Short-Term Feed Deprivation Rapidly Induces the Protein Degradation Pathway in Skeletal Muscles of Young Mice$^{1,2}$

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Abstract

Analysis of protein kinase B (AKT) and S6 kinase1 (p70S6K) activity is widely used to assess the efficacy of interventions designed to increase or maintain skeletal muscle mass; these studies are often performed on feed deprived mice. One problem associated with feed deprivation is that it promotes catabolism, and young or metabolically compromised mice may have less tolerance. The aim of our study was to determine the effect of various times of feed deprivation on the activity of AKT and p70S6K signaling and markers of protein catabolism in young, growing mice compared with adult mice. Young 23-d-old and adult 3-mo-old mice were feed deprived for 8, 10, and 12 h starting at 0700 h. In addition, adult mice were feed deprived for 24 h. AKT(Ser473) phosphorylation decreased by 50 and 76% from fed amounts by 10 and 12 h of feed deprivation, respectively, in young but not adult muscles. In adult muscles, feed deprivation for 24 h reduced AKT (Ser473) phosphorylation by 70%. Significant de-phosphorylation of p70S6K(Thr389) occurred in all feed deprived young and adult mice. There was an increase in muscle RING-finger protein-1 (Murf1; 133–1245%) and muscle atrophy F-box protein or Atrogin-1 (Fbxo32; 210–2420%) mRNA in all young but not adult groups deprived of feed for 8–12 h, and there was a trend ($P = 0.08$) toward increased MURF1 associated with the contractile protein-enriched fraction isolated from young muscles of mice feed deprived for 12 h. This study demonstrates that skeletal muscles of young mice respond rapidly to feed deprivation by decreasing AKT activity and upregulating the protein degradation program. J. Nutr. 143: 1–7, 2013.

Introduction

There is intense interest in developing interventions to promote muscle growth (hypertrophy) and prevent loss of muscle mass (atrophy) in vivo and much information comes from mouse models. Activity of signaling molecules downstream of the insulin-like growth factor-1 (IGF-1)$^3$ and insulin receptors is widely studied to assess the efficacy of such interventions, because activation of these molecules is implicated in the increased protein synthesis associated with hypertrophy and may counteract muscle atrophy [reviewed in (1,2)]. Skeletal muscles express high amounts of both insulin and IGF-1 receptors and feeding-induced protein synthesis is highly pronounced in skeletal muscle (3,4). One of the pathways activated by binding of IGF-1 or insulin to their respective trans-membrane tyrosine kinase receptors is protein kinase B (AKT) signaling that is important for regulation of protein metabolism in skeletal muscle [reviewed in (1)]. A particular emphasis has been placed on this pathway, because it is also regulated by exercise, which can prevent the loss of muscle mass (5–7). Another target that becomes activated downstream from AKT is mammalian target of rapamycin (mTOR), which promotes protein synthesis by phosphorylating 2 major targets: S6 kinase 1 (p70S6K) and eukaryotic initiation factor 4E-binding protein-1 (8). Apart from the stimulus of growth factor-initiated signaling, mTOR can be directly activated by nutrients and the availability of energy in the cell (9).

Activation of the AKT signaling pathway not only stimulates protein synthesis but may also counteract protein degradation in catabolic states. In skeletal muscle atrophy models, 2 genes that are widely studied are the E3 ubiquitin-protein ligases: muscle RING-finger protein-1 (Murf1) and muscle atrophy F-box protein or Atrogin-1 (Fbxo32) [reviewed in (10,11)]. These genes appear to be partially regulated by AKT; they become suppressed when AKT is activated (phosphorylated) and this process impedes protein degradation in skeletal muscle cells. The expression amounts of Murf1 and Fbxo32 have been used to determine the efficacy of interventions that aim to prevent muscle atrophy (12,13).

Experiments to investigate interventions that are expected to act on signaling molecules downstream from the IGF-1 or insulin receptors in vivo are often carried out in feed deprived animals to test the effects on the basal phosphorylation state of signaling molecules in the absence of the anabolic impact of...
endogenous insulin and amino acids. Because of convenience, many studies routinely use an overnight feed deprivation regimen for 14–16 h. Overnight fasting for a similar time duration is a standard procedure for human metabolic studies; however, the metabolic rate of mice is higher compared with humans (14) and such prolonged feed deprivation may induce a catabolic state in rodents (15). For instance, in normal adult mice, overnight feed deprivation leads to a complete depletion of hepatic glycogen stores and reduction of glucose and insulin to concentrations that resemble a state of starvation (15).

Another issue that needs to be considered is the age of mice. The vast majority of intervention studies use adult (8 wk and older) mice and there is no information available on how feed deprivation affects signaling events in very young mice. The availability of such information is important, because young mice represent a model for physiological muscle growth with a high response to hypertrophic stimuli (16). One important reason for testing interventions on growing muscle is because this situation represents a particularly sensitive read-out to test the ability of a specific factor to promote increase in myofiber size; if an intervention is effective in growing but not adult muscle, then for benefits in clinical situations, the intervention would need to be combined with a growth stimulus like exercise. Young growing muscles are more responsive to growth stimuli (e.g., feeding and growth factors) and have a heightened capacity to activate signaling cascades that promote protein translation (16,17); thus, it is expected that they are also more sensitive to the absence of food.

We looked at the effect of increasing feed deprivation time on the activity (phosphorylation) of AKT and downstream signaling and the markers of proteasomal proteolysis in young (actively growing) 23-d-old and adult 3-mo-old mice. The 23-d-old mice were selected, because in laboratory mice, intensive postnatal muscle growth occurs up to 26 d (3).

Methods

Mouse housing. Young (21-d-old) weight-matched and adult (3-mo-old) C57BL/6J male mice were purchased from the Animal Resource Centre in Perth, Western Australia. The animals were housed in groups of 4 in standard animal cages and were maintained at a 12-h-light-dark cycle (lights turned on at 0700 h) with free access to meat-free rat and mouse diet (protein, 20%; total fat, 4.8%; total fiber, 28.8%; total carbohydrate, 59.4%) fortified with vitamins and minerals (Speciality Feeds) and drinking water for 2 d to allow for acclimatization. One-half of the mice from each age group were randomly chosen for feed deprivation for various times [feed deprived (FD) groups]. The young mice (reached 23 d of age) were feed deprived for 8, 10, and 12 h and the adult mice were feed deprived for 8, 10, 12, and 24 h. Young mice were not feed deprived for 24 h due to animal ethics regulations. Feed was removed at 0700 h for all FD groups, which corresponds to the start of the daytime and inactive phase for mice; however, mice had free access to water. For each FD group, freely fed (FF) mice served as a control. For each experimental group (FF or FD), 4–6 mice were used. Feed intake was recorded in the FF groups for the following 3 intervals: 1900 to 0700, 0700 to 1500, and 1500 to 1900 h. All experiments were conducted in accordance with the guidelines of the National Health and Medical Research Council, Australia and were approved by the animal ethics committee of the University of Western Australia.

Blood glucose measurement and tissue collection. At the end of the experiment, FD mice and the respective FF mice were anesthetized with a gaseous mixture of 2% isoflurane (Bomac), 400 mL NO₂, and 1.5 L O₂. Blood samples were collected via heart puncture and blood glucose measurements made using Accu-check Performa (Roche). The mice were killed by cervical dislocation and gastrocnemius and quadriceps muscles were removed from the lower limbs and snap-frozen in liquid nitrogen for protein and RNA extraction.

Protein extraction and Western blotting. Frozen gastrocnemius muscles were ground in liquid nitrogen and lysed in 5 volumes of PhosphoSafe reagent (MERCK) supplemented with protease inhibitor tablets (Roche) followed by a brief homogenization with a Polytron homogenizer. Muscle lysates were incubated on ice for 20 min, centrifuged at 13,000 × g for 20 min and supernatant was stored at −80°C. The resultant pellets were solubilized in a buffer containing 20 mmol/L HEPES and 4% SDS supplemented with protease and phosphatase inhibitor tablets (Roche) by sonication, followed by centrifugation at 19,600 × g for 10 min (18). Protein samples were resolved on 4–15% TGX gels (Bio-Rad) and protein was transferred onto Nitrocellulose membrane using a Trans Turbo Blot system (Bio-Rad). Western blotting was performed with antibodies for phospho-AKT(Ser473) (9271), total AKT (9272), phospho-p70S6K(Thr389) (9205), total p70S6K (9202) phospho-ribosomal protein S6(Ser235/236) (4858), and ribosomal protein S6 (2217), all from Cell Signaling, in addition to MURF1/ TRIM63 (AF5366) from R&D systems and mouse anti-a-tubulin (Sigma T5169). Donkey anti-rabbit, donkey anti-mouse, and rabbit anti-goat HRP-conjugated secondary antibodies (Pierce) were used to detect primary antibodies. Chemiluminescence imaging was done on ChemiDoc MP Imaging System (Bio-Rad). Resultant images were quantified using ImageJ software. Each blot contained at least one sample from every FF and FD group. Values were standardized to the 0700-h FF value that was assumed as 100%. All Western blot images in the figures represent samples immunoblotted on the same membrane; however, in some cases, the sequence of the samples was rearranged to match the quantification data presented in the graphs.

RNA extraction and qPCR. RNA was extracted from quadriceps muscles using Tri-Reagent (Sigma) and reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase (Promega). The qPCR was performed on a Rotor Gene 6000 real-time rotary analyzer using a QuantFast SYBR Green qPCR kit (Qiagen). The QuantiTec primers for the genes of interest were Murf1 (QT00291991) and Hoxa3 (QT00158543). Actb (beta-actin; QT01136772) was used as a reference gene.

Statistical analysis. Statistical analyses were performed with GenStat Software, 14th edition. Data were assessed for normal distribution and non-normally distributed data were log₁₀-transformed. Repeated-measures 2-way ANOVA was used to analyze feed intake data in young and adult mice across time. Feed intake within young or adult groups across time was compared with repeated-measures 1-way ANOVA and comparisons between young and adult groups at specific time points were made by 2-tail t tests. All other data from FF and FD mice were analyzed with a 2-way ANOVA using time and state (FF or FD) as sources of variance. If no interaction between time and state was found, post hoc tests of least significant differences were used for direct mean comparisons. Where an interaction between factors (time and state) was found, differences within FF or FD groups were determined with a 1-way ANOVA followed by post hoc least significant difference tests and differences between FF and FD groups were determined by 2-tail t tests. Differences were accepted as significant at P ≤ 0.05. Data were generated on n = 4–6 mice/each analytical group. Values in the figures are reported as mean ± SEM.

Results

Feed intake. Feed intake decreased during light hours (0700–1900 h) in both young (P < 0.01) and adult (P < 0.001) mice (Fig. 1A). From 1900 to 0700 h (dark hours) and 0700 to 1500 h (light hours), the feed intakes of young mice, standardized to body weight, were greater by 28 and 65%, respectively, compared with adults (P < 0.001). However, from 1500 to 1900 h, feed intake in young mice decreased by ~90% (P < 0.001), while feed intake in adult mice remained constant. Overall, from 1500 to 1900 h, adult mice consumed 300% more feed compared with young mice (P < 0.005).
Blood glucose. Blood glucose gradually decreased in young FF mice during the light hours (Fig. 1B) and 76.2%, respectively (P < 0.05) (Fig. 2A). In adult muscles, AKT(Ser473) amounts were not significantly reduced after 8–12 h of feed deprivation compared with the FF state (Fig. 2B). Adult mice feed deprived for 24 h had 70% less AKT(Ser473) phosphorylation compared with time-matched FF mice (P < 0.05) (Fig. 2B).

Because the mTOR-mediated pathway is tightly controlled by the availability of nutrients (9), we measured the amount of p70S6K(Thr389) phosphorylation, because the Thr389 site is a downstream phosphorylation target of mTORC1 (19,20). We also measured phosphorylation of ribosomal protein S6(Ser235/236) as a specific downstream target of p70S6K (21).

In young FF mice, phosphorylated p70S6K(Thr389) amounts did not change from 0700 to 1900 h (Fig. 3A). In young mice feed deprived for 8, 10, and 12 h, phosphorylation of p70S6K

FIGURE 1 Feed intake (A) and blood glucose in young 23-d-old (B) and adult 3-mo-old (C) mice. Feed intake was standardized to BW and calculated on an hourly basis. Means for feed intake are based on n = 4, i.e., 4 cages of young, and 4 cages of adult FF mice with 4 mice/cage. Means for blood glucose are based on n = 6 individual mice. Young mice (B) were not feed deprived for 24 h; thus, the 0700-FD value was not determined (indicated by ND). All values are mean ± SEM. Letters indicate differences within each group (young or adult (A); FF or FD (B, C) across time and means without a common letter differ, P < 0.05. *Different between groups [young vs. adult (A); FF vs. FD (B, C)] at a specific time point, P < 0.05. BW, body weight; FD, feed deprived; FF, freely fed; ND, not determined.

Blood glucose. Blood glucose gradually decreased in young FF mice during the light hours (P < 0.05) (Fig. 1B). In young FF mice, glucose concentrations were similar at 0700 h (9.53 ± 0.76 mmol/L) and 1500 h (9.92 ± 0.95 mmol/L) but decreased from 1500 to 1900 h (7.17 ± 0.25 mmol/L) (P < 0.05) (Fig. 1B). Blood glucose did not change from 0700 to 1900 h in FF adult mice (Fig. 1C). Blood glucose concentrations were significantly reduced in young and adult mice feed deprived for 12 h and in adult mice feed deprived for 24 h compared with time-matched FF concentrations (P < 0.01) (Fig. 1B,C). It is noted that in young mice feed deprived for 12 h, blood glucose was reduced to 3.67 ± 0.31 mmol/L, whereas adult mice feed deprived for 12 and 24 h maintained blood glucose at 6.65 ± 0.41 and 4.72 ± 0.11 mmol/L respectively.

AKT, p70S6K, and ribosomal protein S6 phosphorylation in young and adult muscles. There was a wide biological variation in AKT(Ser473) phosphorylation among FF mice and overall amounts were similar in muscles within the young (Fig. 2A) and adult (Fig. 2B) FF groups from 0700 to 1900 h. Amounts of AKT(Ser473) phosphorylation were lower in young mice feed deprived for 10 and 12 h compared with time-matched FF mice by 49.5 and 76.2%, respectively (P < 0.05) (Fig. 2A). In adult muscles, AKT(Ser473) amounts were not significantly reduced after 8–12 h of feed deprivation compared with the FF state (Fig. 2B). Adult mice feed deprived for 24 h had 70% less AKT(Ser473) phosphorylation compared with time-matched FF mice (P < 0.05) (Fig. 2B).

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FIGURE 2 P-AKT(Ser473) relative to T-AKT in skeletal muscles of young (A) and adult (B) mice in the FF and FD groups. For Western blots, α-tubulin was used to confirm equal protein loading. Values are mean ± SEM, n = 4–6. Letters indicate differences within FF or FD groups across time, and means without a common letter differ, P < 0.05. *Different between groups (FF and FD) at a specific time point, P < 0.05. FD, feed deprived; FF, freely fed; ND, not determined; P-AKT, phosphorylated protein kinase B; T-AKT, total protein kinase B.
was reduced by 57.2, 96.4, and 91.5% \((P < 0.05)\), respectively, compared with time-matched FF amounts (Fig. 3A). Phosphorylation of S6\((\text{Ser}235/236)\) was greater in young FF mice at 0700 h compared with later time points \((P < 0.05)\), probably reflecting intensive feeding during dark hours (Fig. 3B). Phosphorylation of S6\((\text{Ser}235/236)\) was not significantly different between young FF and FD mice at 1500 h \((8 \text{ h of feed deprivation})\); however, S6\((\text{Ser}235/236)\) was dephosphorylated by 85.9 and 97.4% in young FD mice after 10 and 12 h of feed deprivation, respectively, compared with the time-matched FF mice \((1700 \text{ and } 1900 \text{ h time points}; P < 0.001)\) (Fig. 3B).

In adult FF mice, amounts of phosphorylated p70S6K \((\text{Thr}389)\) and S6\((\text{Ser}235/236)\) were overall similar from 0700 to 1900 h (Fig. 4A, B). There was a dramatic decrease in phosphorylation of p70S6K\((\text{Thr}389)\) and S6\((\text{Ser}235/236)\) \((up\text{ to }97.5\%\text{ reduction from the FF amounts})\) in FD compared with FF mice at all time points \((P < 0.05)\) (Fig. 4A, B).

Expression of Murf1 and Fbxo32 in young and adult muscles. In young FF mice, amounts of Murf1 and Fbxo32 mRNA did not change from 0700 to 1900 h (Fig. 5A, B). Murf1 mRNA amounts were greater in young FD mice compared with FF mice 8, 10, and 12 h after feed deprivation by 133 \((P < 0.05)\), 724, and 1245\% \((P < 0.005)\), respectively (Fig. 5A). Fbxo32 mRNA amounts were also greater in FD mice compared with FF mice at 8, 10, and 12 h after feed deprivation by 210 \((P < 0.05)\), 789, and 2420\% \((P < 0.005)\) respectively (Fig. 5B).

In adult FF mice, Murf1 mRNA amounts did not change from 0700 to 1900 h (Fig. 5C). The Fbxo32 mRNA amount decreased in the adult FF group from 0700 to 1500 h by 55\% \((P < 0.05)\) and increased over time from 1500 to 1900 h by 73\% \((P < 0.05)\) (Fig. 5D); such changes in Fbxo32 mRNA amounts within FF mice over time may reflect circadian variation in the expression of this gene. In adult mice feed deprived for 24 h, Murf1 and Fbxo32 mRNA amounts were greater than FF...
amounts by 930 and 524%, respectively ($P < 0.05$) (Fig. 5C, D). Feed deprivation did not result in the increased expression of Murf1 and Fbxo32 mRNA at earlier times. The amount of Fbxo32 mRNA was greater in adult mice fed deprived for 8 h compared with time-matched FF mice; however, this is probably due to the fact that at 1500 h, the FF mice had a lower amount of Fbxo32 mRNA compared with the 0700- and 1900-h FF time points ($P < 0.05$) (Fig. 5D).

MURF1 is a muscle-specific ubiquitin ligase and it facilitates degradation of thick filaments during muscle atrophy; among these are myosin heavy and light chains (22,23). We measured the amount of MURF1 protein associated with the PhosphoSafe-insoluble muscle protein fraction, which is enriched with contractile proteins including myosin chains. In young mice, there was a trend toward increased amounts of MURF1 bound to PhosphoSafe insoluble fraction at 12 h after feed deprivation ($P = 0.08$) (Fig. 6A). In adult mice, the amount of MURF1 remained unchanged at 12 h after feed deprivation but increased by 1121% at 24 h ($P < 0.05$) (Fig. 6B).

**Discussion**

We observed that adult mice tend to remain more active and consume more feed during daytime compared with young mice. The average daily blood glucose concentration in the young and adult male C57Bl/6J mice was in the range of that reported in the literature for this particular strain: $\sim$8.8 mmol/L (24). This same...
study showed that blood glucose is maintained at a concentra-
tion comparable with the fed state after daytime feed depriva-
tion for 6 h; however, it is reduced to 6 mmol/L when mice are
fed deprived overnight (24). In our study, 12 h of feed
depression during daytime was sufficient to significantly reduce
blood glucose concentrations in all mice, with a more dramatic
reduction in young animals.

As expected, feed deprivation reduced phosphorylation of
AKT, p70S6K, and S6 in both young and adult skeletal muscles.
In young mice, phosphorylation of AKT decreased more rapidly
following feed removal compared with adults. This was not
unexpected, because heightened sensitivity of AKT signaling to
feeding has been shown in very young pigs and such sensitivity
decreased as animals matured (17). Although overall food
depprivation reduced AKT phosphorylation in both young and
adult muscles, some phosphorylated AKT was detectable by
Western blotting even 24 h after feed deprivation in adults. This
persistent low amount of AKT phosphorylation may be due to
many factors that activate AKT in skeletal muscles, including
growth factors, e.g., IGF-1 (16), cytokines, and mechanical
stimulation (25).

Phosphorylation of p70S6K(Thr389), which serves as a
marker for activation of mTORC1 signaling, and its down-
stream target S6(Ser235/236) were reduced in all FD mice.
Regulation of mTORC1 signaling is very complex and depends
on the availability of nutrients, growth factors and insulin, and
the energy status of the cell (9,17).

The less dramatic decrease in phosphorylation of p70S6K
(Thr389) and S6(Ser235/236) in young mice compared with
adults after a relatively short-term (8 h) food deprivation may
be explained by the fact that young growing muscle is highly
responsive to IGF-1 and here activity of mTORC1/p70S6K
signaling will be more dependent on IGF-1 compared with the
adult muscle (16). Accordingly, we have previously shown that
transient overexpression of IGF-1 in skeletal muscles maintains
phosphorylation of S6 in 23-d-old mice deprived of feed
overnight (16).

Expression of the E3 ubiquitin-protein ligases, Murf1 and
Fbxo32, mRNA has been shown to consistently increase in
models of skeletal muscle atrophy and these are sometimes
referred to as atrophy-related genes. These 2 E3 ligases seem to
have distinct roles (10): Murf1 facilitates breakdown of
myosin heavy chain and other myofibrillar proteins (22,23),
while FBXO32 has a role in the control of protein synthesis (26).
In rodents, long-term (24–51 h) feed deprivation results in
upregulation of these atrophy-related genes (27,28). For exam-
ple, in 5-wk-old Wistar rats, feed deprivation for 51 h produced a
5.8-fold increase in Fbxo32 mRNA expression and a 16% decrease in
weight of the tibialis anterior muscle (28). Similar to rats, in
6-wk-old C57BL6 mice, feed deprivation for 1 or 2 d increased
expression of Fbxo32 and decreased body mass by 15–
20% (27). To our knowledge, there are no reports that describe
changes in atrophy-related gene expression within a shorter
time (6–12 h) of feed deprivation in young and adult mouse muscles,
although this type of feed deprivation protocol is widely used to
assess the metabolic state of the muscle and investigate anabolic
and catabolic signaling. Upregulation of Murf1 and Fbxo32 was
rapid in FD young mice, with significant increases by 8, 10, and
12 h. In addition, there was a trend toward an increased amount
of MURF1 protein associated with contractile protein-enriched
fraction isolated from young muscles following feed deprivation
for 12 h. There were no increases in Murf1 and Fbxo32 mRNA
and MURF1 protein in adult muscles of mice fed deprived for
12 h. The likely mechanism for the upregulation of these
atrophy-related genes in skeletal muscles of feed deprived mice
is inactivation of AKT signaling, because it has been proposed
that activation (phosphorylation) of AKT is a necessary step to
block the expression of Murf1 and Fbxo32. A significant
reduction in AKT phosphorylation following short-term feed
depprivation (up to 12 h) in young mice is consistent with the
steady increase in expression of Murf1 and Fbxo32. Inter-
restingly, in young mice, Murf1 and Fbxo32 mRNA amounts
increased as early as 8 h following feed deprivation, when
phosphorylated AKT amount is not significantly reduced com-
pared with the fed amount. This may mean that either young
mice are very sensitive to feed deprivation and even a slight
decrease in AKT phosphorylation affects expression of atrophy-
related genes, or there is another mechanism that regulates
expression of these genes in addition to AKT.

This study demonstrates that young muscles are more
sensitive to feeding compared with adults and they respond
rapidly to feed deprivation by inactivating AKT and upregu-
larizing the protein degradation program.

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