STRESS HORMONE SECRETION AND GUT SIGNAL TRANSDUCER (STAT) PROTEINS AFTER BURN INJURY IN RATS

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ABSTRACT

A burn injury triggers traumatic reactions characteristic of a stress. Here we investigated the early responses of prolactin (PRL), corticosterone (CS), and signal transducer and activator of transcription 5 (STAT5) in male Sprague-Dawley rats after burn injury. PRL and CS levels were determined in blood serum. STAT5 and phospho-STAT5 levels were determined in jejunum total protein extracts. The results confirmed an expected increase of CS between 4 and 6 h after the burn injury. Unexpectedly, PRL secretion was suppressed during the same time frame. These hormone levels returned to normal 6 to 8 h after burn injury. STAT5 was increased in the jejunum after burn injury, and its phosphorylation was increased between 8 and 11 h after burn injury. These changes in STAT5 were not temporally correlated with either the hormone changes that we observed or with previously documented changes of the gut function after burns.

KEYWORDS: Glucocorticoids; prolactin; STAT5; radioimmunoassay

INTRODUCTION

A burn injury is a physiological stress that provokes traumatic reactions at both systemic and tissue levels (1-4). The first and immediately most important effect is located at the burn site itself and is due to the reaction of living tissues to the heat, in the case of thermal burn, or chemicals, in the case of chemical burns. Neurally mediated effects, such as pain and a psychological shock status, happen simultaneously. Burn site-generated molecular products reach the blood circulation and spread systemically (5, 6). All these factors converge and trigger numerous systemic and local effects in tissues and organs that are not directly affected by the burn. Meaningful examples of these target organs are the pituitary gland, the adrenal glands, and the gut. Each of them, affected by the burn-generated systemic reaction, is a source of hormones and cytokines that affect other important aspects of the post-burn physiology, contributing to the burn-generated trauma at both systemic and local levels.

The pituitary gland responds to physiological stresses by altered secretion of prolactin (PRL), growth hormone (GH), and adrenocorticotropic hormone (ACTH) (7, 8). PRL and GH may be immunostimulatory (9). In contrast, the adrenal glands are the main secreting organs for immunosuppressing glucocorticoids (GC), under the control of pituitary-derived ACTH. Gut cells (macrophages and enterocytes) produce interleukin-6 (IL-6), and its production is significantly increased shortly after thermal injury (10). This proinflammatory cytokine is known to act, as GH and PRL, through a Jak2-STAT5 (Janus kinase 2: signal transducer and activator of transcription 5) intracellular pathway leading to a dimerization of phosphorylated STAT5, entry of the dimer in the nucleus, and binding of the dimer to GAS (gamma activation sites) response elements triggering specific gene expression (11). Glucocorticoid receptor (GC-R) also binds STAT5 and acts as coactivator of STAT5-related signal transduction (12 for review). Reciprocally, STAT5 reduces GC-R effectiveness, leading to reduction of GC-mediated gene induction (13). STAT5 phosphorylation can also be activated by numerous soluble factors (e.g., IL-3, IL-7, granulocyte macrophage colony-stimulating factor, or erythropoietin), and STATS is present in both hematopoietic and epithelial gut cells that are affected by burn injuries. Thus, STAT5 may be a critical link between endocrine hormones and cytokine signaling.

The integrity of the gut is compromised after a burn injury, allowing translocation of bacteria to the interstitium and circulation, contributing to septicemia (14). The profound reactions in the gut after burn injuries imply a role
for stress-induced endocrine factors in this gut response, and PRL/GH receptors are present in the gut (15, 16). Consequently, GH, PRL, and other molecules, such as IL-6 or glucocorticoids, could play an active role in these phenomena.

Anabolic hormones such as GH or PRL have been suggested to improve the current clinical protocols used after a burn injury. Indeed, the results of several studies suggest that a GH treatment can help to reduce the mortality (17-20). PRL seems to help as a treatment of the immunodepression after hemorrhage (21, 22). But Alzeer et al. (7) showed that a high level of GH during the first 6 h after a burn injury was correlated with a lower patient survival ratio. This result is challenging because it is opposite to the proposed hypothesis that GH and PRL will improve patient outcomes. However, there are very few data regarding the pattern of pituitary hormone secretion during the first hours after a burn injury, which could help to explain such contradictions. Because PRL may have fewer undesirable side effects than GH the lack of information regarding PRL after traumas is a particularly important knowledge gap.

Figure 1 summarizes the hypothesis we will test in this article. This hypothesis proposes that after burn injury, the pituitary synthesis and/or release of PRL and ACTH are increased, under the control of the hypothalamus and systemic regulators. ACTH increases the glucocorticoid secretion from the adrenal glands. The proinflammatory cytokine IL-6 production is increased in the gut, through a mechanism yet to be discovered. The increases of PRL, GC, and IL-6 synthesis are then integrated at the STAT5 phosphorylation levels in gut cells, and the resulting effect contributes to the loss of tissue integrity observed in gut after burn injury. In addition, IL-6 also plays an important role in the acute phase response, which includes a complex set of host reactions to prevent ongoing tissue damage and activate repair processes through changes in protein production by the liver and other organs (23). Some of these IL-6 actions are mediated by STAT5 (24).

To help answer these questions, we have investigated in the present study the serum levels of corticosterone (CS) and PRL in male Sprague-Dawley rats during the first 24 h after burn injury, for which no data are available in the literature. To investigate if hormones could act directly through STAT5 on the enterocytes bordering the gut, we have also followed the variations of the amount of STAT5 and phosphorylated STAT5 in total protein extracts from gut cells during the 24 h after burn injury.

**Fig. 1. Working hypothesis.** Burn injury increases ACTH and PRL secretion from the pituitary gland. ACTH increases GC secretion from the adrenal glands. Increase of IL-6 synthesis in the gut contributes to the acute phase response (thus synthesis of acute phase proteins, or APP) in the liver. Then PRL, GC, and IL-6 act on STAT5 synthesis and/or phosphorylation in the gut, which contribute to the loss of gut tissue integrity and could be involved in an acute phase response in the gut.
MATERIALS AND METHODS

Rat treatments and sample collecting

Male Sprague-Dawley rats (Jackson Laboratories, Bar Harbor, MA), 250-300g. were adapted to a 12-h light (6 a.m. to 6 p.m.)-12-h darkness (6 p.m. to 6 a.m.) cycle during 1 week and then were divided into 3 groups: 1) no treatment and no handling before killing (control animals); 2) rats anesthetized and prepared for the burn but not burned (sham-treated animals); and 3) rats anesthetized and burned (burned animals). Water and food were provided ad libitum before and after the treatments. All experiments are performed according to the University of Cincinnati Institutional Animal Care and Use guidelines. All experiments adhered to the National Institutes of Health Guidelines for the Care and Use of Experimental Animals.

The control animals were taken at specific times of the day (9:30 a.m., 12 p.m., 4 p.m., and 7 p.m. ± 30 min) front the animal quarter and immediately killed by CO₂ inhalation. Their abdomen and thorax were shaved and opened, and 1.4 mL of blood was obtained from the heart and placed on ice overnight. Blood serum was obtained after centrifugation (3000 g, 10 min., 4°C) and was stored at -20°C. The jejunum was isolated, its content was washed with saline and opened, and the internal face on the jejunum was harvested by scraping with a glass microscope slide. The tissue sample was then homogenized in standard RIPA buffer (in 100 ml PBS; 1 ml NP40, 0.3 g Na deoxycholate, 0.1 g sodium dodecyl sulfate, 1 ml phenylmethylsulfonyl fluoride at 10 mg/mL in isopropanol, 2.3 mL aprotinin at 2 mg/mL, 1 mL Na orthovanadate at 100 nM). Total jejunal protein extract was obtained after a 15-min centrifugation (13,000 g, 4°C) of the homogenate, and stored at -20°C. These control animals provided normal circadian cycle data. Four animals were used per condition. As controls for neutrophils and Mo/Ma detection, spleen cells were isolated after spleen grinding between frozen glass plates, bone marrow cells were harvested after flushing of the femoral cavity, and CHO D6 cells were obtained from culture. Their total proteins were extracted by using RIPA buffer as described for the jejunal. Four animals were used per condition.

The sham-treated animals were taken from the animal quarter at 7 a.m. They were anesthetized by pentobarbital (Abbott Laboratories. Abbott Park, IL) i.p. injections (first 2 mL of a 5 mg/mL solution in saline/kg, and then additional injections of 1 mL and 0.5 mL were administered when needed). When asleep, their back was shaved, and they received an i.p. injection of saline (100 mL/kg). At 8 a.m. (0 time point), the animals were placed on a metallic grid. The burn site, representing 25-30% of total body surface according to weight surface standards, was limited by wet tissues covering both of their sides, the limbs, and the shoulders, but the animals were not burned. They recovered thereafter on a warming pad. The sham-treated animals were killed at specific times (±30 min) during day 1 (9:30 a.m., 12 p.m., 4 p.m., and 7 p.m.) and day 2 (8 a.m.) after the sham treatment. Serum samples and jejunum protein extracts were obtained for each animal. Three animals were used per condition.

The burned animals were handled exactly like the sham-treated rats, but for the burn itself. Briefly, they were removed from the animal quarter at 7 a.m. and anesthetized by using pentobarbital. Their back was shaved and saline was injected i.p. At 8 a.m. (0 time point), they were placed on a metallic grid with the burning site limited by wet tissues. Kerosene-soaked gauze (cut to 25-30% of the total body surface area) was applied on the site and was allowed to burn for 30 s. This burn is a full-thickness third-degree burn. The rats recovered thereafter on a warming pad and were killed like the sham-treated animals at specific times (±30 min) during day 1 (9:30 a.m., 12 p.m., 4 p.m., and 7 p.m.) and day 2 (8 a.m.) after the burn. Serum samples and jejunum whole-cell protein extracts were obtained for each animal. Three animals were used per condition.

Cell line

Chinese hamster ovary cells (CHO D6, an epithelial cell line) are maintained in Ham's F-12 culture medium supplemented by 10% fetal bovine serum, 100 U/ml. penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine and 250 µg/mL G418 (Sigma, St. Louis, MO).

Rat serum PRL

The concentrations of prolactin in the blood sera were determined by a modified radioimmunoassay (RIA). Rat PRL (RP-3 from NIDDK, Bethesda. MD) was used as reference preparation. Briefly, serum aliquots were diluted in PBS containing 0.1 % BSA in opaque while 96-well plates (Packard, Meriden, CT) to a final volume of 100 µL. After adding 50 µL each of primary antibody and iodinated hormone, the plates were incubated for 2 days at 4°C. Protein A (50 µL) was then added, and the plates were centrifuged at 4000 g for 10min.
supernatant was aspirated, and the pellet was dissolved in 20 µL of 0.1 N NaOH followed by 200 µL of scintillation fluid (Microscint 20, Packard). The plates were sealed with TopSeal (Packard), and after vigorous mixing, radioactivity was counted by using a Packard TopCount.

**Rat serum corticosterone**

The concentrations of corticosterone in the serum samples were determined by a commercial solid-phase RIA validated for the rat (Rat Corticosterone Coat-A-Count, Diagnostic Products. Los Angeles, CA). The analysis was performed by the Endocrinology Laboratory in the Diagnostic Laboratory, at the College of Veterinary Medicine, Cornell University.

**Rat jejunum cells STAT5 and phospho-STAT5**

The amount of STAT5 and phospho-STAT5 (pSTAT5) in the jejunum cell protein extracts were determined by Western blotting. The jejunum whole-cell protein extracts (15 µg) were resolved on 7% acrylamide gels by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) along with a molecular weight marker (Bio-Rad Laboratories. Hercules, CA), followed by electrophoretic transfer to Protran nitrocellulose membranes (Schleicher and Schuell, Valley Park, MO). For STAT5, membranes were blocked by incubation for 30 min at room temperature with 5% nonfat milk in TBS-T (Tris-buffered saline-Tween: 20 mM Tris pH 7.5, 140 mM NaCl, 0.1% Tween-20), followed by 1-h incubation with rabbit anti-mouse STAT5h polyclonal antibody (N-20, able to bind both a and b forms of STAT5, from Santa Cruz Biotechnology. Santa Cruz, CA) 1:1000 in 5% milk and two 5-min washes in TBS-T. Blots were then incubated with 1:1000 of horseradish peroxidase-linked goat anti-rabbit IgG monoclonal antibody (Sigma), followed by two 5-min washes with TBS-T.

For pSTAT5, membranes were blocked by incubation for 1 h at room temperature with 5% BSA in modified TBS-T (mTBS-T: 10 mM Tris pH 7.5, 50 mM NaCl, 0.1% Tween-20), followed by a 1-h incubation with rabbit anti-mouse pSTAT5 polyclonal antibody (Zymed, San Francisco, CA) 1:250 in 5% BSA and four 10-min washes in mTBS-T. Blots were then incubated with horseradish peroxidase-linked goat anti-rabbit IgG monoclonal antibody (Sigma) 1:1000 in mTBS-T, followed by four 10-min washes with mTBS-T.

Enhanced chemiluminescence (ECL) detection was performed according to the manufacturer’s (Amersham Pharmacia Biotech, Piscataway, NJ) specifications. Quantification of digitally scanned Western blots was performed by using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

**Rat jejunum cells Mo/Ma**

Jejunum monocytes/macrophages (Mo/Ma) invasion was investigated by Western blotting. The jejunum whole-cell protein extracts (15 µg) were resolved on 7% acrylamide gels by reducing SDS-PAGE, followed by electrophoretic transfer to Protran nitrocellulose membranes. Membranes were blocked by incubation for 30 min at room temperature with 5% nonfat milk in TBS-T (20 mM Tris pH 7.5, 140 mM NaCl, 0.1% Tween-20), followed by a 1-h incubation with mouse anti-rat monocytes/macrophages monoclonal antibody (Biosource, Camarillo, CA) 1:400 in 5% milk and two 5-min washes in TBS-T. Blots were then incubated with 1:1000 of horseradish peroxidase-linked goal anti-mouse IgG polyclonal antibody (Cappel, Durham. NC), followed by two 5-min washes with TBS-T. ECL detection was performed according to the manufacturer’s specifications.

**Rat myeloperoxidase assay**

Jejunum neutrophil invasion was investigated by measuring tissue myeloperoxidase (MPO) levels. Jejunum cell protein extracts were obtained as described here before, and the MPO levels were measured by a 30-min H2O2-dependent oxidation of 3,3’5,5’-tetramethylbenzidine (Sigma), which generates a blue colorimetric reaction shifted to yellow using H2SO4. Spectrophotometry absorbance was read at 450 nm and compared with a linear standard curve.

**Statistics**

Statistical analyses were performed by analysis of variance (ANOVA), with post hoc comparison among individual treatment means. Significance was defined as $P < 0.05$. 

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**RESULTS**

*Serum hormone levels after burn injury*

The normal circadian cycle of CS, which peaks early in the dark phase (25), resulted in the lowest level being recorded at 9:30 a.m. and the highest at 4 p.m. When the burn injury was performed, CS levels were elevated 10-fold by 1.5 h (9:30 a.m.) after the injury, reaching a maximum of 600 ng/mL. The serum concentration progressively declined to normal levels by 6 h after the burn injury and followed the normal pattern thereafter. There was also a stress-induced alteration in CS concentrations in sham-treated animals, but the maximum elevation was lower (5-6-fold) and returned to normal concentration after only 4 h (Fig. 2).

The normal circadian cycle of PRL, resulled in highest levels at 8 a.m. In male rats, PRL normally peaks late in
the dark phase (26). Consequently, the sampling schedule would miss the expected peak. Because PRL secretion is pulsatile (27), the PRL level in the normal controls at the S a.m. sample was highly variable. PRL secretion was dramatically inhibited after burn injury (below 20 ng/mL) and stayed below control levels for longer than 4 h after the burn injury. In sham-treated animals, PRL was also temporarily suppressed, but in contrast to the burned animals, PRL returned to normal by 4 h after the sham treatment (Fig. 3).

**Neutrophils and Mo/Ma in the jejunum**

Neutrophils and monocyte/macrophage populations in the jejunum were quantified at several time points (day 1: 12 p.m. and 7 p.m.; day 2: 8 a.m.). This control was thought to be important because invading cells could influence the apparent STAT5 quantities in gut protein extracts. Positive and negative controls were used in each case. Neutrophil and Mo/Ma positive controls were, respectively, bone marrow and spleen cell protein extracts, whereas negative controls were, respectively. CHO D6 cells protein extract and primary antibody-omitted Western blotting. Myeloperoxidase levels (neutrophil marker) were similar to the negative control at all times of day. Similarly, anti-rat Mo/Ma staining was low at all times of day (maximum 2% of spleen control). Therefore, we conclude that invasive cells did not contribute significantly to the quantification of gut STAT5 proteins.

**STAT5 in the jejunum of burn-injured rats**

STAT5 levels in the jejunum of control animals varied among the time points but did not show an organized circadian pattern. STAT5 levels were at each time higher in the burned animal than in the sham-treated animals (Fig. 4). Analysis of variance showed an overall treatment effect of burn injury \((P < 0.01\) compared with sham), but there was no significant time of day effect. Among the individual sampling times, only the first time point \((1.5 \text{ h})\) showed statistical significance.

There was no evident circadian cycle obtained for phospho-STAT5 at the experimental times examined. Considering the binned animal data, the pSTAT5 content was higher than in control animal 8 and 11 h after the burn injury. The pattern for the sham-treated animals was similar to the burned animals except at 7 p.m., when the burned animals had a higher level of pSTAT5 (Fig. 5).

Figure 6 shows representative immunoblots for pSTAT5 and STAT5 in samples from mice 11 h after the burn injury.

**Fig. 4. Gut (jejunum) STAT5 levels.** CTRL shows the normal STAT5 pattern in the gut. SHAM shows the effect of anesthesia and animal handling on the STAT5 levels. BURN shows the effects of the burn injury on the STAT5 levels. No evidence of an organized STAT5 circadian cycle was observed in the gut. Values represent the means ± SEM. Bar: dark-light 24-h cycle; dark bar for dark. *Differs from CTRL \((P < 0.05)\); **differs from SHAM \((P < 0.01)\).
**Fig. 5. Gut (jejunum) phospho-STAT5 levels.** CTRL shows the normal pSTAT5 pattern in the gut. SHAM shows the effect of anesthesia and animal handling on the pSTAT5 levels. BURN shows the effects of the burn injury on the pSTAT5 levels. No evidence of an organized phospho-STAT5 circadian cycle was observed in the gut. BURN values significantly differ from CTRL at 4PM (P < 0.01), 7 p.m. (P < 0.05), day 2 8 a.m. (P < 0.01). SHAM values significantly differ from CTRL at 4 p.m. (P < 0.05) and 7 p.m. (P < 0.01), and from BURN only at 9 a.m. (P < 0.05) and 7 p.m. (P < 0.01). Values represent the means ± SEM. *Differs from CTRL (P < 0.05); **differs from CTRL (P < 0.01); ●● differs from SHAM (P < 0.01).

**Fig. 6. Representative pSTAT5 and STAT5 labeling in Western blotting.** Extracts were obtained 11 h after the burn injury. The molecular weight of the bands was 92-94 kD, compared with simultaneously run molecular weight markers.

**DISCUSSION**

We tested in this article the hypothesis that during the first 24 h after a burn injury, the secretion of PRL and glucocorticoids is increased. We also tested whether the resulting hormonal changes could contribute through STAT5 to the loss of tissue integrity observed in the post-burn gut.

The serum concentrations of PRL and CS were measured by RIA at several time points during the first 24 h after burn injury. In our model, CS levels were elevated after the burn injury. Indeed, the results obtained here for CS serum levels show a large increase by 90 min after the burn injury and then returning gradually back to normal. CS reached the normal circadian pattern by 6 h after the burn injury. The normal circadian pattern continued through 48 h after burn injury (data not shown). The stress control (SHAM-treated animals) showed that the response pattern is typical of a stress response but is higher and longer lasting when a burn injury is applied. This implies a higher level of stress or other mechanisms acting in parallel.

Concerning PRL, our model supposed also a rise in serum PRL after burn injury. However, the results showed a decreased PRL level in serum by 90 min which lasted at least 4 h after the burn injury. This result may seem to contradict published literature, which has shown that stress causes increased PRL secretion (8). However, data in the literature have been mainly concerned with PRL levels from 24 h to several weeks after burn injuries, and none have measured PRL levels during the first 24 h after the burn injury.
Other classic experiments regarding stress-induced PRL secretion focused on the rapid secretion of stored PRL during the first few minutes after stresses such as ether or restraint (28 and references therein). We concerned ourselves with PRL levels during the time frame that coincides with changes in GC secretion, i.e., hours rather than either minutes or days. This time frame has special relevance because of the proven interactions between PRL and GC signal transduction (12). PRL-R activation in neutrophils triggers elevated superoxide anion secretion (29), which is important for the antibacterial response, but which also causes tissue damage (30). Consequently, a temporary reduction in PRL secretion after burn trauma may minimize the tissue damage resulting from neutrophils released from the bone marrow in response to the injury (31). In human, the GH molecule binds to and activates the PRL-R in neutrophils (29). This interaction may explain the observation that high serum hGH during the first 6 h after a burn are associated with a poorer patient prognosis (7).

In a previous study using mice, Neely et al. (32) saw a raise of serum IL-6 levels 8 h after burn injury. As for GH, high post-burn IL-6 levels are correlated with higher patient mortality (33). GC is known to inhibit IL-6 mRNA expression in the rat (34), but IL-6 production is significantly increased by both gut-derived macrophages and enterocytes after thermal injury (35). The effects of GH and PRL on IL-6 expression are less clear. GH reduced gut bacteria translocation or spreading and hypermetabolic response through IGF-1 stimulation (36, 37). IGF-1 could thus also be considered as a therapeutic molecule.

The resulting effects of such interactions could be mediated by STAT5 and STAT5 phosphorylation in the gut. STAT5 activity was assessed by quantifying total STAT5 and phosphorylated STAT5 in total protein extracts from the jejunum.

First, the absence of a well-defined circadian pattern for STAT5 and pSTAT5 seemed to indicate that STAT5 synthesis and activation in the gut are probably not dependent on one predominant factor (PRL or CS for example). This would be consistent with the hypothesis that STAT5 is an integrator of several factors. Also, levels of STAT5 were significantly reduced in sham-treated animals and increased in burn-treated animals 1.5 h after burn injury. Although STAT5 tended to be higher in the gut of burned animals at all times of the day, the values were quite variable in the burned animals. In contrast with CS and PRL, STAT5 does not seem to be modified after burn injury in a way typical of a stress response. The levels of phospho-STAT5 were very similar in control, sham-treated, and burn-treated animals, except during a period of time between 8 and 24 h after burn injury. At those times, burn injury seems to cause an elevation of pSTAT5 levels above the control levels. This may reflect a generally higher level of cytokine stimulation that occurs several hours after the burn but not during the initial stress phase.

These results could be interpreted as follows. By 8 h after burn injury or sham treatment, when CS levels are reduced and PRL levels are back to normal, STAT5 phosphorylation is increased. Loss of gut barrier integrity occurs by 2 h after burns (38), which coincides with a high ratio of CS to PRL, but does not correlate with a modified STAT5 phosphorylation level. We conclude that STAT5 activity is unlikely to be an important regulator of gut pathology during the first few hours after a burn.

In conclusion, we showed in male Sprague-Dawley rats first that hormonal response to burn injury during the first hours after burn injury is partially typical of a response to stress. We showed a rise of CS levels and a reduction of PRL levels in the serum during 4-6 h after burn injury. A link with the immune effects of these hormones could suggest that it would be inappropriate to use PRL timing the first hours after burn injury in a therapeutic protocol, because it could trigger undesired neutrophil activation in the blood and inflammatory processes. We also showed that the changes in STAT5 and phospho-STAT5 are probably not more than minor contributors to the burn-induced gut modifications. Nevertheless, closely related factors could be modulated by burn injuries. For example, the suppressor of cytokine signaling-3 (SOCS-3) factor, a STAT5 inhibitor, is modified post-burn (39) and could exert significant effects on the STAT5 signaling and activity.

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REFERENCES


