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Actions of Short-Term Fasting on Human Skeletal Muscle Myogenic and Atrogenic Gene Expression

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Key Words
Myostatin · Atrogin-1 · myoD · Myogenin · Atrophy · mRNA

Abstract
Background: Skeletal muscle mass is governed by multiple IGF-1-sensitive positive regulators of muscle-specific protein synthesis (myogenic regulatory factors which includes myoD, myogenin and Myf5) and negative regulators, including the atrogenic proteins myostatin, atrogin-1 and muscle ring finger 1 (MuRF-1). The coordinated control of these myogenic and atrogenic factors in human skeletal muscle following short-term fasting is currently unknown. ***Method:*** Healthy adults (n = 6, age 27.6 years) undertook a 40-hour fast. Skeletal muscle biopsy (vastus lateralis) and venous blood samples were taken 3, 15 and 40 h into the fast after an initial standard high-carbohydrate meal. Gene expression of the myogenic regulator factors (myoD, myogenin and Myf5) and the atrogenic factors (myostatin, atrogin-1 and MuRF-1) were determined by real-time PCR analysis. Plasma myostatin and IGF-1 were determined by ELISA. ***Results:*** There were no significant alterations in either the positive or negative regulators of muscle mass at either 15 or 40 h, when compared to gene expression measured 3 h after a meal. Similarly, plasma myostatin and IGF-1 were also unaltered at these times. ***Conclusions:*** Unlike previous observations in catabolic and cachexic diseased states, short-term fasting (40 h) fails to elicit marked alteration of the genes regulating both muscle-specific protein synthesis or atrophy. Greater periods of fasting may be required to initiate coordinated inhibition of myogenic and atrogenic gene expression.

Introduction

Muscle atrophy occurs in response to protein-energy malnourishment and fasting, but is also a characteristic feature of immobility, weightlessness, diabetes, cancer cachexia, sepsis and AIDS [1]. In fasting and many systemic diseases, the protein catabolism of muscle provides a strategy to repartition amino acids to other body fractions or to provide oxidative substrates for the maintenance of metabolic function [2, 3]. Despite the varied aetiologies leading to muscle atrophy, the cellular mechanisms underpinning the increased proteolysis and decreased protein synthesis have many common elements. During atrophy, there is an increase in the amount of components of the ubiquitin-protein conjugates and increased transcription of components of the ubiquitin degradation pathway [4, 5]. Two of the most sensitive markers of muscle atrophy are the mRNA expression of two muscle-specific ubiquitin-ligases, MAFbx (also called atrogin-1) and MuRF-1 (muscle ring finger 1) [6,

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7]. An increased expression of these genes has been observed in models of muscle wasting, including fasting [8]. Furthermore, rapid suppression of atrogin-1 and MuRF-1 mRNA synthesis has been demonstrated following IGF-1-stimulated hypertrophy in vitro suggesting inhibition of the protein degradation pathway is an important component of muscle growth [9].

In addition to ubiquitin proteasome activation, skeletal muscle mass is subject to further inhibitory regulatory mechanisms. Myostatin (growth/differentiation factor 8), a member of the TGF-β family [10], is a powerful endogenous negative regulator of muscle hypertrophy [11]. Myostatin is synthesized and secreted (largely from skeletal muscle) as an inactive complex of the N-terminus propeptide and active C-terminus and circulates bound to and inhibited by follistatin. Once cleaved, mature myostatin exerts its effects via interaction with the activin/TGF-β ActIIb receptor and subsequent activation of the SMAD signalling pathway to control gene transcription [12, 13]. Myostatin exerts its powerful inhibition of hypertrophy by signalling satellite cell (muscle-specific stem cell) quiescence and thus reducing the differentiation potential of these cells [14]. In atrophic conditions including HIV infection [15], prolonged bed rest and thyroxine administration [16], plasma myostatin is increased. Conversely, mice, cattle and humans with inactive or absent myostatin proteins exhibit a gross hypermuscular phenotype [17–19].

Counterbalancing the atrogenic pathways are series of basic helix-loop-helix transcription factors known collectively as the myogenic regulatory factors (MRFs). Of the MRF members, MyoD, myogenin and Myf5 exert crucial roles in the hypertrophic mechanisms governing the recruitment and maturation of satellite cells to mature myofibres [20]. Central to the transcriptional activation of the MRFs is the insulin-like growth factor-1 (IGF-1) signalling pathway which mediates skeletal muscle growth, at least in part, through the expression of these factors [21]. More recently, MyoD and myogenin have also been shown to be expressed in mature myofibres where they direct and maintain characteristics of the muscle phenotype [22].

Given the central role of the myogenic factors (MyoD, myogenin and Myf5) for the maintenance of muscle cells mass and the converse roles of the atrogenic factors (atrogin-1, MuRF-1 and myostatin), the skeletal muscle gene expression of these factors might be hypothesized to be regulated in a coordinated fashion in skeletal muscle. Thus, we aimed to establish the impact of short-term fasting (15 and 40 h) on the gene expression of these factors within human skeletal muscle. For these studies we used healthy subjects who commenced the study with a standard meal. Repeated muscle biopsies were performed 3 h after the last meal (fed state) and again 15 and 40 h after this meal. Blood samples were taken at these times for analysis of plasma mature myostatin and IGF-1 concentrations.

Table 1. Subject characteristics

	Males (n = 3)	Females (n = 3)
Age, years	27.6 ± 1.5	25.3 ± 2.8
Mass, kg	79.4 ± 6.7	63.7 ± 5.0*
BMI, kg · m^{-2}	24.5 ± 0.9	21.6 ± 0.7

* $p < 0.05$.

Materials and Methods

Subject Characteristics

Six healthy adults (3 females and 3 males) volunteered to participate in the study. The mean age, height, weight, and BMI prior to the commencement of the study are shown in table 1. All female subjects were pre-menopausal and no control was made for the phase of the menstrual cycle. Written informed consent was obtained from all subjects before their participation in the study, which was approved by the Deakin University Human Research Ethics Committee. An analysis of metabolic factors has previously been published on these subjects [23].

Study Design

Subjects presented to the laboratory in the afternoon, and consumed a standard high-carbohydrate meal containing 61% energy from carbohydrate, 24% energy from fat, and 15% energy from protein. Subjects then fasted for 40 h, returning to the laboratory at 3, 15, and 40 h during the fast for blood and muscle sampling. During the fasting period, subjects were instructed to only consume water ad libitum or non-energy-containing beverages without caffeine.

Blood Sampling and Analysis

Venous blood (~6 ml) was obtained from an antecubital vein at 3, 15 and 40 h during the fast for the analysis of plasma myostatin by ELISA. Briefly, maxisorp ELISA plates (Nunc #439454) were pre-coated with 100 μl of 2.0 μg/ml recombinant myostatin. Samples were first prepared by the addition of 100 μl of standard recombinant myostatin or sample (diluted 1 in 10 in PBS-T-PVP; 0.01 M phosphate buffer with 0.15 M NaCl, 0.05% Tween-20 and 0.1% PVP, pH 7.4) and 100 μl of primary antibody, rabbit anti-myostatin (diluted 1 in 50,000 in PBS-T-PVP) was added into a Nunc microtitre plate (product #26920 96F; Invitrogen NZ Ltd).

Plates were incubated overnight in a humidified container at 4°C. The ELISA plates were washed 3 times with PBS-T and blocked, whilst the mixing plate was equilibrated to room temperate. 100 μl of solution was transferred from the mixing plate to the ELISA plate, incubated at room temperature for 2 h and washed 3 times with PBS-T. 100 μl of secondary antibody was added to the ELISA plate with Dako Goat anti-rabbit IgG-HRP (diluted 1 in 5,000 in PBS-T-PVP) and incubated at room temperature for 2 h. The ELISA plate was washed twice with PBS-T and twice with PBS (0.01 M phosphate buffer with 0.15 M NaCl, pH 7.4). 100 μl of TMB working solution was added to each well. The plate was covered and incubated for 30 min on a shaker at room temperature. Reaction was stopped by adding 50 μl 2 M sulphuric acid and mixed briefly and read at a wavelength of 450 nm. Plasma IGF-1 was assayed via a solid-phase, enzyme-labelled chemiluminescent immunometric assay on the Immulite® analyser (Immulite®, Diagnostics Products, USA) according to the manufacturer's instructions.

Muscle Sampling

A muscle sample was obtained under local anaesthesia (xylocaine 1%) from the vastus lateralis using the percutaneous needle biopsy technique modified to include suction at 3, 15, and 40 h during the fast. Tissue was stored in liquid nitrogen until subsequent analysis.

Total RNA Isolation and Reverse Transcription

Total RNA from ~15 mg of muscle was isolated using FastRNA Kit-Green (BIO 101, Vista, Calif., USA) protocol and reagents. Total RNA concentration was determined spectrophotometrically at 260 nm. First-strand cDNA was generated from 1 μg RNA using AMV RT (Promega, Madison, Wisc., USA) as previously described [23]. The cDNA was stored at –20°C for subsequent analysis.

Real-Time PCR Analysis

To perform PCR, specific primers were designed for all genes using Primer Express software (Applied Biosystems, Foster City, Calif., USA) on sequences obtained from GenBank. The primer sequences used were: atrogin-1, NM_058229: forward primer – 5′-GAC AGG ATG CAG AAG GAG-3′, reverse primer – 5′-TGA TCC ACA TCT GCT GGA AGG T-3′; MuRF-1, NM_032588: forward primer – 5′-GGC GTG GCT CTC ATT CCTT-3′, reverse primer – 5′-CGG TGT AGA TGC ACA GCT TCT C-3′; myostatin, NM_005259: forward primer – 5′-CCA GGA GAA GAT GGG CTG AA-3′, reverse primer – 5′-CAA GAC CAA AAT CCC TTC TGG AT-3′; myogenin, NM_002479: forward primer – 5′-GGT GCC CAG CGA ATG C-3′, reverse primer – 5′-TGA TGC TGT CCA CGA TGG A-3′; MyoD, NM_002478: forward primer – 5′-CCG CCT GAG CAA AGT AAA TGA-3′, reverse primer – 5′-GCA ACC GCT GGT TTG GAT T-3′; Myf5, NM_005593: forward primer – 5′-TTC TAC GAC GGC TCC TGC ATA-3′, reverse primer – 5′-CCA CTC GCG GCA CAA ACT-3′; β-actin, X00351: forward primer – 5′-GAC AGG ATG CAG AAG GAG-3′, reverse primer – 5′-TGA TCC ACA TCT GCT GGA AGG T-3′. Where possible, primers were designed spanning intron-exon boundaries to prevent amplification of the target region from any contaminating DNA. Quantification of mRNA expression was performed (in duplicate) by real-time RT-PCR using the ABI PRISM 5700 sequence detection system (Applied Biosystems) as described previously [23]. For the PCR step, reaction volumes of 20 μl contained SYBR Green 1 Buffer (Applied Biosystems), forward and reverse primers and cDNA template (diluted 1:40). Fluorescent emission data were captured and mRNA levels were quantitated using the critical threshold (C_T) value. To compensate for variations in input RNA amounts and efficiency of reverse transcription, β-actin (GenBank Accession No. X00351) mRNA was quantitated and results were normalized to these values.

Statistical Analysis

All data are presented as means ± SEM. Changes in genes and plasma myostatin over 40 h were analyzed using one-way repeated measures ANOVA. Differences between subject characteristics were determined with an unpaired t test. An α error of $p < 0.05$ was used to determine statistical significance in all analyses. Data was analyzed using SPSS Statistical Software (Version 11.5).

Results

Expression of Myogenic mRNA during Fasting

Expression of muscle myogenic and atrophy gene mRNA during the 40-hour fast is shown in figure 1. Assessment of gene expression after 3, 15 and 40 h of fasting showed no significant change in expression of genes responsible for myogenesis (MyoD, Myf5, myogenin and myostatin) or muscle atrophy (atrogin-1 and MuRF-1). A 60% decrease in MyoD expression was observed from 3 to 40 h, however this failed to reach the level of significance. Overall, a consistent trend for a decrease in expression over the 40 h was seen for the three positive promoters of myogenesis (MyoD, Myf5 and myogenin) while no observable change was seen in myostatin mRNA expression. Expression of the two ubiquitin ligase genes involved in muscle atrophy (atrogin-1 and MuRF-1) did not change at any point during the fast.

Plasma Myostatin and IGF-1

Measurement of plasma myostatin and IGF-1 over 3, 15 and 40 h fasting is shown in figure 2. Circulating levels of plasma myostatin and IGF-1 remained stable over the 40-hour fasting period.

Discussion

Fasting elicits rapid and pronounced alterations in skeletal muscle substrate metabolism, primarily with the aim of minimizing total energy metabolism and preserving the limited carbohydrate supplied. Previous studies from our laboratory have shown marked activation of the gene transcription of metabolic genes associated with

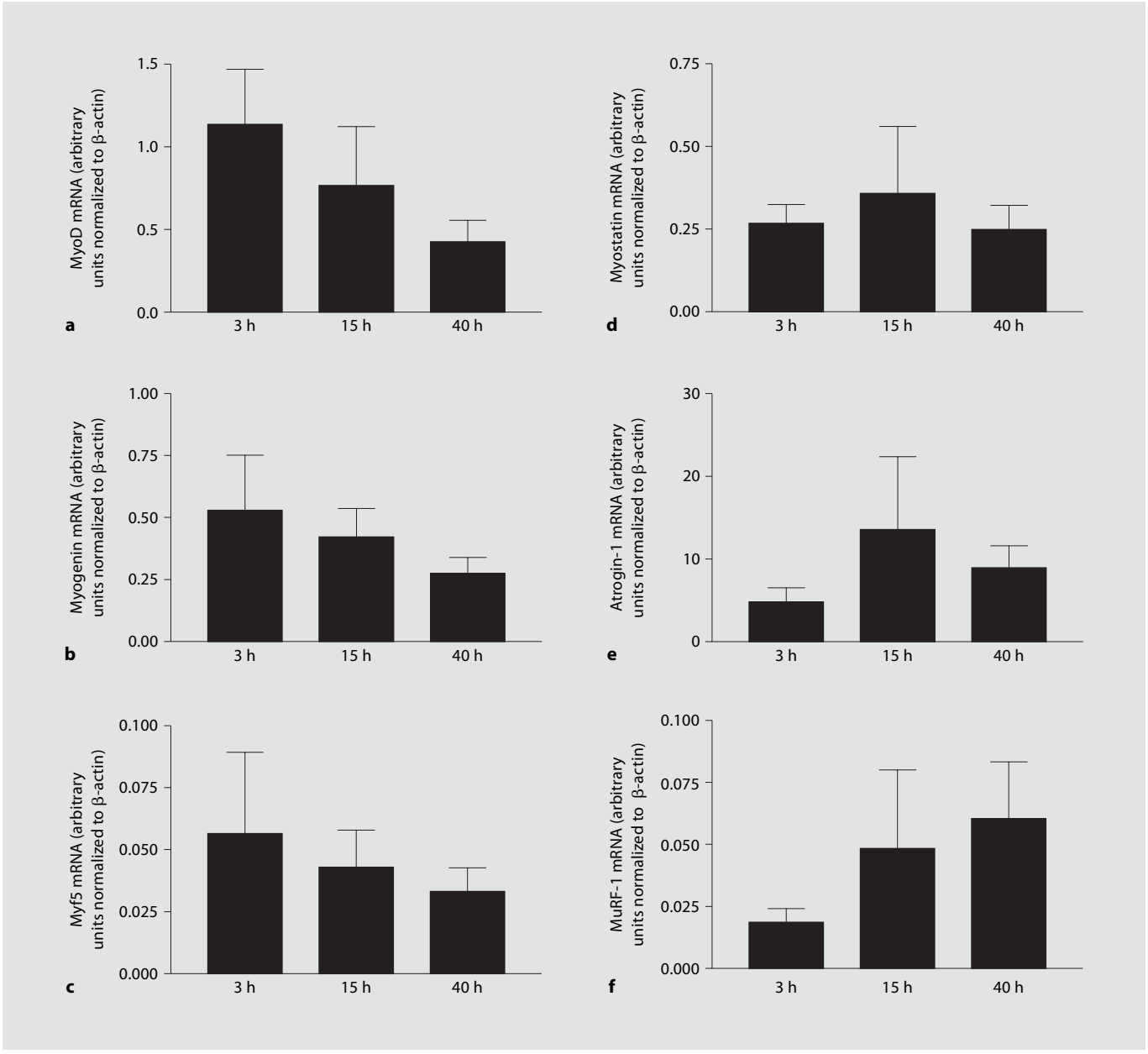

Fig. 1. Effect of fasting on gene expression of **a** MyoD, **b** myogenin, **c** Myf5, **d** myostatin, **e** atrogin-1, and **f** MuRF-1. Values are means ± SEM, n = 6. Expressed as duration of fast (hours).

these alterations in macronutrient homeostasis [23]. Muscle atrophy is a physiologic response to fasting as well as a wide range of systemic diseases including cancer, AIDS, sepsis and hyperthyroidism [1]. In the study presented here, we used a human model of fasting to approximate a catabolic disease process. We hypothesized that selected myogenic genes would be downregulated as an adaptive response in human skeletal muscle to fasting, whilst genes involved in muscle atrophy (acting downstream of the ubiquitin proteasome pathway) would be upregulated. We also hypothesized that both plasma myostatin and skeletal muscle gene expression would also increase in response to fasting. Somewhat surprisingly, no significant changes in any of the mRNA or plasma measures were observed after 15 and 40 h of fasting. Whilst underfeeding of sheep has previously been dem-

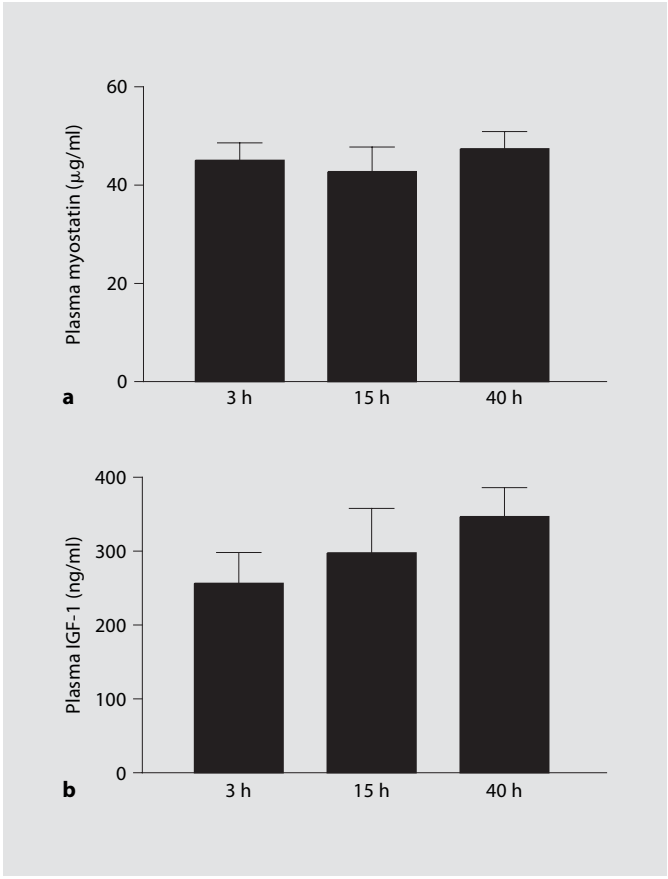

Fig. 2. Effect of 40 h fasting on **a** myostatin and **b** IGF-1 plasma levels. Values are means ± SEM, n = 6. Expressed as duration of fast.

onstrated to reduce the expression of MyoD, Myf5 and myogenin within 1 week [3], this is the first study to examine the impact of fasting on the expression of the MRFs in human skeletal muscle. The MRFs that are central to satellite cell proliferation (Myf5), proliferation arrest (MyoD) and progression towards terminal differentiation of satellite cells did not change within skeletal muscle at 15 or 40 h of fasting.

Elevated circulating levels of myostatin have been associated with muscle atrophy in cases such as prolonged bed rest [24] and thyroxine administration [24], while decreased levels of circulating myostatin have been associated with exercise-induced muscle hypertrophy [25] and weight loss [26]. In the present study, short-term fasting had no impact on either circulating myostatin levels or skeletal muscle mRNA expression. Previous studies investigating the effects of fasting or underfeeding on myostatin levels have shown conflicting results. In some studies, short-term fasting in various species has resulted in an increase in myostatin mRNA in skeletal muscle [27–29] whilst other studies have demonstrated either an increase [30] or no change [27] in myostatin plasma levels during fasting. However, species differences, age of the animals investigated as well as different fasting conditions make direct comparisons difficult.

During fasting, skeletal muscle undergoes proteolytic processes mediated in part by the atrophy-related ubiquitin ligases atrogin-1 and MuRF-1. Studies have previously described increased mRNA expression of atrogin-1 during energy deprivation in rodent muscle [31, 32]; however, to our knowledge this is the first study to investigate the expression of MuRF-1 and atrogin-1 in human skeletal muscle during short-term fasting. The present study demonstrated that neither atrogin-1 nor MuRF-1 expression appeared to change over the 40 h. Consistent with the gene results, no change in plasma IGF-1 was observed at any point during the fast. These ubiquitin ligases are transcriptionally suppressed by IGF-1 through the PI3K-Akt pathway [1]. Thus the lack of change in plasma IGF-1 would have likely contributed to the unchanged expression patterns of MuRF-1 and atrogin-1.

In summary, the present study demonstrates that transcription of myogenic and atrophy-specific genes does not change with short-term fasting in human skeletal muscle. Similarly, skeletal muscle myostatin gene expression and plasma levels were not altered throughout the fasted state. Longer periods of fasting are possibly needed to elicit marked changes in expression of these genes. Whilst no measure of muscle mass changes was observed in this study, it is likely 40 h would be insufficient time to stimulate marked catabolic processes and subsequent atrophy within skeletal muscle.

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