

# Porcine leptin inhibits protein breakdown and stimulates fatty acid oxidation in C<sub>2</sub>C<sub>12</sub> myotubes<sup>1</sup>

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**ABSTRACT:** This study evaluated the potential mechanism(s) by which leptin treatment inhibits loss of muscle mass with fasting. Cultures of C<sub>2</sub>C<sub>12</sub> myoblasts were differentiated into myotubes with 5% (vol/vol) horse serum in Dulbecco's modified Eagle's medium/F12. These myotubes were used to assess <sup>3</sup>H-tyrosine incorporation and release following incubation with recombinant porcine leptin (0 to 500 ng/mL). Protein synthesis in myotubes, as measured by <sup>3</sup>H-tyrosine incorporation, was not affected by leptin treatment ( $P > 0.05$ ). Protein breakdown in C<sub>2</sub>C<sub>12</sub> myotubes, as measured by <sup>3</sup>H-tyrosine release, was inhibited by leptin treatment. A leptin concentration of 0.5 ng/mL was sufficient to inhibit <sup>3</sup>H-tyrosine release by 3.5% ( $P < 0.05$ ); 50 ng/mL produced a maximal inhibition of 10.2% ( $P < 0.05$ ). Dexamethasone (1  $\mu$ M) was used to maximally stimulate protein breakdown. Leptin (50 ng/mL leptin) de-

creased dexamethasone-induced <sup>3</sup>H-tyrosine release by 32% ( $P < 0.05$ ). The inhibition of <sup>3</sup>H-tyrosine release in C<sub>2</sub>C<sub>12</sub> myotubes suggests that leptin produces a protein-sparing effect in vitro by inhibiting protein breakdown. Fatty acid metabolism also was investigated because fatty acids are a major energy source for muscle during periods of reduced intake, as occurs with leptin treatment. Acute (4 h) and chronic (24 h) exposures to porcine leptin (0 to 500 ng/mL) were used to evaluate <sup>14</sup>C-palmitate oxidation. Acute leptin treatment had no effect ( $P > 0.05$ ) on palmitate metabolism. Chronic leptin exposure resulted in up to a 26% increase in palmitate oxidation ( $P < 0.05$ ). The stimulation of fatty acid oxidation with chronic leptin treatment suggests that leptin spares other energy sources in muscle from oxidation during periods of a leptin-induced decrease in feed intake.

Key Words: Fatty Acid Metabolism, Leptin, Protein Breakdown, Protein Synthesis

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## Introduction

Several studies have demonstrated that leptin treatment of rodents causes a severe reduction in feed intake and a loss in body weight within a week (Campfield et al., 1995; Pelleymounter et al., 1995). A single intracerebroventricular administration of porcine leptin has also been demonstrated to severely reduce feed intake (Barb et al., 1998). Unfortunately, inadequate quantities of porcine leptin are available for repeated administration over time. The rapid decline in body weight in leptin-treated mice is almost exclusively a reduction in

fat mass. Analysis of total body composition demonstrates that leptin does not produce a proportional weight loss in all tissue compartments during this rapid weight loss (Pelleymounter et al., 1995; Levin et al., 1996; Farooqi et al., 1999). The loss of fat mass far exceeds the loss in protein or muscle. This is in contrast with the loss of lean mass before loss of fat mass during feed restriction or stress (Zhou et al., 1999). This "protective" effect of leptin on muscle has not been previously examined.

Leptin has specific effects on muscle tissue, based upon previous studies. Leptin can stimulate glycogen synthesis by rodent skeletal muscle in vitro (Berti et al., 1997; Ceddia et al., 1998) or in vivo (Harris, 1998), although leptin stimulation of glucose transport is debated (Harris, 2000). Mouse leptin has been shown to increase fatty acid oxidation and reduce triglyceride synthesis in skeletal muscle in vitro, although at pharmacological levels (Muoio et al., 1997). Even though the effects of leptin on glucose and fat metabolism in skeletal muscle have been investigated, the effects on protein metabolism and turnover have not. The present study was designed to determine whether porcine leptin

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partitions energy for muscle use and reduces muscle protein loss using the C<sub>2</sub>C<sub>12</sub> myoblast cell line.

## Materials and Methods

### *Culture Conditions*

Mouse C<sub>2</sub>C<sub>12</sub> myoblasts (Collection #CRL-1772, American Type Culture Collection, Manassas, VA) were used as an *in vitro* model for skeletal muscle. Cells were used between the third and seventh passage. The C<sub>2</sub>C<sub>12</sub> cells were seeded into six well plates ( $0.5 \times 10^5$  cells) for protein turnover experiments. Plating medium contained Dulbecco's modified Eagle's medium/F12 (DMEM/F12) medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum and antibiotics (100 units penicillin/mL, 100  $\mu$ g streptomycin/mL, and 250 ng amphotericin/mL). The C<sub>2</sub>C<sub>12</sub> myoblasts were grown to confluency in this medium. Myoblasts were then fused into myotubes using DMEM/F12 medium containing 5% horse serum and antibiotics. Fusion occurred over the next several days. Cultures containing myotubes were used for tyrosine incorporation/release experiments 4 d after fusion began. Greater than 80% of the cells were fused based on microscopic analysis by the fourth day after fusion, which agrees with Desler et al. (1996). All experiments were performed with triplicate wells and repeated on four separate occasions.

### *Protein Synthesis*

Protein synthesis was measured by monitoring the incorporation of <sup>3</sup>H-tyrosine into cell proteins in the presence of treatment media according to the procedures of White et al. (1988), as modified by Desler et al. (1996). Cultures were exposed to fusion medium from confluence until d 4 following fusion. Next, cultures were washed with three changes of serum-free medium (DMEM/F12). Cultures were then exposed to test media for 4 h, followed by addition of 0.25  $\mu$ Ci/mL <sup>3</sup>H-tyrosine and another 4 h of incubation. Test media were comprised of DMEM/F12, 2% horse serum, and a range of porcine leptin concentrations (0, 0.5, 5.0, 50, and 500 ng/mL). Recombinant porcine leptin was prepared and acquired from Arieh Gertler (Raver et al., 2000).

Plates were washed with 10% trichloroacetic acid and permitted to sit overnight following the 8-h exposure to test media (the last 4 h used for measurement of protein synthesis). Cells were removed from the plates using a rubber policeman and transferred to microfuge tubes. Tubes were spun for 5 min at  $12,000 \times g$ . The trichloroacetic acid was removed and 1 mL of 0.5 M NaOH + 0.1% Triton X was added to each microfuge tube. The tubes were then heated in a 50°C water bath for 1 h. A 500- $\mu$ L sample was transferred to a scintillation vial containing 5 mL of cocktail and neutralized with 400  $\mu$ L of 1 N acetic acid. Triplicate wells were used for each treatment, and the experiment was re-

peated on four separate occasions. Data were calculated as nanomoles of tyrosine incorporated per 4 h. Data were converted then to percentages of basal activity to account for culture-to-culture variation. Synthetic activity from cultures incubated with 0 ng/mL porcine leptin represented basal activity and was defined as 100%.

### *Protein Degradation*

Protein degradation was measured by quantifying release of <sup>3</sup>H-tyrosine into culture medium following a pulse-labeling with <sup>3</sup>H-tyrosine, according to the procedures of Ballard (Ballard, 1982), as modified by Desler et al. (1996). Myotube-containing cultures were washed with serum-free medium 4 d after fusion. Medium was then replaced with fresh fusion medium containing 0.25  $\mu$ Ci <sup>3</sup>H-tyrosine/mL medium and incubated for 24 h. Cultures were again washed, and chase medium (DMEM/F12 + 2 mM tyrosine, serum-free) was added for a 4-h incubation. Chase medium was removed, and test media were then added. Cultures were incubated with test media for 20 h before harvest. Test media were comprised of serum-free DMEM/F12 and increasing concentrations of porcine leptin (0, 0.5, 5.0, 50, and 500 ng/mL) or 1  $\mu$ M dexamethasone (positive control). The medium was then sampled, and <sup>3</sup>H-tyrosine released into the medium was counted in a scintillation counter.

An additional experiment was performed to determine whether leptin could inhibit dexamethasone (1  $\mu$ M)-induced proteolysis. Test media for this experiment were comprised of serum-free DMEM/F12 and increasing concentrations of porcine leptin (0, 0.5, 5.0, 50, and 500 ng/mL) in combination with 1  $\mu$ M dexamethasone. Triplicate wells were used for each treatment, and the experiment was repeated on four separate occasions. Data were calculated as nanomoles tyrosine released per hour. Data were then converted to percentages relative to basal tyrosine release to account for culture-to-culture variation.

### *Palmitate Oxidation*

The fourth experiment was designed to determine whether porcine leptin could alter C<sub>2</sub>C<sub>12</sub> myotube lipid metabolism. These experiments were designed to determine whether porcine leptin has acute or chronic effects on palmitate metabolism in C<sub>2</sub>C<sub>12</sub> myotubes. Acute effects were measured using a 4-h incubation period because this is generally considered to be insufficient for transcription, translation, and post-translational modification of autocrine peptides, thus permitting analysis of leptin's direct effect on metabolism. Incubation medium was comprised of recombinant porcine leptin at concentrations of 0, 5, 50, or 500 ng/mL medium added to Medium 199 (Invitrogen) supplemented with 25 mM HEPES, 3% BSA, 1 mM sodium palmitate, and 0.5  $\mu$ Ci 1-[<sup>14</sup>C]-palmitic acid/mL (Moravsek Biochemicals, Brea,

CA). Basal medium (0 ng/mL leptin) served as a negative control. Insulin (100 nM) served as a positive control. Two milliliters of these incubation media was added to flasks for measurement of palmitate oxidation.

Following addition of radiolabeled medium, flasks were gassed for 1 min with 95% air:5% carbon dioxide and then capped with rubber stoppers containing center wells, according to procedures previously described (Ramsay et al., 1989). Injection of 500  $\mu$ L 1 N H<sub>2</sub>SO<sub>4</sub> into the medium killed the metabolic activity of the cells following 4 h of incubation. Ten minutes later, flasks were placed vertically and 250  $\mu$ L of methylbenzethonium hydroxide (Sigma, St. Louis, MO) was injected into the center wells. Carbon dioxide was captured during a 30-min incubation. Stoppers were then removed and the center wells were transferred to scintillation vials for counting.

Chronic effects of leptin were measured using a 48-h incubation, which provides time for leptin to stimulate the synthesis and secretion of other autocrine or paracrine factors from the myotubes. These induced autocrine or paracrine factors may then alter cellular metabolism. Therefore, chronic leptin treatment permits analysis of the indirect effect of leptin on metabolism. Porcine leptin at concentrations of 0, 5, 50, and 500 ng/mL medium were added to DMEM/F12 supplemented with 2.5% horse serum. Basal medium (0 ng/mL) served as a negative control. Insulin (100 nM) served as a positive control. Duplicate flasks of C<sub>2</sub>C<sub>12</sub> myotubes (4 d after the onset of differentiation) were incubated with these media for 20 h. At 20 h, medium was changed to the <sup>14</sup>C-palmitic acid radiolabeled incubation media described above. Incubations were continued for 4 h; therefore, these myotube cultures were exposed to supplemental leptin for a total of 24 h.

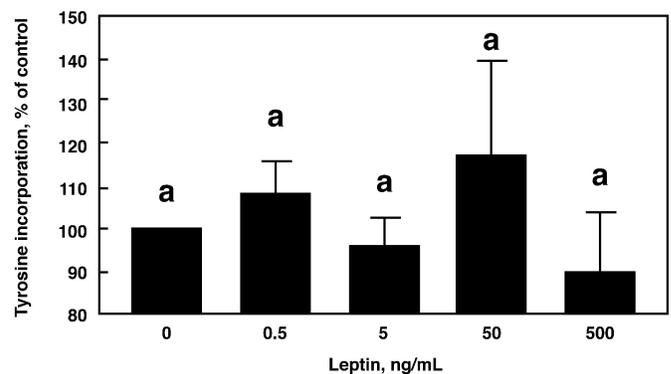
Duplicate flasks were used for each treatment and the experiments were repeated with four separate cultures of C<sub>2</sub>C<sub>12</sub> cells. Data were calculated as nanomoles of palmitate converted to CO<sub>2</sub> per flask per 4 h.

### Statistical Analyses

The experimental model for these experiments was a completely randomized design. Blocking was accomplished by converting data to percentages, relative to basal medium (0 ng/mL leptin) to account for culture-to-culture variation. Data were analyzed by one-way analysis of variance using SigmaStat software (SPSS Science, Chicago, IL). Mean separation was analyzed using a Student-Newman-Keuls test. Means were defined as significantly different at  $P < 0.05$ .

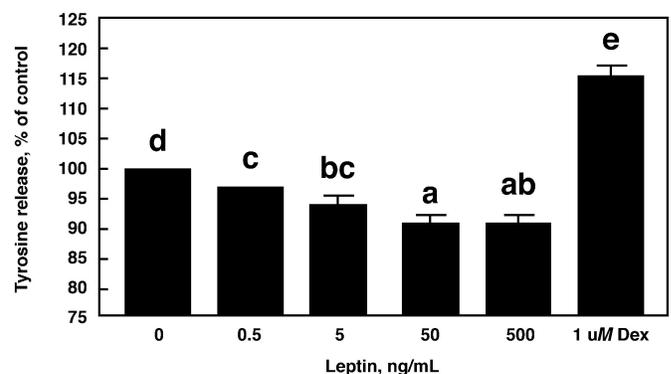
## Results

Protein synthesis in myotubes was unaffected by leptin treatment within the concentration range of 0.5 to 500 ng/mL medium (Figure 1;  $P > 0.05$ ). Proteolysis in all cultures as measured by <sup>3</sup>H-tyrosine release was

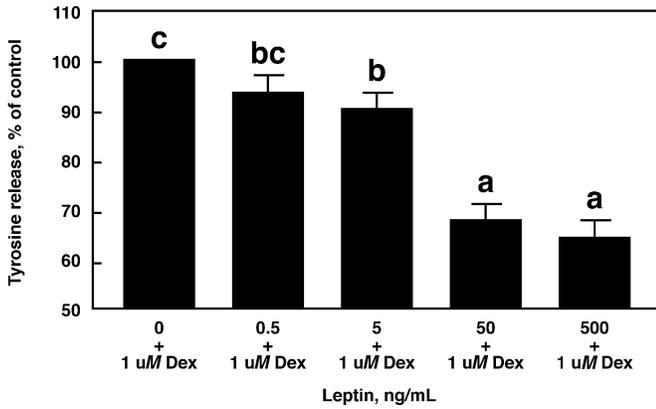


**Figure 1.** Relative percentage of tyrosine incorporation into C<sub>2</sub>C<sub>12</sub> myotubes in response to acute (8 h) incubation with leptin. Cultures of C<sub>2</sub>C<sub>12</sub> myotubes were incubated with 0 to 500 ng porcine leptin/mL medium (DMEM/F12, serum-free) for 8 h. Protein synthesis was measured during the last 4 h following introduction of <sup>3</sup>H-tyrosine into the medium. Values are expressed as a percentage of the tyrosine incorporation of the baseline group (0 ng leptin, 100% = 10,866  $\pm$  402 dpm/well) in a bar graph as means  $\pm$  SE. No significant differences ( $P > 0.05$ ,  $n = 4$ ).

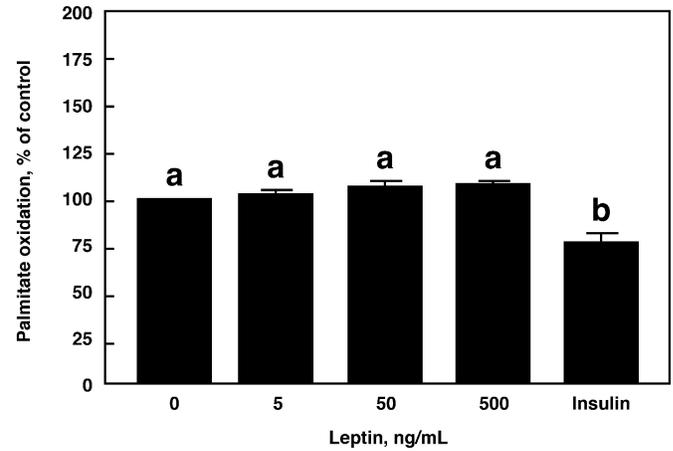
quantified relative to proteolysis in the presence of 0 ng/mL leptin medium (Figure 2). Porcine leptin treatment reduced <sup>3</sup>H-tyrosine release by up to 10% ( $P < 0.05$ ). A leptin concentration of 0.5 ng/mL was sufficient to inhibit <sup>3</sup>H-tyrosine release by 3.5% ( $P < 0.05$ ). The data indicate that 50 ng/mL produced the greatest inhibitory response (10.2%,  $P < 0.05$ ) of those leptin concentrations tested.



**Figure 2.** Relative percentage of change in tyrosine release from C<sub>2</sub>C<sub>12</sub> myotubes with chronic (24 h) leptin treatment. Myotubes were prelabeled with <sup>3</sup>H-tyrosine before the experiment. Cultures were incubated with serum-free test media containing 0 to 500 ng/mL leptin for 20 h before sample collection. Dexamethasone (Dex) was used as a positive control. Values are expressed as a percentage of the tyrosine released by the baseline group (0 ng leptin, 100% = 63,040  $\pm$  2,560 dpm/well) in a bar graph as means  $\pm$  SE. Means that do not have a common superscript letter differ ( $P < 0.05$ ;  $n = 4$ ).



**Figure 3.** Relative percentage of change in tyrosine release from  $C_2C_{12}$  myotubes with chronic (24 h) leptin treatment. Myotubes were prelabeled with H-tyrosine before the experiment. Cultures were incubated with serum-free test media containing 0 to 500 ng/mL leptin + 1  $\mu$ M dexamethasone for 20 h before sample collection. Dexamethasone was used as a positive control. Values are expressed as a percentage of the tyrosine released by the dexamethasone treatment group (0 ng/mL leptin, 1  $\mu$ M Dex, 100% = 75,344  $\pm$  3,176 dpm/well) in a bar graph as means  $\pm$  SE. Means not having a common superscript letter differ ( $P < 0.05$ ;  $n = 4$ ). Dex = 1  $\mu$ M dexamethasone.



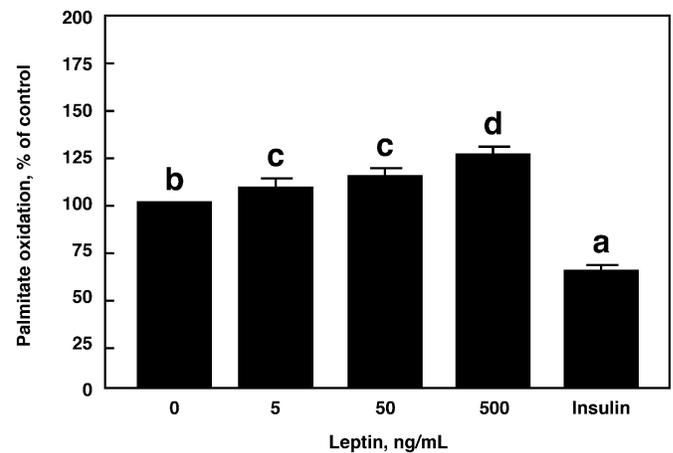
**Figure 4.** Relative palmitate oxidation in  $C_2C_{12}$  myotubes in response to acute (4 h) porcine leptin exposure. Myotube-containing  $C_2C_{12}$  cultures were incubated for 4 h in a medium containing 0.5  $\mu$ Ci  $1\text{-}^{14}\text{C}$ -palmitate/mL  $\pm$  porcine leptin (0 to 500 ng/mL medium) or 100 nM insulin, followed by collection of  $^{14}\text{CO}_2$  for analysis of palmitate oxidation. Data are expressed relative to cultures incubated without leptin (0 ng leptin/mL, 100% = 1,076  $\pm$  55 nmol incorporated/flask) in a bar graph as means  $\pm$  SE. Means not sharing a common superscript letter are different ( $P < 0.05$ ;  $n = 4$ ).

Preliminary experiments demonstrated that 1  $\mu$ M dexamethasone produces a maximal rate of protein breakdown in these cultures (data not shown). Dexamethasone elevated protein breakdown by 15% ( $P < 0.05$ ; Figure 2). Leptin exposure could inhibit this dexamethasone-stimulated protein degradation by up to 32 to 36% (Figure 3;  $P < 0.05$ ). A leptin concentration of 5.0 ng/mL was sufficient to inhibit  $^3\text{H}$ -tyrosine release by 9.7% in dexamethasone-treated myotubes ( $P < 0.05$ ). The data indicate that 50 ng/mL produced the maximal inhibitory response (32%,  $P < 0.05$ ) in dexamethasone-treated myotubes, of those leptin concentrations tested.

Acute incubation with leptin did not alter the palmitate oxidation rate (Figure 4;  $P > 0.05$ ). Insulin reduced the oxidation rate by 24% ( $P < 0.05$ ). Chronic exposure to porcine leptin increased the relative rate of palmitate oxidation by up to 26% at 500 ng/mL leptin medium ( $P < 0.05$ , Figure 5). Lower concentrations of leptin (5 to 50 ng/mL) increased the oxidation rate by 8 to 14% ( $P < 0.05$ ). Chronic exposure to insulin reduced palmitate oxidation by 37% ( $P < 0.05$ ).

## Discussion

The  $C_2C_{12}$  myogenic cell line is derived from adult mouse skeletal muscle, presumably from the satellite cell population. Recent studies have demonstrated that  $C_2C_{12}$  cells express both the long (ObRb) and short (ObRa) forms of leptin receptors (Berti and Gammeltoft, 1999). The functional expression of the long form of the receptor permits activation of the signaling cascade



**Figure 5.** Relative palmitate oxidation in  $C_2C_{12}$  myotubes in response to chronic (24 h) porcine leptin exposure. Myotube-containing  $C_2C_{12}$  cultures were incubated  $\pm$  porcine leptin (0 to 500 ng/mL medium) or 100 nM insulin for 20 h. This was followed by a 4-h incubation in a medium containing 0.5  $\mu$ Ci  $1\text{-}^{14}\text{C}$ -palmitate/mL  $\pm$  porcine leptin (0 to 500 ng/mL medium) or 100 nM insulin, followed by collection of  $^{14}\text{CO}_2$  for analysis of palmitate oxidation. Data are expressed relative to cultures incubated without leptin (0 ng leptin/mL, 100% = 1,119  $\pm$  31 nmol incorporated/flask) in a bar graph as means  $\pm$  SE. Means that do not have a common superscript letter are different ( $P < 0.05$ ;  $n = 4$ ).

necessary for leptin to affect metabolism (Kellerer et al., 1997; Berti and Gammeltoft, 1999).

Previous studies have demonstrated that leptin can alter glucose uptake and metabolism in skeletal muscle (Harris, 1998; Ceddia et al., 1999; Yaspelkis et al., 1999) and C<sub>2</sub>C<sub>12</sub> myotubes (Berti et al., 1997; Berti and Gammeltoft, 1999). Leptin may also stimulate skeletal muscle fatty acid metabolism (Muoio et al., 1998, 1999). However, there have been no reports of an effect of leptin on amino acid metabolism in skeletal muscle. Of greater significance, there have been no reports of the effect of leptin on protein synthesis or breakdown in skeletal muscle, despite the significant role of muscle in overall metabolism.

The release of <sup>3</sup>H-tyrosine from C<sub>2</sub>C<sub>12</sub> myotubes was sensitive to leptin (0.5 ng/mL) within the physiological range. Dexamethasone was used to stimulate a relative maximal rate of protein breakdown. Yet leptin was still able to inhibit proteolysis. Muscle contains multiple proteolytic systems that may contribute to overall protein breakdown, including ubiquitin Ub-ATP-dependent (Fagan et al., 1987), lysosomal (Bird et al., 1980), and calcium-dependent (Dayton et al., 1976). It cannot be ascertained from the present data which of these pathways is affected by leptin.

Leptin's inhibitory effect on protein breakdown may contribute to the relative resistance to loss of muscle mass with leptin-induced negative energy balance and body weight loss. The dramatic weight loss that results from leptin treatment is primarily a loss of fat mass. Protein mass is disproportionately maintained (Pelley-mounter et al., 1995; Levin et al., 1996; Farooqi et al., 1999), despite what can be a significant negative energy balance.

Rapid weight loss is often associated with a proportional decrease in lean body mass (Forbes, 2000; Harris, 2000). However, the loss of lean body mass declines with weight reduction as the percentage of initial body fat increases (Forbes, 2000), so that, in some studies, the severely obese do not lose lean mass with dieting (Koyama et al., 1990; Hill and DiGirolamo, 1991; De Lorenzo et al., 1999). The obese pig expresses higher levels of leptin than the nonobese due to the greater fat mass (Ramsay et al., 1998). Thus, it may be hypothesized that leptin is functioning to prevent a loss of muscle mass in the obese undergoing weight loss. Leptin may function during periods of stress to reduce muscle wasting in the nonobese state. This may be a problem for the fast-growing, extremely lean lines of pigs now being produced. These animals may be more susceptible to muscle loss with stress, due to the lower leptin levels produced from the small amount of adipose in these lines.

Muscle cells primarily use glucose as an energy source although fatty acids can supply energy to skeletal muscle during periods of fasting or nutrient deprivation (Owen et al., 1979). Leptin treatment of mice results in a decrease in serum lipids (Pelley-mounter et al., 1995; Schwartz et al., 1996), which may suggest an

increase in lipid metabolism. Previous studies have demonstrated that mouse leptin can stimulate an acute increase in fatty acid oxidation by mouse skeletal muscle, although concentrations of 10 µg/mL were necessary (Muoio et al., 1997, 1999; Steinberg and Dyck, 2000; Lau et al., 2001). The present study only used concentrations of porcine leptin up to 500 ng/mL. Mouse C<sub>2</sub>C<sub>12</sub> myotubes in the present study required chronic exposure to porcine leptin to alter fatty acid oxidation. These metabolic effects suggest an indirect response to porcine leptin. This adaptive response suggests that synthesis and secretion of additional autocrine or paracrine factors may be necessary before changes in fatty acid oxidation. Previous studies used tissue explants rather than cell cultures to quantify a metabolic response to leptin. This difference in methodology may account for an inability to detect an acute response in the present study. In any case, this experiment demonstrates that porcine leptin can alter skeletal muscle fatty acid metabolism, although using a mouse myoblast cell line.

## Implications

Leptin treatment causes a severe reduction in feed intake and a rapid loss in body weight in mice. This rapid decrease in weight is almost exclusively a reduction in fat mass. This is in contrast with the loss of lean mass before loss of fat mass during feed restriction or stress. This "protective" effect of leptin on muscle was examined in the present study. Pig leptin decreased protein breakdown in a muscle cell line (mouse C<sub>2</sub>C<sub>12</sub>) by up to 10%. Using conditions to maximize the rate of protein breakdown, leptin decreased the rate by 32%. In addition, pig leptin increased fatty acid metabolism by C<sub>2</sub>C<sub>12</sub> muscle cells by up to 26%. These results indicate that pig leptin can decrease protein breakdown and increase fatty acid utilization.

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