

Hyperglycemia Exacerbates Burn-Induced Liver Inflammation via Noncanonical Nuclear Factor- κ B Pathway Activation

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Hyperglycemia and inflammation are hallmarks of burn injury. In this study, we used a rat model of hyperglycemia and burn injury to investigate the effects of hyperglycemia on inflammatory responses in the liver. Hyperglycemia was induced in male Sprague-Dawley rats with streptozotocin (STZ) (35–40 mg/kg), followed by a 60% third-degree scald burn injury. Cytokine levels (by multiplex, in cytosolic liver extracts), hormones (by enzyme-linked immunosorbent assay (ELISA), in serum), nuclear factor (NF)- κ B protein deoxyribonucleic acid (DNA) binding (by ELISA, in nuclear liver extracts) and liver functional panel (using VetScan, in serum) were measured at different time points up to 7 d after burn injury. Blood glucose significantly increased after burn injury in both groups with different temporal patterns. Hyperglycemic rats were capable of endogenous insulin secretion, which was enhanced significantly versus controls 12 h after burn injury. DNA binding data of liver nuclear extracts showed a robust and significant activation of the noncanonical NF- κ B pathway in the hyperglycemic versus control burn animals, including increased NF- κ B-inducing kinase expression ($p < 0.05$). Liver acute-phase proteins and cytokine expression were increased, whereas secretion of constitutive proteins was decreased after burn injury in hyperglycemic versus control animals ($p < 0.05$). These results indicate that burn injury to the skin rapidly activated canonical and noncanonical NF- κ B pathways in the liver. Robust activation of the NF- κ B noncanonical pathway was associated with increased expression of inflammatory markers and acute-phase proteins, and impaired glucose metabolism. Hyperglycemia is detrimental to burn outcome by augmenting inflammation mediated by hepatic noncanonical NF- κ B pathway activation.

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INTRODUCTION

Hyperglycemia occurs in critically ill patients (transplant, surgical, trauma, burns) and has been associated with adverse clinical outcomes (1). Tight euglycemic control in these patient populations has been shown to be beneficial,

with decreased incidence of infection, decreased acute hospital stay and decreased morbidity and mortality (1). Critically ill patients with hyperglycemia have a higher incidence of infection and sepsis, both in the adult population (18–55 years old) and in children (2,3). In

patients with extensive burn injury, control of hyperglycemia is associated with better graft survival and a decrease in resting energy expenditure (4,5). Hyperglycemia has been associated with significant risk for wound infection, pneumonia and bacteremia in burn injury patients (6). Hyperglycemia was shown to be associated with impaired wound healing by decreasing tensile wound strength (7) and with reduced graft success in patients with hyperglycemia compared with patients with adequate glucose control (8,9). Massive release of cytokines in systemic circulation after burn injury can lead to distant organ damage (10).

The correlation between glucose control and outcomes of critically ill patients

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has also been studied in animal models. Heuer *et al.* (11) reported that hyperglycemic and septic rodents had higher levels of cytokines/chemokines, serum organ damage markers and reduced survival. In a rabbit model of critical illness, elevated blood glucose induced by alloxan administration evoked cellular glucose overload, inducing mitochondrial dysfunction (12). Maintenance of normoglycemia, but not hyperinsulinemia, protected against mitochondrial damage in the liver, myocardium and kidney (12). Zhang *et al.* (13) reported that insulin administration increased wound protein and deoxyribonucleic acid (DNA) synthesis in a rabbit model of burn injury. Consequently, tight euglycemic control has become the standard of care in intensive care units worldwide, although the desirable target range for glucose in these patients remains controversial. Importantly, whether insulin administered to maintain euglycemia or the reduced glucose burden on cells (glucose cytotoxicity) is responsible for beneficial effects is still unknown.

The literature investigating the effects of burn on NF- κ B activation in the liver is scarce. Chen *et al.* (14) showed increased DNA binding activity of NF- κ B proteins in the liver after 30% total body surface area burn in rats and morphological alterations. A study from our group (15) showed an increase of NF- κ B proteins in hepatocytes harvested from rats that received a 40% burn injury. Nishiura *et al.* (16) showed activation of the NF- κ B pathway within 30 min after burn injury in mice. The liver plays central roles in metabolism, inflammation and acute response to trauma or injury and is pivotal to patient survival and recovery. Therefore, the current study focuses on effects after burn injury in the liver (17). Here, we used an animal model of streptozotocin (STZ)-induced hyperglycemia to further explore mechanisms of how hyperglycemia interacts with burn injury (a two-hit model) to activate inflammation. We found significant alterations of the noncanonical NF- κ B signaling pathway, leading to in-

creases in acute-phase protein production, decreases in constitutive proteins and massive increases in cytokine secretion. These phenomena were augmented by hyperglycemia. Severe burn leads to profound dysregulations in many organs for prolonged time periods that may be linked to increased activation of the NF- κ B noncanonical pathway.

MATERIALS AND METHODS

Animal Model

We used a two-hit animal model, including STZ-induced diabetes plus burn, to investigate relationships between hyperglycemia and inflammation after severe burn injury. Our animal use protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch (Galveston, TX, USA). Animal care and handling was in accord with National Institutes of Health guidelines. Male Sprague-Dawley rats (Harlan Laboratories, Houston, TX, USA), 250–300 g, were allowed to acclimatize for 5–7 d before induction of diabetes with STZ (Sigma Aldrich, St. Louis, MO, USA). The optimal STZ dose was determined in preliminary studies as a compromise between increased mortality at the time of burn injury and severity of hyperglycemia. Hyperglycemia was induced by injecting 37 mg/kg STZ in ice-cold sodium (Na) citrate buffer (pH 4.5) intraperitoneally twice. We conducted preliminary studies (data not shown) in which we administered various doses of STZ (55, 45 and 35 mg/kg). We chose 37 mg/kg because it induced hyperglycemia without the dramatic body weight loss associated with higher doses, which is a major confounding variable that we did not want to add to the study, since burned animals usually lose 10–20% of their body weight. Our model is a hyperglycemia model, since our STZ did not destroy all insulin secretion. This was important to us, since we wanted to study the effect of preexisting hyperglycemia on burn responses.

Nonfasting blood glucose (measured by tail snip and glucometer) and body

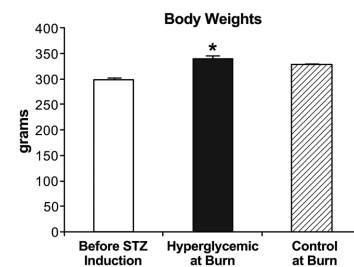


Figure 1. Body weights. The induction of hyperglycemia produced a mild model of type 1 diabetes with a 14% weight increase during the 6 wks from induction of hyperglycemia with STZ to the burn injury. At the time of burn, weights in the hyperglycemic group were significantly higher than at baseline (*t* test $p < 0.001$). To produce a uniform burn injury, the experimental design used “weight-matched” rather than “age-matched” experimental groups. *Significant difference for hyperglycemia versus control.

weights were monitored weekly for 6 wks after induction of diabetes, at which time, one-half of the control and hyperglycemic rats were randomized into burn groups. Rats were subjected to 60% total body surface area scald injury under deep anesthesia with ketamine and xylazine mixture (50–90 and 5–10 mg/kg, respectively, injected intraperitoneally) as described previously (18). The animals were immobilized in a prefabricated mold to expose approximately 30% of the body surface area and immersed in a water bath at 94–98°C for 10 s on the back and 2 s on the abdomen, resulting in a 60% total body surface area (TBSA) burn. Lactated Ringer solution at 40–50 mL/kg was administered by intraperitoneal injection for resuscitation. Animals were allowed to recover in 100% oxygen in a warm environment. Buprenorphine (0.05 mg/kg injected subcutaneously) was used for analgesia. Supplemental buprenorphine was administered if rats displayed signs of pain or discomfort, according to an Institutional Animal Care and Use Committee (IACUC)-approved rat health score. Rats were transferred to individual cages when fully recovered and were monitored twice daily after the burn injury for the

remainder of the study. Rats from each experimental group (n = 5–12 animals) were euthanized at different time points after burn injury by decapitation without anesthesia (19), and blood and organs were harvested for biochemical analysis. Samples were flash-frozen in liquid nitrogen and stored at -80°C until assayed.

Protein, Glucose and Insulin Analysis

A panel of functional parameters consisting of albumin, alkaline phosphatase, calcium, blood urea nitrogen (BUN), glucose and total protein were analyzed in serum samples using a VetScan 2 instrument (Abaxis, Union City, CA, USA). Endogenous insulin levels were measured in serum samples using an enzyme-linked immunosorbent assay (ELISA) kit (Millipore, Billerica, MA, USA) specific for rat insulin, according to the manufacturer's instructions.

Preparation of Subcellular Extracts

Cytosolic and nuclear fractions were extracted from liver tissue using a Dounce homogenizer followed by sequential resuspension in nondetergent low-salt sucrose and high-salt solutions to obtain cytosolic and highly purified nuclear extracts as previously described (20). Total protein content of the samples was measured using bicinchoninic acid (BCA) protein assay (Pierce/Thermo-Fisher, Rockford, IL, USA).

Western Blotting

Whole tissue extracts were separated on NuPAGE[®] Bis-Tris (Invitrogen, Carlsbad, CA, USA) gradient gels, transferred to nitrocellulose membrane and probed with antibodies as previously described (18). Detection was accomplished using enhanced chemiluminescence (ECL) reagents (Pierce/Thermo-Fisher), and imaging and analysis of the blots was performed using an UltraLum Omega 12Ci instrument and Ultraquant software (Claremont, CA, USA).

DNA Binding Assay

DNA binding capacity of the NF- κ B proteins in the nuclear fraction was mea-

sured using a Universal transcription factor assay (Millipore). Briefly, nuclear fractions are incubated with annealed oligonucleotides containing the consensus binding sequence for *RelA*, *RelB*, *p50* and *p52*. An appropriate primary antibody was added for 1 h, followed by a secondary antibody conjugated with horseradish peroxidase. Colorimetric reaction is initiated by adding tetramethylbenzidine to the plate for 10–15 min, followed by 2N sulfuric acid. Optical density of the solution was read at 450 nm with correction at 570 nm. Performance of the assay was assessed by comparing optical density of the sample wells with optical density of competitor, positive and negative probes.

Cytokine Assay

Cytokine content of the cytosolic fraction was measured using a multiplex kit (Millipore) according to the manufacturer's instructions. We used a custom kit containing beads coated with antibodies against rat interleukin (IL)-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p70), IL-13, IL-17, IL-18, granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (IFN)- γ , eotaxin, granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein-1 (MCP-1), leptin, macrophage inflammatory protein-1 α (MIP-1 α), IFN- γ -induced protein 10 kDa (IP-10), Gro/KC, RANTES and vascular endothelial growth factor (VEGF). Briefly, beads loaded with specific antibodies were incubated with 50 μL cytosolic extract overnight at 4°C in the dark on a shaker. The plate was washed three times with wash buffer using a vacuum manifold, and detection antibodies were added for 2 h at room temperature. The plate was washed three times, and streptavidin-R-phycoerythrin was added for 30 min. After a final wash step, beads were resuspended in 150 μL sheath fluid and mixed for 30 s at maximum speed on the shaker. Fluorescence intensity was measured using a Luminex 100 instrument (Austin, TX, USA) using the manufacturer's suggested settings.

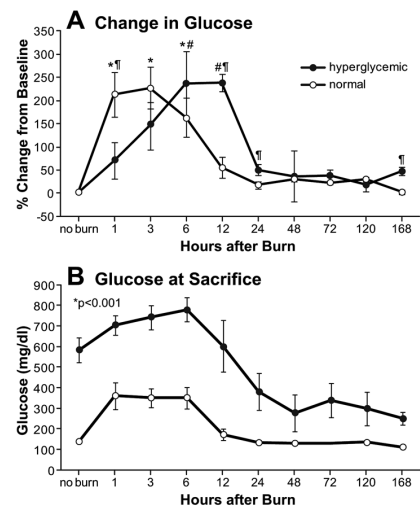


Figure 2. Changes in serum glucose after burn (A). Glucose was measured in a serum sample at the time of euthanasia using a VetScan 2 instrument (B). Each time point represents n = 5–10 separate animals per experimental group. Data from hyperglycemic and control groups were compared over time by two-way ANOVA. Changes within each group were analyzed by one-way ANOVA with *post hoc* correction and compared with sham, no burn (* for hyperglycemic group and # for control group). Differences in change in glucose at each time point between hyperglycemic and control animals were analyzed by Tukey *t* test (¶).

Statistical Analysis

Statistical analysis was performed by using (a) two-way analysis of variance (ANOVA) between hyperglycemic and normal groups over time, (b) one-way ANOVA with *post hoc* correction for the data within groups over time and (c) one-way ANOVA with pair-wise comparison where appropriate. The latter was used to compare values between hyperglycemic and normal values at individual time points. Statistical significance was accepted at $p < 0.05$. The software package SigmaPlot 11.0 (Systat Software, Chicago, IL, USA) was used for all analyses. Data are presented as the mean \pm standard error of the mean.

All supplementary materials are available online at www.molmed.org.

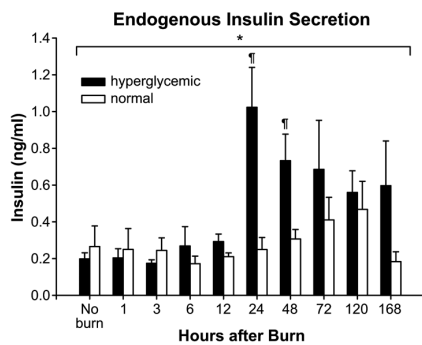


Figure 3. Endogenous insulin at time of euthanasia. Insulin levels were measured in the serum from blood collected at euthanasia by ELISA. Each time point represents $n = 5-10$ separate animals per experimental group. Data from hyperglycemic and control groups were compared over time by two-way ANOVA (*). Changes within each group were analyzed by one-way ANOVA with *post hoc* correction and compared with sham, no burn (¶ for hyperglycemic group).

RESULTS

Body Weight

Hyperglycemic rats gained 14% body weight during the 6–8 wks from STZ injection to the burn procedure (Figure 1), which was significantly different (t test, $p < 0.001$). Animal body weights at the time of burn injury were similar in both the control and hyperglycemic groups, indicating adequate matching-pairing on the basis of body weight and not on age. Our primary aim for weight matching was that the percent skin area burn inflicted with our prefabricated molds is calculated on the basis of weight. We also showed in clinical studies that a main factor determining the outcomes of burn injury is burn size. To have the same severity of injury, we decided to weight-match the experimental groups. At the time of burn injury, animals in the normal burned group were 10–11 wks old, whereas hyperglycemic rats were 12–14 wks old.

Plasma Glucose and Insulin Secretion

As shown in Figure 2, there were comparable changes in serum glucose levels

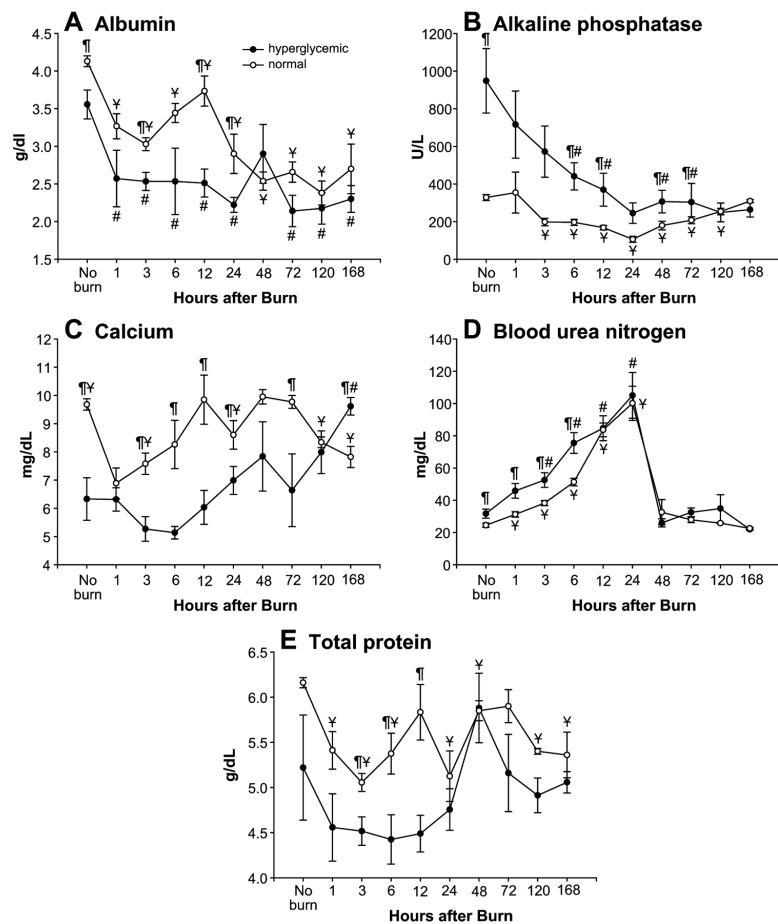


Figure 4. Liver function markers. Liver proteins were measured in the serum collected at the time of euthanasia using a VetScan 2 instrument. Each time point represents separate animals ($n = 5-10$) per experimental group. Data from hyperglycemic and control groups were compared over time by two-way ANOVA (*). Changes within each group were analyzed by one-way ANOVA with *post hoc* correction and compared with sham, no burn (# for hyperglycemic group and x for control group). Differences at each time point between hyperglycemic and control animals were analyzed by Tukey t test (¶¶).

expressed as percent change from baseline after burn injury in both groups, although the baseline value in the STZ-treated group vastly exceeded the control group (shown as a bar graph on the left side of Figure 2), and the peak response was delayed in the hyperglycemic group. At the time of burn injury, non-fasting blood glucose values were 405 ± 22 mg/dL for hyperglycemic animals and 89 ± 3 mg/dL for normal animals.

There were significant differences in blood glucose values of hyperglycemic and normal animals (measured at the time of sacrifice) throughout the study

period. An increase in glucose levels occurred within 1 h after burn injury in normal animals, reaching a peak at 3 h. In hyperglycemic animals, the peak in glucose levels occurred 6 h after burn.

Although the high-dose STZ-induced hyperglycemic rat is normally the prototypical model for type 1 diabetes, the animals in our study that received low-dose STZ still had endogenous insulin secretion after STZ injection (Figure 3), indicating that the STZ dose did not destroy all pancreatic β -cells, resulting in a hyperglycemic rat model. After burn injury, there was an increase in endoge-

nous insulin release, which was significantly greater and occurred more abruptly at 24 h in the STZ-treated versus control rats. We observed no significant difference in insulin levels between hyperglycemic nonburned and normal nonburned animals at baseline.

Liver Markers

Thermal injury produced profound alterations in hepatic homeostasis, with increases in liver acute-phase proteins and decreases in constitutive protein (such as albumin; Figure 4A). Markers of liver damage, such as liver enzymes (alkaline phosphatase, Figure 4B) were significantly increased in hyperglycemic animals before burn injury but decreased to normal values by the end of the study period. There were significant changes in serum levels of calcium (Figure 4C) in both groups over time. Baseline calcium levels were lower in the hyperglycemic group. Impaired glucose metabolism and depletion of amino acid pools led to protein degradation for fuel, as shown by increased BUN (Figure 4D). In both experimental groups, BUN values rapidly returned to baseline levels 24 h after burn injury. Albumin and total protein content (Figures 4A and E) in the hyperglycemic group were significantly lower compared with normal animals.

DNA Binding Assay

There was no difference over time between hyperglycemic and normal groups in the DNA binding of the canonical pathway proteins *RelA* and *p50* (two-way ANOVA). DNA binding data, confirmed by Western blotting (data not shown), demonstrated a robust and rapid increase in DNA binding and expression of the noncanonical NF- κ B pathway proteins *RelB* and *p52*, before returning to control levels within 6–8 h after burn injury. Baseline binding for *RelB* and *p52* was not different between groups, but there were significant differences between binding of NF- κ B proteins at individual time points (Student *t* test) (Figure 5).

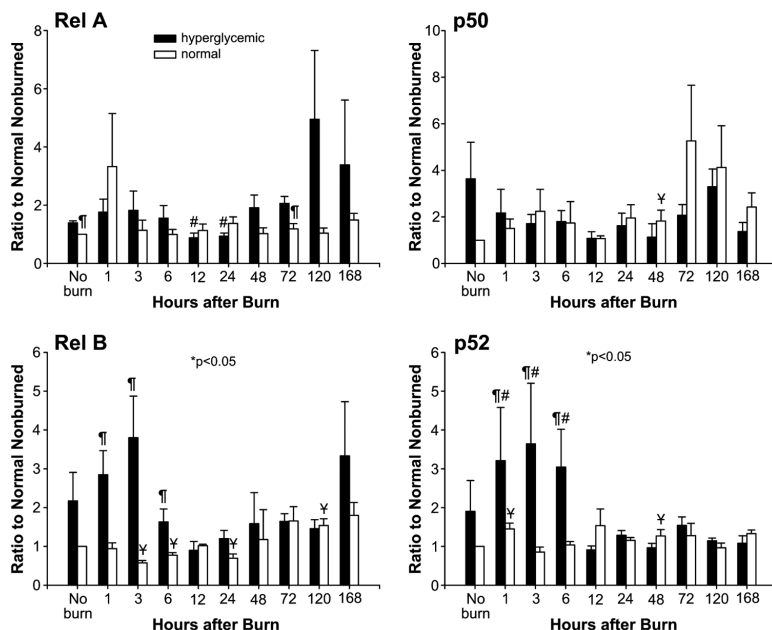


Figure 5. DNA binding assay. DNA binding was assayed in the liver nuclear fractions harvested at different time points after burn injury (n = 4–8 separate animals for each group per time point). Data from hyperglycemic and control groups were compared over time by two-way ANOVA (*). Changes within each group were analyzed by one-way ANOVA with *post hoc* correction and compared with sham, no burn (# for hyperglycemic group and x for control group). Differences at each time point between hyperglycemic and control animals were analyzed by Tukey *t* test (¶).

Western Blot Data

Western blot analysis was used to assess expression of NF- κ B-inducing kinase (NIK). Figure 6 quantifies four to eight Western blots for each time point. NIK levels in the liver demonstrated a significant increase over time in the hyperglycemic group after burn injury, which was attenuated in the control group (see Figure 6).

Liver Cytosolic Cytokines

NF- κ B-mediated cytokine levels were analyzed in the liver cytosolic fraction. These data demonstrated that the NF- κ B response in the liver to burn injury on the skin occurred within 1 h after burn injury. Eight of the cytokines analyzed (IL-1 α , IL-6, IFN- γ , TNF- α , eotaxin, G-CSF, RANTES and VEGF) are shown in Figure 7. The response to burn injury was an immediate and highly significant increase in cytokine production in the hyperglycemic versus control animals,

which persisted for up to 24–48 h before returning to control and baseline levels. Interestingly, the cytokine production profile for RANTES demonstrated an opposite pattern, with levels higher over time after burn injury in the control group, but decreased significantly over time in the hyperglycemic group. RANTES is transcribed from a non-canonical κ B inhibitory promoter. Maximum decreases in the latter group were evident between 48 and 72 h post-burn. The response to burn injury within the control group was more gradual, with some cytokines showing significant increases over the first 24 h (IL-1 α , IL-6, eotaxin, RANTES, VEGF), whereas other cytokines were unaffected (IFN- γ , TNF- α , G-CSF). Supplementary Figure S1 demonstrates that other cytokine profiles were also significantly dysregulated between groups, including IL-4, IL-5, IL-9, IL-10, GM-CSF, IL-13, IL-17 and IL-18. No significant differences existed in the

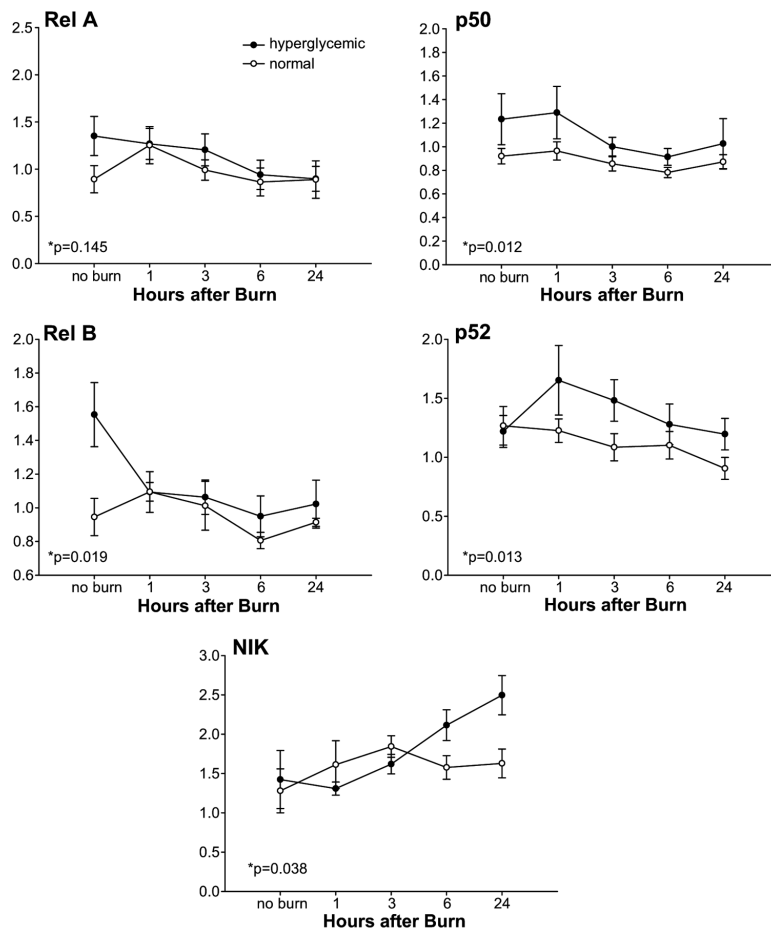


Figure 6. NIK protein levels in the same nuclear extracts used for DNA binding assay were compared by Western blot ($n = 4-8$ blots, each sampled from a separate animal per group per time point). Data from hyperglycemic and control groups were compared over time by two-way ANOVA (*). Changes within each group were analyzed by one-way ANOVA with *post hoc* correction and compared with sham, no burn (# for hyperglycemic group and \times for control group). Differences at each time point between hyperglycemic and control animals were analyzed by Tukey t test (\square).

expression of MIP-1 α , leptin, MCP-1, IL-16, IL-2, IL12(p70), IP-10 and Gro/KC over the study period.

DISCUSSION

In this study, we evaluated the effects of a thermal injury on the liver of hyperglycemic animals and investigated whether this two-hit animal model more closely mimicked human clinical findings than the one-hit model of burn injury. We explored the expression of NF- κ B proteins in the acute phase post-burn. NF- κ B is a fast-response, inflammatory signaling pathway because the proteins

are constitutively expressed in the cells, and activation of NF- κ B does not require *de novo* protein synthesis. Activation of the NF- κ B canonical pathway leads to transcription of inhibitory proteins I κ B to terminate the response, whereas the non-canonical pathway is I κ B independent. Our experimental design used animals that were weight-matched rather than age-matched because our prefabricated molds used for burn injury have the area exposed calculated from total body surface area and weight. To produce the same degree of injury, animals must be weight-matched. Because of the impaired

rate of weight gain in the hyperglycemic animals, this meant that our nonhyperglycemic animals were slightly (2–4 wks) younger in age.

Our two-hit model included hyperglycemia, which was induced by STZ injection, and skin burn injury. The dose of STZ represented a compromise between the degree of hyperglycemia produced and the extent of mortality after burn injury. The burn injury in this model was 60% of total body surface area, and up to 80% of the animals with this burn injury and with nonfasting blood glucose levels >400 mg/dL at the time of burn injury did not survive (unpublished observations, GA Kulp, RG Tilton, DN Herndon, and MG Jeschke) with STZ doses >50 mg/kg. In this study, the mortality of the two-hit model was <10%. Plasma insulin levels measured at euthanasia demonstrated that STZ-treated animals were capable of endogenous production and were able to elicit an increase in plasma insulin levels after the burn injury. There was no difference between hyperglycemic nonburned and normal nonburned animals. One possible explanation is that the hyperglycemic animals have hyperphagia (overeating), so insulin secretion in this case might be stimulated by food intake more than in normal rats. STZ-treated animals had very high glucose levels after burn (on average, 779 ± 151 mg/dL at 6 h after burn). Such high glucose levels are associated with dehydration and hypovolemia. In this study, glucose values peaked at 6 h after burn, before returning to preburn levels. In our experience, adequate resuscitation after burn (50 mL/kg lactated Ringer) compensates dehydration until the animals resume normal drinking and feeding (about 3–4 h after burn). We did not observe any animals that were “dry” at euthanasia (meaning low blood volume collected by decapitation), and they did not show signs of major pain or discomfort. Water consumption for the animals euthanized at later time points was monitored visually twice a day during rounds, and bottles were refilled if necessary.

Similar to our findings in a patient population (21), liver homeostasis was profoundly affected after burn injury to the skin, as shown by increases in acute-phase protein secretion and decreases in constitutive protein production. Liver is the main organ where constitutive and acute-phase protein synthesis, gluconeogenesis and glycogenolysis occur. Perturbations of homeostasis in the liver result in concomitant activation of multiple signaling pathways as a response to the burn injury and recovery (18,22). In the acute phase after injury, there is a shift in the protein secretory profile of the liver, toward acute-phase proteins, leading to an increased burden on the folding capacity of endoplasmic reticulum (18), contributing to hepatic insulin resistance.

We found significant differences between the two burn groups in NIK expression during the first 24 h after burn. NIK is an upstream kinase that plays an important initiating role in activation of both canonical and noncanonical NF- κ B pathway (23), and it has been shown to be phosphorylated by various kinases, such as MKK3/6. MKK3/6 may serve as a link between the very early, burn-induced catecholamine signaling and NF- κ B activation, which could partly explain the fast response through these pathways. It also has been shown that NF- κ B pathways can potentiate each other's activity (24). Massive release of catecholamine in the circulation is a hallmark of burn injury and persists for a prolonged period of time (25). This result might explain the prolonged increases in cytokine expression seen in clinical studies.

NF- κ B is a family of highly inducible cytoplasmic DNA binding proteins that includes the transactivating subunits RelA, RelB and c-Rel and the post-translationally processed DNA binding subunits NF- κ B1 (p50) and NF- κ B2 (p52) (26). The NF- κ B dimers remain sequestered in the cytoplasm by interacting with inhibitory ankyrin repeat-containing proteins, collectively called I κ Bs (I κ B α , I κ B β , I κ B γ , p100 and p105) (27). Recently, it was shown that NF- κ B activation can be controlled by at least two

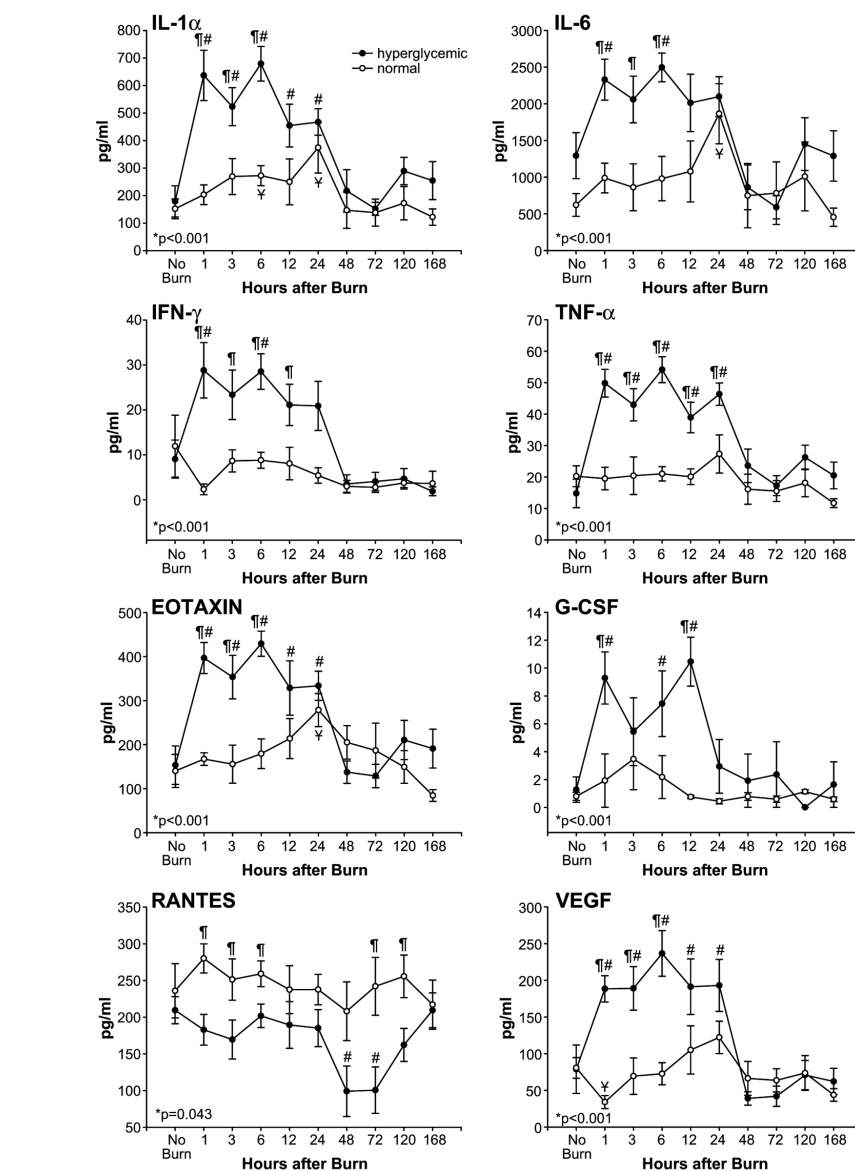


Figure 7. Liver cytosolic cytokine levels. Cytokine levels were measured in cytosolic extracts of liver harvested at multiple times after burn injury using a 24-plex assay (n = 5–10 separate animals for each group per time point). Although most of the 24 cytokines measured showed significant changes between the two groups over time (two-way ANOVA), we report results for eight representative cytokines. Data from hyperglycemic and control groups were compared over time by two-way ANOVA (*). Changes within each group were analyzed by one-way ANOVA with *post hoc* correction and compared with sham, no burn (# for hyperglycemic group and x for control group). Differences at each time point between hyperglycemic and control animals were analyzed by Tukey *t* test (∇).

separate and independent pathways: the “canonical pathway” mediated by the I κ B kinase (IKK, a complex of two catalytic subunits, IKK α and IKK β , and the regulatory subunit, IKK γ) (28) and the “noncanonical pathway” mediated by a

complex of IKK α and NIK. The former plays an important role in the host innate immune response by inducing the expression of numerous proinflammatory cytokines, chemokines, adhesion molecules, inducible enzymes and pro-

angiogenic growth factors, as well as inhibiting apoptosis (29–32). The latter, more recently identified noncanonical pathway plays an important role in the adaptive immune response, including secondary lymphoid organogenesis, the induction of genes involved in this process and lymphocyte maturation (33–35). Stimuli activating NF- κ B via the canonical pathway stimulate IKK, resulting in I κ B α phosphorylation at specific N-terminal serine residues targeting them for proteasomal degradation (36). This process releases sequestered RelA:p50 to enter the nucleus. In contrast, the noncanonical pathway produces RelB:p52 complexes that enter the nucleus. Recently, it was observed that the noncanonical pathway can be activated in response to specific stimuli, including lymphotoxin β (37,38), CD40 ligand (39), DNA virus infection (40) and B-cell-activating factor (35,38,41,42). Interestingly, neither IKK γ nor IKK β , key regulators of the canonical pathway, are required for activation of the noncanonical pathway (35,40). Rather, a kinase complex consisting of NIK and IKK α activates posttranslational processing of the p52 precursor, p100, into the 52-kDa active DNA binding isoform. Activators of the noncanonical pathway stimulate IKK α activity that, in turn, phosphorylates specific serine residues in p100, targeting its COOH terminus for ubiquitination and proteasomal processing to generate active p52. Newly formed p52 then dimerizes with cytoplasmic RelB and translocates into the nucleus. In this pathway, NIK serves to activate IKK α as well as providing a docking site to recruit both p100 and IKK α into a complex (38). NIK therefore is an essential component of the noncanonical NF- κ B activation pathway.

Correlations between temporal patterns of expression of acute-phase proteins, cytokines and insulin were observed in this dataset. One possible explanation is that the time point around 24 h after injury is crucial for the recovery of animals. It will be interesting to study in more detail what hap-

pens in these animals at this time point. In clinical (5) and animal (43) studies, we showed that insulin administration has antiinflammatory effects in burned patients (decrease in cytokine) and also decreases resting energy expenditure (decrease in BUN levels, for example, which are an indicator of the amount of protein that is used as fuel for energy production). The explanation for these observations remains unknown at this time. Changes in glucose found in this study show that hyperglycemic animals still elicit the same glucose response to burn injury as normal animals. At the same time, these changes were accompanied by a significant increase in cytokine production in hyperglycemic animals compared with normal burned rats. This correlation suggests that hyperglycemia in response to burn injury exacerbates inflammatory response. In support of this hypothesis, we have shown that administration of insulin after burn in both patients and animals (22,43) reduces the secretion of inflammatory serum markers.

Rodent models of burn injury reproduce the acute-phase physiologic response to severe burn injury but not the prolonged increase in inflammatory markers, such as cytokines and acute-phase proteins, observed in humans. In burn wound healing studies, reepithelialization is complete in 14–18 d in rodents (unpublished observations, GA Kulp, DN Herndon, and MG Jeschke; 44), even without acute escharotomies. In contrast to burned patients who require years of reconstructive surgery and rehabilitation, rodents do not develop hypertrophic scars or contractures. More detailed studies in rodent models of severe burn injury might provide insights into mechanisms to counteract the detrimental long-term effects seen in patients.

Inflammation and hyperglycemia/insulin resistance are concomitant events occurring after burn injury. It is not well understood if inflammation causes hyperglycemia, with subsequent insulin resistance, or hyperglycemia occurs first and causes inflammation. We developed

a “two-hit” model to explore the question of whether hyperglycemia induces inflammation or inflammation is responsible for the impaired glucose metabolism in critical illness. On the basis of the temporal expression patterns of cytokines and glucose changes after severe burns, our data suggest that hyperglycemia and inflammation might be independent processes that converge, resulting in insulin resistance and impaired glucose metabolism. On the basis of our results, we cannot rule out if stress hyperglycemia is a result of inflammation. Even when hyperglycemia is a preexisting condition, as in our hyperglycemic rats, there is still an increase in glucose levels after burn injury, concomitant with an increase in cytokine expression. Preexisting hyperglycemia only exacerbates this response after a burn.

There are other signaling pathways responsible for the persistence of hypermetabolism, inflammation and insulin resistance after burn injury, including endoplasmic reticulum stress, unfolded protein response and jun-c-kinase pathway activation (18). The effects seen clinically are the result of these concomitant events, but the individual contributions of these pathways and their time sequence have not yet been elucidated. Our data suggest that NF- κ B activation is the earliest event, followed by induction of other inflammatory pathways. It has been shown that inflammatory pathways can potentiate each other, contributing to a vicious cycle of inflammation.

CONCLUSION

We have developed a two-hit rodent model of hyperglycemia plus burn injury that mimics early clinical symptoms found in burned patients. We have demonstrated a robust activation of the canonical NF- κ B signaling pathway, as shown by significant increases in NF- κ B-driven cytokine production and increased secretion of inflammatory markers. Surprisingly, we also have demonstrated a role for the NF- κ B non-canonical activation pathway, as evidenced by increased NIK levels and in-

creased RelB and p52 DNA binding in liver nuclear extracts from hyperglycemic burned animals. Activation of this latter NF- κ B pathway may be responsible for burn-induced dysregulations in glucose metabolism and may contribute to the prolonged inflammatory response observed in burn patients.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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