousel to be lifted safely out of the instrument.

**Data Analysis**

There are two different methods of quantification i.e. calculating the crossing points on the LightCycler: the fit point method and the second derivative maximum method.

**Fit point method**

This method allows the user to set the 'noise band' according to the threshold in the log linear phase of PCR. 'Fit points' are defined as a number of reading points in the log linear phase, used for calculation of a straight line that represents the linear portion of the amplification plot. It is possible to change the number of fit points. The crossing point in this case is the cycle number at which the straight line (calculated using fit points) crosses the noise band.

**Second derivative maximum method**

The point at which the maximal increase of fluorescence within the log linear phase takes place is calculated by determining what is referred to as the second derivative maxima of the amplification curves. The cycle number at which this point is reached is calculated and recorded. With this method, it is not the operator that sets the noise
band and thus is the preferred method of analysis, as it avoids any operator bias. This method was therefore used when analysing data from all the studies.
Figure 2.7. An example of some of the different screens presented to the user. (A) shows an example of the screen the user is presented with once a run is complete. The top half of the screen shows a summary of the temperature cycling profile i.e. the 1 cycle activation step, the 35-40 cycle amplification step, and the 1 cycle melting curve step. The stage at which fluorescence is acquired is shown in purple. The bottom half of the screen displays the amplification values obtained relative to the standard curve and (C) – which shows the melting curve profile of the specific products formed during the PCR.
**Gel Electrophoresis**

PCR products were recovered from the glass capillaries by removing the plastic caps and inverting them into a 1.5ml eppendorf tube, which were then spun down in a bench top centrifuge at no higher than 3000 rpm. 20μl of these PCR products were mixed with 2μl of 6X Loading buffer (Fermentas, UK). The 2% agarose gel was prepared by adding 2g agarose to 100ml tris/acetate buffer (TAE) and heating in the microwave for approximately 2 mins to dissolve the agarose. After cooling slightly, ethidium bromide (10mg/ml) was added to the mix, which was then poured/cast into a gel tank and left to set. The PCR products + loading buffer were then carefully loaded into the wells as was a size marker (DNA ladder, Fermentas, UK). The gel was left to run for approximately 45 mins at 100 volts until good separation of the dye fronts had been achieved. Target specificity was further confirmed by visualising the product bands under UV light.

**Making standards**

If making standards for use in real-time quantitative PCR, the bands were briefly visualised using a hand held trans-illuminator and then carefully excised from the gel using a clean blade (exposure to short-wave ultraviolet light was minimal in order to avoid the formation of pyrimidine dimers). These gel pieces containing the product of interest were then purified as described in the next section.

**DNA purification**

DNA was purified using Wizard PCR Preps DNA purification system, Promega, UK. Briefly, approximately 300 mg of the agarose slice was transferred to a 1.5ml microcentrifuge tube, to which 1ml of resin was added. This was heated to 65°C in a
water bath until the agarose was completely melted. For each gel sample one Wizard Minicolumn was prepared into which the resin/DNA mix was pipetted. A plunger was slowly pushed through the syringe barrel gently pushing the mix into the minicolumn. 2ml of 80% isopropanol was then pipetted and pushed through the syringe barrel to wash through the column. The syringe was then removed from the minicolumn and this was placed into a 1.5ml microcentrifuge tube. The minicolumn was then centrifuged at 10 000 x g to dry the resin. The minicolumn was then transferred to a new microcentrifuge tube into which 50µl was applied. After waiting for one minute, the minicolumn was centrifuged at 10 000 x g for 20 seconds to elute the DNA fragment. The dsDNA concentration of the purified products was determined by absorbency at 260nm where absorbency of 1 cm path length cuvette at 260nm is equivalent to 50µg/ml DNA. The purified DNA could be stored in the microcentrifuge tube at -20°C.
2.4 Technique Development

The following section of this chapter will focus on optimisation strategies employed in developing a sensitive, reliable and reproducible quantitative PCR assay using LightCycler technology, for the study of IGF-I isoform expression in human skeletal muscle.

Optimisation Strategies

As mentioned earlier in this chapter, real-time quantitative PCR is a highly sensitive and versatile technique. However, for it to be reliable and reproducible requires extensive optimisation. How reliable and how reproducible depends largely on the target genes of interest and the levels at which they are expressed in the tissue being studied. Abundantly expressed genes prove least problematic to quantify compared with genes expressed at low levels, which can require extensive optimisation, particularly on the LightCycler set-up. The work in this thesis was concerned with the study of IGF-I and its isoforms in muscle. The technique therefore required extensive optimisation to ensure reliable and reproducible quantification of these, in particular MGF, as this proved to be the most problematic. The following section details optimisation strategies performed to ensure specific, sensitive and reliable quantification of IGF-I and its three isoforms in human skeletal muscle.

Primer Design

Primers for real-time PCR ideally need to generate a product of between 100-250 bp, the advantage of this being that shorter products amplify more efficiently than longer ones (Bustin, 2001). Smaller products are also more tolerant of reactions conditions, as they have a higher chance of being fully denatured during the initial denaturation step of
PCR, thereby allowing the primers to bind more successfully to their complementary targets. Furthermore, with short products the extension time can be kept as little as 15 seconds (5 seconds per 100bp, with a minimum extension time of 10 seconds), thereby considerably reducing the time it takes to complete a run and the chances of amplifying genomic DNA. Primers that were used for real-time quantitative PCR on the LightCycler were designed using Omiga version 2.0 primer design software (Oxford Molecular, UK). The optimal length for individual primers is approximately 15-20 bp and the Guanine/Cytosine (GC) content should be between 40-60% with an optimum of approximately 50%. Ideally, the Tm of primer pairs should not differ by more than 1-2 °C. When designing primers, it was ensured that the forward and reverse primers were located in different exons of the gene and that they preferably spanned an intron/exon boundary. This is one way of ensuring that the primers do not amplify genomic DNA. As an additional precaution, all RNA samples were treated with RNase-free DNase during the RNA extraction step. Specificity of the primer sets for the product of interest is essential. It was found that primers, which work well when using conventional PCR, don't necessarily work well on the LightCycler system. Therefore, in some cases it was worthwhile designing 3-4 optimal primer sets simultaneously and screening them to determine the single most efficient pair. Figure 2.8 shows the melting curve profile of two different sets of primers for the same gene during the screening process. It should be noted that the single, sharp product peak seen in Figure 6B is the desired result. Any double peaks relate to primers miss-priming and annealing to non-specific products. All primer sets used were synthesised by Sigma Genosys (UK), HPLC purified and made to a concentration of 0.05μmol.
Figure 2.8. Melting curve peaks for two MGF primer sets (A and B) generated by the LightCycler software. (A) shows the curve generated from a sub-optimal primer set which gives rise to multiple peaks. These double peaks relate to the primers miss-priming. (B) shows the melting peak curve for an optimal pair of primers. The sharp and narrow single product peak relates to the primers annealing to a specific product. There is evidence of a small amount of primer-dimer in some samples. The larger primer-dimer peak relates to the ‘no-template’ control sample (negative control).

Total RNA concentration

Because of the nature of these studies, tissue size was always a limitation. It was therefore necessary to determine the lowest starting concentration of total RNA that could be used to make cDNA, with which it was still possible to attain reliable and reproducible results. Different concentrations of total RNA ranging from 0.1μg, 0.2μg to 1μg were used to make cDNA. This cDNA was then used to amplify the abundantly expressed target, GAPDH, and the low expression level target, MGF in duplicate runs on the LightCycler (see Table 2.5). The study showed that 0.5μg of total RNA was the lowest starting concentration of RNA that could be used to obtain reproducible results, particularly when amplifying low expression level targets such as MGF.
RNA Cone (fig)

<table>
<thead>
<tr>
<th>RNA Conc (μg)</th>
<th>GAPDH Run 1 (ng)</th>
<th>GAPDH Run 2 (ng)</th>
<th>MGF Run 1 (ng)</th>
<th>MGF Run 2 (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>X</td>
<td>X</td>
<td>X</td>
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</tr>
<tr>
<td>0.2</td>
<td>2.3x10^-3</td>
<td>2.7x10^-3</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>0.5</td>
<td>5.2x10^-3</td>
<td>5.1x10^-3</td>
<td>3.3x10^-8</td>
<td>3.2x10^-8</td>
</tr>
<tr>
<td>1.0</td>
<td>8.6x10^-3</td>
<td>8.6x10^-3</td>
<td>5x10^-8</td>
<td>5.1x10^-8</td>
</tr>
</tbody>
</table>

Table 2.5 Table showing quantitative values from duplicate runs for GAPDH and MGF made from cDNA containing different amounts of starting material. Values are expressed as ng of ss PCR product. ‘X’ denotes where the value was below the limit of detection.

Magnesium Concentration

The PCR buffer used in the DNA Master SYBR Green I kits (Roche, UK) required that a magnesium titration be carried out. The optimum Mg concentration at which the primers worked most efficiently was determined by carrying out Mg titrations between a range of 1-5mM, for each of the genes of interest.

Annealing Temperature and Annealing Time

An appropriate annealing temperature (T_a) can be worked out using the following formula:

\[ T_a = 2(A + T) + 4(G + C) \]

(A=adenine T=thymine G=guanine C=cytosine)

However, it was often the case that the optimum annealing temperature for each gene of interest needed to be determined by carrying out a series of runs in which the annealing temperature was increased or decreased in 1°C or 2°C increments. The annealing time also contributed to the efficiency with which the primers annealed optimally to the
product of interest and minimally to any non-specific products. When using the Roche DNA Master SYBR Green I kits, the annealing time can lie anywhere between 1-5 seconds for any gene analysed. Therefore, once the optimal annealing temperature was determined the optimal annealing time was decided by performing a series of runs with different annealing times.

**Transfer RNA concentration**

Transfer RNA (tRNA) reduces the formation of primer artefacts during quantitative PCR (Sturzenbaum, 1999) and was added to the LightCycler reaction mixture to avoid non-specific binding of reagents to the Lightcycler glass capillaries. It was found that adding tRNA to the reaction mix also increased the efficiency of amplification of low-level transcripts such as MGF, by reducing any primer dimer formation. An optimum concentration of tRNA was determined by performing a tRNA titration at different concentrations.

**Sequence Specific RT Priming**

To facilitate the efficiency of reverse transcription (RT) in transcripts expressed at low levels (e.g. MGF), short specific primers 50-100 base pairs downstream of the PCR reverse primers were used. A specific RT primer (dodecamer) was designed downstream from the specific reverse primers for IGF-1Ea and MGF to enhance the amplification of both of these isoforms in the samples. The position of the specific RT primer that was designed in relation to the reverse primers for MGF and IGF-1Ea is shown in Figure 2.9.
**Exon 3** (157), **Exon 4** (182), **Exon 5** (49 bp) **Exon 6** (344)

**Figure 2.9.** Part of the mRNA sequence of exons 3, 4, 5 and 6 of the human IGF-I gene. Reverse primers for IGF-Ie and MGF are highlighted in yellow and blue respectively. The position of the specific RT primer in relation to reverse primers for IGF-Ie and MGF is shown highlighted in red.
Generating standard curves

In order to generate a standard curve, at least 5 different concentrations of the standard DNA should be measured and the amount of unknown target should fall within the range tested. To ensure reproducibility, reactions should be carried out in duplicate.

Standards

When using ‘absolute’ quantification, as in this study, the copy number or concentration of the nucleic acids used as standards must be known. Furthermore, standards should have the following properties: (i) primer binding sites identical to the target being quantified (ii) sequence between primer binding sites identical or highly similar to target sequence and (iii) sequences upstream and downstream from the amplified sequence identical or similar to natural target.

The ‘type’ of standard used depends entirely on what type of nucleic acid is being quantified. RNA or DNA molecules can be used to quantify RNA or DNA respectively as can genomic DNA, plasmid DNA or PCR products. It was found that purified PCR products (see Methods and Materials) have the most comparable amplification efficiencies as samples. This may be because they have essentially undergone the same prior experimental treatment (i.e. RNA extraction and cDNA synthesis) as the sample being quantified.

Reproducibility of standard curves

With absolute quantification, it is essential to understand the importance of the quality of the standards used to generate the standard curve from which the
concentration of unknown samples is determined. The amount of care that needs to be taken when designing, making and calibrating standards really depends on the research question being asked. If only relative changes are important, for example changes in gene expression in response to a treatment, it is probably not as important to know the actual concentration of the standards, it only matters that the standards are consistent. It is generally accepted that determining the concentration by absorbance at 260nm is a consistent method of calibrating standards (as long as the same purification procedure is always used). If the absolute quantity of target, rather than the ratios is of interest, greater care must be taken in determining the specific RNA levels.

**Calibration of standard curves**

As this work focuses largely on the changes in expression of the IGF-I isoforms in human muscle with exercise, it is important that the standards used to generate the curve are consistent and reproducible. As mentioned above, standards were made from purified PCR products. The concentration of these purified PCR products was determined by absorbance at 260nm. To avoid the effects of repeated freezing and thawing, after purification, the DNA was aliquoted into smaller volumes and stored at -20°C. On the day that quantitative PCR was performed, one of these aliquots was thawed, its concentration re-determined to see if there had been any changes to the concentration and then serially diluted for use in PCR. To determine the consistency of the standards, slope values from 6 separate standard curves used in the same study were compared (see Table 2.6). As the LightCycler carousel only allows a maximum of 32 samples to be loaded at one time, it was sometimes necessary to split analysis of samples into two or three, depending on how many samples there were for a
particular study. In such cases it is essential that the standard curves between runs are directly comparable.

<table>
<thead>
<tr>
<th>Separate runs</th>
<th>Slope values</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-3.579</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>-3.537</td>
<td>1.9</td>
</tr>
<tr>
<td>3</td>
<td>-3.430</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>-3.483</td>
<td>1.9</td>
</tr>
<tr>
<td>5</td>
<td>-3.443</td>
<td>2.0</td>
</tr>
<tr>
<td>6</td>
<td>-3.508</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Table 2.6 Variability of standard curves from 6 different runs from the same study.

2.2.1 Ensuring that standards and samples run with similar efficiency

To calculate the efficiency of a PCR run the following equation can be used:

**Efficiency = 10^{-1/slope}**

Ideally, the PCR efficiency should be as close to '2' as this would suggest a perfect PCR doubling with each cycle. It is possible to determine whether samples and standards are running at similar PCR efficiencies. This can be achieved by serially diluting a cDNA sample in the same dilution series as a set of standards (e.g. 10 fold dilutions). When programming the LightCycler, the cDNA samples are programmed in as standards with a 'known' concentration and then both dilution series of samples and standards are run within the same run. It is then possible to determine the slope values for each individual dilution series and these can be compared with one another.
Fully optimised MGF run using Roche chemistry

A fully optimised run using the Roche reagents for a less abundantly expressed target such as MGF is shown in Figure 2.10. To achieve this level of target specificity, the PCR reaction included the addition of tRNA, addition of an optimal amount of MgCl₂ and fine-tuning of the annealing temperature and time used. Although the level of primer dimer is minimal it will still interfere with the true quantitative values. The LightCycler software provides an option to take into account any background fluorescence generated from the amplification of primer dimers, by enabling quantification to be carried above their melting temperature. However, this does not eliminate the fact that the PCR reaction is sub-optimal and primer dimers are still being formed.

![Figure 2.10. A fully optimised run using Roche chemistry. The broader peaks denote primer dimers whereas the narrower peaks at a higher melting temperature the specific MGF product.](image)
Inconsistencies with Roche chemistries

Thus far all optimisation had been carried out using Roche chemistries. However, it was found that there were inconsistencies in the absolute values attained from the same sample when amplified using different stocks of SYBR Green I from the same batch, and these inconsistencies were even further compounded when using SYBR Green I from a different batch. It was also determined that a kit which could be used to amplify one gene e.g. GAPDH, would not necessarily work to amplify another gene e.g. IGF-1Ea. A further problem was then detected with the DNA Master SYBR Green I Fast Start kits. It was found that using different concentrations of MgCl₂ in the reaction mix as a part of the optimisation procedure would cause varying degrees of white precipitate formation in the glass capillary, over the course of the PCR. This undoubtedly interfered with the true quantitative values. In light of all of these problems it became necessary to investigate using an alternative chemistry for real time PCR.

Adapting the protocol for use with a different chemistry

All analysis for the studies detailed in Chapters 3–5 was performed using QuantiTect SYBR Green PCR kits (Qiagen, UK). These kits became available on the market in November 2001 and were specifically designed for use with two real-time PCR systems, one of which was the Roche LightCycler. Switching chemistries did require having to completely re-optimise the reaction conditions. However, the results obtained with these new kits proved to be highly reproducible, even when amplifying low copy number targets, which had previously proved to be most problematic.
Optimisation of quantitative PCR using Qiagen QuantiTect kits

Unlike the Roche kits, these kits required less optimisation and were altogether more consistent. The most important consideration seemed to be the size and specificity of the primers. The annealing temperatures for all targets were also found to be considerably lower (sometimes more than 10°C) than had previously been the case with the Roche kits e.g. to amplify IGF-IEa using the Roche kits required an annealing temperature of 61°C. Whereas, with the Qiagen kits the best annealing temperature for this target was 52°C. It was also no longer necessary to add and optimise the MgCl₂ concentration with the Qiagen kits, as the QuantiTect SYBR Green PCR buffer employs a different system that contains a balanced combination of KCl and (NH₄)₂SO₄. This is believed to promote a high ratio of specific to non-specific primer binding during the annealing step of each PCR cycle, creating stringent primer annealing conditions, leading to increased PCR specificity. When using this buffer system, primer annealing is only marginally influenced by the addition of MgCl₂.

Fully optimised runs using Qiagen QuantiTect kits

Fully optimised runs for targets IGF-IEa and MGF are shown in Figure 2.11 below. The melting curve profiles of the quantitative runs show only a sharp narrow peak relating to the specific target of interest with no primer dimer peaks.
**Figure 2.11.** Fully optimised runs for IGF-IEa and MGF using Qiagen QuantiTect chemistry. For each target of interest, the conditions are such that there is only amplification of specific product and no primer dimmer (shown by the series of coloured lines forming the peak). The no template (negative) control (flat blue lines), with no target mRNA contains no specific product hence is denoted by a flat line.


2.5 Initial Studies using the LightCycler

Having established a fully optimised method for the quantification of IGF-I and its isoforms in human muscle, it was necessary to answer two critical questions. Firstly, how reproducible was the method on the LightCycler set-up? Secondly, given the low levels of expression of genes such as MGF, what was the optimal amount of cDNA to add to the reaction mix to increase the reproducibility within the run? The following two studies detailed below were performed to address these questions. Also, a third study was performed to determine whether different starting concentrations of total RNA were accurately reflected by the quantitative values calculated for a particular target. Details of RNA extraction, first strand cDNA synthesis, generating standards and real time PCR are given earlier in this chapter in section entitled General Methods and Materials.
Study 1: Intra-run reproducibility on the LightCycler

Aim
To determine the intra-run reproducibility at different concentrations of GAPDH plasmid DNA ranging from $1 \times 10^{-2} - 1 \times 10^{-8}$ ng / μl.

Methods
5 identical volumes (2μl) of the same concentration of GAPDH plasmid DNA were included in the same run. 7 separate runs containing different concentrations of plasmid DNA ranging from $1 \times 10^{-2}$ to $1 \times 10^{-8}$ ng / μl were then performed using the same reagents (SYBR Green I master-mix, Mg$^{2+}$ and primers). From the quantitative values obtained for all 7 different GAPDH concentrations, the coefficient of variation of the 5 samples was calculated for each different concentration used.

Results
The results from this study are shown in Figure 2.12. The coefficient of variation decreases as the concentration of plasmid DNA increases. This shows that the intra-run reproducibility is therefore higher and more consistent at higher DNA concentrations.
Figure 2.12. Intra-run reproducibility within a LightCycler run.

Conclusion

It was found that the reproducibility within a run was higher and more consistent at higher target concentrations, which was to be expected. However, this posed a problem, as some of the transcripts of the genes of interest were inevitably going to be present at very low concentrations, such as MGF. It was therefore important to carefully optimise conditions so that rare targets could be quantified reliably and reproducibly on the LightCycler system. The next phase was therefore to perform an experiment to determine how the intra-run reproducibility could be improved.
Study II: Improving the intra-run reproducibility

Aim

To determine the effects of increasing the amount of DNA added to the reaction mix on the intra-run reproducibility, in particular at low concentrations of target.

Methods

Three separate runs were carried out in which the reaction mix contained three different volumes of plasmid DNA, these being 2μl, 5μl and 10μl respectively. These runs were then performed with 5 capillaries that contained the same amount of DNA i.e. the 5 tubes in run 1 contained 2μl of plasmid DNA, the 5 tubes in run 2 contained 5μl and the 5 tubes in run 3 contained 10μl. These three runs were then repeated using three different concentrations of plasmid DNA: 1x10^6ng / μl, 1x10^7ng / μl and 1x10^8ng / μl. The coefficient of variation was then determined for each volume of DNA added, and this was done for all three concentrations of target.

Results

The results from the run containing the three different volumes of plasmid DNA at a concentration of 1x10^6ng / μl can be seen in Figure 2.13. It was observed that the intra-run reproducibility increased by proportionally increasing the DNA volume added to the reaction mix.
Figure 2.13. The effects of increasing DNA volume on the reproducibility within a run. Each point represents a mean of 5 different PCR reactions within the same run.

**Conclusion**

It would seem that when analysing samples where the target concentration is low such as with MGF, a proportionally larger volume of cDNA could be added to the reaction mix to ensure good reproducibility of results. In this case, as Roche buffers were used to make the cDNA (Expand Reverse Transcriptase, Roche Diagnostics) and to carry out the PCR, both systems were highly compatible with one another. It was therefore possible to add a cDNA volume which constituted up to 50% of the reaction volume (i.e.10μl) without interfering with the PCR or causing any kind of PCR inhibition. However, it should be noted that for the studies reported in Chapters 3-5 a different type of chemistry was used (see section on ‘Adapting the protocol for use with a different chemistry’), which produced more consistent and reproducible measurements, even at low target concentrations.
Study III: Correlation between total RNA concentration and quantitative values

Aim
To establish whether mRNA concentrations of GAPDH determined by real time quantitative PCR on the LightCycler correspond to the original total RNA concentration used to make cDNA.

Methods
Three different starting concentrations of total RNA from a resting muscle sample were used to make cDNA, these were: 0.2μg, 0.5μg and 1μg. First strand cDNA synthesis was performed at the same time using the same reagents and conditions for all three samples. Levels of expression of GAPDH mRNA were quantified using real-time quantitative RT-PCR relative to a set of standards, serially diluted in 10 fold dilutions ranging from 1ng / μl to 0.00001ng / μl (1x10^{-5}ng / μl). Duplicate runs were performed. The ratios between GAPDH mRNA concentrations for the three samples were then compared with the original total RNA concentration added. Linear regression analysis was used to determine if there was any relationship between the initial concentration of total RNA used to make cDNA and the final GAPDH mRNA concentration determined by quantitative PCR.

Results
The ratios between GAPDH mRNA concentrations from the three different samples were found to be highly comparable to the original total RNA concentration used to make cDNA in both runs. These results are shown in Table 2.7 below.
Ratios are calculated as follows:
For example, if $1.8 \times 10^3 \text{ng} = 1$ as it is the highest concentration value.
A concentration of $1.2 \times 10^3 \text{ng}$ is therefore $1.2 \times 10^3 \div 1.8 \times 10^3 = 0.66$, of this.

<table>
<thead>
<tr>
<th>Total RNA</th>
<th>GAPDH concentration (Run 1)</th>
<th>Ratio</th>
<th>GAPDH concentration (Run 2)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µg</td>
<td>$1.8 \times 10^3 \text{ng}$</td>
<td>1</td>
<td>$2.7 \times 10^3 \text{ng}$</td>
<td>1</td>
</tr>
<tr>
<td>0.5 µg</td>
<td>$1.2 \times 10^3 \text{ng}$</td>
<td>0.66</td>
<td>$1.8 \times 10^3 \text{ng}$</td>
<td>0.68</td>
</tr>
<tr>
<td>0.2 µg</td>
<td>$1.7 \times 10^{-4} \text{ng}$</td>
<td>0.1</td>
<td>$2.9 \times 10^{-4} \text{ng}$</td>
<td>0.11</td>
</tr>
</tbody>
</table>

**Table 2.7** Comparison of starting RNA concentration with ratios of GAPDH mRNA concentration after quantitative PCR using the LightCycler. The ratios of GAPDH mRNA concentration are shown in red for 2 duplicate runs.

![Graph](image)

**Figure 2.14.** Correlation between total RNA concentration and mean GAPDH mRNA concentration from 2 duplicate runs ($r = 0.98$).
Conclusion

This study has shown that the original total RNA concentration used to make cDNA can be quite accurately reflected in the mRNA concentration of an abundantly expressed target such as GAPDH. The significant relationship ($r = 0.98, P<0.05$) between initial total RNA concentration and GAPDH mRNA concentration (Figure 2.14) also highlights that performing first strand cDNA synthesis of samples at one time, using the same reagents and conditions is important to minimise operator error when analysing multiple samples. Even if the data is not normalised to a control gene such as GAPDH, it is beneficial to know that the reverse transcription procedure has been successful, and that there is essentially an equal concentration of mRNA between all samples. Control genes can therefore also prove useful as an initial check of the quality and concentration of cDNA, and should be amplified from the same sample that will subsequently be used for analysis of other targets of interest. Running an initial check in this way is extremely useful, as it is difficult to determine the concentration of cDNA by absorbance at 260nm due to the high concentration of interfering dNTPs.
Chapter 3

Expression of IGF-I splice variants in young and old human skeletal muscle after a single bout of high resistance exercise
3.1 Introduction

The ageing process is associated with a progressive decrease in skeletal muscle mass. This time dependent muscle loss is a major cause of disability, frailty and loss of independence in the elderly, due mainly to the associated loss of muscle strength and power (Harridge and Young, 2000). We know that strength training exercise increases muscle mass and strength in young individuals. The responsiveness of aged muscle to strength training has also been confirmed by longitudinal studies of previously non-active elderly people. Progressive resistance training trials in healthy elderly women aged 76-93 years (Skelton et al. 1995) and frail, institutionalised subjects aged 72-98 years (Fiatarone et al. 1994, Harridge et al. 1999), confirm that elderly muscle remains responsive to training.

The mechanisms involved in increasing muscle mass with strength training are still not fully understood. However, IGF-I has been shown to have an important role in the hypertrophic adaptation of muscle to overload. Studies in support of this have been detailed in Chapter 1. Briefly, the role of IGF-I as a potential hypertrophic agent in both young and older muscle was shown in studies where a 15% increase in muscle size and strength of young animals was reported following injection of a viral construct containing IGF-I (Ea isoform) cDNA. In the older animals, the injected muscles were 27% stronger than controls and there was also evidence of a prevention of age related loss of muscle mass (Barton-Davies et al. 1998). Furthermore, a recent study in which a transgenic mouse model overexpressing the same isoform (termed m.IGF-I in this case) in skeletal muscle was shown to have pronounced muscle hypertrophy (Musaro et al. 2001). The role of the different isoforms of IGF-I in muscle hypertrophy has been less extensively studied. The two muscle isoforms of
IGF-I: IGF-IEa and MGF, have however been shown to be positive regulators of muscle growth, as their expression has been shown to increase in animal muscles which have been subject to stretch and stimulation (Yang et al. 1996; McKoy et al. 1999; Hill et al. 2003). IGF-I is thought to increase muscle mass through two main mechanisms. The first of which is through the stimulation of protein synthesis and decreasing the rate of protein degradation in muscle tissue (Bark et al. 1998), and the second is through the activation of satellite cells. Upon activation, these cells rapidly upregulate MyoD, a member of the myogenic regulatory transcription factors (MRFs) (Cornelison and Wold, 1997).

Apart from the preliminary finding that MGF is expressed in man (Yang et al. 1996), little is known about the regulation of different IGF-I isoforms in human skeletal muscle. The present study was therefore undertaken to study the effects of an acute bout of high resistance weightlifting exercise (the type known to result in muscle hypertrophy) on the different IGF-I transcripts in human skeletal muscle, shortly after the end of exercise. This was in relation to age, MyoD expression and MyHC isoform composition of the muscle.

The main aims of this study were:

(i) To determine whether IGF-IEa and MGF mRNAs responded differently to high resistance exercise in human skeletal muscle.

(ii) To determine whether there was an age related impairment in the expression of IGF-IEa or MGF mRNA after high resistance exercise.

(iii) To confirm the localisation of MGF mRNA to within the muscle fibres.
(iv) To ascertain if there were any correlations between the mRNA expression of IGF-IEa or MGF and MyoD in the 'short-term' response to high load exercise.

(v) To compare the MyHC isoform composition of young and old individuals and to determine whether there was any relationship between muscle composition and IGF-I isoform expression.
3.2 Methods and Materials

Subjects

Eight healthy young men (age 29.5 ± 1.5 years, body mass 81.1 ± 2.4 kg, height 179.3 ± 1.8 cm) and 7 elderly men (age 74.4 ± 1.8 years, body mass 74.7 ± 2.1 kg, height 177 ± 2.3 cm) defined as healthy according to the criteria of Greig et al (1994), participated in this study. All subjects were given an oral and written briefing of the study before signing informed consent forms. The study was approved by the Ethics Committee of the Royal Free Hospital and all procedures were performed according to the Declaration of Helsinki.

Study design

After habituation to the knee extensor weight lifting apparatus (Ultimate Workout, Nottingham, UK), each subject completed a one repetition maximum (1-RM) test one week before the study day to determine the maximum load that could be lifted with one leg when carrying out knee extensor exercise (Figure 3.1). On the day on which measurements were made, subjects performed a standardised warm up which comprised cycling at 50 watts for 5 minutes on a cycle ergometer (Cybex – Metabolic Systems) and light stretching exercise. With the test leg they then performed 10 sets of 6 repetitions of the knee extensor muscles at 80% of their 1-RM. A 2-minute rest period was given between sets and the subjects were encouraged throughout. If it was apparent that the subjects were not going to complete 10 sets of 6 repetitions, then the weight was lowered to ensure completion of the exercise protocol. The contra-lateral leg served as a control. Following 2½ hours of recovery, a muscle biopsy was taken from the exercised (test) and non-exercised (control) leg.
Figure 3.1. A young subject performing knee extensor weightlifting exercise.

Muscle biopsy procedure

Following local anaesthesia (1% lignocaine), muscle biopsies were taken from the vastus lateralis muscle using the percutaneous needle biopsy technique (Bergström, 1962) with applied suction. The sample obtained from each biopsy was divided into two pieces. The first piece was quick frozen in liquid nitrogen for use in quantitative RT-PCR analysis and for electrophoretic separation of the MyHC isoforms. The second piece was orientated longitudinally, mounted in embedding medium (optimum cutting temperature compound (OCT)), and frozen in isopentane cooled to the temperature of liquid nitrogen.
RNA extraction

Total RNA was extracted from the test and control muscle samples using Tri Reagent (Sigma) as described in Chapter 2, Methods and Materials.

Measurement of total RNA concentration

The quality of the RNA was assessed as described in Chapter 2, Methods and Materials.

Electrophoresis of RNA

To ensure the integrity of the RNA samples, total RNA was separated under denaturing conditions using formaldehyde agarose gel electrophoresis. This procedure has been detailed in Chapter 2, Methods and Materials.

Primers used for cDNA synthesis and quantitative PCR

To facilitate the efficiency of reverse transcription (RT) in transcripts expressed at low levels, such as MGF, short sequence specific dodecamers 50-100 base pairs downstream of the reverse PCR primers for both MGF and IGF-IEa were specifically designed from the mRNA sequences. The oligonucleotide sequences for this specific RT primer and those used in real time quantitative PCR are given in Table 2.4 Chapter 2. For amplification of all other targets, random hexamers (dN)_6 (Roche) were used for first strand cDNA synthesis.

First strand cDNA synthesis

0.5µg total RNA was reverse transcribed into cDNA as described in Chapter 2, Methods and Materials.
Real time quantitative PCR

Quantification of the mRNA message coding for α-actin, IGF-IeA, and IGF-IeC (MGF) and the myogenic regulatory factor MyoD was performed using LightCycler technology (Roche Diagnostics, UK). Details of this method are given in Chapter 2, Methods and Materials. Runs were performed in duplicate and mean values were subsequently used for analysis.

Gel electrophoresis

PCR products were recovered from the glass capillaries by removing the plastic caps and inverting them into a 1.5ml eppendorf tube, which were then spun down in a bench top centrifuge at no higher than 3000 rpm. Target specificity was further confirmed by running these samples on a 2% agarose gel as described in Chapter 2, Methods and Materials.

DNA purification

Initially, to confirm the identity of the PCR products, these were purified using Wizard PCR Purification kit (Promega,USA), details of which are given in Chapter 2, Methods and Materials.

Cloning of PCR products

To confirm their identity, PCR products were cloned into specifically designed vector systems such as pGEM-T (Promega, USA) following DNA purification before being sent off for sequence analysis.
Ligation

3µl of purified PCR product was ligated to 50ng of PGEM-T vector in a final volume of 10µl, containing ligation buffer, T4 DNA ligase (3 Weiss units/µl) (Promega kit). Ligation reactions were mixed by pipetting then incubated overnight at 4°C to produce the maximum number of transformants.

Transformation

Transformation of JM109 High Efficiency Competent Cells (Promega, USA) was carried out as follows: 50µl of cells were directly pipetted into a fresh 1.5ml eppendorf tube containing 2µl of the ligation reaction. The vials were gently flicked to mix and then incubated on ice for 20 minutes. The cells were then heat-shocked for 45-50 seconds in a water bath at exactly 42°C, followed by incubation on ice for 2 minutes. 950µl of SOC medium (at room temperature) was added to each vial and these were incubated in a rotary shaker (~150 rpm) for 1 hour at 37°C. The transformation culture was plated on LB agar plates containing 50µg/ml ampicillin, which had been coated with 40µl X-gal (40mg/ml) and 40µl isopropyl-β-D-galactoside (IPTG). The plates were incubated at 37°C overnight.

Colony selection

Positive transformants were initially identified by blue/white colony selection. Colony PCR was performed on a selection of white colonies. Those that were found to contain the insert of interest were cultured overnight using a small-scale plasmid preparation system - Wizard Minipreps DNA Purification System (Promega, UK). 10µg of each mini-prep DNA was then sent off for sequence analysis. After confirmation of the identity of the PCR products it was not necessary to run the PCR
products on an agarose gel. Target specificity confirmed using the melting curve profile feature.

**SDS PAGE of MyHC isoforms**

The relative proportions of the three MyHC isoforms (MyHC 1, 2a and 2x) present in the vastus lateralis muscle from each of the young and old subjects was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) (Andersen and Aagard, 2000).

**Preparation of solutions and samples for SDS PAGE**

**Sample buffer stock solution**

The sample buffer stock solution contained 6.25ml of 0.5M Tris (pH 6.8), 10ml of 10% SDS, 10ml glycerol and 2.5ml β-mercaptoethanol. The solution was made up to 50ml using distilled water. Bromophenol (25mg) was added in order to stain the solution.

**Muscle tissue preparation**

Before adding the stock solution, the muscle tissue was homogenised manually with a pestle and mortar. Each sample had 500μl of sample buffer added to the tube. The mixture was then heated to 97° C for 3 minutes.

**SDS gel preparation**

The SDS PAGE gel electrophoresis system is a discontinuous system consisting of a stacking gel and a resolving gel. For the stacking gel a 4% solution was used in order to align the contractile proteins prior to separation.
Resolving gel preparation

A 7% resolving gel was made up with the following reagents: Acrylamide – Bis (100:1) (3.13ml), Lower Tris (pH 6.8) (1.34ml), Distilled H2O (1.05ml), Glycine (1.0ml), 10% SDS (0.4ml), Glycerol (3.0ml), APS (100μl) and TEMED (8μl). In order to set the resolving gel a set of glass plates was positioned in a cradle designed to hold the resolving gel plates. Approximately 4ml of the solution was poured into the space between the glass plates. The solution was allowed to set for approximately 2 hours prior to loading the stacking gel.

Stacking gel preparation

A stacking gel was made up using the following reagents: Acrylamide – Bis (37:5:1) (0.5ml), Upper Tris (pH 8.8) (1.25ml), Distilled H2O (3.25ml), APS (50μl) and TEMED (10μl). This solution was poured on top of the resolving gel and allowed to set for a further 1 hour.

A running buffer consisting of 50ml buffer concentrate (BioRad Tris, Glycine) added to 450ml distilled H2O pre cooled to approximately 5° C was placed into the running tank and the samples are loaded into the stacking gel wells. A further 200μl of β-mercaptoethanol was added to the centre of the tank prior to running. The gels were set to run for approximately 26 hours at 5° C at 80v.

Sample staining

The gels were removed from the glass plates and placed into a small container. The gels were rinsed for approximately 10 minutes in distilled water this was then repeated. Next, the gels were covered with 20ml ‘simply blue safe stain’ (Invitrogen,
UK) (LC6060) and were left to stain for approximately 1 hour, depending on the
required intensity and the amount of protein present. The stained gels were then
rinsed again in distilled water for approximately 2 hours, which decreased the
background staining. This improved protein band resolution during light
densitometry.

**Light densitometry**

After staining, the resolving gels were placed into a BioRad densitometer (BioRad
Geldoc 2000). The gels were scanned and the density map produced along with a
calculated proportional percentage with respect to the separate myosin isoform bands
using the associated software (BioRad Quantity 1, version 4.2). The gel background
was subtracted from the protein band density in order to align the baseline to zero.
In-situ hybridisation

Tissue preparation

Transverse serial sections (10-μm thick) of the vastus lateralis muscle were cut using a cryostat microtome (LEICA CM3050) at -20°C and applied to super-frost premium plus-coated glass slides (BDH). All sections were allowed to air-dry for at least 10 minutes. The sections were then fixed in DEPC-treated PBS containing 4% paraformaldehyde pH 7.5 for 5 minutes at 4°C, washed with DEPC-treated PBS pH 7.4 and stored at -70°C until further processing.

Probe preparation

Primers were synthesized (Sigma-Genosys) using T7 and SP6 promoter sequences with part of the 49bp MGF sequence from exon 5 of the IGF-I gene included in each primer.

The sense (forward) primer used was SP6 + MGF part of sequence:

TGATTTAGGTGACACTATAGAATCTAGAACACTGACATGCCCAAGA

The antisense (reverse) primer used was T7 + MGF part of sequence:

TGTAATACGACTCACTATAGGGAAGCTTTGCACTTCCTCTACTTTGTGT

RT-PCR was performed in order to amplify MGF flanked by T7 and SP6 promoter sequences. 1μl of purified MGF plasmid DNA prepared as described in Chapter 2, Methods and Materials was amplified using the primers above in 2x50 μl reactions, as described in Chapter 2, Methods and Materials. PCR amplification consisted 35 cycles of denaturation at 94°C for 1 minute followed by annealing at 61°C for 1
minute and elongation at 72°C for 1 minute. 2μl of the PCR products were then run on a 1.2% agarose gel to confirm the size of the bands (figure 3.2). Following gel electrophoresis, the PCR products were purified (Wizard PCR Preps DNA purification, Promega) and their concentration determined by spectroscopy at 260 nm using the Gene Spec I (Naka Instruments).

**Figure 3.2.** 1% agarose gel showing purified MGF plasmid flanked by T7 and SP6 promoter sequences, following PCR amplification. PCR consisted 35 cycles of denaturation at 94° for 1 minute, annealing at 60°C for 1 minute and elongation at 72°C for 1 minute. The picture shows PCR products at approximately 100bp obtained using T7 and SP6 primers with part of MGF sequence included. Lane 1 - 100bp DNA size marker, lanes 2 and 3 MGF PCR product.
RNA labelling with DIG-UTP by \textit{in vitro} transcription with SP6 and T7 RNA polymerases

PCR-fragments with RNA polymerase promoters ligated at their 5' terminus act as templates for transcription. DIG-11-UTP is incorporated by SP6, T7 at approximately every 20-25th nucleotide of the transcript. The standard labelling reactions for SP6 (sense) and T7 (antisense) digoxigenin-labelled RNA probes accordingly, were as follows: the following reagents were added to a microfuge tube on ice: 200ng of PCR product, 2μl of 10xDIG RNA Labelling mix, 2μl of 10x transcription buffer (400mM Tris-Cl, pH 8.0; 60mM MgCl₂, 100mM DTT, 20mM spermidin), sterile RNase-free water to a final volume of 18μl and 2μl RNA polymerase (SP6 or T7 respectively). After mixing and centrifuging briefly, the reactions were incubated at 37°C for 2 hours. The reactions were stopped by adding 2μl 0.2M EDTA-solution, pH 8.0 on ice. The protocol and reagents used were by Boehringer Mannheim.

\textit{In-situ} hybridisation standard procedure (Boehringer Mannheim)

\textbf{pre-hybridisation}

Sections were washed 2x5 minutes with DEPC-treated PBS, pH 7.4 and then washed further 2x5 minutes with DEPC-treated PBS containing 100mM glycine. These were then treated for 15 minutes with DEPC-treated PBS containing 0.3% Triton-X-100 and then washed 2x5 minutes with DEPC-treated PBS. Permeabilization of the sections was performed for 30 minutes at 37°C with TE buffer (100mM Tris-HCl, 50mM EDTA, pH 8.0) containing 1μg/ml RNase-free Proteinase- K (Sigma). They were then post-fixed for 5 minutes at 4°C with DEPC-treated PBS containing 4%
paraformaldehyde and washed 2x5 minutes with DEPC-treated PBS. To acetylate, the slides were placed in containers on a rocking platform and incubated with 0.1M triethanolamine (TEA) buffer (Sigma), pH 8.0 containing 0.25% (v/v) acetic anhydride for 2x5 minutes. Acetic anhydride (Sigma) is highly unstable so it was added just before incubation. The sections were then incubated at 37°C for an hour with pre-hybridisation buffer (4xSSC (1xSSC= 150mM NaCl, 15mM sodium citrate, pH 7.2- Sigma) containing 50% v/v deionised formamide (Sigma)). Pre-hybridisation buffer was drained from the slides and hybridisation buffer was applied containing 10µg per 100ml of hybridisation solution of the DIG-labelled SP6 and T7 RNA probes. Sections were covered with cover slips (BDH) and incubated at 42°C overnight in a humid chamber.

**Preparation of hybridisation buffer**

This consisted: 40% deionised formamide, 10% dextran sulfate, 1x Denhartd’s solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 10µg.ml RNase-free bovine serum albumin), 4x SSC, 10mM DTT, 1mg/ml yeast t-RNA and 1mg/ml denatured salmon sperm DNA and was added shortly before hybridisation. (All reagents used were provided by Sigma).

**Post-hybridisation**

Coverslips were removed by immersing the slides in 2xSSC for 5-10 minutes in different containers for the two RNA probes and the sections were washed in a shaking water bath at 37°C 2x15 minutes with 2xSSC and 2x15 minutes with 1xSSC. To digest any single-stranded (unbound) RNA probe the sections were incubated for 30 minutes at 37°C in NTE buffer (500mM NaCl, 10mM Tris, 1mM EDTA, pH 8.0)
containing 20μg/ml RNase A (Sigma). They were then washed 2x30 minutes in a shaking water bath at 37°C with 0.1xSSC.

Immunological detection

Sections were washed 2x10 minutes in a shaking platform with Buffer 1 (100mM Tris-HCl, pH7.5 and 150mM NaCl). They were then incubated for 30 minutes with blocking solution (Buffer 1 containing 0.1% Triton X-100 and 2% normal sheep serum (Sigma)). The Blocking solution was taken off and sections were incubated for 2 hours in a humid chamber with Buffer 1 containing 0.1% Triton X-100, 1% normal sheep serum and 1 in 500 dilution of sheep anti-DIG-alkaline phosphatase (Fab fragments) (Boehringer Mannheim). They were then washed 2x10 minutes with Buffer 1 in a shaking platform. After which the sections were incubated for 10 minutes with Buffer 2 (100mM Tris-HCl, pH 9.5, 100mM NaCl and 50mM MgCl₂).

The sections were covered with colour solution containing 10ml of Buffer 2, 45μl nitroblue tetrazolium (NBT), 35μl 5-bromo-4-chloro-3-indolyl-phosphate (BCIP or X-phosphate), both by Boehringer Mannheim and 1mM levamisole (Sigma) and incubated for 6 hours (following optimisation) in a humid chamber in the dark.

The colour reaction was stopped by incubating the slides in Buffer 3 (10mM Tris-HCl, pH8.1 and 1mM EDTA) and then washed in distilled water. Sections were mounted with an aqueous mounting solution.
Negative controls

Negative controls used: (i) tissue mRNA was digested with RNase prior to in-situ hybridisation, (2) hybridisation was also performed with SP6 sense probe and (2i) the anti-DIG antibody was omitted.

Care was taken to prepare all solutions with DEPC-treated distilled water (0.1% diethylpyrocarbonate-DEPC, Sigma). Different glassware was used for pre and post hybridisation steps and baked at 180°C to avoid RNase contamination. Gloves were used throughout the procedure.

Statistical analysis

Data are given as means ±SE. Comparisons between paired and unpaired data were made using the Wilcoxon’s signed rank test and the Mann Whitney test respectively. Non-parametric tests were chosen because inspection of the raw data suggested that the distribution of values or change in values did not follow a normal distribution. Linear regression analysis (Spearman’s product moment) was used to compare association between variables. The level of statistical significance was taken as P<0.05.

It should be noted that the measurement error will increase the random variation when comparing groups of subjects (as done in this chapter and chapter 4 where old subjects are compared with young subjects). Taking the average of duplicate measurements will therefore decrease the random variation and increase the power to show true differences.
3.3 Results

Differences in the weight lifted by young and old subjects

The mean (+SE) 1-RM for the young subjects was 41.9±4.3 kg, which was significantly greater than for the old subjects (20.6±1.6 kg). The mean weight lifted by the two groups on the first and last sets was 34.5+3.6 and 33.4+3.5 and 16.5+1.3 and 15.2+1.2 kg for the young and elderly subjects respectively.

Figure 3.3. Mean 1-RM lifted by young (n=7) and old subjects (n=7). Values are expressed in kg (+SEM). * Significant difference from old (P<0.05).
Integrity of total RNA

Total RNA was extracted from the vastus lateralis muscle of young and old subjects from the control and exercised legs. The integrity of RNA was assessed by running a 1.2% agarose formaldehyde gel and visual inspection of ethidium bromide stained 18s and 28s ribosomal RNA bands was performed under ultra violet light. An example of an ethidium bromide stained gel is shown in Figure 3.4.

Electrophoresis of PCR products

The PCR products were recovered at the end of the PCR reaction and specificity of the real time quantitative PCR reaction was further confirmed by running the PCR products on a 2% agarose gel. Visual inspection of the ethidium bromide stained bands was performed under ultra violet light. The sizes of the products relative to a standard size marker (100bp DNA ladder), which was also run alongside the samples on the gel, were determined as were any non-specific PCR amplifications such as primer dimers. An agarose gel picture of all four PCR products (IGF-Ia, MGF, α-actin and MyoD) is shown in Figure 3.5. The specificity of the PCR conditions can be seen by the absence of any primer dimers. If present, these would have been a lot smaller than the specific products and approximately 40-50bp.
**Figure 3.4.** Electrophoresis of total RNA extracted from the vastus lateralis muscle of a young and an old subject. Lanes 1 and 2 are pre and post exercise samples from a young subject and lanes 3 and 4, control and exercised samples from an older subject.

**Figure 3.5.** 2% agarose gel picture showing PCR products for each of the targets measured. 4 products are shown for each target - these correspond to a young subject (control and exercised leg) and an old subject (control and exercised leg). Lanes 1 and 10 - 100bp DNA size marker, lanes 2-5 - IGF-1Ea, lanes 6-9 - MGF, lanes 11-14 - α-actin and lanes 15-18 - MyoD.
**Alpha actin expression**

To assess that the percentages of muscle mRNA to total RNA had not changed within the 2.5 hour period and that there was equal RNA and cDNA loading between samples, α-actin mRNA levels were measured and found not to be significantly different between the control and exercised legs. This would suggest that for the purpose of this study, α-actin could have been used as a suitable control gene to which the data could have been normalised.

**Expression of IGF-I isoforms after exercise**

**MGF**

Levels of MGF mRNA are shown in Figure 3.6. At rest, MGF mRNA levels averaged 6.6x10^8 ng/μg RNA in the young subjects. This was not different to the elderly subjects. However, in the young subjects a significantly higher level of MGF mRNA was observed in the exercised leg when compared with the control leg. The increase ranged from 2% to 864%. In contrast to the young subjects, three of the elderly subjects showed lower values in the exercised compared to the control leg, therefore there was no significant difference observed in MGF mRNA levels between their exercised and control legs.

**IGF-IEa**

The levels of IGF-IEa mRNA are shown in Figure 3.7. IGF-IEa values were higher than that of MGF by 2-3 orders of magnitude. (10^{-5} vs 10^{-8}). As with MGF, baseline levels of IGF-IEa at rest were not significantly different between the young and older subjects. However, in contrast to MGF there was no significant change in IGF-IEa expression observed in either the young or the older individuals.
Figure 3.6 Individual MGF mRNA levels in young (n=8) and old (n=7) subjects in the control and exercised (Ex) muscles. Values are expressed as ng of mRNA per µg total RNA. Black line represents subject RS who showed the greatest increase in MGF mRNA after exercise. * Significant increase in MGF mRNA 2.5 hours after exercise in the young subjects (P<0.05).
Figure 3.7. Individual IGF-IEa mRNA levels in young and old subjects in the control and exercised (Ex) muscles. Values are expressed as ng of mRNA per μg total RNA.
Figure 3.8. The MGF and IGF-IEa responses in the control and exercised leg for subject RS. Values are expressed as ng of mRNA per µg total RNA.
MyoD expression after exercise in young and old subjects

The resting values of MyoD in older subjects were significantly higher than those observed in the young subjects (P<0.05). Despite there being an ~80% increase in MyoD mRNA after exercise in the young subjects, this was not statistically significant.

![Bar graph showing MyoD mRNA levels in young and old subjects in control and exercised muscles. Values are expressed as ng of mRNA per μg total RNA (mean +SE). * Significant difference between baseline levels of MyoD compared with old subjects (P<0.05).](image)

Figure 3.9. MyoD mRNA levels in young and old subjects in control and exercised muscles. Values are expressed as ng of mRNA per μg total RNA (mean +SE). * Significant difference between baseline levels of MyoD compared with old subjects (P<0.05).
MyHC composition

For each of the subjects MyHC isoform composition of the quadriceps muscle was determined by SDS PAGE. Only one subject (RS) expressed any observable amounts of the fast MyHC-2x isoform (see Figure 3.10). As a result the data are expressed as a percentage of the total MyHC-1 and MyHC-2 (MyHC-2a and 2x) content and is summarised in Table 3.1.

The quadriceps muscle of the elderly subjects was found to contain a significantly lower proportion of fast MyHC-2 isoforms than the young subjects. The relative percentage of MyHC-2 for the young and elderly subjects was 44% and 27% respectively (Table 3.1). No relationship was observed between muscle myosin heavy chain isoform composition and IGF-I isoform expression, either at rest or after the change induced by the exercise challenge.
Table 3.1 Relative MyHC isoform composition of the vastus lateralis muscle in young (n=8) and old (n=7) subjects as determined by SDS-PAGE. Values are expressed as mean ± SE.
* Significantly different from young

![Table 3.1](image)

Figure 3.10. Electrophoretic separation of MyHC isoforms using SDS PAGE. Lane 1 shows a typical example of the MyHC isoform composition of the vastus lateralis muscle observed in young and old subjects. Lane 2 shows subject RS with a total 33% MyHC-2x. None of the other subjects showed any detectable amounts of this isoform.
Localisation of MGF mRNA in human muscle

MGF mRNA expression within the muscle fibres studied by *in situ* hybridisation is shown in Figure 3.11. Hybridisation with the MGF antisense probe resulted in staining of MGF within the muscle fibres themselves, with staining appearing to be more concentrated around the periphery of the fibre. MGF mRNA was present in both the control and exercised muscle samples as shown by the quantitative PCR data. In sections treated with the sense probe, no such staining was observed.
Figure 3.11. *In situ* hybridisation of antisense (A) and sense (B) DIG labelled MGF cRNA probe through the vastus lateralis muscle of a young subject 2½ hours after the end of exercise. The use of fresh frozen tissue has resulted in diminished tissue preservation, which caused a high background staining. The arrows marked on Figure 3.11A point to specific staining (dark purple) and show that it was localised to the periphery of the muscle fibres.
3.4 Discussion
This study has shown that resting human skeletal muscle expresses at least two isoforms of IGF-I, the first being the main isoform expressed by the liver, IGF-IEa, and the second a splice variant, MGF. It has also shown that they differ markedly in their relative expression, with the mRNA of MGF being expressed at lower levels than that of IGF-IEa by two to three orders of magnitude. In the young subjects, expression of MGF mRNA was significantly increased after the single bout of high load weightlifting exercise, whilst there was no such increase observed in the older subjects. In contrast to MGF, IGF-IEa mRNA showed no significant change with exercise. Previous studies on animal muscle have shown that following overload there is an increase in the expression of IGF-I mRNA in muscle (DeVol et al. 1990; Czerwinski et al. 1994; Adams and Haddad. 1996, Yang et al. 1996; Adams, 1998). More recently, Bamman et al (2001) reported a 62% increase in IGF-I mRNA concentration in human muscle 48 hours after a single bout of eccentric resistance type exercise. However, this study did not differentiate between the different isoforms of IGF-I expressed in muscle. In the main, this study was concerned with the short-term response to exercise, therefore expression of these muscle IGF-I isoforms was studied 2½ hours after the end of an acute exercise bout both in young and old individuals. The results from the present study suggest that the expression of these gene products is independently controlled. This is evidenced by the fact that in contrast to MGF, no significant increase in IGF-IEa mRNA was observed after exercise in the young subjects and secondly, no association between the expression of the two mRNAs was observed.
Independent expression patterns of IGF-IEa and MGF in response to exercise

Sensitivity of MGF to mechanical signals has been highlighted in animal studies where passive stretch (Yang et al. 1996), and to a greater extent stretch combined with electrical stimulation (McKoy et al. 1999), increases MGF mRNA expression in muscle. However, the effects of MGF mRNA levels on subsequent protein expression levels and the degree to which these levels affect muscle growth remain to be determined. Studies in animal muscle have also shown that IGF-IEa and MGF behave independently of each other in response to overload, and support the suggestion that MGF is a transient and activity sensitive growth factor (Yang et al. 1996; McKoy et al. 1999; Owino et al. 2001; Haddad and Adams, 2002; Cheema et al, unpublished observations). For example, the study by Owino et al (2001) in which the soleus and plantaris muscles of rats were overloaded using the technique of muscle tendon ablation, showed that IGF-IEa and MGF mRNAs responded differently to this challenge. Following 5 days of overload, MGF mRNA showed a marked increase in the overloaded muscles when compared to control, whereas IGF-IEa appeared relatively insensitive to the challenge. A more recent study which determined the effects of a single or two bouts of resistance exercise in rats, on the expression IGF-I and MGF mRNA, concluded that a single bout of resistance exercise stimulated a relatively rapid increase in MGF and, to a lesser extent that of IGF-I. Two bouts of exercise resulted in sustained increases in MGF and IGF-I mRNAs that were more pronounced when the rest intervals were longer (Adams and Haddad, 2002). Work by Hill and Goldspink (2003) supported the idea of this quite independent expression pattern of the two isoforms, with MGF being upregulated as an initial response to injury, followed the upregulation and sustained expression of IGF-IEa. Furthermore, in vitro studies in differentiated myoblasts, which have been embedded in a collagen matrix have also shown that the two isoforms behave independently of one
another after being subjected to varying degrees of strain (Cheema et al. unpublished observations). In contrast to animal muscle, this study has shown that there is a considerable degree of variation between individuals in the response to exercise, for example one young subject showed a particularly dramatic increase in MGF mRNA with exercise, whilst no change in IGF-IEa mRNA levels were seen (comparison of MGF vs IGF-IEa mRNA response in this subject RS is shown in Figure 3.8). The findings of the present study therefore support the notion that IGF-IEa and MGF are differentially regulated.

Expression of IGF-I and its isoforms in aged muscle

The muscles of older animals have been shown to recover less well from contraction-induced injury, when compared to young animals (Brooks and Faulkner, 1990; Faulkner et al. 1995). In these studies isometric force was used to characterise the force deficit obtained following a number of high force pliometric (eccentric contractions) in young and old mice. This is in accord with a recent report in which the muscles of old rats overloaded by synergistic muscle tendon ablation showed an attenuated MGF response (Owino et al. 2001). In the young animals, MGF expression increased by ~1000% after 5 days of muscle overload as compared with only ~250% for the 24 month old animals. The magnitude and duration of the challenge and likely local tissue damage caused is greater than that seen in the present study, but the data are in general agreement, with the older men in the present study not showing any significant increase in MGF mRNA levels after exercise. In the work by Faulkner and colleagues, the mice performed extensive eccentric (damaging) contractions, whereas subjects in the present study performed both eccentric and concentric contractions. The comparatively smaller magnitude of response observed in the present study is likely to be explained by the fact
the overall challenge to the human muscles was not as severe as that given in the animal experiments. The effects of performing eccentric only exercise in humans, on the expression of these growth factors in muscle is described in the next chapter.

In a study investigating IGF-I peptide levels in human muscle following 10 weeks of strength training in old men and women (aged 72 – 98 years), it was shown that there was a ~500% increase in the levels of IGF-I within the muscle fibres of these subjects after the training period, as determined using immunohistochemistry (Fiatarone-Singh et al. 1999). This demonstrates that IGF-I peptide levels are increased in older muscles as a result of longer-term exercise training. Indeed, the results of longitudinal strength training studies have confirmed that the muscles of even very elderly people are able to hypertrophy response to resistance exercise (Fiatarone et al. 1990; 1994; 1999; Harridge et al. 1999). The 12 weeks of training contrasts with the single exercise bout in the present study. This matter is explored later in Chapter V. Furthermore, in the study by Fiatarone-Singh et al (1999), the effects of exercise training on IGF-I peptide levels in young individuals were not investigated to determine if there was an age related difference in the muscle IGF-I response to exercise. In addition, the antibody used for immunohistochemical analysis was a general IGF-I antibody, which made no distinctions between the different isoforms of IGF-I. It was therefore not possible to determine the relative contributions of the different IGF-I isoforms to the increase seen in muscle mass in that study.

**MyoD Expression in Aged and Exercised Muscle**

Like IGF-IEa, MGF contains exons 3 and 4 which encode the signalling peptide. It is therefore likely to play a role in increasing protein synthesis but possibly not to the same
extent, as reflected by the lower expression levels of MGF (10^8 ng) when compared to IGF-Ie (10^6 ng). There is some evidence that MGF may be involved in the initial activation of satellite cells, with the E domain of the protein thought to be responsible for this action. In this regard, recent cell culture studies on C2/C12 cells treated with either MGF or IGF-I peptides suggest that MGF may play a different role in the process of satellite cell activation to IGF-I. In this study, MGF was shown to initiate satellite cell proliferation, whilst IGF-I initiated proliferation but then also promoted their differentiation into myotubes (Yang and Goldspink, 2002). The early induction of MGF mRNA in muscle reported in the present study following exercise would support this idea. As detailed in Chapter I, satellite cells are small mononucleate muscle stem cells located between the sarcolema and basal lamina of muscle fibres. Recently, the link between satellite cell number and myofibre size has been demonstrated in both untrained and hypertrophied human muscle fibres (Kadi and Thornell, 2000). These cells, when activated, are believed to proliferate and differentiate into myoblasts which then fuse with existing fibres, thus providing new nuclei to maintain the DNA to protein ratios of fibres undergoing hypertrophy.

As the present study was concerned with the short-term response to exercise, the MRF MyoD was believed to be the most suitable marker of satellite cell activation following local injury to the muscle. This was based on findings that MyoD is the first and most abundantly expressed myogenic regulatory gene in activated satellite cells (Smith et al. 1993). Further studies in satellite primary cell culture found that MyoD mRNA increased before proliferation, whereas the appearance of myogenin mRNA appeared to coincide with the process of differentiation (Smith et al. 1994). A later study by Cornelison and Wold (1997) analysed the expression of the MRF genes in cultures of
mouse satellite cells prepared from isolated myofibres. In this *in vitro* study they concluded that activated satellite cells began by either expressing MyoD or myf5. A more recent *in vivo* study confirmed these findings, highlighting that MyoD was expressed by activated satellite cells as early as 3 hours after muscle damage (Cooper *et al.* 1999). Furthermore, MyoD has been reported to be a reliable marker of activated satellite cells (Koishi *et al.* 1995), as it has only been shown to be present in detectable amounts in proliferating but not quiescent satellite cells (Yablonka-Reuveni and Rivera, 1994). Since MyoD mRNA levels in the present study were measured in total RNA extracted from homogenised muscle samples, it was not possible to determine whether expression of this MRF was derived exclusively from satellite cells. Since MyoD expression is primarily thought to originate from satellite cells, this was believed to be highly likely. Nevertheless, the possibility that some of the MyoD mRNA measured may have originated from the muscle fibre nuclei cannot be excluded.

In agreement with previous observations, we report that baseline levels of the transcription factor MyoD are higher in the muscles of older individuals when compared with young (Musaro *et al.* 1995; Marsh *et al.* 1997; Owino *et al.* 2001). It is not fully understood why aged muscle should express elevated baseline levels of myogenic factors such as MyoD, although it has been suggested that it may reflect a continued attempt to maintain muscle specific expression and ameliorate muscle atrophy (Marsh *et al.* 1997). In the young subjects there was a tendency for an increased (~80%) expression of MyoD following exercise whilst in the old subjects MyoD values were essentially unchanged. The myogenic response to a single bout of weightlifting exercise has been shown to be impaired in old muscle (Tamaki *et al.* 2000). Muscles, which were sampled through out the post exercise period showed that MyoD mRNA levels were
lower in older rats when compared to young. The results of the present study are in general agreement with this, although the shorter time point (2.5 hours) at which the samples were taken after exercise may have been insufficient time to allow for completion of the myogenic regulatory response to the exercise stimulus.

**MyHC isoform expression in young and old muscle**

The hypertrophic response to strength training exercise has been reported to be greater in type 2 fibres, which express the MyHC-2a and 2x isoforms (Tesch et al. 1985; Kadi et al. 1999; Aagaard et al. 2001; Andersen and Aagaard, 2001). The quadriceps muscles of the old subjects in the present study comprised a significantly lower proportion of MyHC-2 isoforms compared to the young subjects (Table 3.1). This is most likely a reflection of a selective atrophy of type 2 fibres (Klitgaard et al. 1990). It might be suggested that it is this pattern of expression, which accounts for the somewhat attenuated response of MGF to the exercise protocol in the older subjects. However, we observed no association between the change in MGF and the MyHC composition of the quadriceps muscle in either young or old individuals, or when both age groups were considered together. Despite this, it was interesting to find that the one young subject (RS) to express a detectable amount of MyHC-2x (33%) also showed the most marked MGF response (864% increase) to the exercise challenge (black line in Figures 3.6). The MyHC-2x isoform is particularly expressed in muscles that have been subject to disuse (Andersen & Aagaard, 2000 Andersen et al. 1996, Harridge et al. 2002). This particular subject, whilst not completely inactive (1 game of football per week) was the least active of all the subjects. It might be speculated that the large increase in MGF mRNA relates to either an isoform specific (i.e. MyHC-2x) response, or a sensitivity to overload, which may reflect the training status of the muscle. Had all the subjects in our study been
relatively inactive, we may have found a relationship between MyHC-2x expression and MGF. In general, those individuals who are unaccustomed to exercise suffer from muscle soreness / damage more so than those who are more active. There would therefore be less muscle damage as a result of exercise and possibly an attenuated MGF response in these more trained individuals. This is in general agreement with the results of a recent study by Tipton et al (1999), which showed that protein synthesis rates were reduced in trained subjects when compared with untrained subjects, following a single bout of eccentric weightlifting exercise. Both these data, and the data from the present study would therefore suggest that trained muscle is in someway less sensitive to the mechanical signals provided by exercise. It would be of interest to study the responses of these muscle IGF-I isoforms in trained vs non-trained individuals, to establish whether the training status of muscle affects the extent of the response.

Conclusion

This study reports the expression of two splice variants of the IGF-I gene present in human skeletal muscle: IGF-IEa, the main isoform expressed hepatically and MGF, an activity sensitive local growth / repair factor. In young subjects, the splicing of these isoforms appears to differ in response to a single bout of high resistance weightlifting exercise, with MGF being upregulated and the IGF-IEa isoform remaining unchanged. The magnitude of the response does not appear to be related to MyHC composition, but may be related to the underlying training status of the muscle. In contrast to the young subjects, older subjects who performed the same relative intensity of exercise showed a tendency for an attenuated MGF response, which would be in agreement with recently reported animal studies. The type of exercise undertaken in the present study, if performed regularly, promotes muscle growth. Whether the MGF response to overload
is selective to the eccentric or lengthening component of the exercise, which is associated with muscle damage, remains to be determined. This question is addressed, in part, in the next chapter, in which the MGF response after an acute bout of eccentric only cycling exercise was investigated in young and old individuals.
Chapter 4

Expression of IGF-I splice variants in young and old human skeletal muscle after a single bout of eccentric cycling exercise
4.1 Introduction

Conventional resistance exercise i.e. lifting and lowering of a weight either 'free' or in the confines of a piece of apparatus, usually comprises a combination of concentric and eccentric contractions. However, as mentioned in Chapter 3, the mechanisms by which resistance training induces myofibre hypertrophy are still not fully understood, but may relate in part to the micro-damage caused by the eccentric component of the exercise. During a concentric contraction, the muscle produces tension while shortening, whereas during an eccentric contraction (also termed lengthening, stretching or pliometric contractions) a contracting muscle is forced to lengthen whilst producing force. The force generated during an eccentric contraction is greater than that of a concentric contraction. High intensity eccentric exercise in untrained subjects is associated with increased levels of myofibre enzymes in plasma, ultrastructural damage of the muscle fibres and an acute inflammatory response in the muscle leading to oedema, infiltration of inflammatory cells and muscle soreness (Evans et al. 1986). For example, a number of studies to date have demonstrated that eccentric exercise results in an increase in serum creatine kinase (CK) levels 24-48 hours after the exercise bout, which may persist for 3-6 days (Manfredi et al. 1991; Bruunsgaard et al. 1997). Changes in muscle ultrastructure, such as sarcolemmal disruption, swelling or disruption of the sarcotubular system, distortion of the myofibrils' contractile components, cytoskeletal damage and abnormalities to the extracellular myofibre matrix, have also been reported in both animal (Armstrong et al. 1983; McCully and Faulkner, 1985; Lieber and Friden 1988) and human (Newham et al. 1983; Friden et al. 1983; Friden et al. 1984) models following eccentric contractions. Recent work by Yu et al., (2004) suggests that many of the signs which are associated with muscle damage are in fact the
hypertrophic invasion of satellite cells into undamaged muscle cells. This is interesting, as it conflicts somewhat with the current dogma that exercise which causes hypertrophy is preceded by muscle damage. The inflammatory response to strenuous exercise has also been extensively studied. During eccentric exercise, myofibres are mechanically damaged and this process stimulates the local production of inflammatory cytokines such as tumour necrosis α (TNF-α), interleukin (IL)1β, IL-6 and IL-1 receptor agonist (IL-1ra) (reviewed by Pedersen et al. 1998). Whether these events are causally related to the growth response remains unknown; however, hypertrophy is blunted if the eccentric component is omitted from conventional resistance training (Hather et al. 1991). In the study by Hather et al. (1991) a combination of concentric/eccentric training induced hypertrophy in both type 1 and type 2 muscle fibres, whereas there was no hypertrophy of the type 1 fibres, and a reduced response in type 2 fibres after concentric only exercise. Thus indicating that the eccentric component of exercise was necessary for maximising the hypertrophy response.

As has been extensively discussed, IGF-I is believed to play a significant role in this hypertrophic adaptation of muscle to exercise; yet the number of studies conducted to date which examine the muscle IGF-I response to resistance exercise in human muscle are limited. Furthermore, human studies, which have investigated the effects of concentric vs eccentric contractions and muscle IGF-I expression, are even less well documented. One such study by Bamman et al. (2000) investigated the effects of concentric vs eccentric loading on muscle IGF-I mRNA concentration. This study found that IGF-I mRNA levels were significantly elevated after eccentric but not
concentric exercise. Thus suggesting that IGF-I mRNA in the muscle was more sensitive to the eccentric component of exercise.

The study described in Chapter 3 utilised a high resistance knee extensor exercise protocol, which comprised a combination of both the concentric and eccentric contractions. Briefly, this study described a significant increase (ranging from 2%-864%) in MGF mRNA in the muscles of young individuals, but no significant increase in the older subjects in the short term after exercise. Eccentric differs from concentric exercise in that it uses less ATP per unit force generated, and is therefore less metabolically demanding. However, eccentric exercise also results in a higher degree of muscle soreness following the exercise bout, as discussed, and there is evidence that this is due to a greater amount of muscle fibre damage. It would therefore be of interest to determine whether carrying out eccentric only exercise elicits a significant MGF response in older muscle given that MGF production is thought to be both mechano-sensitive and extremely sensitive to damage (Hill and Goldspink, 2003).

The main aims of this study were:

(i) To determine the IGF-IEa and MGF mRNA responses after a single bout of eccentric cycling exercise.

(ii) To determine whether the response in elderly individuals differs to that observed in young subjects.
4.2 Methods and Materials

This study was carried out in collaboration with Professor Bente Klarlund Pedersen, Department of Infectious Diseases, Copenhagen Muscle Research Centre, Rigshospitalet, Copenhagen, Denmark, as a part of a European collaborative project, the Pan European Network into Ageing Muscle (PENAM). The amount of tissue available for analysis was limited. The MGF and IGF-IEa mRNA data presented in this chapter therefore relates to fewer subjects than those investigated in the original study by Toft et al. 2002. Subject recruitment, exercise training, and muscle sampling procedures were carried out in their laboratory. Any data generated in their laboratory is clearly indicated and has been reproduced with their permission.

Subjects

10 healthy young (age 20-27 years, height 176-187 cm, weight 72-89 kg) and 10 healthy elderly (age 67-75 years, height 172-191 cm, weight 70-89 kg) subjects participated in this study. Subjects were not specifically trained but they were recreationally active, performing exercise 1-3 days per week for 1-2 hours. Before inclusion subjects were screened for systemic disorders by blood sample analysis and physical examination. All elderly subjects underwent an electrocardiogram (ECG) and were screened for arthrosis of lower limb joints by X-ray and physical examination. Informed consent was obtained from all subjects, and studies were performed in accordance with the Declaration of Helsinki. The experimental protocol was approved by the local ethics committee for Copenhagen and Frederiksberg Communities, Denmark.
**Study Design**

2 weeks before the experimental day, maximal oxygen uptake ($\text{VO}_2\text{ max}$), maximal heart rate, and maximal concentric workload were determined during an incremental concentric exercise test on the same cycle ergometer as used for the eccentric exercise (Figure 4.1). A concentric exercise protocol was used in this test so as to avoid causing any muscle damage. Values for muscle mass, $\text{VO}_2\text{ max}$, maximal heart rate, and maximal concentric workload in young and old subjects are given in Table 4.1.

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle mass, 2 legs (kg)</td>
<td>17.3 (15.6-18.3)</td>
<td>20.9 (18.8-22.2)</td>
</tr>
<tr>
<td>$\text{VO}_2\text{ max}$ (ml.kg$^{-1}$.min$^{-1}$)</td>
<td>56.1 (37.4-64.1)</td>
<td>29.3 (19.3-33.7)</td>
</tr>
<tr>
<td>Max HR concentric (beats/min)</td>
<td>186 (175-198)</td>
<td>155 (132-167)</td>
</tr>
<tr>
<td>Concentric workload at $\text{VO}_2\text{ max}$ (W)</td>
<td>265 (230-366)</td>
<td>119 (94-214)</td>
</tr>
</tbody>
</table>

**Table 4.1** Subject values for muscle mass, $\text{VO}_2\text{ max}$, maximal heart rate, and maximal concentric workload. Values are medians and ranges are given in parentheses. Abbreviations: HR – heart rate, $\text{VO}_2\text{ max}$ – maximal O$_2$ uptake. (Toft *et al.* 2002)

**Eccentric Exercise Protocol**

The eccentric exercise consisted of 60 minutes of opposing the rotation of the pedals down to 60 rpm. Subjects performed the following program of six working intervals: 0-6 minutes at 50%, 6-12 minutes at 75%, 12-20 minutes at 100%, 20-25 minutes at 130%, 25-40 minutes at 100%, and 40-60 minutes at 75% of the load eliciting concentric $\text{VO}_2\text{ max}$, as described by Bruunsgaard *et al* (1997). The workloads were chosen to give the same relative increases in $\text{VO}_2\text{ max}$ and heart rate. An example of the cycle ergometer used for this type of exercise is shown in Figure 4.1.
Experimental Protocol

Subjects were asked to refrain from any strenuous exercise for 7 days before the experimental day. On the study day, subjects reported to the laboratory after an overnight fast. After resting for 10 minutes on the cycle ergometer, subjects performed the eccentric exercise program. ECG and heart rate were continuously monitored throughout the exercise. VO\(_2\) was recorded after 30 minutes (during a steady state period of 100% of the load eliciting concentric VO\(_2\) \text{max} ), and workload was noted continuously. After completion of the exercise, subjects rested and were provided with carbohydrate-rich food and drinks for 4 hours after the exercise. Subjects were advised to refrain from any strenuous physical activity for 5 days after the exercise.

Figure 4.1. An elderly subject performing eccentric ‘reverse pedal cycling’ exercise, while continuously monitoring heart rate and O\(_2\) consumption.
Muscle Biopsies

Following local anaesthesia (1% lignocaine), muscle biopsies were taken from the vastus lateralis muscle before exercise, then 2¼ hours after the end of the exercise bout. Samples were immediately frozen and stored at -70°C.

RNA Extraction

Total RNA was extracted from the muscle samples using Tri Reagent (Sigma) and the quality of the RNA was assessed as described in Chapter 2, Methods and Materials.

First strand cDNA synthesis

0.5µg total RNA was reverse transcribed into cDNA as described in Chapter 2, Methods and Materials.

Real Time Quantitative PCR

Quantification of the mRNA message encoding the isoforms IGF-IeA and IGF-IeC (MGF) was performed using LightCycler technology (Roche Diagnostics) with SYBR green I as the method of detection as described earlier in Chapter 2, Methods and Materials. The sequences of the primers used are given in Table 2.4 in Chapter 2. Quantitative PCR runs were performed in duplicate and mean values were subsequently used for analysis.
**Gel Electrophoresis**

PCR products were recovered from the glass capillaries by removing the plastic caps and inverting them into a 1.5ml eppendorf tube, which were then spun down in a bench top centrifuge at no higher than 3000 rpm. Target specificity was further confirmed by running these samples on a 2% agarose gel as described in Chapter 2, Methods and Materials.

**Creatine Kinase**

Blood samples were obtained pre exercise then 2 hours after the end of exercise from the antecubital vein. Levels of CK were measured in lithium heparinized plasma with automated enzyme reactions (Hitachi Systems 717: Boehringer Manheim Diagnostica, Germany). Details of this method are given in Toft et al. 2002.

**Statistical Analysis**

Data are given as means ±SE. Comparisons between paired and unpaired data were made using the Wilcoxon’s signed rank test and the Mann Whitney test respectively. CK data were analysed using a paired t-test. The level of statistical significance was taken as P<0.05.
4.3 Results

Serum creatine kinase levels in young and old subjects after the exercise

The mean (+SE) values for serum creatine kinase before exercise were 157.2±33.5 U/l and 113.3±19.6 U/l in young and old subjects respectively. These levels were significantly elevated to 506.5±71 U/l and 149.6±22.4 U/l in the 2 hours post exercise samples in young and old subjects.

Figure 4.2. Mean levels of creatine kinase in young and old subjects. Values are expressed in U/l (+SEM). * Significant difference from before exercise (Control) (P<0.05). Data reproduced by kind permission of Dr Anders Toft.
Expression of IGF-I isoforms after eccentric cycling exercise

**MGF**

Levels of MGF mRNA are shown in Figure 4.3. Before exercise, MGF mRNA levels averaged $9.9 \times 10^{-8}$ ng in the young subjects. This was not different to the elderly subjects. In both young and old subjects, significantly higher levels of MGF mRNA were observed in samples obtained 2.25 hours after the end of exercise when compared with the before exercise samples.

**IGF-IeA**

The levels of IGF-IeA mRNA are shown in Figure 4.4. IGF-IeA values were higher than that of MGF by 2-3 orders of magnitude ($10^{-5}$ ng vs $10^{-8}$ ng). As with MGF, levels of IGF-IeA before exercise were not significantly different between the young and old subjects. However, in contrast to MGF, there was no significant change in IGF-IeA expression observed in either the young or the older individuals as a result of the exercise challenge.
Figure 4.3. Individual MGF mRNA levels in young (n=9) and old (n=8) subjects in the control (Ctrl) and exercised (Ex) muscles. Values are expressed as ng of mRNA per μg total RNA. * Significant increase in MGF mRNA 2¼ hours after the end of exercise in both young and old subjects (P<0.05).
Figure 4.4. Individual IGF-IEmRNA levels in young (n=8) and old (n=10) subjects in the control (Ctrl) and exercised (Ex) muscles. Values are expressed as ng of mRNA per µg total RNA. Colours reflect same subjects in Figure 4.3.
4.4 Discussion

This study has shown that levels of MGF mRNA increase in both young and old individuals in the short term following a single bout of eccentric exercise. The type of exercise performed in the present study induced a large degree of muscle soreness and damage, as indicated by the elevated levels of serum CK in the post exercise samples. However, with this prolonged protocol (i.e. lasted one hour) the absolute forces generated were not as high as those that can be obtained with conventional resistance training, and might therefore not result in muscle hypertrophy. Similar to the study in Chapter 3, mRNA levels of IGF-IEa showed no significant change with eccentric only exercise in either age group. Thus supporting the hypothesis that both isoforms are differentially regulated.

IGF-I expression after muscle damaging exercise

In a study of young military recruits, Hellsten et al (1996) reported increased numbers of IGF-I immunoreactive capillaries and satellite cells in the vastus lateralis muscle after 7 days of intense military training, that included 150 km of terrain marching carrying a 30 kg load (gear). In this study, the increased IGF-I staining was also accompanied by a six-fold increase in serum CK activity. Thus indicating that there was a large degree of muscle damage as a result of the exercise undertaken. Studies investigating the muscle IGF-I response following concentric vs eccentric training in human muscle are rare. One such study by Bamman et al (2000) used leg squatting exercise with resistance to concentrically or eccentrically train the quadriceps muscles in young subjects, before determining their muscle IGF-I response 48 hours after the end of exercise. Subjects performed 8 sets of 8 repetitions with resistance at 85% and 110% of their 1-RM for the concentric and eccentric
exercise protocols, respectively. Mean levels of IGF-I mRNA increased after both
congentric and eccentric loading. However, it was only after eccentric loading that
IGF-I mRNA levels were significantly increased from baseline (~62%). Thereby
suggesting that overall IGF-I mRNA in muscle was more sensitive to the eccentric
component of exercise. The method used for measuring levels of IGF-I mRNA in
this study was not strictly quantitative, and was described by the authors as a
qualitative RT-PCR method. It involved performing conventional RT-PCR, then
running the PCR products on a Southern gel and measuring the band densities using
densitometry. For standardisation, data was normalised to GAPDH. As discussed in
Chapter 2, when normalising to a control gene, the first thing that needs to be
determined is whether the chosen gene is affected in any way by the experimental
treatment and also if its expression levels are similar to those of the mRNA being
studied. Because no trustworthy control gene exists within muscle tissue, attempts to
validate the chosen control should always be performed to ensure that it does not
change with the particular intervention being studied (Schjerling, 2001). Otherwise
normalisation to total RNA has been suggested to be the most reliable method,
particularly in human muscle biopsy samples (Tricarico et al. 2002). Bamman et al
do not describe any experiments performed to validate the use of GAPDH as a
control gene. Furthermore, the primers used for detection of IGF-I in that study were
general primers that did not differentiate between the different isoforms.

The much larger increase observed in levels of MGF mRNA after eccentric exercise
in the present study (~186% from baseline in young subjects) can possibly be
explained by different time points at which the biopsies were taken. Samples were
taken in the short term following exercise in the present study, whereas in the study
by Bamman et al samples were taken 48 hours after the end of exercise. The varied responses could also possibly be explained by the different exercise protocols performed by the subjects in both studies. In the present study one hour of eccentric cycling exercise induced severe muscle damage and soreness, but the absolute forces generated would have been much lower compared with the heavy resistance type eccentric exercise performed in the study by Bamman et al, which is known to result in hypertrophy. Thus it is difficult to strictly compare the two exercise modes directly as the exercise used in the present study could probably be described as a severe muscle damaging protocol as opposed to a more 'hypertrophic’ protocol in the study by Bamman et al.

The optimum time point for an acute post-exercise elevation in muscle IGF-I is unknown. However, the smaller response observed in this study could also, in part, be attributed to the previously reported speed with which MGF mRNA increased in response to exercise, also evidenced by the study in Chapter 3. It is therefore possible that the MGF response had peaked soon after exercise and was beginning to diminish by 48 hours. Furthermore, the present study made no comparison with the IGF-I mRNA response following a comparable bout of concentric exercise.

Several studies have indicated that older animals are more susceptible to acute eccentric exercise-induced muscle damage than are young animals (Brooks and Faulkner, 1990; McBride et al. 1995), whereas the results from studies in human muscle are less clear. For example, Manfredi et al (1991) reported that following an acute bout of eccentric exercise designed to elicit muscle damage; older men had higher levels of muscle damage than compared with younger men. In this study,
>90% of muscle fibres from the older subjects exhibited muscle damage. This was compared with 5-50% damaged muscle fibres reported in a study of young subjects after a similar exercise protocol (O’Reilly *et al.* 1987). More recent electron micrography studies, assessing ultrastructural muscle damage in young and old individuals have reported a higher degree of muscle damage in elderly women when compared with young women, after a 9 week period of heavy resistance strength training (Roth *et al.* 2000). Interestingly however, a similar study by the same group comparing muscle damage in young vs old men concluded that both young and older men exhibited a similar increase in muscle damage after 9 weeks of heavy resistance strength training (Roth *et al.* 1999).

CK is an intramuscular enzyme responsible for maintaining adequate ATP levels during muscle contraction. Increased serum CK levels following periods of activity are indicative of an increased permeability or breakdown of the membrane surrounding the muscle cell. The severity of muscle damage caused by the exercise in the present study was reflected by the increased levels of serum CK in both young and old individuals 2 hours after the end of the eccentric cycling exercise bout. However, despite showing an increase, the CK response was somewhat blunted in the older subjects when compared with the young. This cannot be explained with certainty, given that some of the elderly subjects required hospitalisation due to the severity of muscle soreness experienced in the days following the exercise (personal communication Bente Klarlund Pedersen). The high degree of muscle damage caused in the present study coupled together with the increase in MGF mRNA observed in both young and old individuals after exercise, would suggest that MGF
activity is somehow linked to the mechanisms involved in tissue regeneration and repair after mechanical damage.

Conclusion

This study has shown that eccentric cycling exercise increases the expression of MGF mRNA in young, but also older individuals in the short term after exercise. It has also confirmed the finding in Chapter 3 that the two isoforms, IGF-IeA and MGF, are differentially regulated in human muscle in response to exercise. The type of exercise undertaken in the present study induced severe muscle damage. This resulted in a significant (32%, P<0.05) increase in MGF mRNA in the old subjects. In contrast, a lower (14%, not significant) increase was observed after the weightlifting exercise. This might suggest that myofibrillar disruption and/or sarcolemmal damage caused by eccentric exercise may play a role in activating MGF expression in older muscle.
Chapter 5

The effect of recombinant human growth hormone (GH) and resistance training on IGF-I mRNA expression in the muscles of elderly men
5.1 Introduction

Circulating IGF-I is predominantly liver derived and is produced in response to growth hormone (GH) secretion from the anterior pituitary, with its primary role believed to be in promoting the differentiation and growth of skeletal muscle during development (Daughaday et al. 1986; reviewed by Cohick and Clemmons, 1993). It is now a generally accepted phenomena that liver derived circulating IGF-I is not essential for normal postnatal body growth (Yakar et al. 1999; Sjogren et al. 1999) and that the modified somatomedin hypothesis (Chapter 1), whereby GH promotes postnatal body growth mainly through the local production of IGF-I in non-hepatic tissues such as muscle, is largely responsible for muscle growth in adulthood.

Studies have shown that local IGF-I synthesis may be partially GH-dependent in some IGF responsive tissues (Isaksson et al. 1982; 1987). Much of the work carried out to date studying the effects of GH regulation of IGF-I gene expression in skeletal muscle has predominantly been in rodent muscle. In vivo studies have shown that injection of GH leads to increased levels of IGF-I mRNA in rat skeletal muscle (Isgaard et al. 1988; 1989; Gosteli-Peter et al. 1994). In support of the idea of a direct effect of GH on skeletal muscle, increases in the tyrosine phosphorylation of the cytoplasmic tyrosine kinase Janus kinase-2 (JAK2), signal transducer and activator of transcriptions factor-5 (Stat5), insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) and SHC have been observed in rat skeletal muscle within 5 minutes of GH injection (Chow et al. 1996; Thirone et al. 1999). Furthermore, there is evidence of a GH-dependent change in chromatin structure of the rat IGF-I gene. The emergence of this GH-inducible deoxyribonuclease-I (DNase-I)-hypersensitivity site (HS), which is located within the second intron of the IGF-I gene, supports the idea
of an enhanced IGF-I gene transcription by GH (Rotwein et al. 1993; Thomas et al. 1995). However, it is still not clear whether GH acts directly on human skeletal muscle to stimulate growth through expression of IGF-I, despite there being evidence to suggest that human skeletal muscle expresses the different isoforms of the GH receptor (Martini et al. 1995; Ballesteros et al. 2000). Furthermore, the extent to which GH regulates the different IGF-I isoforms in human skeletal muscle has yet to be determined.

Characteristically, with increasing age there is a loss of muscle mass and strength, as well as a gradual decline in circulating GH levels (Rudman et al. 1981). There are reports that GH administration increases fat free mass and decreases fat mass in elderly individuals (Rudman et al. 1990; Holloway et al. 1994; Yarasheski et al. 1995; Lange et al. 2000). However, its effects in further augmenting the strength gains seen with resistance training alone are still a matter of much debate. So far, there have been a limited number of animal (Andersen et al. 2000) and human (Welle et al. 1996) studies that have shown an additional effect of GH supplementation compared with just training. Exercise has been shown to influence both circulating (Brahm et al. 1997) and muscle IGF-I expression levels as evidenced by the studies in the two previous chapters. It is also known that the muscles of even very elderly individuals can hypertrophy in response to resistance training (Fiatarone et al. 1990; 1994; 1999; Harridge et al. 1999), but the coupling to local growth factor expression still remains to be determined. The present study was therefore conducted to study the effects of GH and resistance training on local growth factor expression in the muscles of elderly subjects.
The main aims of this study were:

(i) To determine which isoforms of IGF-I in muscle are influenced by GH.

(ii) To see how GH administration might interact with mechanical signals provided by high resistance exercise in elderly individuals.

(iii) To determine the extent to which the mRNA of the different isoforms of IGF-I could be upregulated in elderly people in response to a period of strength training, with the addition of studying the effects of exercise training on an, as yet little studied, IGF-I isoform (IGF-IEb).

To achieve these aims, muscle samples were studied which had been obtained from 3 groups of elderly men who had either i) received daily injections of recombinant human GH ii) had in addition to receiving GH, undertaken 12 weeks of resistance training or iii) undertaken 12 weeks of resistance training without receiving GH injections (placebo).
5.2 Methods and Materials

This study was carried out in collaboration with Professor Michael Kjaer's group at the Sports Medicine Research Unit, Bispebjerg Hospital, Copenhagen, Denmark. All subject recruitment, training, GH administration and muscle sampling procedures were carried out in their laboratory and are reported in Lange et al (2002). Any data generated in their laboratory is clearly indicated and has been reproduced with their permission.

Subjects

31 healthy male subjects participated in the original study reported by Lange et al (2002). However, for the present study tissue was available for 19 of these subjects. The original study also included a placebo only group from whom muscle biopsies were not obtained.

19 healthy, elderly male subjects participated in the study; age 74 ± 1 year (70-82) [mean ± SEM (range)]; height, 174 ± 1cm (163-186); body weight, 80.8 ± 1.8kg (63.1-100.3); body mass index, 26.7 ± 0.5kg m⁻² (20.9-32.8); body fat mass, 22.9 ± 1.2kg (10.9-38.9), body fat percentage 28.0 ± 1.0% (14.9-40.0). Before inclusion, each subject underwent a medical evaluation, including medical history, physical examination, routine blood tests, and an exercise electrocardiogram. Exclusion criteria were metabolic, cardiac and malignant disease, anaemia, hormonal replacement therapy, and medication with α or β blockers. Informed consent was obtained from all subjects and procedures were performed according to the Declaration of Helsinki II. The study was approved by the Ethics Committee for Medical Research in Copenhagen and by the Danish National Board of Health.
Study design

Subjects were assigned to either resistance training (3 sessions / week, 3-5 sets of 8-12 repetition maximum per session) with placebo (RT group, n=6), resistance training combined with rhGH administration (RT + GH group, n=6) or rhGH alone (GH group, n=7) in a randomised, placebo-controlled and double-blinded experimental design.

Administration of recombinant human GH

Recombinant human GH or placebo (GH - Norditropin PenSet 24; placebo - Norditropin PenSet 24 Placebo; both from Novo Nordisk, Denmark) was administered daily through subcutaneous injection in the thigh. After careful instruction subjects were able to self-administer either GH or placebo at home in the evening. The dose of GH administered was 0.5 IU·m⁻² rising to 1.5 IU·m⁻². Subjects were weighed, checked for oedema and questioned about side effects on a weekly basis. If any side effects were apparent, the dose of GH was reduced by 50% until the side effects had disappeared or were deemed acceptable by both the subject and the investigator.

IGF-I

Blood was sampled at baseline and after 12 weeks from the antecubital vein into sealed vials without any additives. The blood was allowed to clot for 15 minutes at room temperature and was then centrifuged for 15 minutes at 4°C. The resulting serum was stored at -80°C until further analysis. Circulating total IGF-I levels were measured using Radio Immuno Assay (RIA) as described by Juul et al. 1994).
Briefly, serum was extracted by acid ethanol and was cryoprecipitated prior to analysis to remove any interfering binding proteins.

**Muscle cross sectional area**

Magnetic resonance imaging (MRI) measurements were made at baseline and after 12 weeks. 2D T1-weighted fast field echo (TR/TE, 500/14 ms; FOV180; matrix 512 x 512; slice thickness, 6 mm) MR images (Philips, Gryoscan ACS-NT 1.5 T, Best, Holland) were obtained at a level positioned two thirds proximal along an axis connecting a fix point at the tibial eminentia intercondylaris with a fix point at the femoral trochanter major. The rectus femoris, vastus lateralis, vastus intermedius, and vastus medialis were identified and their circumference and cross sectional area (CSA) were determined. Total quadriceps CSA was calculated by adding the area of the four individual muscle groups together. An estimate of subcutaneous fat CSA was determined by subtracting the leg CSA within the muscle fascia from total leg CSA.

**Muscle Biopsies**

Following local anaesthesia of the overlying skin (1% lidocaine), muscle biopsies were obtained from the right vastus lateralis muscle at baseline, 5 weeks and 12 weeks. In the two training groups biopsy samples were obtained 24h after completion of the last training session. Samples were immediately frozen in liquid nitrogen and stored at −70°C.
RNA extraction

Total RNA was extracted from the muscle samples using Tri Reagent (Sigma) and the quality of the RNA was assessed as described in Chapter 2, Methods and Materials.

First strand cDNA synthesis

0.5 μg total RNA was reverse transcribed into cDNA as described in Chapter 2, Methods and Materials.

Real time quantitative PCR

Quantification of the mRNA message encoding the two isoforms IGF-IEa and IGF-IEc (MGF) and a third isoform IGF-IEb (details of optimisation given below) was performed using LightCycler technology (Roche Diagnostics) with SYBR green I as the method of detection as described in detail in Chapter 2, Methods and Materials. The sequences of the primers used are given in Table 2.4 in chapter 2. Quantitative PCR runs were performed in duplicate and mean values were subsequently used for analysis.

To ensure unbiased analysis, real time quantitative PCR was carried out blind and the identity of the samples was only revealed after the mRNA measurements had been made.

Gel electrophoresis

PCR products were recovered from the glass capillaries by removing the plastic caps and inverting them into a 1.5ml eppendorf tube, which were then spun down in a bench top centrifuge at no higher than 3000 rpm. Target specificity was further confirmed by
running these samples on a 2% agarose gel as described in Chapter 2, Methods and Materials.

**Optimisation of IGF-IEb**

Expression levels of a third isoform of the IGF-I gene were also investigated in the present study. Primers suitable for use on the LightCycler system were designed from the human mRNA sequence of IGF-IEb. Both accession numbers and primer sequences are given in Tables 2.3 and 2.4 in Chapter 2. Quantification of mRNA encoding this isoform in muscle biopsy samples was optimised using the QuantiTect chemistries (Qiagen, UK), given that this was the first time that IGF-IEb was being investigated in human muscle. The optimisation strategies described in Chapter 2 were used to ensure amplification of a specific product with no interfering primer dimer. PCR products from initial runs were run on an agarose gel, gel extracted, DNA purified and sent off for DNA sequence analysis to ensure specificity of the amplification. The methods for these procedures are described in Chapter 2, Methods and Materials. The melting curve profile of a fully optimised IGF-IEb run is shown in Figure 5.1. Due to a shortage of material, measurements of IGF-IEb mRNA were only made in the group that carried out resistance training only.
Statistical analysis

Data are presented as means ±SEM. ANOVA followed by the Student-Newman-Keuls post hoc test was used to determine whether MGF and IGF-Ia expression levels were significantly different between the RT only, RT+GH and GH only groups and also within groups at each time point studied. Statistical significance was accepted at the P<0.05 level. Linear regression studies were used to describe the relationship between levels of MGF mRNA and muscle CSA at baseline and between levels of serum total IGF-I and IGF-Ia mRNA and MGF mRNA in muscle.
5.3 Results

Absolute values of IGF-IEa and MGF mRNA

Baseline levels of both IGF-IEa and MGF mRNA in the group that received GH treatment alone were found to be significantly higher than baseline levels for these isoforms in the other two treatment groups. These values have been highlighted in blue in Table 5.1. For the purpose of clarity, the data has been analysed and discussed as a percentage change from baseline after 5 and then 12 weeks of treatment.

<table>
<thead>
<tr>
<th></th>
<th>IGF-IEa</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>5 weeks</td>
<td>12 weeks</td>
</tr>
<tr>
<td>RT</td>
<td>5.3 ± 0.7  ᵃ</td>
<td>9.4 ± 1.9  ᵆᵃ</td>
<td>10.2 ± 1.0  ᵆᵃ</td>
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<tr>
<td>RT+GH</td>
<td>6.1 ± 0.9  ᵃ</td>
<td>15.3 ± 2.5  ᵆᵃ</td>
<td>14.8 ± 2.8  ᵆᵃ</td>
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<tr>
<td>GH</td>
<td>10.5 ± 1.5</td>
<td>34.7 ± 7.4  ᵇ</td>
<td>23.1 ± 2.2</td>
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<table>
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<tr>
<th></th>
<th>MGF</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>5 weeks</td>
<td>12 weeks</td>
</tr>
<tr>
<td>RT</td>
<td>1.7 ± 0.2  ᵃ</td>
<td>4.7 ± 1.1  ᵆᵇ</td>
<td>4.1 ± 0.4  ᵆ</td>
</tr>
<tr>
<td>RT+GH</td>
<td>2.2 ± 0.5  ᵃ</td>
<td>10.1 ± 1.3  ᵇ</td>
<td>7.8 ± 1.3  ᵇ</td>
</tr>
<tr>
<td>GH</td>
<td>4.9 ± 1.2</td>
<td>5.8 ± 1.2  ᵋ</td>
<td>7.8 ± 1.6  ᵇ</td>
</tr>
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</table>

Table 5.1 Mean values of IGF-IEa and MGF mRNA at baseline, after 5 weeks and after 12 weeks of RT, RT+GH or GH administration alone. Data are expressed as ng of single stranded (ss) PCR product / μg total RNA. Levels of expression for IGF-IEa are 10⁻⁵ ng and for MGF, 10⁻⁸ ng. ᵆ Significant difference from baseline, ᵇ from 5 weeks (p<0.05).

ᵃ Significant difference from GH, ᵋ from RT+GH at given time point (p<0.05)
**Effect of GH administration on IGF-IEa and MGF mRNA**

GH administration alone was shown to have a marked effect on IGF-IEa mRNA levels. This isoform was increased by 237% (p<0.05) after 5 weeks of GH treatment and remained elevated from baseline after 12 weeks. In contrast, levels of MGF mRNA showed very little change after 5 weeks of GH administration but showed a significant increase from both the baseline (80%, P<0.05) and the 5 week time point after 12 weeks, although this was still to a much lesser degree than the change observed in levels of IGF-IEa.

![Graph showing % change from baseline in IGF-IEa and MGF mRNA levels in the GH group.](image)

**Figure 5.2.** % Change from baseline in IGF-IEa and MGF mRNA levels in the GH group. Values are +SEM: n=7. * Significant difference from baseline † from 5 weeks (P<0.05). ‡ Significant difference in % change between isoforms (P<0.05).
Effect of resistance training on IGF-IEa and MGF mRNA

After 5 weeks of resistance training (RT group), both IGF-IEa (68%) and MGF (163%) transcripts were significantly elevated when compared to baseline. Interestingly however, the change in levels of MGF mRNA at this time point was significantly greater than the change in IGF-IEa. mRNA levels of both MGF and IGF-IEa remained significantly elevated from baseline after 12 weeks, but there were no further significant changes observed at this time point.

**Figure 5.3.** % Change from baseline in IGF-IEa and MGF mRNA levels in the RT group. Values are ±SEM: n=6. * Significant difference from baseline. † Significant difference in % change between isoforms (P<0.05).
Effect of resistance training and GH on IGF-I\text{Ea} and MGF mRNA

In the group where resistance training was combined with GH administration (RT+GH group), substantially greater increases in both IGF-I\text{Ea} (167\%) and MGF mRNA expression (456\%) were observed at the 5-week time point compared with the group that carried out training alone. Again, there were no further significant changes seen after 12 weeks. Furthermore, when GH administration was combined with resistance training, the change in MGF mRNA levels was greater than the change in IGF-I\text{Ea} mRNA, although the difference did not quite reach significance (P<0.066).

Figure 5.4. % Change from baseline in IGF-I\text{Ea} and MGF mRNA levels in RT+GH group. Values are +SEM: n=6. * Significant difference from baseline.
Comparison between treatment groups

Analysis of the combined responses of IGF-IEa and MGF mRNAs in the 3 treatment groups highlights the smaller MGF mRNA response to GH treatment alone, but comparative sensitivity to training and furthermore to training combined with GH. The somewhat reciprocal response of IGF-IEa, with its mRNA being highly responsive to GH treatment alone but comparatively less sensitive to training is also highlighted.

Figure 5.5. Comparison of the MGF and IGF-IEa mRNA responses in the 3 treatment groups. Values are ±SEM: * Significant difference from baseline. † Significant difference in % change between isoforms (P<0.05).
IGF-IEb mRNA Expression after Resistance Training

Expression levels of this isoform were low and in the $10^8$ ng range. Baseline levels of IGF-IEb mRNA ($4.2 \times 10^8$ ng) were significantly increased by approximately 75% ($P<0.01$) after 5 weeks of training ($7.1 \times 10^8$ ng). By the 12 week time point, they had declined to levels similar to those observed at baseline ($4.7 \times 10^8$ ng, $P<0.01$). A comparison of the IGF-IEb response compared with those of IGF-IEa and MGF in the same subjects are shown in Figure 5.7.

Figure 5.6. Levels of IGF-IEb mRNA at baseline, after 5 and 12 weeks of RT. Values are +SEM: $n=5$. * Significant difference from baseline. # from 5 weeks ($P<0.01$).
Figure 5.7 Comparison of the IGF-IEb mRNA response with those of IGF-IEa and MGF after 5 and 12 weeks in the same subjects. Values are +SEM (n = 4).

There were differences in the absolute levels of expression between IGF-IEa, IGF-IEb and MGF transcripts. IGF-IEa mRNA was expressed at higher levels when compared to IGF-IEb and MGF by 2-3 orders of magnitude (10^{-5} ng vs 10^{-8} ng).
Circulating IGF-I
Measurements of circulating levels of total IGF-I were made in all subjects from all three intervention groups at baseline, 5 and then 12 weeks. There were no significant differences observed in baseline levels of IGF-I between the three groups. In the two groups receiving GH, serum IGF-I levels increased significantly from baseline to 12 weeks, whereas no change was observed in the resistance training only group from baseline to 12 weeks. (Lange et al. 2002).

Figure 5.8. Levels of circulating IGF-I at baseline, 5 weeks and 12 weeks in the 3 intervention groups (n = 24): RT, RT+GH and GH. Values are expressed as +SEM.
* Significant difference from baseline (P<0.05). Data reproduced by kind permission of Dr Kai Lange.
Muscle cross sectional area

Measurements of muscle cross sectional area were made in all subjects from the three intervention groups at baseline and then after 12 weeks. There were no significant differences observed in baseline measurements of muscle cross sectional area between the RT and RT+GH groups. However, the group that received GH alone had significantly higher muscle CSA at baseline compared with the other two groups. This was unusual and could not be explained with certainty, as the subjects had not received any GH prior to the start of the study. Furthermore, the samples were analysed in a blind fashion and only decoded after analysis. In the two resistance training groups (RT and RT+GH), muscle CSA increased significantly from baseline to 12 weeks, whereas no change was observed in the group that received only GH (Lange et al. 2002).
Figure 5.9. Measurements of muscle CSA at baseline and 12 weeks in the 3 intervention groups (n = 24): RT, RT+GH and GH. Values are expressed as ±SEM.

* Significant difference from baseline # between baseline levels (P<0.05). Data reproduced by kind permission of Dr Kai Lange.
Correlation between circulating IGF-I and IGF-IEa and MGF mRNA in muscle

After 12 weeks of GH treatment alone, there was a significant correlation \((r = 0.81, p<0.05)\) between the change in levels of serum IGF-I and the change in levels of IGF-IEa produced by the muscle (Figure 5.9) but not with MGF \((r = 0.43)\). However, after 12 weeks of GH treatment in combination with RT, there was no significant relationship between the change in serum IGF-I and the change in muscle IGF-IEa \((r = 0.31)\), but a significant relationship with the change in muscle MGF mRNA \((r=0.83, p<0.05)\) (Figure 5.10). There were no significant correlations observed between the change in serum IGF-I concentrations and either of the muscle growth factors IGF-IEa and MGF in the RT only group.
Figure 5.10. Relationship between the change in serum IGF-I levels and the change in muscle IGF-IeA after 12 weeks of GH administration alone ($r = 0.81, P<0.05$).

$$Y = -0.006 + 0.062 \times X$$

Figure 5.11. Relationship between the change in serum IGF-I levels and the change in MGF mRNA after 12 weeks of GH combined with RT ($r = 0.83, P<0.05$).

$$Y = 0.815 + 0.022 \times X$$
**Correlation between muscle CSA and MGF mRNA at baseline**

Significant differences in baseline levels of both IGF-IeA and MGF mRNA were observed between the three groups RT, RT+GH and GH only. Levels of both IGF-IeA and MGF mRNA were higher in the group that received GH treatment only when compared to the other two training groups. When related to muscle size, as determined by nuclear magnetic resonance imaging (NMRI) (Lange et al. 2002), a significant correlation was observed between baseline levels of MGF mRNA and quadriceps cross sectional area (CSA) ($r = 0.58, P<0.05$). There appeared to be no significant relationship between muscle size and levels of IGF-IeA and no further correlations were observed between changes in IGF-I mRNA expression and muscle morphology over the training period.

![Graph showing the relationship between MGF mRNA and muscle CSA](image)

*Figure 5.12 Relationship between baseline levels of MGF mRNA and muscle CSA ($r = 0.58, P<0.01$).*
5.4 Discussion

This study has revealed three key findings. Firstly, GH administration in elderly people appears to have differential effects on the mRNA expression of the isoforms of IGF-I expressed in muscle. GH seems to upregulate IGF-I expression, in an isoform and time specific manner with the preferential upregulation of the IGF-IEa isoform. However, in contrast, when exercise is combined with GH administration the mechano-sensitive isoform, MGF, is more markedly upregulated. Secondly, this study demonstrates that older muscles are able to upregulate both IGF-IEa and MGF mRNA in muscle in response to a period of prolonged resistance training. This is in contrast to the results shown in Chapter 3 where a single bout of high resistance exercise had no effect on either of these isoforms in older subjects, at least when measured 2½ hours after the end of the exercise bout. Thirdly, this study has also shown that the exercise training intervention upregulates a third isoform of the IGF-I gene, IGF-IEb.

GH regulated IGF-I expression in muscle

GH stimulates post-natal growth of the body in general through induction of IGF-I gene expression (Sadowski et al. 2001). Although the liver is the major site of GH-regulated IGF-I production, there is evidence to suggest that GH is able to regulate IGF-I gene expression in other non-hepatic tissues including the kidney, bone, intestine and skeletal muscle in an autocrine / paracrine fashion (Isaksson et al. 1982; 1987). Increased IGF-I mRNA levels have been observed in the muscles of GH-treated hypophysectomized rats (Murphy et al. 1987; Isgaard et al. 1989; Gosteli-Peter et al. 1994) and the muscles of rats implanted with GH-secreting cells (Turner et al. 1988). In vitro studies have also shown that C2C12 skeletal muscle cells
respond rapidly to GH administration with increased tyrosine phosphorylation of the GH receptor (GHR) and increases in IGF-I mRNA expression (Sadowski et al. 2001). However, whether GH directly stimulates IGF-I mRNA expression in human skeletal muscle has remained somewhat unclear (Taaffe et al. 1996; Welle and Thornton, 1997). The present study therefore aimed to determine whether GH differentially affects the different isoforms of IGF-I.

The effects of GH administration in elderly people

GH release in response to resistance exercise is markedly attenuated with increasing age both in the untrained and trained state (Pyka et al. 1994; Zaccaria et al. 1999). Several studies have shown that GH administration in elderly people decreases fat mass and increases fat free mass (Rudman et al. 1990; Papadakis et al. 1996; Taaffe et al. 1994; Yarasheshki et al. 1995; Lange et al. 2000; Lange et al. 2002). However, despite increasing fat free mass, GH administration has not been shown to increase muscle mass or strength in healthy elderly individuals (Papadakis et al. 1996; Lange et al. 2002). Furthermore, trials in elderly people where GH administration has been combined with resistance training, GH does not seem to augment the strength gains obtained from exercise training (Taaffe et al. 1994; Yarasheshki et al. 1995; Lange et al. 2002). This is in contrast to studies in aged rats, which have reported gains in muscle mass and tetanic tension when GH administration is combined with cycling exercise (Andersen et al. 2000).
Differential regulation of IGF-IEa and MGF by GH

The findings in the present study confirm the increase in IGF-I mRNA in the muscles of older people reported by Brill et al. (2002), who administered daily injections of GH over a one month period. However, the present study shows that there is an isoform specific response, with IGF-IEa (main liver isoform) being the most responsive to GH administration. Earlier studies investigating GH-regulated IGF-I mRNA expression in human skeletal muscle of elderly subjects had reported no consistent increases in IGF-I mRNA levels after 10 weeks of GH administration (Taaffe et al. 1996) or in muscle biopsy samples taken 10 hours after a single subcutaneous injection of GH (Welle and Thornton, 1997). However, these previous studies did not distinguish between the different isoforms of IGF-I expressed in muscle. As previously discussed, the IGF-I gene has two promoter regions, with transcripts initiating at promoter 2 believed to be GH sensitive (Lyall, 1996). Therefore, the suggestion that the two isoforms are regulated differently, with IGF-IEa being GH responsive and MGF appearing relatively insensitive to GH treatment would further support the results of the study in Chapter 3, which suggested that the two isoforms behave independently from one another. In support of this, another study showed that when rat muscles are exercised, MGF mRNA is expressed earlier than IGF-IEa (Haddad and Adams, 2002). Also, a later study showed that following local muscle injury the IGF-I gene is spliced to produce the MGF transcript, which precedes muscle satellite cell activation (Hill and Goldspink, 2003). After a few days, it appears that it is then spliced towards IGF-IEa. Thereby highlighting the idea that these are different growth factors with different functions and expression kinetics.
Baseline differences in IGF-IEa and MGF expression

Baseline levels of both IGF-IEa and MGF mRNA in the group that received GH treatment alone were found to be significantly higher than baseline levels for these isoforms in the other two treatment groups. This cannot be explained with certainty, as all of the samples were analysed randomly and in a blind fashion and only re-coded once quantification and analysis were complete. The possibility that this treatment group had received a dose of GH prior to the baseline biopsy being taken was also explored, however, all biopsy samples were taken before any GH had been given.

Changes in the expression of IGF-IEa and MGF mRNA in elderly people with prolonged resistance training

The study in Chapter 3 suggested that older muscles are less able to upregulate MGF mRNA when compared to young muscle following mechanical loading. Expression of MGF mRNA in the young subjects in that study significantly increased 2½ hours after the end of a single bout of high resistance exercise whereas levels of IGF-IEa in both young and older subject groups showed no significant change with exercise. The present study however has clearly shown that following a 12-week period of progressive resistance training, even muscles of elderly individuals are able to significantly upregulate both IGF-IEa and MGF mRNAs. Levels of IGF-IEa were significantly increased after 5 weeks of resistance training and further increased at the 12-week time point when compared to baseline (68% and 103%). Interestingly, MGF levels in the present study were more sensitive to the training regime than IGF-IEa (168% and 169% from baseline). The comparative sensitivity of MGF compared with IGF-IEa to training would add weight to the suggestion made earlier in Chapter
3, that being, when other studies such as the one by Fiatarone-Singh et al (1999) report an increase in IGF-I levels after resistance training, this might reflect an increase in MGF rather than IGF-IEa. As with the aforementioned study, the present study did not evaluate young subjects within the same study. This would be an invaluable exercise if we were to determine if there is an age related impairment to the upregulation of the different IGF-I isoforms in muscle with training. At present however no conclusions can be drawn on this issue.

Changes in the expression of IGF-IEa and MGF mRNA in elderly people with resistance training in combination with GH supplementation

Previous studies have investigated the effects of combining resistance training with GH supplementation on muscle mass and strength in elderly individuals (Taaffe et al. 1994; Yarasheshki et al. 1995). The present study also combined these two stimuli. In this group of individuals, levels of IGF-IEa mRNA were no greater than those observed with either resistance training or GH administration groups. In contrast however, levels of MGF mRNA observed at both 5 and 12 weeks were markedly higher when compared with the GH only and RT only groups respectively. This suggests that GH administration results in a greater upregulation of the IGF-I gene in muscle which, when combined with high resistance exercise causes splicing towards the MGF isoform. Due to the dramatic increase observed in levels of MGF in response to resistance training combined with GH, it could be speculated that the long term effects (i.e. beyond 12 weeks) of combining these treatments may lead to an increase in muscle mass. Indeed, even in the GH only group it is possible that the increased levels of MGF mRNA observed after 12 weeks may have resulted from more of the primary transcript being available for splicing as a result of the.
mechanical activity caused by habitual levels of physical activity. Furthermore, a
significant association was observed between basal levels of MGF and quadriceps
CSA as determined by MRI (Figure 5.12). This might provide some tentative
evidence for the long-term regulation of muscle mass by the expression of local
growth factors.

The relationship between circulating and muscle IGF-I

Circulating IGF-I is almost exclusively derived from the liver in response to GH
secretion from the anterior pituitary. However, it has also been shown that the
exercising muscle itself takes up GH during exercise and consequently releases IGF-
I, thus providing a local contribution to circulating IGF-I (Brahm et al. 1997). In the
present study, an increase in resting levels of circulating IGF-I was seen from
baseline to 12 weeks in subjects receiving exogenous GH (Lange et al. 2002), but no
further increase in IGF-I levels were seen in the RT+GH groups. Interestingly, the
change in circulating IGF-I levels in the GH only group correlated with the change in
IGF-Ieα mRNA (r = 0.81, P<0.05), but not MGF mRNA (r = 0.43), in muscle. There
was no such relationship between the change in circulating IGF-I and the mRNA of
IGF-Ieα (r = 0.45) or MGF (r = -0.53) in muscle, in the RT only group. In contrast,
in the RT+GH group the change in circulating IGF-I was related to the change in
MGF (r = 0.83, P<0.05), but not IGF-I eα (r = 0.31). This suggests that GH
administration may lead to an overall upregulation of the IGF-I gene in muscle, as
well as in the circulation. Also, that isoform expression is regulated by physical
activity, such that when combined with mechanical overload the IGF-I gene favours
splicing towards the MGF isoform. The contribution of IGF-Ieα and MGF to the
circulating IGF-I levels may be a simple reflection of their increased expression in
the different experimental groups. Thus suggesting that under certain conditions MGF might contribute significantly to circulating IGF-I levels.

GH and strength training

Measurements made by Lange et al. (2002) in subjects from the present study showed that after 12 weeks of training, the gain in muscle mass and strength was actually no different in the group that strength trained whilst receiving GH (RT+GH) compared with the group that strength trained only. This finding is in agreement with previous studies in both old (Taaffe et al. 1994; Yarasheski et al. 1995) and young men (Yarasheski et al. 1992). Although one such study by Welle et al. (1996), reported that GH treatment did further improve training induced muscle strength in healthy elderly men. In the present study (Lange et al. 2002) the increase in muscle mass and strength tended to be higher than in the group that trained whilst receiving GH treatment compared with the group that carried out strength training alone, however these changes were not significant. The possibility that a longer training period would have resulted in a greater increase in muscle mass and strength in the group that trained in combination with receiving GH cannot be excluded, and might be suggested by the MGF mRNA response seen in this group in the present study. Alternatively, it might be that the overload provided by training provides a sufficient stimulus for the upregulation of MGF at levels, which are optimal for muscle repair and adaptation, at least in healthy older people undergoing exercise training.

Changes in the expression of IGF-IEb with resistance training

An additional finding in the present study is the change in expression of a third isoform of the IGF-I gene, IGF-IEb. There are three alternative splicing sites at the
3' end of IGF-I transcripts, which generate different E peptides with a common 16 amino acid N-terminal sequence and alternative C-terminal sequences. Transcripts containing Eb are more abundant in the liver, whereas transcripts containing Ea are commonly expressed in extra-hepatic tissues (Adamo et al. 1993; Stewart and Rotwein, 1996). It was possible to measure levels of this isoform as a part of this study as the optimisation process for different primers on the LightCycler was an ongoing process, with quantification of this isoform proving most difficult. A number of different primer sets were designed before a suitable pair was found. As with MGF and IGF-IEa, IGF-IEb is upregulated as a result of strength training exercise. The precise role of this isoform in muscle is unclear and further studies are required to elucidate its role.

**Conclusion**

This study demonstrates that elderly people can upregulate three isoforms of IGF-I in muscle as a result of high resistance training. The administration of recombinant GH alone, and in combination with exercise, has demonstrated that there is differential regulation of at least two isoforms of IGF-I, IGF-IEa and MGF. The data supports the argument that the IGF-I gene favours splicing towards the MGF isoform with increased mechanical activity provided by high resistance exercise. This might provide some evidence for the long-term regulation of muscle mass by the expression of local growth factors. These effects have been summarised in Figure 5.13 below. Whether higher levels of MGF induced as a result of combining GH with exercise induce further adaptations in muscle beyond 12 weeks remains to be determined.
GH administration

local cell damage

GH administration RT

GH

JAK2

P

PP

JAK2

PP

PP

PP

PP

PP

PP

PP

IRS-ive

STAT 1,3,5

P

P

P

SHP-2

homo/heterodimerization

STAT 1,3,5

P

P

P

P

SOCS

nuclear translocation

IGF-I

RNA splicing

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Figure 5.13 Diagram showing some of the possible signalling pathways involved leading to IGF-I gene transcription if GH were to regulate its expression in muscle following either I. GH treatment alone or II. GH+RT. III shows how RT alone may affect MGF and IGF-IEa mRNA levels. Preferential splicing towards IGF-IEa ( ) or MGF ( ) after each intervention is shown by the relative proportion of each isoform present, after splicing of the IGF-I gene in the nucleus. Abbreviations: JAK, Janus kinase; STAT, signal transducers and activators of transcription; SOCS, suppressors of cytokine signalling; IRS, insulin receptor substrate; SHP2, protein tyrosine phosphatase. (Adapted from Le Roith et al. 2001).
Chapter 6

Summary and Conclusions
Summary and Conclusions

The aim of this thesis was to study the expression of splice variants of IGF-I in human skeletal muscle, with particular reference to age and exercise. To this end a new technique for the detection and accurate quantification of these mRNA transcripts was developed. Preliminary experiments in young subjects which used conventional PCR methods and which utilised the syringe needle aspiration technique of muscle sampling, revealed that the isoform most commonly expressed in the liver IGF-IEa, as well as the mechano sensitive isoform, MGF, were present in both resting and post exercise muscle samples (see Appendix 1). This was contrary to an earlier experiment performed in one subject, which had not been able to detect MGF in resting muscle (Yang et al. 1996). This finding coupled with technological advancements lead to the development of a new method for both detecting and quantifying these IGF-I transcripts using a real time quantitative PCR technique based on LightCycler technology. Time was taken to develop and optimise this method so that it would be possible to measure changes in expression of IGF-I isoforms in muscle with exercise. Initial experiments, had utilised the syringe needle aspiration technique. This is somewhat less invasive than the conventional needle biopsy technique, and has the advantage of allowing multiple sampling. However, it became clear that the target mRNA concentration in these samples was below a reliable detection limit of the real time PCR set-up (see Appendix 2). Thus, subsequent experiments were performed using the conventional needle biopsy method.

The expression of IGF-IEa and MGF mRNAs were studied in young and old subjects 2½ hours after the end of an acute bout of high resistance weightlifting exercise.
Based on earlier animal experiments (Owino et al. 2001), it was hypothesised that older muscle would show a reduced MGF response after the exercise challenge. This study revealed that in young subjects, there was a distinct difference in the expression pattern of the two IGF-I isoforms in response to a single bout of high resistance weightlifting exercise, with MGF being upregulated and the IGF-IEa isoform remaining unchanged. In contrast, and in general agreement with the animal experiments, older subjects were less responsive. It was concluded that the two isoforms of IGF-I were differentially regulated in young muscle in response to a single bout of high resistance weightlifting exercise. There did not appear to be any relationship between the magnitude of the response and the MyHC composition of the muscle, but it was suggested that the underlying training status of the muscle might play some part in determining the degree of responsiveness. The type of exercise performed in this study is known to result in muscle hypertrophy. It is known that IGF-I has an anabolic action and that it can increase rates of muscle protein synthesis and that the activation of satellite cells is required for hypertrophied fibres to maintain their DNA to protein ratios (Kadi and Thornell, 2000). The role of MGF in satellite cell activation was highlighted in a recent cell culture study where C2C12 cells were treated with either MGF or IGF-I peptides. In that study MGF appeared to initiate satellite cell proliferation, whilst IGF-I promoted the differentiation of these cells into myotubes (Yang and Goldspink, 2002). Also that MGF expression precedes that of IGF-IEa and markers of satellite cell activation following muscle damage (Hill and Goldspink, 2003). However, to link these events it was important to consider the type of exercise in relation to muscle hypertrophy.
In order to determine whether the MGF response was selective to the eccentric lengthening component of a muscle contraction, the mRNA responses of both IGF-IeA and MGF were studied in young and old subjects that had performed one hour of reverse pedal cycling eccentric only exercise. This type of exercise evoked a high degree of muscle soreness and damage, as was indicated by the elevated serum CK levels in the subjects after the exercise challenge. However, due to the relatively low absolute forces generated with this prolonged exercise protocol, it was unlikely to result in muscle hypertrophy. Interestingly, 2.25 hours after the end of this acute bout of this highly damaging exercise, this study revealed that both young and old subjects exhibited a marked increase in their MGF mRNA levels. While mRNA levels of IGF-IeA showed no significant change at the same time point. These data supported the findings in Chapter 3 that these isoforms were differentially regulated in human muscle shortly after the end of exercise. In contrast to the weightlifting exercise, the older subjects showed a significant upregulation of MGF. These data might therefore suggest that MGF expression in muscle may, in part, be linked to the myofibrillar disruption and/or sarcolemmal damage caused by eccentric exercise.

Although, the data reported in Chapter 3 implies a lack of response on the part of the older subjects, it is now established that progressive resistance training can increase muscle mass in elderly individuals. Furthermore, previous studies in elderly subjects have also shown an increase in IGF-I protein levels following a 10 week period of resistance training (Fiatarone et al. 1999). Thus, it may be that there is an age-related change in the sensitivity to a single exercise bout, or that there is slower expression (i.e. greater then 2.5 hours) in the older compared to the young muscle. The experiments conducted in Chapter 5 were designed to measure the effects of a period
of strength training on these different IGF-I splice variants. These experiments also allowed an investigation into whether the local IGF-I response in human skeletal muscle is in any way under the control of GH. In this experiment elderly subjects were randomly assigned into one of three intervention groups: a group that received daily injections of GH, and two groups that underwent a 12 week resistance training programme either with or without (placebo) GH treatment. In addition, it was now possible to study the response of a third isoform, IGF-IEb in relation to resistance training. The results of the study demonstrated that elderly people could significantly upregulate all three isoforms of IGF-I in muscle after a period of high resistance training. Interestingly, GH administration was shown to have differential effects on the mRNA expression of the two isoforms IGF-IEa and MGF in muscle, with GH administration seeming to favour upregulation of the IGF-IEa isoform. This is perhaps not unexpected as in the liver it is the IGF-IEa isoform which is essentially under the control of growth hormone. However, with increased mechanical activity in the form of resistance training, there was marked upregulation of the MGF isoform. This response was further enhanced in the group where resistance training was combined with GH. These data would therefore suggest that GH induced an overall upregulation of the IGF-I gene, and that splicing towards the MGF isoform was favoured with increased mechanical activity.

In conclusion, the development of a real time quantitative PCR method that can measure changes at the mRNA level has allowed both short-term and long-term IGF-I isoform responses to exercise to be assessed in young and old human skeletal muscle. It is becoming more apparent that local growth factors are not only essential for muscle growth and repair, but that within the IGF-I gene family different
isoforms exist as a result of alternative splicing, which may have different physiological roles. Whilst the work in this thesis has concentrated on the expression of IGF-I transcripts in human muscle studied \textit{in vivo}, future work will need to measure the resultant levels of protein expression. The combination of both \textit{in vivo} and \textit{in vitro} studies should help characterise the precise physiological roles of these different isoforms.
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Appendix 1
**Human muscle sampling using syringe needle aspiration**

Initial experiments involved using the syringe needle aspiration technique to obtain muscle tissue. This technique is similar to that of an intra-muscular injection. It involves inserting a 19-gauge needle into the belly of the muscle, creating a vacuum with a 20ml syringe and then agitating the needle three to four times before withdrawing the needle. A 'tiny' amount of muscle tissue is recovered using this technique, but there is enough to extract total RNA and consequently make cDNA for RT-PCR.

**Expression of IGF-I isoforms in human skeletal muscle**

**Rationale**

An initial experiment by Yang *et al*, 1996, where MGF was detected in human muscle for the first time was performed in one subject. In this experiment, the individual performed half an hour of intense eccentric exercise of the forearm flexor muscles. Using the syringe needle aspiration technique, a muscle sample was taken from the biceps brachii muscle 2 hours after completion of the exercise. A second sample was also taken from the contralateral muscle, which served as a non-exercised control. This study detected MGF mRNA in the eccentrically exercised arm, but not in the contralateral control muscle. Thereby suggesting that MGF was not present in resting human muscle. Since this study was performed in only one subject it was necessary to repeat the experiment in a number of subjects before any conclusions could be drawn.

To perform a pilot study, 4 young subjects were used to determine the expression profile of this isoform in the vastus lateralis muscle after a single bout of eccentric box-stepping exercise. Syringe needle aspiration samples were obtained at multiple time points following the exercise bout, details of which are given below. A number of factors had to be taken into consideration when designing this pilot study. Firstly, the experiment
described by Yang et al (1996) sampled the biceps brachii muscle. Since this is not a naturally weight bearing muscle such as the vastus lateralis, it is possible that there may have been baseline differences in the levels of MGF mRNA simply as a result of the vastus lateralis being a weight bearing, locomotory muscle.

Study protocol
In order to test the validity of the syringe needle aspiration technique as a reliable method of muscle sampling, we conducted a pilot study in four healthy young female volunteers (aged 22-25 years), using a box stepping model of damaging eccentric exercise. The subjects performed 40 minutes of repetitive box-stepping exercise on a box 47 cm high in time with a metronome. Duplicate syringe needle aspiration samples were taken from the vastus lateralis muscle of both the concentrically and eccentrically exercised legs before exercise, then 2 hours, 6 hours and 2 days after the end of the exercise bout. Total RNA was extracted from the muscle sample. Since the samples were so small, it was not possible to quantify total RNA concentration using spectrophotometry. All of the RNA extracted was therefore reverse transcribed using oligo(dT)17 to make first strand cDNA. Details of RNA extraction and first strand cDNA synthesis are given in Chapter 2, Methods and Materials. The cDNA was then amplified using conventional PCR as described in Methods and Materials. The primers used to amplify the MGF product were taken from Chew et al (1995). The forward and reverse primer sequences are given in Chapter 2, Table 2.4. These primers yielded a 449bp product.
Results

α-actin and myosin screening

Samples were initially screened using both myosin and α-actin primers to confirm the presence of muscle mRNA. Muscle sampling using the syringe needle aspiration technique was successful in obtaining muscle tissue, as following RT-PCR all samples were found to contain both myosin and α-actin.

Key Finding

The results from this pilot study revealed that MGF mRNA was present in muscle samples taken both before and post exercise (Figure A 1.1). This study also revealed that conventional PCR methods could be used quite reliably to determine the presence of this isoform in human muscle in samples obtained using the syringe needle aspiration technique. The original idea behind performing this study was to determine the time point after exercise that MGF mRNA could be detected, and this approach would have proved useful had we not detected MGF mRNA in the samples taken before exercise. Since MGF mRNA was detected in both before and post exercise samples, it became necessary to develop a technique that would allow quantification of the mRNA levels of isoforms such as MGF, in order to establish whether there was a change in their expression with exercise.
Figure A 1.1. 2% agarose gel picture showing amplification of MGF mRNA following 40 minutes box-stepping exercise.
Appendix 2
Quantitative PCR of muscle samples obtained using syringe needle aspiration

Aim
The purpose of this pilot study was to determine whether muscle samples obtained using the syringe needle aspiration technique could be used for quantitative PCR on the LightCycler system.

Background
Since the amount of tissue obtained when using this muscle sampling technique was so small (less than 1mg), the concentration of total RNA could not be determined using spectrophotometry prior to generating cDNA. It was therefore not possible to normalise to total RNA concentration. The alternative was to normalise levels of IGF-I isoform mRNA to a control gene (i.e. relative quantification). Finding a suitable control gene, which is unchanged by the intervention being studied, is always a lengthy procedure and can involve testing the validity of many genes before a suitable one is found. Initial experiments looked at the possibility of using GAPDH for this purpose. Previous experiments to optimise the GAPDH primers on the LightCycler system were performed using tissue that had been obtained using the needle biopsy technique (which yields approximately 50mg of tissue). Here, the starting RNA concentration used to make cDNA was typically 0.5 μg. These early optimisation experiments had revealed that GAPDH mRNA was abundantly expressed in muscle tissue and also that the primer pairs designed worked efficiently to yield a specific PCR product with no primer dimer. A typical example of a melting curve profile of GAPDH is shown in Figure A 2.1 below.
Figure A 2.1. A fully optimised GAPDH run on the LightCycler showing a sharp narrow specific product peak and no primer dimer, except that generated in the no template control sample.

Measurement of GAPDH mRNA in samples from box-stepping study

Using real time quantitative PCR to analyse these samples proved extremely problematic. Although it was possible to amplify the abundantly expressed gene GAPDH to a certain degree using this approach, the results were inconsistent and unreliable. An example of the amplification profile obtained from a run with 4 standards and 8 samples is shown in Figure A 2.2. The standards amplified with few problems and yielded the desired specific product, as reflected by the melting curve profile shown in Figure A 2.2. However, the ‘unknown’ cDNA samples proved difficult to amplify, and amplified less efficiently at much later cycle numbers. The resulting melting peaks of the samples were also broad and out of sync with those of the standards. Adding more cDNA (up to 10 µl in the 20 µl PCR reaction) made very
little difference to the quality of the PCR, thus suggesting that the initial target concentration was below the detection limit of the LightCycler.

**Figure A 2.2.** (A) amplification profile and (B) melting curve profile obtained after amplification of GAPDH mRNA in samples from box-stepping study.
Measurement of IGF-IEa mRNA in samples from box-stepping study

Unlike GAPDH mRNA, which could be amplified to a certain degree, IGF-IEa mRNA could barely be amplified at all using this method. Figure A 2.3 shows the amplification profile of a run with 6 IGF-IEa standards and the same 8 cDNA samples used to amplify GAPDH. The amplification efficiencies between standards and samples were clearly very different, which would immediately suggest that any quantitative values obtained were inaccurate. Furthermore, the melting curve profile shown in Figure A 2.3 revealed that there was a large degree of primer dimer for both the standards and samples and barely any specific product being amplified.
Figure A 2.3 (A) amplification profile and (B) melting curve profile obtained after amplification of IGF-I\(\text{Ea}\) mRNA in samples from box-stepping study.
Conclusion

These pilot studies revealed that real time quantitative PCR could not be used reliably to measure levels of either GAPDH or IGF-IEa mRNA in tissue samples that had been obtained using syringe needle aspiration. It is likely that because of the small sample size and subsequently low starting total RNA concentration, that the target concentration in the cDNA samples was below a reliable detection limit for the LightCycler set-up. As it was believed that these problems were largely related to the small tissue size, it became necessary to change the method used to obtain tissue. The needle biopsy technique described by Bergstrom et al. 1962 was subsequently used to obtain muscle tissue.
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Weightlifting exercise increases the mRNA expression of a mechanosensitive IGF-1 (MGF) in the muscles of young, but not old men

M. Hamre*, T. R. Orrellt, M. Cobbold*, G. Goldspink† and S.D.R. Harridge*

Departments of *Physiology, †Neurology and ‡MCR Unit, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, UK

Muscle mass is lost in later life and the mechanisms underlying this phenomenon remain unclear. It is possible that older muscles are less sensitive to mechanical signals. Skeletal muscle is known to express at least two isoforms of IGF-1 and it has been reported that in response to overload the muscles of aged rats are less able to upregulate the splice variant of IGF-1 termed MGF (Owino et al. 2001). In the present study, approved by the local ethics committee, we aimed to determine whether there were any age-related differences in MGF mRNA expression in human muscle shortly after a bout of high resistance exercise.

Following prior determination of the maximum one-legged knee extensor lift (1-RM) at least 1 week before the experimental day, eight healthy young (26–36 years) and seven healthy older (76–82 years) men performed ten sets of six repetitions of knee extensor exercise, lifting and lowering a mass equal to 80% of their 1-RM. Two minutes passive recovery was given between each set. Following local anaesthesia (1% lignocaine), muscle biopsies were obtained from the vastus lateralis muscle of the exercised (test) and non-exercised (control) legs 2.5 h after the end of the exercise session using the needle technique. Samples were immediately frozen. Subsequent quantification of the mRNA of the two IGF-1 isoforms in muscle (MGF and IGF-1Ea) was performed using a quantitative reverse transcription-polymerase chain reaction (RT-PCR) method (LightCycler, Roche UK).

Figure 1. MGF mRNA expression (mean ± S.E.M.) in muscles of young (n = 8) and older (n = 7) men before and after weight-lifting exercise. *Significant difference between control and exercise samples (P < 0.05, Wilcoxon’s signed rank test).

The mean (± S.E.M.) 1-RM for the young subjects was 41.9 ± 4.3 versus 20.6 ± 1.6 kg for the older subjects (P < 0.05). When normalised to total RNA content, levels of IGF-1Ea were not different between the young and old subjects (6.3 ± 0.9 versus 6.9 ± 1.0 × 10⁻⁵ ng mRNA/μg RNA) and no change was observed as a result of the exercise bout. MGF mRNA levels at rest were lower than those of IGF-1Ea, but were also not different between the young and old subjects (Fig. 1). In the young subjects, a significantly higher level of MGF was observed in the test leg when compared with the control leg (P < 0.05). However, no difference was observed between the test and control legs in the elderly subjects.

These data show that following a standardised bout of high resistance weight-lifting exercise, there is an age-related difference in MGF gene expression. The data in young subjects also suggest that the MGF and IGF-1Ea isoforms are differentially regulated in human skeletal muscle.


The financial support of REMEDI and the European Union (Framework V) is gratefully acknowledged. S.D.R.H. is a Wellcome Trust Research Fellow.

**All procedures accord with current local guidelines and the Declaration of Helsinki.**

The influence of fatigued antagonists on net static knee extension torque

J.G.M. Beltsman, A. de Haan, A.J. Sargeant, C.N. Manganaris and D. Ball

Centre for Biophysical and Clinical Research into Human Movement, Manchester Metropolitan University, Alsager, UK and Institute for Fundamental and Clinical Human Movement Sciences, Vrije Universiteit, Amsterdam, The Netherlands

Net knee extension torque is a function of agonist extension torque and opposing antagonistic torque. It has been proposed that antagonistic activity may be functional in stabilizing and/or protecting the joint. The present study has examined the hypothesis that, after fatiguing the antagonist hamstring muscles, a higher net extension torque would be found, which would be related to the degree of fatigue. Moreover, it was investigated whether such an increase was different between knee angles.

Seven, recreationally active males performed isometric MVCs at the following knee angles: 90, 70, 50 and 30 deg knee flexion. Thereafter, subjects performed maximal effort dynamic flexion contractions (180 deg s⁻¹) until torque had declined ~80%; this was immediately followed by a knee extension MVC. This sequence was repeated four times and randomised for all knee angles. EMG measurements of m. biceps femoris were taken in extension contractions. Antagonist average rectified value (EMGₐ) was normalized to the maximal flexion EMGₐ at the same knee angle. Voluntary activation (VA) and maximal torque generating capacity (MTGC) of the m. quadriceps were determined using superimposed electrical stimulation.

Dynamic torque decline after the fatiguung bout of flexion contractions was 48 ± 11%. The Wilcoxon signed rank test showed no significant effect of fatigue at the different knee angles on EMGₐ, MVC torque and MTGC, nor was there a significant effect of knee angle on EMGₐ (P > 0.05). EMGₐ was
The effect of growth hormone administration and strength training on IGF-I (Ea) and MGF mRNA expression in elderly men

M. Hameed*, t, K.H.W. Lang,et, J.L. Andersen,§, P. Schjerling,§, M. Kjaert, G. Goldspink*, t and S.D.R. Harridge* t

*Department of Physiology, fMCR Unit Royal Free and University College Medical School, London, tSports Medicine Research Unit, Bispebjerg Hospital, Copenhagen, Denmark and §Department of Molecular Muscle Biology, Copenhagen Muscle Research Centre, Denmark

The mRNA of two isoforms of the IGF-I gene has been shown to be expressed in human muscle. One, IGF-I(Ea), is similar to the systemic, liver-type IGF-I, whilst a second, MGF, is produced in muscle response to mechanical overload or damage. Recent studies of human muscle samples obtained after a single bout of high-resistance exercise suggest that IGF-I(Ea) and MGF mRNA transcripts are differentially regulated [Hameed et al. 2002]. The present study was aimed at determining the effects of recombinant growth hormone (rhGH) administration with and without resistance training in elderly subjects on the mRNA expression of the two different isoforms of IGF-I.

Healthy elderly men (aged 74 ± 1 years, mean ± S.E.M.), were assigned to either resistance training (3 sessions/week, 3–5 sets of 8–12 repetition maximum per session) with placebo (RT group, n = 6), RT combined with rhGH administration (RT + GH group, n = 6) or rhGH alone (GH group, n = 7) in a randomised, placebo-controlled, double-blinded design (Lange et al. 2002). Administration of GH occurred daily through subcutaneous injection in the thigh (0.5 IU m−2 rising to 1.5 IU m−2). Following local anaesthesia (1% lidocaine), muscle biopsies were obtained from the right vastus lateralis muscle at baseline, 5 weeks and 12 weeks. In the two training groups the biopsies were obtained 24 h after completion of the last training session. Samples were immediately frozen in liquid nitrogen. IGF-I(Ea) and MGF mRNA transcripts were analysed using a quantitative reverse transcription-polymerase chain reaction (RT-PCR) method (LightCycler, Roche UK).

After 5 weeks of GH administration without exercise (GH), IGF-I(Ea) had increased, by on average 226%, in contrast to MGF mRNA levels, which were unchanged (Table I). However, there was a significant increase in both IGF-I(Ea) (77 and 145%) and MGF mRNA levels (200 and 354%) in the RT and RT + GH groups, respectively, at this time point. A further 7 weeks of GH administration resulted in a significant increase in MGF (63% relative to baseline), but no other significant changes in MGF or IGF-I(Ea) mRNA were observed between 5 and 12 weeks.

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>Post 5 weeks</th>
<th>Post 12 weeks</th>
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<tbody>
<tr>
<td>IGF-I(Ea)</td>
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<tr>
<td>RT</td>
<td>5.3 ± 0.7*</td>
<td>9.4 ± 2.01*</td>
<td>10.2 ± 1.01*</td>
</tr>
<tr>
<td>RT + GH</td>
<td>6.2 ± 0.8*</td>
<td>15.2 ± 2.61*</td>
<td>14.8 ± 2.89*</td>
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<tr>
<td>GH</td>
<td>10.6 ± 1.4</td>
<td>34.6 ± 7.4†</td>
<td>23.2 ± 2.2</td>
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<tr>
<td>MGF</td>
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<tr>
<td>RT</td>
<td>1.6 ± 0.2</td>
<td>4.8 ± 1.2†</td>
<td>4.2 ± 0.4†</td>
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<tr>
<td>RT + GH</td>
<td>2.2 ± 0.6†</td>
<td>10.1 ± 1.4†</td>
<td>7.8 ± 1.2†</td>
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<tr>
<td>GH</td>
<td>4.8 ± 1.2</td>
<td>5.8 ± 1.2†</td>
<td>7.8 ± 1.6†</td>
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</table>

Table I. MGF and IGF-I(Ea) mRNA levels pre, after 5 weeks and after 12 weeks of either resistance training (RT), resistance training and rhGH administration (RT + GH) or rhGH administration only (GH)

Data are expressed as ng mRNA/μg RNA. For IGF-I(Ea) 10−4 ng, for MGF 10−6 ng. *Significant difference from 0 weeks, †from RT + GH at given time point (P < 0.05). Data are means ± S.E.M. and analysed using ANOVA followed by Student-Newman-Keuls post-hoc tests.

The results suggest that MGF mRNA expression in muscle is less sensitive to GH administration than IGF-I(Ea), at least in elderly subjects. However, when mechanical loading in the form of resistance training is combined with GH (RT + GH), both MGF and IGF-I(Ea) mRNA levels are enhanced, which may reflect an overall up-regulation of transcription of the IGF-I gene prior to splicing. Previously reported data from this study showed no greater increase in muscle strength and cross-sectional area with RT + GH compared with RT (Lange et al. 2002).


This work was supported by grants from WADA, European Union (Framework V-PENAM) and The Wellcome Trust.

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with substrate breakdown, protein turnover, glycolysis and oxidative phosphorylation. Interestingly, several novel genes associated with mRNA and protein isoforms in a limb muscle growth suppression were also identified. Their possible involvement with IGF signalling is being examined.

**VIII.9: Poster presentation**

**Differential expression of equine myosin heavy chain mRNA and protein isoforms in a limb muscle**

K. EIZEMA, M. VAN DEN BURG, E.D. GINGBOOM and W.A. WEIJS

Division of Anatomy, Department of Pathobiology, Faculty of Veterinary Science, Yalelaan 1, 3584 CL Utrecht, The Netherlands

The horse is one of the few animals kept and bred for its athletic performance. The major structural protein in skeletal muscle, myosin heavy chain (MyHC) is thought to be primarily transcriptionally controlled. To investigate the expression of the MyHC genes on the transcriptional level we isolated cDNAs encoding the adult equine MyHC isoforms. The mRNA expression was compared to the protein expression on a fiber-to-fiber basis using in situ hybridization (non-radioactive) and immunohistochemistry in a biopsy (m. glutaeus medius) from an adult untrained horse. Marked differences were detected between the expression of MyHC transcripts as compared to MyHC protein isoforms. Mismatches were primarily due to the presence of hybrid fibers expressing two fast (2ad) MyHC isoforms, but only one fast (mainly 2a) MyHC RNA isoform. This discrepancy was most likely not due to differential mRNA expression of myonuclei. Currently we are extending these analyses to horses of different ages and training status.

**VIII.10: Poster presentation**

**The effects of eccentric exercise on IGF-I isoform expression in the muscles of young and elderly people**

M. HAMEED1, A.D. TOFT2, B.K. PEDERSEN2, S.D.R. HARRIDGE3 and G. GOLDSPIK3

1Royal Free & University College Medical School, Department of Surgery, Rowland Hill Street, London NW3 2PF, UK; 2Department of Infectious Diseases 7641, Copenhagen Muscle Research Centre, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark; 3Royal Free & University College Medical School, Department of Physiology, Rowland Hill Street, London NW3 2PF, UK

The mRNA expression levels of two splice variants of the insulin-like growth factor-I (IGF-I) gene (IGF-I^A and MGF) were studied following muscle damaging eccentric exercise. Subjects (10 young, 20-27 years and 10 elderly, 67-75 years) completed 60 min of reverse pedal cycling at the same relative oxygen uptake. Muscle biopsy samples were obtained from the quadriceps muscle before and after completion of the exercise. Expression levels of the IGF-I transcripts were determined using real-time quantitative RT-PCR using specifically designed primers. No difference was observed between the resting levels of the two isoforms between the two age groups. Eccentric cycling exercise resulted in a significant increase in MGF mRNA in the young, but not in the elderly subjects. No changes in IGF-I^A mRNA levels were observed as a result of the exercise in either group. These data are in general agreement with a previous study (Hameed _et al._, 2003) in which a one off bout of high resistance weightlifting exercise was performed. The studies suggest that there is an impaired MGF response in elderly muscle. The response in young subjects also supports the suggestion that the two isoforms MGF and IGF-I^A are differentially regulated in human skeletal muscle following exercise.

**VIII.11: Poster presentation**

**Adaptation of fast-twitch hindlimb muscle to exercise training in mice**

J.A.L. JENESON, A. SCHOT, B.J.L.J. JOOSTEN, A. DOORNENBAL and M.E. EVERTS

Department of Physiology, Division of Pathobiology, School of Veterinary Medicine, Utrecht University, The Netherlands

The adaptive response of fast-twitch hindlimb muscle to mild exercise training was investigated in C57BL/6WT mice. Mice ran 0.5 km per day on a treadmill at a speed of 16 m/min against an 11° incline for 6 weeks. Intact EDL muscles of CON and EXE mice were isolated and characterized mechanically and energetically at 20°C (Jenesson _et al._, _J Muscle Res Cell Motil_ 23: 22) Na^+K^-ATPase in CON and EXE tibialis anterior and gastrocnemius muscles were determined by 3H-ouabain-binding (Kjeldsen _et al._, 1986, _Pflugers Arch_ 406: 529-535.). No change in Na^+K^-ATPase activity was found (CON: 386 ± 70 pmol/g wet wt) Peak tetanic, but not twitch force of EXE EDL was lower than CON (22.8 ± 1.5 vs. 29.0 ± 4.9 N/g (n = 5)). Serial stimulation at 2 Hz caused progressive fatigue and slowed relaxation in CON, but less so in EXE. Oxygen consumption during contraction at 0.5, 1 and 2 Hz was higher in EXE than CON at all frequencies. Nonlinear curve fitting predicted a 1.5-fold higher mitochondrial V_mmax for EXE EDL than CON (4.5 ± 0.3 vs. 3.1 ± 0.3 mmol O_2/g/s). These results suggest that adaptations of mouse hindlimb FT muscle to mild endurance training are restricted to the supply-side of cellular energy balance.

**VIII.12: Poster presentation**

**The effect of mechanical overload on the composition and turnover of regulatory proteins in skeletal muscle**

P. KAASIK, K. ALEV, A. PEHME, K. REISBERG and T. SEENE

Jakobi 5., University of Tartu, Chair of Functional Morphology, Tartu, 51014, Estonia

The purpose of this study was to find changes in tropomyosin (TM), troponin (Tn) and their relations with MyHC isoforms' composition and turnover rate during 2-week overload in skeletal muscle. 16-17-week-old male rats of the Wistar strain were used. M. Plantaris of one hindlimb was overloaded by unilateral removal of one its synergists m. Gastrocnemius. TM subunits and TnT isoforms were separated by 7.2% SDS-PAGE. Our results showed that in overloaded m. Plantaris the development of compensatory hypertrophy caused increase in the relative content of b-TM and decrease in the relative content of a-TM. The turnover rate of TM increased from 0.50 ± 0.03 to 0.62 ± 0.04; P < 0.05 during overload. During the chronic overload the relative content of the TnT2f decreased significantly and the content of the TnT3f increased. At the same time the TnT1f showed the tendency to decrease. The decrease of the TnT2f and the increase of TnT3f may show the changes in the skeletal muscle contractile properties towards the slow-twitch, as the concomitant change in the MyHC isoform pattern towards the increase of MyHC 1, Ila was found in our study. The turnover rate of TnT did not change significantly (from 0.98 ± 0.6 to 0.90 ± 0.7) during overloading. Taking also into account the increase of b-TM subunits during chronic overload, we
Sarcopenia and Hypertrophy: A Role for Insulin-Like Growth Factor-1 in Aged Muscle?

Mahjabeen Hameed,1 Stephen D.R. Harridge,2 and Geoffrey Goldspink1

Departments of 1Anatomy and Developmental Biology and 2Physiology, Royal Free & University College Medical School, University College London, London, England

HAMEED, M., S.D.R. HARRIDGE, and G. GOLDSPIINK. Sarcopenia and hypertrophy: A role for insulin-like growth factor-1 in aged muscle? Exerc. Sport Sci. Rev., Vol. 30, No. 1, pp 15-19, 2002. Sarcopenia is the loss of muscle mass associated with the aging process. Although systemic or circulating growth hormone and insulin-like growth factor-1 levels fall as we age, this is likely to be of lesser importance in regard to muscle mass than the role of locally produced insulin-like growth factor-1 generated in the muscle in response to exercise. Keywords: muscle, strength, growth factors, aging, protein synthesis

INTRODUCTION

Sarcopenia is the loss of muscle mass that is associated with the aging process. The loss of muscle mass is, in part, responsible for the decline in the functioning of muscle as we get older. This decline might be in its mechanical function (i.e., generating force, power, or braking movement) or in its roles as a dynamic metabolic store, a generator of heat, or as a form of protective padding. In addition, the decline in mass also accounts for a significant proportion of the decline observed in maximal aerobic power with increasing age. As physical function declines with age, simple daily tasks such as rising from a chair unaided or climbing stairs become increasingly more difficult and, eventually, impossible to perform. This has important consequences for older people whose quality of life is reduced through a loss of independence and increased reliance upon others and also for the strain put upon health and social services charged with the care of an increasingly aging population.

So why is muscle lost with increasing age? At the anatomical level it is clear that the loss of muscle is a reflection of a reduction in the number of muscle fibers and a reduction in the size of individual muscle fibers, notably the fast Type II fibers. However, although the aging muscle has lower rates of muscle protein synthesis (2,11), the mechanisms that may underlie this process of sarcopenia are unclear. This short review will focus upon a possible role for insulin-like growth factor (IGF-1) in sarcopenia.

POSSIBLE CAUSES OF SARCOPENIA

Despite the fact that age-related muscle loss and weakness seem synonymous to the changes associated with decreased physical activity, it is clear that this is not necessarily the case. The atrophy associated with muscle unloading results in reversible atrophy of individual fibers and not a loss of fibers, whereas a loss of muscle mass is seen even in older people who maintain very high levels of physical training (i.e., master athletes).

The rate at which muscle protein is synthesized is reduced in later life. Balagopal et al. (3) showed that whole body protein synthesis was 19% lower in older individuals (77 ± 2 yrs) when compared with younger individuals (23 ± 1 yr). More specifically, muscle protein synthesis was 55% lower, whereas synthesis rates of nonmuscle proteins remained the same. This was in general agreement with the results of an earlier study by Welle et al. (11) who showed that the fractional rate of myofibrillar protein synthesis was 28% lower in older (62-81 yrs) compared with younger (21-31 yrs) men. In addition to overall changes in protein synthesis, there are a number of structural and compositional differences that are characteristic of the aging muscle. A loss of contractile proteins relative to other cell components and increased amounts of connective tissue and intramuscular fat have been reported. Whether there is a change in the relative distribution of the main histochemically determined fiber types with aging remains somewhat unclear, although it is
certain that there are less fast myosin heavy chain isoforms (in part due to the atrophy of the faster Type II fibers).

Hypertrophy is the increase in muscle size that occurs as an adaptation to increased muscle loading, which in humans is most easily achieved through strength training and results from a net increase in protein turnover. It is clear that because hypertrophy is limited to the muscle exercised and not to all muscles, there are "local" factors regulating this process. There is now good evidence to suggest that IGF-1 plays a key role in the hypertrophic adaptation of muscle to exercise.

**INSULIN GROWTH FACTOR-1 (IGF-1)**

Mature IGF-1 is a 70-amino acid single-chained polypeptide with many three-dimensional structural similarities to the proinsulin molecule. It has an important role in the regulation of somatic growth, development, and differentiation. IGF-1 is synthesized in the liver as a consequence of growth hormone (GH) secretion from the anterior pituitary gland. This "systemic" IGF-1 promotes cell division and is generally responsible for normal growth and development. However, it is now known that IGF-1 is synthesized "locally" and has potential autocrine/paracrine actions. Local IGF-1 synthesis is thought to be partially GH dependent in some IGF-responsive tissues. However, in other tissues such as skeletal muscle, local IGF-1 expression seems to be GH independent. It is now known that IGF-1 exists in multiple isoforms that may have specific functions. The following sections will briefly introduce these locally acting isoforms of IGF-1. IGF-1 receptors through which IGF acts on target tissues, and the binding proteins by which IGF-1 is transported and whose action may be regulated.

**MUSCLE-SPECIFIC ISOFORMS OF IGF-1 AND MECHANO-GROWTH FACTOR (MGF)**

Two known isoforms of the IGF-1 gene are expressed by muscle when it is subjected to mechanical stimulation. These are derived from the IGF-1 gene by a process of alternative splicing. This is a complex mechanism by which exons are arranged in different combinations from pre-mRNA. It is an important and common process for generating protein diversity and regulating gene expression in higher eukaryotes. The IGF-1 gene contains two promoters initiating at the 5' exons 1 and 2. Transcripts initiating at exon 2 are common in the liver and are highly GH dependent, whereas transcripts initiating at exon 1 are widely expressed in extrahepatic tissues. Alternative splicing of the IGF-1 gene generates three different E peptides that have a common N-terminal sequence but alternative C-terminal sequences. A schematic representation of the exon arrangement of the IGF-1 gene and its splice variants can be seen in Figure 1.

The first isoform is similar to the hepatic endocrine type of IGF-1. Several different abbreviations have emerged for this isoform: IGF-1Ea (10), L.IGF-1 (14), and mM.IGF-1 (8). Transgenic mice that overexpress this particular isoform in skeletal muscle have been shown to have pronounced muscle hypertrophy (8), and it seemed that the older animals showed signs of protection against the normal loss of muscle mass associated with aging. It was concluded that the localized mIGF-1 transgene expression preserved muscle architecture and age-independent regenerative capacity of muscle.

The second isoform, IGF-1Eb (humans), is thought to be predominately expressed in the liver, and its role in muscle is unknown. The third isoform is a splice variant resulting from a novel splice acceptor site in the intron preceding exon 6 and is generated in muscle subjected to stretch and overload (14). This isoform is termed MGF, IGF-1Ec (human), and IGF-Eb (rat) in the literature. Structurally, the MGF gene differs from its liver type counterpart because of the presence of a 49-base pair insert (52 base pairs in the rat). It is thought not to be glycosylated, therefore it is expected to have a shorter half-life than the liver IGFs. MGF is thus likely to be designed to act in an autocrine/paracrine, rather than systemic, mode of action. As a result, it binds to its own muscle-specific binding protein, in the interstitial tissue spaces.

**INSULIN GROWTH FACTOR-1 RECEPTOR (IGF-1R)**

The cellular effects of IGF-1 are mediated by the activation of a specific tyrosine kinase receptor. IGF-1 binds with high affinity to the Type 1 dimeric receptor, which is structurally and functionally homologous to the insulin receptor. It is composed of two ligand binding α subunits and two transmembrane β subunits. It is known that the biological activity of a hormone or growth factor does not simply reflect the level of hormone or growth factor, but also depends on the concentration of the receptor and the affinity of its interaction. There is evidence that there is an impairment of both IGF-1R density and signaling in aged skeletal muscle. However, IGF-1R number, binding capacity, and affinity were shown to increase in the soleus muscle of old mice after physical exercise (13), suggesting that old animals retain plasticity for IGF-1R.

**IGF-BINDING PROTEINS (IGFBP)**

The interaction between IGFs and their binding proteins (IGFBP) represents prereceptor regulation. Excluding an MGF binding protein, six IGFBPs have been characterized to date. Together, these act as modulators of IGF-1 action. Their main functions are to 1) stabilize and transport IGFs...
from the circulation to peripheral tissues, 2) maintain a reservoir of IGFs in the circulation, 3) potentiate or inhibit IGF action, and 4) mediate IGF-independent biological effects. Of the six systemic-type binding proteins, IGFBP-3 is primarily responsible for maintaining IGF-1 levels in the circulation in conjunction with another protein called acid labile subunit. There is some evidence that alterations in IGFBP expression, namely IGFBP-4 and IGFBP-5, occur in unloaded or overloaded soleus muscle and that this may play a role in skeletal muscle adaptation to changes in loading (3).

MECHANICAL ACTIVITY AND THE EXPRESSION OF LOCALLY PRODUCED GROWTH FACTORS

The expression of the two main muscle isoforms of IGF-1, namely IGF-1Ea and IGF-1Ec (MGF), seem to be affected in different ways by physical activity. An early study by DeVol et al. (5) demonstrated that there was a threefold increase in total IGF-1 mRNA levels in the soleus and plantaris muscle in 11- to 12-wk-old female rats after tenotomy-induced hypertrophy. These levels were maintained 2, 4, and 8 d after the experimental procedure, thereby suggesting a relationship between local stimulation of skeletal muscle growth and IGF-1 gene expression.

Yang et al. (14) showed that MGF was markedly up-regulated in rabbit extensor digitorum longus muscle, which had been subject to acute stretch by immobilizing the hind limb in the extended position. In the same study it was shown that the expression of the IGF-1Ea isoform was also induced by mechanical stimulation.

This work was further supported in a study in which mRNA expression of the muscle-specific isoforms of IGF-1 was investigated in rabbit muscle after different mechanical stimuli regimens (7). The study showed that within 4 d of stretch using plaster cast immobilization, with the limb in the plantar flexed position, that a marked up-regulation of both IGF-1Ea and MGF was apparent. Electrical stimulation at 10 Hz combined with stretch (overload) resulted in an even greater increase of both types of IGF-1 transcript. Electrical stimulation alone, without stretch, resulted in no significant increase compared with muscle from sham-operated controls. The expression of both systemic and autocrine IGF-1 in muscle provides an interesting link between the mechanical signal and a marked increase in structural gene expression involved in tissue remodeling and repair.

GROWTH HORMONE AND IGF-1 IN OLD AGE

The GH-IGF-1 axis plays an essential role in postnatal growth and development. A summary of this process is schematically represented in Figure 2. Circulating GH and IGF-1 are required for growth in adolescence and for the maintenance of muscle mass and strength in adulthood. Levels of these hormones reach their peak during adolescence and are maintained at somewhat lower levels during adulthood. In later life, there is a further decline in the production of GH and IGF-1. It was suggested that a close relationship existed between the age-related decline in the GH and IGF-1 axis and a loss of muscle mass and strength. In young adults who were GH deficient, the administration of recombinant GH had positive effects in terms of muscle growth. It was thus hypothesized that older individuals with decreased levels of circulating GH and IGF-1 would benefit from such treatment. The results, however, have been disappointing, with rates of protein synthesis being no different in older men who have undergone strength training with recombinant GH supplementation when compared with those who only underwent strength training (15). In this regard, and in support of the argument that systemic growth factors may be of relatively minor importance in hypertrophy, is one study that showed that the response to a single injection of GH in older men increased plasma IGF-1 concentrations but resulted in no increase in the mRNA for IGF-1 in muscle (12).

THE ROLE OF SATELLITE CELLS IN MUSCLE GROWTH AND REPAIR

If locally produced IGF-1 plays a role in the hypertrophic process, how might it exert its action? One likely mechanism is through its influence on satellite cells. Satellite cells are small mononucleate precursor cells that are located between the basal lamina and sarcolemma of muscle fibers (Fig. 3). These cells are believed to remain mitotically inactive but are mobilized by increased mechanical loading or damage, playing a role in both adaptation and repair of muscle. It is believed that these cells proliferate and differentiate into myoblasts, which then fuse with existing fibers, providing new nuclei to maintain the DNA to protein ratios of fibers undergoing hypertrophy. Satellite cells are important for postnatal growth of muscle and are the primary means by
which adult muscle mass is formed. Their number decreases gradually after birth, and there is some recent evidence from studies of human satellite cells in culture that the replicative potential of these cells may be reduced in later life (4).

DeVol et al. (5) suggested that during muscle growth, locally produced IGF-1 had two distinct functions with respect to satellite cell involvement in that it stimulated both proliferation and differentiation of satellite cells. The role of IGF-1 in satellite cell activation was further supported by experiments in which direct infusion of IGF-1 into the tibialis anterior muscle of adult rats resulted in an increased total muscle protein and DNA content, demonstrating skeletal muscle hypertrophy related to satellite cell activation (1). Further evidence for the role of these cells in hypertrophy is provided by an experimental approach whereby satellite cells were prevented from proliferating after treatment with γ-radiation. The subsequent overloading of the adult rat soleus muscle failed to evoke hypertrophy of the overloaded irradiated muscles (9).

Furthermore, Musaro et al. (8) have recently reported that IGF-1 is able to induce the calcineurin nuclear factor of activated T cells. This signaling pathway leads to activation of GATA-2, a transcription factor whose up-regulation is related to muscle fiber hypertrophy. IGF-1 is therefore thought to play an important role in the stimulation of both myogenic precursor cell proliferation and muscle hypertrophy during muscle regeneration. Whether IGF-1 is involved in the "direct" stimulation of satellite cell activation has yet to be determined. Effective therapeutic strategies aimed at the maintenance of skeletal muscle strength in aging and neuromuscular disease might depend on the development of methods to increase the muscle satellite cell population. Expanding mature satellite cell populations by administering miGF-1 genes may provide an effective clinical strategy for the prevention, reduction, or reversal of age and disease-related muscle frailty (8).

ADAPTABILITY OF AGING HUMAN MUSCLE

Despite the potential age-related impairments in growth factors, a number of recent studies have shown that the muscles of even very frail, very elderly people (≥85 yrs) are able to adapt to muscle-strengthening exercise (Fig. 4). The observation that GH seems relatively ineffective in stimulating muscle growth, raises questions as to the role systemic growth factors may play in the adaptation to exercise. Fiatarone-Singh et al. (6) recently used an immunohistochemical technique to study IGF-1 expression in muscle biopsy samples obtained from older men and women (aged 72–98 yrs) after 10 wk of strength training. They used a general IGF-1 antibody and showed a ~500% increase in the levels of IGF-1 within the muscle fibers of these subjects after the training period. The antibody used for immunohistochemical analysis was nonisofrom specific, hence it cross-reacted with all three IGF-1 isoforms. It was therefore difficult to determine from this study exactly which IGF-1 isoforms had increased in the muscle after strength training. In addition, in the posttraining biopsy samples, they observed increased ultrastructural damage and an increase in the number of fibers expressing embryonic myosin heavy chain isoforms. This would be in support of the hypothesis that muscle damage provokes the "initial" step in the remodeling process, which ultimately leads to muscle regeneration. The increase in amount of weight that could be lifted after training was proportional to the significant increase in IGF-1 immunoreactivity in muscle after training. However, the effect of a similar training regimen on younger individuals was not investigated in this study. Therefore the question as to whether a similar or smaller percentage change in IGF-1 peptide levels occurs in older individuals remains to be determined.
SUMMARY

It is clear that the aging process, which results in a loss of muscle mass, is associated with a reduction in systemic GH/IGF-I muscle. However, it is also clear that hypertrophy is linked to the production of local growth factors, and the effects of the aging process on the local regulation of IGF-I and its isoforms and its coupling to satellite cell activation has yet to be fully determined. The potency of IGF-I, and particularly MGF, may provide the source for potential therapies for older people to increase muscle mass, particularly for those who may not be able to provide their muscles with appropriate physical stimuli through exercise.

The superior mass and function of master strength and power athletes provides evidence of the benefits of long-term mechanical signals. Yet, even these highly trained athletes are not immune to the effects of the aging process, albeit having a considerable advantage over their nonactive counterparts. The fact that frail, nonactive older people seem to retain the ability to hypertrophy their muscles in response to strengthening exercise suggests that mechanisms triggering the growth of muscle remain in place. However, it might be speculated that the activation threshold of mechanical stimuli for IGF-I increases with age such that mechanical signals provided by tasks of daily living are no longer sufficient to maintain muscle mass.

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