Insulin Attenuates Atrophy of Unweighted Soleus Muscle by Amplified Inhibition of Protein Degradation

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Unweighting atrophy of immature soleus muscle occurs rapidly over the first several days, followed by slower atrophy coinciding with increased sensitivity to insulin of in vitro protein metabolism. This study determined whether this increased sensitivity might account for the diminution of atrophy after 3 days of tail-cast hindlimb suspension. The physiological significance of the increased response to insulin in unweighted muscle was evaluated by analyzing in vivo protein metabolism for day 3 (48 to 72 hours) and day 4 (72 to 96 hours) of unweighting in diabetic animals either injected with insulin or not treated. Soleus from nontreated diabetic animals showed a similar loss of protein during day 3 (−16.2%) and day 4 (−14.5%) of unweighting, whereas muscle from insulin-treated animals showed rapid atrophy (−14.5%) during day 3 only, declining to just −3.1% the next day. Since fractional protein synthesis was similar for both day 3 (6.6%/d) and day 4 (7.0%/d) of unweighting in insulin-treated animals, the reduction in protein loss must be accounted for by a slowing of protein degradation due to circulating insulin. Intramuscular (IM) injection of insulin (500 nmol/L) stimulated in situ protein synthesis similarly in 4-day unweighted (+56%) and weight-bearing (+90%) soleus, even though unweighted muscle showed a greater in situ response of 2-deoxy-[^{3}H]glucose uptake to IM injection of either insulin (133 nmol/L) or insulin-like growth factor-I (IGF-I) (200 nmol/L) than control muscle. These findings suggest that unweighted muscle is selectively more responsive in vivo to insulin, and that the slower atrophy after 3 days of unweighting was due to an increased effect of insulin on inhibiting protein degradation.

ATROPHY OF THE SOLEUS muscle by unweighting is associated with decreased protein synthesis and increased protein degradation as demonstrated both in vivo[1-5] and in vitro.[3,6,7] In adult animals, protein degradation increases after an initial rapid decrease in protein synthesis.[8] This increase in proteolysis is transient, peaking after 2 weeks.[4] Glucocorticoids seem to play a minimal role in the accelerated protein degradation of unweighting atrophy.[7,9,10]

Some studies have examined specific contributions of myofibrillar proteins to unweighting atrophy. A relative loss of myofibrillar proteins has been observed over periods of unweighting ranging from several days to 8 weeks.[5,11-14] Both myofibrillar and sarcoplasmic proteins show a decreased rate of synthesis, but only myofibrillar proteins undergo increased protein degradation.[5] In contrast, sarcoplasmic proteins showed slower protein degradation to offset the decrease in their synthesis. In accordance with this observation, receptors for insulin and for β-adrenergic agonists, both components of the sarcoplasmic protein pool, are spared during unweighting atrophy of the soleus.[15,16]

In a previous study, we evaluated the time course of changes in soleus muscle protein degradation in situ following hindlimb unweighting of juvenile rats.[5] Whereas protein synthesis declined markedly within the first 24 hours, the increase in protein degradation was not appreciable until the second and third days of unweighting. Thereafter, protein degradation declined, but without any change in protein synthesis. Coincidental with the decline in protein degradation in the soleus is the onset of increased binding and responsiveness to insulin.[15,17] Previous studies have shown that once atrophy becomes significant, the unweighted soleus muscle shows increased in vitro effects of insulin on protein degradation and protein synthesis,[18] and on the uptake of glucose and its subsequent metabolism.[15,17-21] Insulin-like growth factor-I (IGF-I), like insulin, also shows increased responses of glucose metabolism in vitro.[20] In contrast, in unweighted soleus, neither hypoxia, contractions, nor caffeine enhanced uptake of glucose to a greater extent than in weight-bearing muscles.[21] In contrast to unweighting atrophy, atrophy caused by denervation,[18,22-24] trauma,[25] or fasting[26] is not associated with increased responses to insulin and may even be characterized by insulin resistance.

Prior results suggested that the decline in protein degradation after unweighting the hindlimbs of juvenile rats for 3 days was a consequence of an increased response of protein breakdown to insulin related to the relative increase in insulin binding.[5,15,27] In this study, we have considered this problem from a more physiological perspective by testing the effects of insulin in vivo. Diabetic animals in which insulin was either withdrawn or injected daily were used to reevaluate the role of circulating insulin in the decline of protein degradation during day 4 (72 to 96 hours) of unweighting compared with day 3 (48 to 72 hours). We hypothesized that unweighted, untreated diabetic animals should not exhibit a decline in soleus atrophy during day 4, largely because of preservation of elevated protein degradation, whereas insulin treatment should restore the decline in atrophy and protein degradation during day 4 normally characteristic of the unweighted juvenile soleus. The prior time-course study suggested that protein synthesis in the 4-day unweighted muscle may not show an increased response to insulin.[5] Therefore, we compared the effect of intramuscularly (IM) injected insulin on in vivo protein synthesis in soleus muscles of normal and 4-day hindlimb-suspended animals.

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MATERIALS AND METHODS

Treatment of Animals

All procedures were approved by The University of Arizona Animal Care and Use Committee. Juvenile female rats (Sasco Sprague-Dawley, Omaha, NE) at body weights indicated in the figures were maintained on food and water ad libitum. Animals were killed by cervical dislocation after anesthetizing by injection in a forelimb muscle with 0.1 mL/100 g body weight 10% (vol/vol) Innovar-vet (4 μg Sublimate and 200 μg Inapsine; Pitman-Moore, Washington Crossing, NJ). During casting or surgical procedures, animals were also anesthetized. For hindlimb unweighting, animals were tail-casted and suspended in a head-down position so that the hindlimbs were elevated above the floor of the cage for the period indicated.[6] Casts consisted of Hexcelite orthopedic tape (Kirschner Medical, Timonium, MD) and 6382 RTS elastomer (Factor II, Lakeside, AZ).

Animals were made diabetic after an overnight fast by intraperitoneal (IP) injection of 8.5 mg streptozotocin/100 g body weight administered in 0.5 mL 0.9% NaCl solution. Animals were then refed. After 3 days, the animals were injected subcutaneously with 5 U protamine zinc insulin (Eli Lilly, Indianapolis, IN) in 0.2 mL 0.9% NaCl, and then remained weight-bearing or were hindlimb-suspended the same day. Thereafter, animals continued to be injected daily either with insulin (diabetic insulin-treated) or with 0.9% saline (diabetic nontreated). Following insulin withdrawal, animals were confirmed to be diabetic as long as plasma glucose at the time of collection was at least 20 mmol/L. Insulin-treated animals were similarly tested to verify the effectiveness of insulin treatment. After excision of the soleus, blood was drawn into a heparinized pipette tip from the severed hepatic artery, added to heparinized tubes, and then centrifuged (20 minutes at 2,000× *g*). Plasma (0.2 mL) was deproteinized with 3.8 mL 79-mmol/L $Ba(OH)_2$/79-mmol/L $ZnSO_4$. Plasma glucose was determined spectrophotometrically.[28]

Protein Accretion or Loss

To study in vivo protein metabolism under conditions of altered weight-bearing and circulating insulin, we measured the protein content and protein synthesis of soleus muscles for weight-bearing diabetic animals without or with insulin treatment and for hindlimb-suspended animals that were normal or diabetic without or with insulin treatment. Protein content and protein synthesis were measured in contralateral muscles from the same animals. For each period studied (48 to 72 hours, day 3; 72 to 96 hours, day 4), the extent of growth (protein accretion) or atrophy (protein loss) was based on the change in protein content expressed as milligrams per muscle. For each muscle, protein content was normalized according to the animal's initial body weight relative to the mean initial body weight (107 ± 1 g) for all of the animals. Maximum correction of protein content was approximately 7% for an animal weighing 100 or 115 g, with most animals (≈80%) being within 5 g or less on either side of the mean initial weight. The fractional protein accretion rate (percent per day) for each 24-hour period studied was calculated from the difference between the protein content measured for each group of animals at the beginning and end of the 24-hour period. For example, the accretion rate for day 3, which is the period commencing at 48 hours and ending at 72 hours, was calculated as the percent difference between 72 and 48 hours of treatment. Where protein atrophy occurred, the protein accretion rate is negative. For measurement of total protein levels, the muscle was homogenized in 10% trichloroacetic acid, washed once with the acid and then once with 95% ethanol:ether (1:1), and finally solubilized in 2 mL 1-mol/L NaOH. Total protein content was determined using the Biuret procedure.[29]

Estimation of In Vivo Protein Synthesis and Degradation

Fractional rates (percent per day) of in vivo protein synthesis and degradation in conjunction with measurements of protein accretion (see previous section) were assessed using a flooding-dose technique[30,31] as adapted for use in our laboratory. The fractional rate of degradation was calculated as the difference between the mean fractional rate of synthesis and the fractional rate of protein accretion for the 24-hour period, as described earlier. Animals were tranquilized as already described and then injected IP with 300 μmol and 40 μCi L-[side chain-^{3}H]phenylalanine (ICN, Costa Mesa, CA) administered in 2 mL 1% saline solution/100 g body weight. Fifteen minutes later, one soleus muscle was removed and frozen in liquid nitrogen to terminate protein synthesis. After excision, the other soleus muscle was placed in 10% trichloroacetic acid to be processed for analysis of total protein as described earlier.

Specific activities of free intracellular (S_i) and protein-bound (S_b) phenylalanine were determined as described elsewhere,[30] with modifications restricted to the volumes used.[3] The fractional rate of synthesis was calculated as $S_b/(0.9 \cdot S_i \cdot t)$, where t is the time in days between injection and muscle excision and 0.9 provides a correction factor for delay of label equilibration in the muscle.[31]

In Situ Insulin-Stimulated Protein Synthesis

To evaluate the effect of insulin on in situ protein synthesis and the linearity of insulin-stimulated protein synthesis, we measured fractional rates by a modification of the flooding-dose technique developed in our laboratory.[5] The modification entailed IM rather than IP injection of a flooding dose of radiolabeled phenylalanine. We have previously used IM injections to successfully test in situ effects of protease inhibitors and hormones and to measure protein metabolism.[5,16,27] To inject the soleus muscle, a 5-mm incision is made in the outer side of the shaved, ethanol-swabbed hindlimb of rats anesthetized as before. After the underlying fascia is cut, the soleus is gently exposed by hooking it with fine curved forceps. The belly of the muscle is injected using a 10-μL Hamilton syringe with a solution of 150 mmol/L NaCl containing ^{3}H-phenylalanine (50 mmol/L, ≈4 μCi) and 500 nmol/L porcine insulin (Eli Lilly) where indicated. The volume injected depended on muscle size: 4 μL/100 g body weight for control (weight-bearing) muscles or 3.2 μL/100 g body weight for 4-day unweighted muscles, based on prior studies of ratios of muscle to body weight.

Based on previous results for distribution of injected radioactivity,[5] only the middle approximately 50% of the muscle was excised. As described previously,[5] the muscle was immediately washed twice for 10 minutes in 3 mL ice-cold 0.84% (wt/vol) NaCl solution containing 5 mmol/L cycloheximide to inhibit protein synthesis[32] and 20 mmol/L cycloleucine to prevent leakage of intracellular phenylalanine by inhibiting its transport on the L-system.[33] Muscles were then processed for analysis of fractional protein synthesis as described earlier, except that the initial step of freezing the muscles was skipped. Because the muscle was injected directly with radiolabeled phenylalanine, fractional rates of synthesis were calculated as $S_b/(S_i \cdot t)$.

In Situ Measurement of 2-Deoxyglucose Uptake

In situ uptake of glucose was determined in soleus muscle of weight-bearing and 3- or 6-day hindlimb-suspended animals, as described previously.[34] Soleus muscles were injected by the method described earlier, with 0.9% (wt/vol) NaCl solution containing 20 mmol/L 2-[1,2-^{3}H]deoxyglucose (300 μCi/mmol), 0.6 μCi/mL [^{14}C]mannitol, and 0.1% (wt/vol) bovine serum albumin. Insulin (133 nmol/L) or IGF-I (200 nmol/L; generously supplied by Lilly Research Laboratories) were injected into the contralateral muscle as indicated, to measure insulin-sensitive transport. Twenty minutes after injection, the middle two thirds of each muscle was excised, blotted, frozen using clamps dipped in liquid nitrogen, and weighed. Muscles were solubilized in 0.6 mL 0.5-mol/L NaOH. Then, 5 mL scintillant (Ecolume) was added for radioactive counting in the ^{3}H and ^{14}C channels. ^{14}C radioactivity and ^{14}C specific activity of the injected solution were used to determine the

extracellular volume. Net uptake of 2-deoxyglucose was calculated by subtracting ^3H activity in the extracellular space from total ^3H activity in each sample. The rate of uptake was then calculated using the specific activity of 2-[^3H]deoxyglucose in the injected solution.

Statistical Analysis

Data are expressed as the mean ± SEM. The significance of differences between groups for measurements of in vivo muscle protein content and protein synthesis was analyzed using one-way ANOVA with Bonferroni correction. *P* values are given as the Bonferroni value, where *P* less than .05 is significant, calculated using the Instat program (Graphpad Software, San Diego, CA). Statistical analyses of protein metabolism during the third day (48 to 72 hours) and fourth day (72 to 96 hours) are not possible, because synthesis is the average of means for the beginning and end of each 24-hour period. Protein accretion or loss is estimated as the difference between mean values for protein content, and degradation is calculated using both of these estimates.

RESULTS

Body Weight Changes

Changes in body weight were measured over a period of 4 days for each of eight groups. Percent changes (mean ± SEM) were compared using ANOVA. Normal weight-bearing animals gained 18% ± 3% in 4 days. Unweighting alone (13% ± 2%) did not significantly affect weight gain. When unweighted animals were made diabetic, both insulin (15% ± 3%)- and saline (11% ± 2%)-injected animals gained significant weight similar to the controls. The value for diabetic saline-treated rats differed from the control value without Bonferroni correction. In contrast, both insulin (16% ± 4%)- and saline (13% ± 2%)-treated diabetic, weight-bearing animals gained weight similar to the controls with or without Bonferroni correction.

In Vivo Protein Metabolism in Response to Circulating Insulin

In a previous study, we showed that after an initial period (3 days) of significant atrophy in the unweighted juvenile muscle, there followed a marked slowing in the extent of loss of total proteins accounted for by a slower degradation of myofibrillar proteins.[5] We proposed that an increased response of protein breakdown to insulin might account for the decreased loss of myofibrillar proteins. To test this idea, we conducted a time-course study for 2 to 4 days using diabetic animals that were either weight-bearing or hindlimb-unweighted and either treated or not treated with insulin. Measurements of muscle mass, protein content, and fractional protein synthesis were made at each time point (Table 1). At the 48-hour time point, the normalized (to initial body weight) protein content was similar in all groups. Diabetic weight-bearing animals, from which insulin was withdrawn at the beginning of the first day and was not replaced thereafter, showed a significant ($P < .05$) decline (11%) in muscle protein content during the third and fourth days, which was prevented by administration of insulin. Insulin also prevented the increase in blood glucose (data not shown). There was a tendency for protein content to increase (≈5% to 6%) in the soleus of insulin-treated animals, but this change was not statistically significant. Administration of insulin to these animals was effective, as illustrated by the restoration of protein synthesis to approximately normal values; in a previous study, the fractional rate of total protein synthesis was 18.4%/d for normal animals.[5]

When nontreated diabetic animals were unweighted, after 48 hours muscle protein synthesis was significantly ($P < .05$) slower (51%) compared with nondiabetic unweighted animals. After 96 hours, there was marginally ($P < .1$) less protein and slower protein synthesis in the muscles of nontreated animals compared with either nondiabetic or insulin-treated animals. Without insulin, unweighted animals tended to show slower protein synthesis at all time points compared with the other unweighted groups (Table 1).

In insulin-treated diabetic animals, protein content was decreased (29% to 34%) significantly by unweighting after 72 and 96 hours, and protein synthesis was significantly slower (51% to 62%) at all time points. Even when insulin was not administered, unweighting tended to produce a lower protein content and slower protein synthesis compared with that in the weight-bearing muscle, especially after 96 hours ($P < .1$).

When hindlimbs of nondiabetic animals were unweighted, there was a significant loss of protein during the third day but not during the fourth day, consistent with the prior observation of slower muscle atrophy after 3 days. Fractional protein synthesis in these muscles did not differ significantly after 3 or 4 days of unweighting compared with 2 days of unweighting. When diabetic animals were unweighted but not treated with insulin, protein content declined steadily during both the third and fourth days of unweighting, even though protein synthesis remained constant. When diabetic unweighted animals were treated with insulin, protein content declined markedly during the third day but showed only a small change during the fourth day, and protein synthesis did not differ at any time point. These results suggest that the presence of circulating insulin could

Table 1. Effects of Unweighting and Insulin on Protein Content and Fractional Protein Synthesis

Time (h)	Protein Content (mg/muscle)					Fractional Protein Synthesis (%/d)				
	Weight-Bearing Diabetic			Unweighted Diabetic		Weight-Bearing Diabetic			Unweighted Diabetic	
	−Insulin	+Insulin	Unweighted	−Insulin	+Insulin	−Insulin	+Insulin	Unweighted	−Insulin	+Insulin
48	8.2 ± 0.3	8.6 ± 0.3	7.4 ± 0.2	7.4 ± 0.4	7.6 ± 0.2	6.7 ± 1.1*	16.9 ± 1.2	8.9 ± 0.9*	4.4 ± 0.7	6.5 ± 0.8*
72	7.3 ± 0.3*†	9.1 ± 0.2	6.4 ± 0.3*†	6.2 ± 0.4†	6.5 ± 0.3*	6.9 ± 1.1*	17.5 ± 1.6	7.0 ± 0.6*	4.1 ± 0.8	6.6 ± 0.8*
96	6.5 ± 0.4*†	9.6 ± 0.4	6.3 ± 0.4*	5.3 ± 0.3†	6.3 ± 0.2*†	6.9 ± 1.8*	15.0 ± 1.6	6.5 ± 0.5*	4.8 ± 0.9	7.4 ± 0.7*

NOTE. Values are the mean ± SE for soleus muscles from 10 animals (100 to 115 g initial body weight) subjected to each condition for the duration indicated. In separate experiments at the end of each period, either muscle protein content or in vivo fractional protein synthesis by an IP injection technique were measured.

*$P < .01$ *v* weight-bearing diabetic + insulin by ANOVA.

†$P < .05$ *v* preceding time point by ANOVA.

Table 2. Effects of Unweighting and Insulin on Protein Metabolism

Time (h)	Fractional Rate (%/d)				
	Weight-Bearing Diabetic			Unweighted Diabetic	
	−Insulin	+Insulin	Unweighted	−Insulin	+Insulin
Protein accretion					
48-72	−11.0	5.8	−13.5	−16.2	−14.5
72-96	−11.0	5.5	−1.6	−14.5	−3.1
Protein synthesis					
48-72	6.8	17.2	8.0	4.3	6.6
72-96	6.9	16.3	6.8	5.0	7.0
Protein degradation					
48-72	17.8	11.4	21.5	20.5	21.1
72-96	17.9	10.8	8.4	19.5	10.1

NOTE. Fractional protein accretion (negative = loss) and protein synthesis were calculated from the data in Table 2. Protein accretion (loss) is calculated as the percent change in protein content between 48 and 72 hours or 72 and 96 hours for the third and fourth days of treatment, respectively. Protein synthesis during the third or fourth day is the mean of values at the beginning and end of each day (ie, the value for the third day, 48 to 72 hours, is the average from 48 and 72 hours of duration). Fractional protein degradation was calculated as protein synthesis minus protein accretion. Statistical analysis is not possible, as these are calculations from mean rather than individual values.

restore the characteristic decline in muscle protein loss after the third day of unweighting.

To assess the relative contributions of protein synthesis and degradation to the changes in protein content, we calculated the mean daily rates of protein accretion, synthesis, and degradation, where atrophy is characterized by a negative protein accretion (Table 2). Protein loss was apparent during the third and fourth days of diabetes in weight-bearing animals. Insulin prevented this loss and even promoted a small amount of protein gain. This effect of insulin could be accounted for by an average daily increase (136% to 153%) in synthesis and a decrease (36% to 40%) in degradation.

During the third day (48 to 72 hours), each group of unweighted animals showed appreciable (14% to 16%) atrophy of the soleus exceeding that observed in muscles of nontreated diabetic weight-bearing animals. Generally, this greater atrophy in the soleus of unweighted animals was associated with greater protein degradation. As would be expected, the greatest extent of atrophy during the third day occurred in the muscles of nontreated diabetic unweighted animals, because of slower protein synthesis rather than greater protein degradation. Therefore, in unweighted animals, the lack of circulating insulin in diabetes seemed to elicit a decrease in synthesis rather than an increase in degradation. In contrast, in weight-bearing animals the lack of circulating insulin affected both processes, although the largest absolute effect also was a decline in synthesis.

During the fourth day of unweighting (72 to 96 hours), the rate of protein loss remained markedly elevated only in the soleus of nontreated diabetic animals. In both nondiabetic and insulin-treated diabetic animals, the decline in muscle atrophy was a consequence of a decrease (52% to 61%) in protein degradation. In nondiabetic animals, protein synthesis even appeared to be lower and therefore could not be linked to the decline in atrophy. In insulin-treated unweighted animals, muscle protein synthesis remained nearly constant. These results are consistent with the idea that the decline in muscle atrophy after 3 days of unweighting is due to an increased effect of insulin on protein degradation, because the response was only apparent when circulating insulin was present. During both the third and fourth days of unweighting, the effect of insulin treatment on protein synthesis was much greater in the muscle of weight-bearing versus unweighted animals. In contrast, during the third day, the effect of insulin treatment on degradation was smaller in unweighted than in weight-bearing diabetic animals (0% *v* −36%), whereas the next day, a similar effect was observed in both groups (−48% *v* −40%). These results are also evidence of an increased responsiveness to insulin of protein degradation but not of synthesis after 3 days of unweighting.

In Situ Responses to Insulin Injected IM

Even though in vitro responsiveness of protein synthesis to insulin is enhanced following 3 days of unweighting,[18] this effect was not apparent in vivo when comparing insulin administration in weight-bearing and hindlimb-suspended animals. To examine this inconsistency further, we tested the effect of insulin injected IM on fractional protein synthesis in the soleus. To evaluate over which period insulin-stimulated protein synthesis was linear, we measured the fractional rate of protein synthesis in normal muscle at 15, 20, 30, and 40 minutes after injection of insulin and ^{3}H-phenylalanine (Table 3). Since the fractional rate measured at each time point did not differ, this procedure seemed to yield constant rates of insulin-stimulated protein synthesis for up to 40 minutes.

Subsequently, we measured the fractional rate of protein synthesis in weight-bearing and 4-day unweighted soleus at 20 minutes after injection of ^{3}H-phenylalanine without or with insulin into contralateral muscles (Table 4). Regardless of whether insulin was injected, unweighted muscles showed a slower (55% to 63%) rate of protein synthesis. This difference in synthesis was consistent with the difference observed between weight-bearing and unweighted soleus of insulin-treated diabetic animals (57% to 62%; Table 2). Even though circulating insulin was present, IM injection of insulin increased protein synthesis by 90% in weight-bearing muscle. In unweighted muscle, insulin increased protein synthesis by 56%. Therefore, as in the studies with diabetic animals, treatment with insulin appeared to have a smaller effect in the soleus of unweighted animals. In any event, there was certainly no evidence of an increased effect of insulin on protein synthesis during the fourth day of unweighting.

Table 3. Linear Insulin-Stimulated Protein Synthesis Using IM Injection

Time After Injection (min)	Fractional Rate (%/d)
15	35 ± 6
20	40 ± 3
30	33 ± 4
40	36 ± 3

NOTE. Values are the mean ± SE for soleus muscles from 7 animals (105 to 120 g) injected with 500 nmol/L insulin and ^{3}H-phenylalanine for the duration indicated. Fractional protein synthesis using the IM injection technique was determined.

Table 4. Effect of IM Insulin Injection on Protein Synthesis

	Fractional Rate (%/d)		
	−Insulin	+Insulin	Difference (%)
Weight-bearing muscle	20 ± 3	38 ± 6	90 ± 21*
Unweighted muscle	9 ± 2	14 ± 3	56 ± 12*
Difference (%)	−55 ± 18†	−64 ± 20†	

NOTE. Values are the mean ± SE for contralateral soleus muscles of 12 weight-bearing or 4-day unweighted animals (105 to 120 g) injected IM for 20 minutes with radiolabeled phenylalanine in the absence or presence of 500 nmol/L insulin.

*$P < .005$ *v* −insulin by paired Student's *t* test.

†$P < .05$ *v* weight-bearing by ANOVA.

Since it was possible that an enhanced effect of insulin on muscle processes due to unweighting might not be measurable in situ, we tested this possibility by measuring insulin-stimulated uptake of 2-deoxyglucose at 3 and 6 days after unweighting (Fig 1). In control muscle, insulin increased the uptake of 2-deoxyglucose nearly threefold, whereas in soleus of unweighted animals, the effect of insulin was even greater (fourfold to fivefold stimulation) after 3 or 6 days. Stimulation with IGF-I produced comparable results: 2.5-fold increase in control compared with 3.5- to 4.5-fold in 3- or 6-day unweighted muscle. Therefore, the failure of insulin to increase protein synthesis to a greater extent in 4-day unweighted versus weight-bearing muscle could not have been due to an inability to detect an increased response to insulin in situ.

DISCUSSION

Overview

The primary goals of this report were to demonstrate that an increased inhibitory response to insulin of protein degradation accounted for the slowing of muscle atrophy subsequent to 3 days of unweighting, and that protein synthesis in vivo was immune to enhanced sensitivity. Diabetic animals were used to address these problems, and these data permitted comparisons

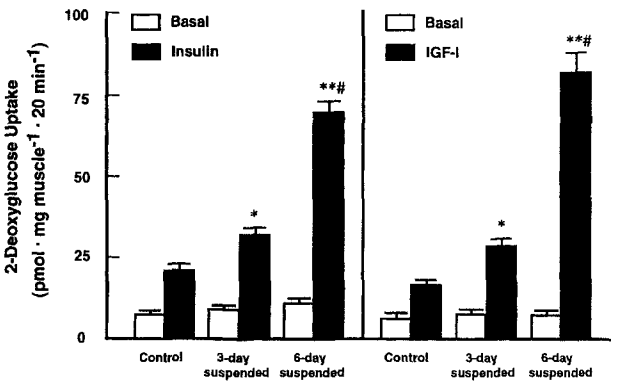

Fig 1. Effect of unweighting on in situ stimulation of glucose transport activity in soleus muscles by insulin or IGF-I. Uptake of 2-deoxy-D-[1,2-^{3}H]glucose was determined following IM injection of vehicle only (□), 133 nmol/L insulin, or 200 nmol/L IGF-I in weight-bearing or 3- and 6-day unweighted soleus. Values are the mean ± SE for 8 muscles in the insulin experiment or 5 muscles in the IGF-I experiment. *$P < .05$, **$P < .001$: *v* control. #$P < .001$ *v* 3-day suspended group.

of in vivo insulin responsiveness of the synthesis and degradation of proteins within this study and relative to other studies.

Protein Metabolism in Weight-Bearing Muscle of Diabetic Animals

Just 48 hours after insulin withdrawal, the protein content of the soleus remained similar to that of insulin-treated animals despite much greater protein synthesis following insulin treatment (Table 1). This result is consistent with a previous study showing that following insulin withdrawal growth of the gastrocnemius continued during the first 24 hours, followed by growth cessation during day 2, and finally a marked reduction in protein mass thereafter.[35] The delay in protein loss by the gastrocnemius was attributed to protein breakdown not exceeding protein synthesis until after day 2, even though there was an appreciable decrease in synthesis after 2 days, just as in our study. Consistent with this conclusion, the results here show considerable protein loss during days 3 and 4 because of slower protein synthesis and faster protein degradation. Other studies[36-38] have yielded similar results. The effect of diabetes on protein degradation has been controversial. One study reported slower proteolysis[39]; however, a later study using 1-day diabetic rats showed increased myofibrillar protein catabolism that was reversed by treatment with insulin.[40] Such discrepancies may be a consequence of differences in the severity, mode of induction, or duration of diabetes.

In our study, insulin treatment had a larger effect on synthesis than on degradation of protein (Table 2). Similarly, daily insulin treatment had a larger effect on protein synthesis in the gastrocnemius of diabetic rats.[35] In contrast, hyperinsulinemia in humans showed degradation to be more responsive than synthesis to insulin in vivo.[41] When the effect of insulin on human muscle was examined in vitro, the results were more consistent with those herein in that synthesis was more responsive to insulin.[42] A possible explanation for this discrepancy is that in studying hyperinsulinemia, basal insulin levels may have already elicited a near-maximal effect on synthesis but not on degradation; thus, degradation appeared more responsive. In contrast, when the basal condition is nontreated diabetes or incubation without insulin, protein synthesis may show more responsiveness because it is sensitive to lower amounts of insulin than protein degradation.

Protein Metabolism in Unweighted Muscle of Diabetic Animals

Unweighting the soleus in nontreated diabetic animals increased the rate of protein loss by 32% to 47% (Table 2). This greater atrophy was accounted for by contributions from both decreased synthesis and increased degradation of protein, with the absolute changes in rates similar for the two processes. Therefore, the lack of circulating insulin did not alter the typical responses of protein synthesis and degradation to unweighting. Similarly, in insulin-treated diabetic animals, the marked atrophy caused by unweighting was a consequence of both a decrease in synthesis and an increase in degradation of total proteins.

The primary purpose for using diabetic rats was to demonstrate in vivo whether increased responsiveness of protein metabolism to insulin might account for the slowing of muscle protein loss. Day 4 of unweighting was characterized by a

marked decline in protein loss in unweighted muscle of nondiabetic or insulin-treated animals but not nontreated diabetic animals. This protein loss occurred solely because protein degradation declined by 52% to 61% when circulating insulin was present. Consequently, during day 4, degradation was considerably more rapid (93% to 132%) in the absence of circulating insulin. Therefore, diminished protein loss after 3 days of unweighting must be a consequence of a reduction in muscle protein degradation owing to an increased ability of circulating insulin to retard this process.

Unlike degradation, synthesis rates were unchanged during day 4 of unweighting (Table 2), even though in vitro data suggested that insulin sensitivity might affect protein synthesis as it does protein degradation.[18] These findings suggested that protein synthesis in vivo may resist the predicted increase in sensitivity. In accordance with this conclusion, comparison of the effects of IM injected insulin on protein synthesis showed a smaller rather than a greater response in unweighted muscle (Table 4), even though under similar conditions the uptake of 2-[^{3}H]deoxyglucose in unweighted soleus showed an increased response to both insulin and IGF-I (Fig 1). This discrepancy between prior in vitro findings and the current in vivo results emphasizes the potential problems associated with drawing firm conclusions from in vitro studies and the need to follow-up such studies in vivo.

The increased responsiveness to insulin seems to be due to the increased binding of insulin by the soleus muscle after at least 3 days of unweighting.[15,19] Other observations support the idea that this is not a specific effect on the insulin receptor, but rather a generalized sparing of membrane components. Ultrastructural studies showed that membranes of fibers of soleus muscle unweighted for 7 days have a wavier appearance.[43] This adaptation created a 29% increase in the ratio of cell membrane to muscle volume, thus representing a relative conservation of the cell membrane. Accordingly, we have shown a general sparing of sarcoplasmic proteins, which include those in the membrane.[5] Furthermore, the binding capacity of the β-adrenergic receptor is also increased,[16] as is the responsiveness of the unweighted soleus to β-adrenergic agonists.[44] Since epinephrine appears to slow muscle proteolysis,[45] it is possible that increased responsiveness to catecholamines may also retard muscle protein loss with chronic unweighting.

Despite the expectation that all insulin responses should be enhanced in conjunction with increased insulin binding capacity, protein synthesis in vivo (Table 4) and amino acid uptake in vitro[18] contradict this supposition. It is noteworthy that these are both processes potentially linked to mitogenic responses in the muscle. Most likely due to altered signal transduction, partial "resistance" of these processes to insulin negates the increased sensitivity associated with greater insulin binding capacity. Resistance of protein synthesis and amino acid transport alone implies that just a portion of the insulin signal transduction pathway was affected by muscle unweighting. The observation of such differential responses is indicative of the multiple signal transduction pathways linked to insulin action.[46] The plausibility of specific resistance of protein synthesis and amino acid transport via a branch of the insulin signal transduction pathway is supported by a recent study in which insulin control of metabolic and mitogenic processes was compared.[47] Analogs of insulin with a broad range of K_d values were used to assess the relative metabolic (3-*O*-[^{3}H]methylglucose uptake) and mitogenic ([^{3}H]thymidine incorporation) responses following analog treatment. Analogs with an increased half-life of the receptor-ligand complex produced a much greater mitogenic response. This was associated with sustained phosphorylation of the Shc protein but not of insulin receptor substrate-1 (IRS-1). These findings lead to the conclusion that phosphorylation of Shc may be associated with mitogenic responses, whereas metabolic responses are linked to phosphorylation of IRS-1. This leads us to speculate that perhaps the responses in unweighted muscle that show increased sensitivity (eg, glucose uptake and metabolism and protein degradation) are mediated through IRS-1, whereas the attenuated responses of protein synthesis and amino acid uptake may use phosphorylated Shc protein.

The effect of unweighting on the insulin receptor was more pronounced in young than in adult rats.[19] Accordingly, the increased responsiveness of glucose uptake and metabolism was less pronounced in adult muscle.[19] One may then predict that insulin would be less effective in slowing protein loss during chronic unweighting of adult muscle. Such a result might explain the different time course of protein metabolism associated with unweighting of juvenile versus adult muscle. In adult muscle, proteolysis reaches a peak after 2 weeks of unweighting, compared with just 3 days in juvenile muscle.[4,5,8] Still, in endeavoring to develop countermeasures to atrophy caused by chronic unweighting of adult muscle, researchers should not overlook taking advantage of even moderate increases in the binding capacity of insulin and β-adrenergic receptors.

In conclusion, some, but not all, processes show an increased in vivo response to insulin when its binding capacity is enhanced in unweighted muscle. This finding has important implications for the long-term rate of atrophy in unweighting compared with other perturbations leading to muscle atrophy. Thus, the selective sparing of certain proteins in unweighting atrophy culminates in ultimately slowing the loss of myofibrillar proteins.[5]

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