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

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Balasubramaniam A, Joshi R, Su C, Friend LA, Sheriff S, Kagan RJ, James JH. Ghrelin inhibits skeletal muscle protein breakdown in rats with thermal injury through normalizing elevated expression of E3 ubiquitin ligases MuRF1 and MAFbfx. 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 MAFbfx were markedly elevated in both EDL and gastrocnemius and were normalized by ghrelin. These results suggest that ghrelin is a powerful antitrophic compound that reduces skeletal muscle protein breakdown through attenuating multiple burn-induced abnormalities.

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 catabolic 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.

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.

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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 animal procedures used in this study were approved by the University of Cincinnati Animal Care and Use Committee. All rats were anesthetized with pentobarbital (50 mg/kg), and their back and abdomen were shaved. The rats were then resuscitated with 0.3 M NaCl (10 ml/100 g body wt ip) to compensate for hypovolemia due to postburn edema. Half of the rats were implanted subcutaneously with a primed Alzet (Durect, Cupertino, CA) osmotic minipump (model 2001D) containing ghrelin (4.8 mg/kg), while the other half were implanted with pumps containing saline. Contents of the minipumps (200 µl) are nominally delivered at 8 µl/h over 24 h. Ghrelin has a short half-life (66), and therefore continuous infusion is required to maintain its effective concentration throughout the experiment. Shortly (<5 min) after implantation, a total of 18 rats (9 burn-ghrelin and 9 burn-saline) were subjected to a 15-s third-degree open flame burn on the back, affecting 30% of total body surface area. The surface area of burn was controlled using a layer of kerosene-soaked gauze applied to the dorsum of the rat (18). The remaining 14 rats, which were not burned, constituted the sham-ghrelin and sham-saline groups (7 per group). All rats were provided with rat chow equivalent to ~10% of their body weight, based on previous observations that burned rats consumed food ≤ 10% of their body weight within the first 24 h after burn injury (13).

After 24 h, rats were reanesthetized with pentobarbital, and blood was collected into a heparinized syringe by cardiac puncture. Gastrocnemius muscles and liver were dissected and rapidly frozen. The dissected extensor digitorum longus (EDL) muscles were used for muscle protein breakdown studies, described below. The above experiment was repeated two more times with a new group of rats to obtain sufficient tissues and plasma for biochemical analysis. In these studies, we used either 32 rats assigned into four groups as above or 25 rats assigned into three groups, sham-saline (n = 7), burn-saline (n = 9) and burn-ghrelin (n = 9). Mortality rate of burned rats was 5–10%. During the latter studies, the EDL muscles were not used for protein breakdown determinations but were rinsed in cold saline, blotted, weighed, and rapidly frozen. This was done to determine 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-
Heneleit bicarbonate buffer (pH 7.4) with 10 mM glucose. Muscles were then transferred to fresh buffer containing 0.5 mM cycloheximide for a 2-h incubation. The total protein (tyrosine) and myofibrillar protein (3-methyl-histidine) breakdown rates were determined by measuring the net production of tyrosine and 3-methyl-histidine during incubation, according to previously published HPLC procedures (12, 13, 27).

The mRNA expression of inflammatory cytokines, ubiquitin E3 ligases, and IGF-I and its binding proteins in liver or skeletal muscle were determined by quantitative real-time RT-PCR. Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the protocol provided by the manufacturer. The yield and the purity of the RNA was determined by absorbance at 260 nm and a 260-to-280 ratio, respectively. Five micrograms of RNA was used to prepare the complementary DNA (cDNA) using SuperScript Reverse Transcriptase (Invitrogen, Carlsbad, CA). Resulting cDNA was subjected to PCR amplification with the appropriate specific forward and reverse primers (Table 1). Real-time RT-PCR was carried out using a SYBR Green-based kit (Brilliant QPCR Master Mix, Stratagene, CA). The threshold cycle, the cycle at which the PCR reaction emits a fluorescence signal greater than background, was used for the quantification of mRNA. The relative quantities of mRNA were calculated using a built-in formula in iCycler IQ from Bio-Rad, and the expression of cyclophilin mRNA was used to normalize the RNA input.

Plasma glucose levels were determined at the clinical chemistry laboratory of the Cincinnati Shriners Hospital for Children. Commercial RIA kits were used to measure plasma total ghrelin (octanoylated + nonoctanoylated; Linco Research, St. Louis, MO) and corticosterone (MP Biomedicals, Orangeberg, NY).

All the results were analyzed for statistical significance (P < 0.05) by ANOVA followed by Tukey’s test for intergroup comparisons.

RESULTS

Preliminary studies were performed to determine the optimal dose of ghrelin to inhibit muscle protein breakdown. These studies revealed that doses of both 0.1 mg·kg⁻¹·h⁻¹ and 1.0 mg·kg⁻¹·h⁻¹ of ghrelin, infused continuously over 24 h, inhibited burn-induced muscle total protein breakdown significantly and nearly to the same degree (results not shown). Therefore, a ghrelin dose of 0.2 mg·kg⁻¹·h⁻¹ was used in these studies.

As estimated by tyrosine release, burn injury increased total muscle protein breakdown in EDL by nearly twofold compared with the sham-saline group (Fig. 1). EDL myofibrillar protein breakdown, as determined by 3-methyl-histidine release, was markedly increased after burn injury. Infusion of ghrelin over 24 h significantly inhibited both total and myofibrillar protein breakdown in EDL of rats with burn injury, but had no significant effects on muscle protein breakdown of sham burned rats. On the other hand, although burn injury decreased EDL weight compared with the sham-saline group (18.2 ± 0.8 mg vs. 21.5 ± 1.4 mg, mean ± SE) and ghrelin treatment of burn rats tended to increase EDL weight (19.4 ± 0.7 mg), these changes did not reach statistical significance. The latter observation is not surprising because previous studies have observed significant increases in muscle weight and protein content in cachectic animal models only on long-term treatment with ghrelin (24, 53). To examine factor(s) potentially mediating the inhibitory effects of ghrelin on skeletal muscle protein breakdown, we also studied changes in the gene expression of hepatic IGF-I and its binding proteins. It should be noted that previous studies have implicated changes in the synthesis and levels of these proteins in the muscle wasting associated with burn injury (32, 41). Moreover, liver is the major source of IGF-I. Burn injury had no significant effect on expression of hepatic IGF-I mRNA compared with the sham-saline group (Fig. 2A), but ghrelin treatment significantly elevated hepatic IGF-I 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).

Fig. 3. Effect of 24-h ghrelin treatment on the mRNA expression of extensor digitorum longus (EDL) IGF-I (A), IGFBP-1 (B), and IGFBP-3 (C) in burn rats; a sham-saline group is included for comparison. A: burn injury reduced EDL IGF-I mRNA expression by ~80% compared with the sham group; ghrelin exhibited a slight tendency to reverse this, but the difference between saline and ghrelin treatment did not reach statistical significance. B: burn injury significantly elevated expression of EDL IGFBP-1, and this was significantly reduced by ghrelin treatment. C: burn injury significantly elevated expression of IGFBP-3, and this was significantly reduced by ghrelin.

Fig. 4. Effect of 24-h ghrelin treatment on the mRNA expression in EDL muscle of TNF-α (A) and IL-6 (B) in sham-burned and burn rats. Burn injury significantly elevated mRNA expression of IL-6, and this was normalized by ghrelin treatment. Similarly, burn injury also elevated mRNA expression of TNF-α, but this did not reach significance (P = 0.08). Again, ghrelin treatment completely normalized the elevated mRNA expression of TNF-α.
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 lowered 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
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 endotoxemic 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-
kines, because infusion of cytokines and dexamethasone in rats induced changes in IGF-I and its binding proteins similar to that of burn injury, while their antagonists or binding proteins attenuated these changes in burn models (e.g., 7, 11, 42, 43, 49). It appears therefore that normalization of glucocorticoid and proinflammatory cytokine responses by ghrelin may, at least in part, contribute to its ability to attenuate burn-induced changes in the mRNA expression of IGF-I and its binding proteins in liver and skeletal muscle. In addition, we have previously shown that ghrelin can normalize the blunted plasma GH levels in burn rats (4). Moreover, GH is known to elevate the synthesis and release of hepatic IGF-I as well as to increase the plasma levels of IGF-I and IGFBP-3 (3, 31, 33). Therefore, direct and indirect (through IGF-I) effects of GH may also have contributed to the attenuation of burn-induced changes of IGF-I and its binding proteins by ghrelin.

The ATP-dependent ubiquitin-proteasome proteolytic pathway involves three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and E3; this pathway has been characterized as playing a crucial role in burn-induced muscle atrophy (19, 26, 30). E3-ligating enzymes impart substrate specificity in that the gene expression of two of these ligases, MuRF1 and MAVbx, has been specifically associated with skeletal muscle atrophy (19, 30). Moreover, the ghrelin receptor agonist, GHRP-2, has been shown to significantly attenuate arthritis-induced elevation of MuRF1 and MAVbx genes in skeletal muscle (23). Therefore, we also investigated the effects of both burn and ghrelin treatment on expression of these genes in two different muscles, EDL and gastrocnemius. As expected, burn injury significantly elevated the expression of MuRF1 and MAVbx genes in both muscles, and ghrelin treatment completely normalized these increases. At about the time we initially reported our findings (35), Lang et al. (40) also confirmed that MuRF1 and MAVbx genes in gastrocnemius muscles were upregulated after burn injury. Individually, IGF-I, glucocorticoid and inflammatory cytokines have already been demonstrated to modulate muscle ubiquitin pathways through regulating MuRF1 and MAVbx genes (46, 59, 64). Therefore, it would appear that the combined effects of burn injury on IGF-I, glucocorticoids and inflammatory cytokines are responsible for the upregulation of these genes (Fig. 6). By the same token, countering each of these changes by ghrelin could have led to normalization of the expression of these genes (Fig. 6). However, there is accumulating evidence to suggest that the pathway(s) mediating muscle proteolysis is (are) more complex than anticipated because there are reports showing that IGF-I downregulated the burn-induced elevation of MuRF1 and MAVbx genes independent of its effect on glucocorticoids (40); 2) that IGF-I could not prevent muscle atrophy induced by proinflammatory cytokines, despite inhibiting MAVbx (9); 3) that TNF-α blockade did not prevent the increase of muscle MuRF1 and MAVbx genes in arthritic rats (21); and 4) that MuRF1 and MAVbx genes could be regulated through multiple pathways including those involving AKT, NF-κB, and p38 MAPK (34). Moreover, as discussed earlier, direct actions of ghrelin on muscle also could have contributed to attenuated expression of the E3 ligase genes and, hence, to reduced muscle proteolysis (10, 20, 45, 51, 67). Therefore, further investigations are warranted to pinpoint the pathway(s) involved in the attenuation of burn-induced muscle proteolysis by ghrelin.

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-1 (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). 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 antica cachetic 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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