Burn injury impairs insulin-stimulated Akt/PKB activation in skeletal muscle

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Sugita, Hiroki, Masao Kaneki, Michiko Sugita, Takashi Yasukawa, Shingo Yasuhara, and J. A. Jeewendra Martyn. Burn injury impairs insulin-stimulated Akt/PKB activation in skeletal muscle. Am J Physiol Endocrinol Metab 288: E585–E591, 2005. First published November 9, 2004; doi:10.1152/ajpendo.00321.2004.—The molecular bases underlying burn- or critical illness-induced insulin resistance still remain unclarified. Muscle protein catabolism is a ubiquitous feature of critical illness. Akt/PKB plays a central role in the metabolic actions of insulin and is a pivotal regulator of hypermetabolism and atrophy of skeletal muscle. We therefore examined the effects of burn injury on insulin-stimulated Akt/PKB activation in skeletal muscle. Insulin-stimulated phosphorylation of Akt/PKB was significantly attenuated in burned compared with sham-burned rats. Insulin-stimulated Akt/PKB kinase activity, as judged by immune complex kinase assay and phosphorylation status of the endogenous substrate of Akt/PKB, glycogen synthase-kinase-3β (GSK-3β), was significantly impaired in burned rats. Furthermore, insulin consistently failed to increase the phosphorylation of p70 S6 kinase, another downstream effector of Akt/PKB, in rats with burn injury, whereas phosphorylation of p70 S6 kinase was increased by insulin in controls. The protein expression of Akt/PKB, GSK-3β, and p70 S6 kinase was unaltered by burn injury. However, insulin-stimulated activation of ERK, a signaling pathway parallel to Akt/PKB, was not affected by burn injury. These results demonstrate that burn injury impairs insulin-stimulated Akt/PKB activation in skeletal muscle and suggest that attenuated Akt/PKB activation may be involved in deranged metabolism and muscle wasting observed after burn injury.

FUNCTIONAL AND METABOLIC ABERRATIONS associated with critical illness such as burn injury include hypermetabolic response, increased protein catabolism, insulin resistance, and muscle wasting. Muscle wasting in critically ill patients leads to muscle weakness, resulting in hypoventilation, difficulties in weaning off respirators, decreased mobilization, prolonged rehabilitation and hospitalization, and even death (2, 3, 4, 6). Insulin resistance is a well-known phenomenon of critical illness and has long been considered to play a cardinal role in the derangements of metabolism and muscle wasting. Binding of insulin to its receptor results in activation of insulin receptor (IR) tyrosine kinase, which in turn phosphorylates the IR substrates (IRSs). Phosphorylation at the tyrosine residues of IRS-1 and IRS-2 transduces signal from IR to phosphatidylinositol 3-kinase (PI3K) (1, 4).

A Ser/Thr protein kinase, Akt/PKB, is a major downstream effector of the IR-IRS-PI3K pathway. Akt/PKB is activated by phosphorylation of Thr308 and Ser473 residues of the kinase (8, 46, 52). The phosphorylation of Akt/PKB is dependent on phosphatidylinositol 3,4,5-triphosphate, a product of PI3K. Akt/PKB drives a major portion of the PI3K-mediated metabolic actions of insulin. Akt/PKB is required for insulin-stimulated glucose uptake and glycogen synthesis (53). Akt/PKB also promotes protein synthesis via activation of the mTOR-p70 S6 kinase pathway (19). Glycogen synthase kinase-3β (GSK-3β), a negative regulator of glycogen synthase, is phosphorylated at Ser65 and inactivated by Akt/PKB, resulting in upregulation of glycogen synthesis (53). Of note, recent studies revealed that Akt/PKB plays a critical role in the regulation of hypometabolism and atrophy in skeletal muscle. Akt1/Akt2 double-knockout mice exhibited skeletal muscle atrophy (39). Phosphorylation (activation) of Akt/PKB was shown to be upregulated during skeletal muscle hypertrophy and downregulated during muscle atrophy in vivo (3).

Impaired IRS-1-mediated signaling and attenuated PI3K activation have been recognized as major contributors to obesity-related insulin resistance and type 2 diabetes. Our previous studies also demonstrated that IRS-1-mediated insulin signaling and PI3K activation are attenuated after burn injury (25), denervation (22), and immobilization (23), all conditions associated with muscle wasting. Notably, the expression of IR was unaltered in all of the above-mentioned conditions (6, 22, 23, 45). Impaired insulin-stimulated Akt/PKB activation has been shown in rodent models (6, 26, 30, 32, 44, 45) and in patients (10, 32) with obesity-related insulin resistance and type 2 diabetes. However, contradictory reports about Akt/PKB activity also exist. Other research groups have demonstrated unaltered insulin-stimulated Akt/PKB activity in rodent models of obesity-related insulin resistance, despite decreased insulin-stimulated PI3K activity (29, 37). Insulin-stimulated Akt/PKB activity has not been investigated in the pathological condition of stress-associated insulin resistance (e.g., burn injury). To address the question of whether reduced Akt/PKB activity is involved in stress-associated insulin resistance, we examined the effects of burn injury on insulin-stimulated Akt/PKB activation.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (176–200 g), purchased from Taconic Farms (Germantown, NY), were used for this study. The Institutional Animal Care Committee approved the study protocol. The animal care facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The rats were

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housed in mesh cages in a room maintained at 25°C, illuminated with 12:12-h light-dark cycles, and provided with standard rodent Chow and water ad libitum. Animals were divided randomly into four groups (sham burn with saline or insulin injection, and burn with saline or insulin injection). Each group consisted of 10 animals. Full-thickness third-degree burn injury comprising 55% of total body surface area was produced, as described previously (25). Briefly, rats were treated by immersing the back of the trunk for 15 s and the abdomen for 8 s in 80°C water under anesthesia with pentobarbital sodium (50 mg/kg body wt, ip). Sham-burned rats were immersed in lukewarm water. Analgesics affect not only insulin sensitivity (9, 35, 49) but also Akt/PKB and ERK activities (35). The responsiveness to opioids is also altered by burn injury (27, 38). Thus administration of equivalent doses (mg/kg) may not have equivalent pharmacological effects in burned animals vs. controls. In view of these considerations, analgesics were not used in our animals. Three days after burn or sham-burn injury, the rectus abdominis muscle was excised under anesthesia for biochemical analyses, and then the animals were immediately euthanized with an overdose of pentobarbital sodium (200 mg/kg body wt, ip).

**Measurement of blood glucose and insulin level.** On the day of the insulin-signaling studies (3 days after burn injury), a blood sample was taken under fasting conditions to measure glucose and insulin concentrations. Blood glucose concentration was measured by Glucometer Elite (Bayer, Elkhart, IN). Plasma insulin concentration was measured by ELISA (Crystal Chem, Chicago, IL), using rat insulin standard.

**Tissue homogenization.** Burned or sham-burned rats were anesthetized with pentobarbital sodium (50 mg/kg body wt, ip). Insulin (10 U/kg body wt Humulin R; Eli Lilly, Indianapolis, IN) or saline was injected via the portal vein, and tissues were harvested 8 min thereafter. Tissue samples were homogenized as described previously (50). Briefly, tissues were powdered under liquid nitrogen and homogenized in ice-cold homogenization buffer (50 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 10% glycerol, 10 mM sodium pyrophosphate, 0.5 mM NaF, and 2 mM sodium vanadate), and then washed with 500 µl of kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl2, 2 mM DTT, and 0.1 mM sodium vanadate). Beads were incubated in kinase buffer containing crosstide as a substrate for Akt/PKB, or ERK substrate peptide (Upstate), in the presence of cold ATP and 1 µCi of [γ-32P]ATP at 30°C for 10 min. The reaction was stopped by adding Laemml sample buffer and boiling for 5 min, and then the samples were centrifuged. Supernatant was transferred to P81 thin cellulose phosphate paper (Upstate). After 1 min, the cellulose phosphate paper was washed with 1% phosphoric acid three times and with water once, and then the radioactivity remaining in the sheet was counted by a liquid scintillation counter.

**Statistical analysis.** The data were compared with one-way ANOVA followed by Fisher’s protected least significant differences test. A value of P < 0.05 was considered statistically significant. All values are expressed as means ± SE.

**RESULTS**

The fasting blood glucose level was significantly increased at 3 days postburn injury compared with sham-burned rats (Fig. 1A). Burn injury-induced fasting hyperglycemia was accompanied by fasting hyperinsulinemia. Fasting plasma insulin concentration was elevated approximately threefold in burned rats compared with sham-burned rats at 3 days after burn injury (Fig. 1B).

To evaluate the insulin-stimulated activation of Akt/PKB, we examined the phosphorylation status of Akt/PKB by immunoblot analysis. In insulin-unstimulated (saline-injected) rats, phosphorylation of Akt/PKB was barely detectable in both burned and sham-burned rats, and no significant difference in basal (insulin-unstimulated) Akt/PKB phosphorylation was found between burned and sham-burned rats. Insulin administration caused a marked phosphorylation of Akt/PKB at both Thr308 and Ser73 in sham-burned rats. However, insulin-stimulated phosphorylation of Akt/PKB at Thr308 and Ser73 in burned rats was reduced to 53 and 44%, respectively, of that obtained in sham-burned rats (Fig. 2). There were no differences in protein expression of Akt/PKB between burned and sham-burned rats (Fig. 2).

![Fig. 1. Fast hyperglycemia and hyperinsulinemia in burned rats. At 3 days after burn injury, fasting blood glucose level (A) and plasma insulin concentration (B) increased in burned rats compared with sham-burned rats. *P < 0.05 and **P < 0.0001 vs. sham-burned rats.](http://ajpendo.physiology.org/ by 10.1210.33.5 on June 23, 2017)
Akt/PKB activity was further assessed by immune complex kinase assay. Consistent with the impaired insulin-stimulated Akt/PKB phosphorylation observed in burned rats (Fig. 2), insulin-stimulated Akt/PKB activity was significantly reduced in burned rats compared with sham-burned rats (Fig. 3). We also assessed Akt/PKB activity by examining the phosphorylation status of GSK-3β, an endogenous substrate of Akt/PKB. In sham-burned rats, insulin administration caused a marked phosphorylation of GSK-3β. However, insulin-stimulated phosphorylation of GSK-3β in burned rats was reduced to 30% of the level obtained in sham-burned rats (Fig. 4). There was no difference in protein expression of GSK-3β between burned and sham-burned rats.

We next examined insulin-stimulated phosphorylation of p70 S6 kinase, a further downstream effector of the PI3K-Akt/PKB pathway. In sham-burned rats, insulin induced a marked phosphorylation of p70 S6 kinase. In burned rats, however, basal (insulin-unstimulated) phosphorylation of p70 S6 kinase was upregulated to 203% of the basal level of sham-burned rats, and insulin injection failed to increase phosphorylated p70 S6 kinase compared with basal (insulin-unstimulated) level (Fig. 5). There was no difference in protein expression of p70 S6 kinase between burned and sham-burned rats.

Next, we investigated the effects of burn on insulin-stimulated activation of ERK, another major insulin-signaling cascade parallel to the PI3K-Akt/PKB pathway. In contrast to Akt/PKB, insulin injection resulted in phosphorylation and activation of ERK in both burned and sham-burned rats, and there was no significant difference in insulin-stimulated ERK activation (both measured by phosphorylation status and immune complex kinase assay; Figs. 6 and 7). The protein expression of ERK did not differ between burned and sham-burned rats.

**DISCUSSION**

We found that burn injury-associated insulin resistance was accompanied by impaired insulin-induced Akt/PKB activation in skeletal muscle, which is the major site of glucose utilization. Reduced activity of Akt/PKB in skeletal muscle of burned rats was confirmed by the phosphorylation status of Akt/PKB.
Burn injury caused both hyperglycemia and hyperinsulinemia (Fig. 1), indicating a state of whole body insulin resistance. In stress- or obesity-mediated insulin resistance of animals or humans, the hyperinsulinemia may potentially accelerate the insulin resistance, thereby creating a vicious circle between insulin resistance and hyperinsulinemia. It is possible, therefore, that hyperinsulinemia in burned rats might contribute to the exacerbation of insulin resistance. However, we believe that hyperinsulinemia and hyperglycemia in burned rats is secondary to insulin resistance rather than primary, because primary hyperinsulinemia would be associated with euglycemia or hypoglycemia but not hyperglycemia.

Akt/PKB is activated by phosphorylation at Thr\(^{308}\) and Ser\(^{473}\), and the phosphorylation at these residues is dependent on phosphatidylinositol 3,4,5-triphosphate, a product of PI3K. Hence, the phosphorylation status of Akt/PKB at Thr\(^{308}\) and Ser\(^{473}\) reflects the intensity of the upstream signal input that activates Akt/PKB. Reduced insulin-stimulated phosphorylation of Akt/PKB at Thr\(^{308}\) and Ser\(^{473}\) (Fig. 2), therefore, is consistent with our previous finding that insulin-stimulated IRS-1-associated PI3K activation was attenuated in skeletal muscle of burned rats (25). These findings indicate that reduced activation of Akt/PKB in burned rats may be attributable to impaired PI3K activation. It must be noted, however, that the burn injury-associated decrease in insulin-stimulated phosphorylation of GSK-3\(^{β}\) appears to be more profound than could be explained by the decrease in insulin-stimulated phosphorylation of Akt/PKB (Figs. 2 and 4). Phosphorylation status of GSK-3\(^{β}\), an endogenous substrate of Akt/PKB, is assumed to reflect the endogenous activity of Akt/PKB. On the basis of these observations, one can speculate that there exists another mechanism to further reduce substrate (GSK-3\(^{β}\)) phosphorylation by Akt/PKB, over and above what can be accounted for by the attenuated PI3K-Akt/PKB activation in burns. Therefore, the present results suggest that insulin-stimulated PI3K-Akt/PKB activation may be impaired by burn injury at the level...
of Akt/PKB, in addition to the impaired PI3K activation explicable by the attenuated IRS-1 tyrosine phosphorylation, documented in our previous study (25). However, further studies will be required to clarify how the effects downstream of Akt/PKB are more profound.

Muscle wasting is a characteristic hypermetabolic response in patients with, and animal models of, burn injury (5, 7, 14, 24, 36). Increased protein degradation after burn injury has been postulated to be a major contributor to muscle wasting (16). Insulin is a well recognized anabolic hormone known to promote protein synthesis and inhibit protein breakdown in normal and burn-injured subjects (17, 42). Akt/PKB also plays a key role in the atrophy and hypertrophy of skeletal muscle cells in vitro and in vivo as well as in most of the metabolic actions of insulin, including protein metabolism. Therefore, our results suggest that impaired activation of Akt/PKB may have a role in the catabolic state and muscle wasting after burn injury.

Akt/PKB is a versatile molecule that exerts its effects on protein synthesis/breakdown and cell survival through a variety of downstream effectors. The multifaceted downstream pathways of Akt/PKB may work in concert to execute the impact of reduced activation of Akt/PKB to produce muscle wasting. Support for this proposed mechanism is threefold. First, recent studies have revealed a close link between apoptotic change and protein catabolism. Activation of caspase-3 has been documented to be an initial step triggering muscle protein breakdown associated with critical illness (13). We have previously demonstrated that burn injury causes an apoptotic change in skeletal muscle (56, 57). Akt/PKB is a major anti-apoptotic molecule. On the basis of our previous findings and the present results, one can conceive that the reduced Akt/PKB activity after burn injury might contribute to burn injury-induced muscle wasting by also promoting apoptotic change in skeletal muscle.

Second, GSK-3β, a downstream signaling molecule of Akt/ PKB, has been highlighted as a negative regulator of skeletal muscle hypertrophy (40, 53), although GSK-3β was originally identified as a kinase that inhibits glycogen synthase. Expression of the kinase-inactive mutant of GSK-3β or pharmacological inhibition of GSK-3β by LiCl caused hypertrophy of cultured skeletal muscle cells (40, 53). Therefore, our finding of attenuated insulin-stimulated phosphorylation of GSK-3β suggests that uninhibited increased GSK-3β activity may also be involved in muscle wasting in burns.

Third, the PI3K-Akt/PKB pathway also regulates protein synthesis by activating p70 S6 kinase. Consistent with impaired insulin-stimulated Akt/PKB activation in burned rats, insulin failed to increase phosphorylation of p70 S6 kinase in burned rats, whereas phosphorylation of p70 S6 kinase was increased by insulin in sham-burned rats (Fig. 5). Of note, basal (insulin-unstimulated) p70 S6 kinase phosphorylation was elevated in burned rats compared with sham-burned rats. Elevated basal activation (phosphorylation) of p70 S6 kinase after burn seems to be in concordance with the observed increase in protein synthesis in patients with burn injury (2, 20). In skeletal muscle of burned patients, intracellular amino acid availability was increased, probably because of accelerated protein breakdown (2, 18). Increased protein synthesis in burned patients has been postulated to be attributed to increased intracellular amino acid availability (2, 18). Intracellular amino acids activate mTOR independently of the PI3K-Akt/PKB pathway; mTOR phosphorylates and activates p70 S6 kinase (31). Therefore, it is possible that increased amino acid availability may increase basal (insulin-unstimulated) protein synthesis by activating mTOR/p70 S6 kinase independently of Akt/PKB in burns.

In addition, Akt/PKB also controls protein degradation. After burn injury, both the synthesis and breakdown of protein are upregulated in skeletal muscle (2, 20). However, the increase in protein breakdown is greater than the increase in protein synthesis in burns, tipping the balance toward muscle atrophy (2). Ubiquitination-dependent, proteasome-mediated protein degradation plays an important role in protein breakdown and muscle atrophy in burns (7, 16). The expression of muscle-specific ubiquitin ligases, atrogin-1 (also termed muscle atrophy F-box), and muscle RING Finger-1 (MuRF-1) is increased during the course of muscle atrophy in various disease conditions, including denervation (47), sepsis (55), and diabetes (34), and is downregulated by the PI3K-Akt/PKB pathway (41, 43, 47). Therefore, impaired activation of Akt/PKB may contribute to increased protein degradation via upregulating expression of the ubiquitin ligases as well.

Our results show that burn-induced impairment in insulin signaling is specific to the Akt/PKB pathway (Figs. 2–5) and that insulin-stimulated ERK activation was spared (Figs. 6 and 7). These findings are consistent with the previous studies showing a specific impairment in the PI3K-Akt/PKB pathway with preserved ERK activation in skeletal muscle from patients with type 2 diabetes (12, 32).

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