Ghrelin inhibits skeletal muscle protein breakdown in rats with thermal injury through normalizing elevated expression of E3 ubiquitin ligases MuRF1 and MAFbx

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Ghrelin inhibits skeletal muscle protein breakdown in rats with thermal injury through normalizing elevated expression of E3 ubiquitin ligases MuRF1 and MAFbx. Am J Physiol Regul Integr Comp Physiol 296: R893–R901, 2009. First published February 11, 2009; doi:10.1152/ajpregu.00015.2008.—We previously determined that ghrelin synthesis was downregulated after burn injury and that exogenous ghrelin retained its ability both to stimulate food intake and to restore plasma growth hormone levels in burned rats. These observations and the finding that anabolic hormones can attenuate skeletal muscle catabolism led us to investigate whether ghrelin could attenuate burn-induced skeletal muscle protein breakdown in rats. These studies were performed in young rats (50–60 g) 24 h after ∼30% total body surface area burn injury. Burn injury increased total and myofibrillar protein breakdown in extensor digitorum longus (EDL) muscles assessed by in vitro tyrosine and 3-methyl-histidine release, respectively. Continuous 24-h administration of ghrelin (0.2 mg·kg−1·h−1) significantly inhibited both total and myofibrillar protein breakdown in burned rats. Ghrelin significantly attenuated burn-induced changes in mRNA expression of IGFBP-1 and IGFBP-3 in liver. In EDL, ghrelin attenuated the increases in mRNA expression of the binding proteins, but had no significant effect on reduced expression of IGF-I. Ghrelin markedly reduced the elevated mRNA expression of TNF-α and IL-6 in EDL muscle that occurred after burn. Moreover, ghrelin normalized plasma glucocorticoid levels, which were elevated after burn. Expression of the muscle-specific ubiquitin-ligating enzyme (E3) ubiquitin ligases MuRF1 and MAFbx were markedly elevated in both EDL and gastrocnemius and were normalized by ghrelin. These results suggest that ghrelin is a powerful anticalculete compound that reduces skeletal muscle protein breakdown through attenuating multiple burn-induced abnormalities.

burn; cachexia; IGF-I; inflammatory cytokines

BURN PATIENTS EXPERIENCE A profound systemic hypermetabolism that persists for long periods even after wound healing (25). This condition has been attributed to increased production of catabolic hormones and inflammatory cytokines combined with a reduction in anabolic hormones (6, 8, 14, 32, 41, 63). This constellation of hormonal changes leads to increased lipolysis, depletion of hepatic glycogen stores, and ultimately to erosion of skeletal muscle to fuel the hypermetabolic response. Muscle loss, in turn, results in decreased strength, making rehabilitation of burn patients difficult. Therefore, many studies have explored potential therapies to curtail long-term hypermetabolism, especially the loss of lean body mass. Several anabolic agents, including growth hormone (GH), IGF-I, insulin, corticosteroid agents, α/β-adrenergic blockers, and glucocorticoid antagonists, have been investigated (28, 55, 58). These agents attenuated skeletal muscle protein loss to some degree, but they also induced adverse effects. Although some of these side effects were lessened by using IGF-I along with either GH or IGFBP-3 (28), to date no satisfactory therapy exists to curtail the loss of skeletal muscle mass associated with burn injury. Therefore, it is imperative to develop novel and alternative therapies to control loss of lean body mass.

Ghrelin is a 28-residue octanoylated peptide initially isolated from the oxyntic glands of rat stomach as the long-sought-after endogenous ligand for the GH secretagogue receptor (38). Ghrelin has been characterized as the most potent GH-releasing (hence, IGF-I-releasing) compound isolated to date (38, 60, 61). Ghrelin also triggers neuronal release of, neuropeptide Y and agouti-related peptide, central signals which promote positive energy balance by stimulating food intake and inhibiting energy expenditure (36, 60, 61). Moreover, chronic central or peripheral administration of ghrelin has been shown to increase food intake, adiposity, and body weight in rodents (62, 65); these effects are not dependent on GH-release because ghrelin also increased body weight in GH-deficient rats (62). The finding that ghrelin stimulates both the orexigenic and anabolic signals, usually downregulated under cachectic conditions, led to the investigation of its usefulness in treating cachexia (1). These studies revealed that ghrelin treatment elevated plasma GH and IGF-I and increased body weight, tibial length, and gastrocnemius muscle protein in rats with cardiac cachexia (52, 53). In nude mice inoculated with human melanoma cells, a model of human cancer cachexia, ghrelin enhanced food intake, white adipose tissue weight, and body weight (24, 47). Ghrelin similarly improved food intake in cancer patients with impaired appetite (54). In arthritic rats, ghrelin exhibited anti-inflammatory effects and decreased external symptoms of the disease (22, 23).

Our initial investigations revealed that burn injury significantly downregulated expression of ghrelin in rat stomach for a 10-day period after burn (4). Total plasma ghrelin was also reduced after burn injury (4). These observations, together with the determination that ghrelin is a major regulator of anabolic hormones and the only known orexigenic signal from the periphery to the brain, would suggest that downregulation of ghrelin may be a key factor in burn-induced loss of lean body mass.

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mass. Moreover, our initial studies showed that peripheral treatment with ghrelin normalized plasma GH levels and stimulated food intake in rats with burn injury (4). In addition, ghrelin has been shown to inhibit the production of inflammatory cytokines (22, 45). We therefore hypothesized that exogenous supplementation with ghrelin may attenuate skeletal muscle protein breakdown in rats by raising anabolic hormone levels (4, 53) and by inhibiting inflammatory cytokine production (10, 22, 45). The present investigations show for the first time that ghrelin can significantly inhibit burn-induced muscle protein breakdown in rats via suppression of the ubiquitin-ligating enzyme (E3) and the ubiquitin ligases muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx), which are elevated in rats with burn injury. These investigations also revealed that this action of ghrelin is due to its ability to counter multiple deleterious signals induced by burn injury, including changes in the production of anabolic and catabolic hormones and inflammatory cytokines.

MATERIALS AND METHODS

Thirty-two Sprague-Dawley male rats weighing 50–60 g (Harlan, Indianapolis, IN) were housed individually in a temperature-controlled room (25°C) under 12:12-h light-dark cycle, and maintained on standard rodent chow (Harlan Teklad Rodent Diet) and water ad libitum for 48 h before experiments were performed. Young rats were used because they possess lower extremity muscles that are thin enough to permit diffusion of oxygen from the medium, thus preventing the development of hypoxic regions in the muscles (12, 13, 27). All animals per group. Whether ghrelin treatment had increased the muscle mass. All biochemical studies were carried out using tissues from six to eight animals per group.

To assess protein breakdown rates, EDL muscles were tied by the tendons at resting length to stainless steel supports and preincubated in a shaking water bath for 30 min at 37°C in individual stoppered 25-ml flasks containing 3-ml oxygenated (95% O₂-5% CO₂) Krebs-
levels of these proteins in the muscle wasting associated with burn injury (32, 41). Moreover, liver is the major source of IGF-1. Burn injury had no significant effect on expression of hepatic IGF-1 mRNA compared with the sham-saline group (Fig. 2A), but ghrelin treatment significantly elevated hepatic IGF-1 mRNA expression in both sham and burn rats to well above normal levels. Although it did not reach significance, there was a clear indication that burn injury substantially increased expression of hepatic IGFBP-1 mRNA, and that this was normalized by ghrelin treatment (Fig. 2B). Burn injury also significantly elevated hepatic IGFBP-3 mRNA expression, and this increase was significantly downregulated by ghrelin (Fig. 2C).

It has previously been reported that changes in mRNA expression of IGF-I in EDL may be more relevant than those...
in liver to muscle protein breakdown (16). Therefore, we investigated the effects of ghrelin on burn-induced changes in EDL of IGF-I, IGFBP-1, and IGFBP-3 mRNA. Burn injury attenuated IGF-I gene expression by nearly 80% compared with sham-saline rats (Fig. 3A). IGF-I mRNA expression remained significantly reduced even after ghrelin treatment. On the other hand, expression of both IGFBP-1 and IGFBP-3 in EDL was increased, respectively, by nearly 60- and 3-fold after burn injury, and these increases were significantly attenuated by ghrelin (Fig. 3, B and C).

It is well known that proinflammatory cytokines play a key role in muscle protein breakdown, and that their plasma concentrations and muscle mRNA levels are elevated under various cachectic conditions, including burn injury (6, 8, 14, 16, 37). We therefore investigated mRNA expression of TNF-α and IL-6 in EDL. Compared with sham-saline rats, burn injury tended to elevate mRNA expression of TNF-α in EDL by twofold, although this did not reach significance ($P = 0.08$). In burned rats, muscle TNF-α expression was significantly reduced by ghrelin treatment (Fig. 4A). Similarly, IL-6 mRNA was significantly elevated twofold by burn injury compared with sham burn controls and this elevation in IL-6 mRNA was also normalized by ghrelin treatment (Fig. 4B).

The gene expression of two muscle-specific E3 ubiquitin ligases, MAFbx and MuRF-1, has been reported to be elevated in a number of cachectic models (30). Consistently, burn injury elevated the expression of both the MAFbx and MuRF-1 genes in EDL by more than 30- and 10-fold, respectively, relative to sham burn controls (Fig. 5, A and B). Similarly, MAFbx and MuRF-1 expression were also elevated in gastrocnemius muscle by 20- and 10-fold after burn injury (Fig. 5, C and D).
Ghrelin treatment nearly normalized expression of these genes in both EDL and gastrocnemius muscles of burn rats. Neither burn injury nor ghrelin treatment had significant effects on expression of these genes in soleus muscles (results not shown).

Since elevation in circulating glucocorticoids has been implicated in burn-induced changes in myostatin, IGF-I, and its binding proteins (29, 42, 43), we also determined the effects of burn and ghrelin treatment on plasma glucocorticoid levels. Burn injury significantly (*P < 0.02) elevated corticosterone levels compared with sham-saline rats, and this was normalized by ghrelin (Table 2). Ghrelin had no significant effect on plasma corticosterone in the sham-saline group.

Ghrelin has been reported to induce hyperglycemia, although the severity depends on the model investigated (5). This finding, and the fact that ghrelin also stimulated release of IGF-I, a known hypoglycemic agent, led us to investigate plasma glucose levels in this study. Although ghrelin significantly (*P < 0.05) enhanced plasma glucose levels in sham groups compared with the sham-saline group, ghrelin did not significantly affect plasma glucose levels in burn rats (Table 2). Moreover, plasma glucose levels in the burn-saline group did not differ significantly from those in the sham-saline group.

We have previously determined that total plasma ghrelin levels are significantly reduced by burn injury in adult rats (4). To determine whether this also occurs in young rats, we compared total plasma ghrelin levels in the four groups of animals used in this study. Consistent with our previous findings, burn injury significantly reduced the plasma levels of total ghrelin compared with sham-saline groups (Table 2). Moreover, the burn-ghrelin group had slightly lower ghrelin levels than the sham-ghrelin group, although this did not reach significance. However, as expected, both ghrelin-infused groups had significantly (8- to 10-fold) higher plasma ghrelin levels than the sham-saline group.

**DISCUSSION**

The present results demonstrate for the first time that ghrelin can significantly attenuate burn-induced total and myofibrillar protein breakdown in rat EDL muscle. Our data are also consistent with previous reports that ghrelin could moderate cachexia, thus promoting savings in muscle protein content in rodents with cardiac cachexia, arthritis, and cancer (1, 22–24, 47, 52–54).

Consistent with our previous findings in adult rats (4), burn injury significantly reduced plasma ghrelin concentrations in young rats used in this study, also. This observation together with the finding in this study that exogenous ghrelin decreased

**Table 2. Effects of burn injury and 24-h infusion of ghrelin on plasma concentrations of corticosterone, glucose, and total ghrelin in burn and sham burned rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham-Saline</th>
<th>Burn-Saline</th>
<th>Sham-Ghrelin</th>
<th>Burn-Ghrelin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosterone</td>
<td>242±40</td>
<td>427±70*</td>
<td>265±33</td>
<td>234±32</td>
</tr>
<tr>
<td>Glucose</td>
<td>136±7</td>
<td>137±6</td>
<td>152±6*</td>
<td>133±5</td>
</tr>
<tr>
<td>Total ghrelin</td>
<td>1190±80‡</td>
<td>920±50‡</td>
<td>10820±990</td>
<td>8120±320</td>
</tr>
</tbody>
</table>

Values are means ± SE. The dose of ghrelin administered over 24 h was 4.8 mg/kg. *P < 0.02 vs. sham-saline or burn-ghrelin; †P < 0.05 vs. sham-saline; ‡P < 0.05 vs. all other groups.
burn-induced muscle protein breakdown, further supports our hypothesis that the reduction in plasma ghrelin concentrations may be, at least in part, responsible for burn-induced muscle cachexia.

In agreement with previous reports (29, 43), burn injury elevated plasma glucocorticoids, and this was completely normalized by ghrelin treatment. This observation is in accord with the report (23) that GHRP-2, a ghrelin receptor agonist, attenuated the arthritis-induced elevation of plasma glucocorticoid levels in rats. Although ghrelin increased the plasma glucocorticoid levels in the controls in the study with arthritic rats (23), it did not have this effect in our investigations in which plasma was collected from anesthetized sham rats. The latter observation is in agreement with the finding that ghrelin had no effect on plasma ACTH and glucocorticoids in anesthetized animals but stimulates their release in conscious animals and humans (39). Although the mechanisms involved are unclear at present, it is possible that ghrelin attenuates plasma glucocorticoid in burn rats through inhibiting ACTH release (23).

Our data also show that burn injury increased mRNA expression of IL-6 and TNF-α in skeletal muscle. This could be due to the elevated plasma levels of inflammatory cytokines and catecholamines that exist after burn injury (6, 8, 14), because previous investigations have shown that circulating cytokines and catecholamines can promote mRNA expression of proinflammatory cytokines in skeletal muscles (2, 16, 17).

The NF-κB pathway appears to be involved in mediating these cytokine-related effects in skeletal muscle (2, 16, 44). It is noteworthy, however, that ghrelin treatment completely reversed increased mRNA expression of TNF-α and IL-6 in EDL in the present studies. This potentially anti-inflammatory effect of ghrelin is consistent with previous findings that ghrelin:

1) attenuated plasma IL-6 levels in cachectic arthritic rats (22);
2) inhibited leptin- and activation-induced proinflammatory cytokine expression by human monocytes and T cells (10);
3) prevented liver inflammatory response in endotoxic rats (20); and
4) inhibited proinflammatory responses and NF-κB activation in human endothelial cells (45). However, it remains to be determined whether the observed anti-inflammatory effects of ghrelin on EDL muscle of burn rats are due to reduction of plasma inflammatory cytokine levels, direct inhibition of NF-κB activation in muscle or to a combination of both pathways. In this regard, it should be pointed out that direct effects of ghrelin on isolated skeletal muscle and C2C12 cells, including the ability to promote differentiation and fusion of muscle cells, have recently been reported (51, 67).

It is well documented that burn injury alters the synthesis of IGF-I and its binding proteins in various tissues, including liver and skeletal muscle (32, 43). In general, the plasma levels of these proteins after burn injury also paralleled changes in their synthesis in liver. Our data revealed that burn injury attenuated mRNA expression of IGF-I by ~80% and ~25% in the skeletal muscle and liver, respectively, while increasing both IGFBP-1 and IGFBP-3 mRNA expression in these tissues. These findings, except that of hepatic IGFBP-3, are in agreement with published reports (41, 42). It should be noted that previous investigations have determined that hepatic IGFBP-3 mRNA expression is downregulated by burn injury (41, 42). At this time, we do not know the reasons for this discrepancy, but adult rats were used in the cited study, whereas we used young rats in the present study. Burn-induced changes in the mRNA expressions of IGF-I and its binding proteins, especially IGFBP-1 and IGFBP-3, have already been shown also to result in similar changes in plasma content of their proteins (41, 42). Therefore, increased mRNA expression of IGFBP-1 in liver and muscle may further dampen the activity of already reduced levels of IGF-I through inhibiting its functions (15, 56, 57). It has been suggested that the increase in skeletal muscle IGFBP-3 mRNA expression, and hence IGFBP-3, may also inhibit IGF-I actions (41, 56). However, it has also been reported that IGFBP-3 facilitates the functions of IGF-I through storage and transport (56) and that combined administration of IGF-I with IGFBP-3 attenuates burn-induced muscle catabolism without the adverse effects usually associated with IGF-I treatment alone (28). Therefore, it is not clear whether the increase in IGFBP-3 synthesis is beneficial or detrimental. We believe that the increase in IGFBP-3 synthesis after burn injury is a compensatory effect to counter the increase in IGFBP-1. It may also be possible that a certain ratio of IGFBP-3/IGFBP-1 is required for the efficient function of IGF-I. Also, elevated IGFBP-3 synthesis may be required to overcome any increased proteolysis of plasma IGFBP-3 under the conditions of burn injury. These possibilities remain to be investigated.

Detailed investigations have previously been carried out to elucidate the pathways mediating the burn-induced changes in plasma and tissue IGF-I and its binding proteins (6, 8, 14–16, 32, 41, 42). These studies have mainly implicated burn-induced elevation of glucocorticoids and proinflammatory cyto-
Consistent with our previous findings that ghrelin normalized plasma GH levels and elevated both hepatic mRNA expression of IGF-I and plasma IGF-I to above-normal levels in rodents with burn injury (3, 4), Nagaya et al. (53) demonstrated that ghrelin administration over 3 wk significantly elevated plasma GH and IGF-I in rats with cardiac cachexia. It appears, therefore, that ghrelin treatment alone will elicit an endogenous hormonal setting similar to that accomplished by combined treatment with both GH and IGF-I (50). Combined treatment with GH and IGF-I has been found to be superior to treatment with either agent individually because it curbs the loss of lean body mass after burn injury without the adverse effects associated with individual treatment with either GH or IGF-I (50). Also, our finding that ghrelin stimulated food intake in burn rats suggests that ghrelin retains the ability in burn rats to upregulate hypothalamic neuropeptide Y and agouti-related peptide signals, which are also known to inhibit energy expenditure (4, 36, 62, 65). Thus, ghrelin treatment might be expected to reduce muscle catabolism by the additional mechanism of lowering overall energy requirements.

**Perspective and Significance**

The results presented in this manuscript demonstrate that ghrelin is a powerful anticachectic compound able to curtail skeletal muscle proteolysis through counteracting multiple burn-induced anomalies. In this respect, ghrelin appears to be superior to other compounds tested to date because those compounds attenuate muscle protein breakdown by directly targeting a single abnormality associated with burn injury. Moreover, as summarized in Fig. 6, a number of pathways triggered by ghrelin contribute to its powerful anticatabolic effects. Ghrelin, unlike other anabolic hormones, exhibited no effect on plasma glucose levels in our burn model. Thus, although long-term studies are required to realize its full clinical potential, ghrelin appears to have great promise as a candidate drug for treating burn-induced cachexia. This possibility is especially important because there are no satisfactory drugs available to date to treat burn-induced dysfunctions.

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**REFERENCES**


6. Cannon JG, Friedberg JS, Gelfand JA, Tompkins RG, Burke JF, Dinarello CA. Circulating interleukin-1β and tumor necrosis factor-α


