

Insulin Protects against Hepatic Damage Postburn

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Burn injury causes hepatic dysfunction associated with endoplasmic reticulum (ER) stress and induction of the unfolded protein response (UPR). ER stress/UPR leads to hepatic apoptosis and activation of the Jun-N-terminal kinase (JNK) signaling pathway, leading to vast metabolic alterations. Insulin has been shown to attenuate hepatic damage and to improve liver function. We therefore hypothesized that insulin administration exerts its effects by attenuating postburn hepatic ER stress and subsequent apoptosis. Male Sprague Dawley rats received a 60% total body surface area (TBSA) burn injury. Animals were randomized to receive saline (controls) or insulin (2.5 IU/kg q. 24 h) and euthanized at 24 and 48 h postburn. Burn injury induced dramatic changes in liver structure and function, including induction of the ER stress response, mitochondrial dysfunction, hepatocyte apoptosis, and up-regulation of inflammatory mediators. Insulin decreased hepatocyte caspase-3 activation and apoptosis significantly at 24 and 48 h postburn. Furthermore, insulin administration decreased ER stress significantly and reversed structural and functional changes in hepatocyte mitochondria. Finally, insulin attenuated the expression of inflammatory mediators IL-6, MCP-1, and CINC-1. Insulin alleviates burn-induced ER stress, hepatocyte apoptosis, mitochondrial abnormalities, and inflammation leading to improved hepatic structure and function significantly. These results support the use of insulin therapy after traumatic injury to improve patient outcomes.

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INTRODUCTION

A burn injury represents one of the most severe forms of trauma and occurs in over two million people in the United States of America per year (1). The liver, with its metabolic, inflammatory, immune and acute-phase functions, plays a pivotal role in patient outcome and recovery by modulating multiple pathways (2–7). It has been suggested that because the liver modulates metabolic pathways, inflammatory processes and acute-phase responses, limiting hepatic damage after traumatic injury may improve patient outcomes significantly (7). In a recent study, Price and colleagues (8)

looked at outcomes of 290 burned patients who suffered from liver disease prior to burn injury. They showed that preexisting liver disease increased mortality risk from 6% (total population) to 27%. The authors concluded that liver impairment worsens the prognosis in patients with thermal injury (8). This suggests that therapeutic agents, which protect against liver dysfunction after traumatic injury, may improve patient morbidity and mortality significantly.

Hepatic dysfunction after burn injury persists over a prolonged time period (3,7,9,10). At the molecular level, we recently have shown that burn injury leads

to gross alterations in hepatocyte calcium homeostasis (7,11). Burn injury causes depletion of endoplasmic reticulum (ER) calcium stores and increased cytosolic calcium, which subsequently induces mitochondrial damage and cytochrome c release (11). Cytochrome c binds to the ER-resident inositol 1,4,5-trisphosphate receptor (IP₃R) calcium channel, augmenting the depletion of ER calcium stores leading to ER stress and induction of the unfolded protein response (UPR) (7,11). ER stress/UPR due to calcium store depletion leads to activation of the JNK signaling pathway and ultimately hepatocyte apoptosis. Burn injury also induces the hepatic acute phase response and a concomitant decrease in the production of constitutive serum proteins such as albumin (3,7,9,12).

The introduction of insulin and the concept of tight euglycemic control represent a cornerstone of modern critical care medicine (13,14). Studies in severely burned patients conducted by our group

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indicated that insulin improves hypermetabolism by decreasing proinflammatory cytokines and hepatic acute-phase protein production (10,15,16). While insulin administration postburn is known to attenuate hepatic damage and improve liver function (5,17), the underlying mechanisms by which insulin exerts its effects on liver structure and function are not understood. The aim of this study was to determine the molecular mechanisms by which insulin administration improves hepatic structure and function postburn. We hypothesized that postburn insulin administration would improve hepatic function by decreasing ER stress and downstream mitochondrial dysfunction and apoptosis, leading to a marked reduction in acute phase protein production and activation of associated inflammatory pathways.

MATERIAL AND METHODS

Animals

All animal manipulations were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston. The National Academy Press *Guide for the Care and Use of Laboratory Animals* (1996) were met. Sprague Dawley rats, 325 to 350 g, were used in these studies and were allowed to acclimate for 1 wk before experiments. Rats were housed in an institutional animal care facility and received a high-protein diet (Ensure, Abbott Laboratories, Waukegan, IL, USA) and water *ad libitum* throughout the study. Ensure (Abbott) was administered 7 d before the study to adjust the animals to the liquid diet.

Burn Injury

A well-established method was used to induce a full-thickness scald burn in our model (18). Animals were anesthetized with general anesthesia (ketamine 40 mg/kg body weight and xylazine 5 mg/kg body weight, both injected intraperitoneally [i.p.]) and received analgesia (buprenorphine 0.05 mg/kg body weight, injected subcu-

taneously). The dorsum of the trunk and the abdomen were shaved, and a 60% total body surface area (TBSA) burn was administered by placing the animals in a mold exposing defined areas of the skin of the back and abdomen under general anesthesia and analgesia. The mold was placed in 96–98°C water, scalding the back for 10 sec and the abdomen for 2 sec. Lactated Ringer's solution (40 mL/kg body weight) was administered i.p. immediately after the burn for resuscitation. After burn and resuscitation, animals were observed, received oxygen and were then placed into cages. Unburned (sham) animals received the same treatment except for the scald burn.

Animals were pair-fed with a liquid high-protein high-amino acid nutrition (Ensure, Abbott Laboratories) and water *ad libitum*. Ensure has a caloric distribution of 24% protein, 21% fat and 55% carbohydrate. All rats were pair-fed according to the caloric intake. The feeding protocol was as follows: 25 calories on the day of burn or sham, 51 calories on the first day postburn, 76 calories on the second, and 101 calories from the third day postburn on, to the end of the study. Nutritional intake was the same in all groups.

Treatment Groups

Animals received a 60% total body surface area (TBSA) burn and animals were injected once daily with either 2.5 IU/kg body weight of insulin glargine (Insulin Lantus, Sanofi Aventis, Kansas City, MO, USA; insulin group), or an equal amount of saline subcutaneous (control group, n = 10). Animals were killed by decapitation 24 and 48 h after burn injury and blood and liver was collected immediately thereafter (n = 10 for each group and time point). No animals were excluded from the study, and there were no significant differences in mortality rate between the groups.

Serum Markers

Serum was separated by centrifugation at 3,000g for 3 min at 4°C and stored at –80°C until analyzed. The levels of IL-6, cytokine-induced neutrophil chemoat-

tractant (CINC)-1 and monocyte chemoattractant protein (MCP)-1 as markers of systemic inflammation were determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc., Minneapolis, MN, USA) according to the protocol of the manufacturer. Absolute cytokine concentrations were determined by comparison to a standard curve.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting

Liver tissue was homogenized and solubilized in 150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.8, 1% (w/v) Triton X-100, 1 mmol/L EDTA, 0.5 mmol/L phenylmethanesulfonyl fluoride and 1 × protease inhibitor cocktail (Roche Molecular Biochemicals, Basel, Switzerland) for 5 min on ice and spun at 12,000g, and 20 µg of the resultant supernatant was run on a 4% to 20% gradient SDS-polyacrylamide gel and subsequently electrotransferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Nitrocellulose sheets then were probed with primary antibody against phosphorylated inositol requiring enzyme-1 (IRE1), c-Jun N-terminal Kinase (JNK), Thr^{183/185}-phosphorylated-JNK, Thr⁹⁸⁰-phosphorylated PKR-like endoplasmic reticulum kinase (PERK), ATF6, Grp78/Bip, and actin. Blots were developed with chemiluminescent substrates (Pierce Biotechnology, Rockford, IL, USA) and were quantified by densitometry (GENE GENIUS, Bio Imaging System, Syngene, Frederick, MD, USA). After quantification, the intensities of the phosphorylated protein bands were divided by the total form of the respective protein or by the intensity of the respective actin band. In those cases where a single blot was probed sequentially with more than one antibody, the nitrocellulose was stripped at 60°C for 30 min in stripping buffer (2% SDS, 100 mmol/L β-mercaptoethanol, 62.5 mmol/L Tris-HCl) before probing with the next antibody.

Liver Apoptosis

Terminal deoxynucleotidyl transferase (TUNEL) (Apoptag, Oncogene, Balti-

Table 1. Blood glucose levels postburn in mg/dL.^a

	Normal	Burn	Burn + insulin
24 h	95 ± 5	145 ± 11	105 ± 6 ^b
48 h	98 ± 6	138 ± 12	103 ± 7 ^b

^aData presented as mean ± SD.^bSignificant difference between burn versus burn + insulin, $P < 0.05$.

more, MD, USA) staining to identify apoptotic hepatocytes *in situ* was performed as suggested by the manufacturer. Six sections of each liver block were obtained at 40- to 50- μ m intervals. Within each section, a blinded observer selected five fields for counting TUNEL-positive cells. Three blinded observers counted TUNEL-positive cells and the data pooled. The data were quantified as the percentage of apoptotic cells per hundred hepatocytes.

Mitochondrial Isolation and Respiration

Liver tissue (400 mg) was minced on ice and transferred (10% w/v) to isolation buffer (250 mmol/L sucrose, 10 mmol/L HEPES, 0.5 mmol/L EGTA, 0.1% bovine serum albumin [BSA], pH 7.4). The sample was homogenized gently by 3 to 4 strokes with a Dounce homogenizer with a loose fitting pestle. The homogenate

was centrifuged at 500g for 5 min at 4°C. The supernatant fraction was retained, whereas the pellet was washed with isolation buffer and centrifuged again (500g for 5 min at 4°C). The combined supernatant fractions were centrifuged at 7800g for 10 min at 4°C to obtain a crude mitochondria pellet. The mitochondria pellet was resuspended in isolation buffer without EGTA and BSA and centrifuged again at 7800g for 10 min. Oxygen consumption of isolated mitochondria was measured at 25°C by using a model 782 oxygen meter system and model 1302 Microcathode oxygen electrode (Strathkelvin, Glasgow, UK).

Statistical Analysis

All data is presented as the mean ± the standard error of the mean. Statistical significance between two groups was examined with a Student *t* test. A P value < 0.05 was determined to be significant. All data were analyzed by using GraphPad Prism software (GraphPad Software, Inc, San Diego, CA, USA).

RESULTS

We first determined whether insulin at a dose of 2.5 units/kg body weight alter serum glucose levels postburn. We found that insulin significantly decreased glucose levels at 24 h and 48 h postburn

when compared with burn, $P < 0.05$ (Table 1).

We next examined whether insulin administration would improve hepatic apoptosis postburn. As shown in Figure 1A, we found that burn induces a 5- to 7-fold increase in hepatic caspase-3 activation. Insulin significantly attenuated caspase-3 activation compared with burn (Figure 1A). Consistent with burn-induced activation of hepatocyte apoptosis, we found a 5- to 6-fold increase in the number of TUNEL-positive hepatocytes after burn injury, which was inhibited significantly by insulin administration (Figure 1B). Burn-induced hepatic apoptosis was associated with activation of the acute phase response as indicated by a drop in production of the constitutive protein albumin (Figure 1C). Importantly, insulin administration increased serum albumin levels significantly postburn (see Figure 1C).

We next asked whether insulin protects against burn-induced induction of the hepatic ER stress response. Confirming previous studies (11,19), we found that burn injury is a potent inducer of the hepatic ER stress response 24 h after injury (Figure 2A). Burn increased levels of phospho-PERK significantly, cleaved ATF-6, phospho-IRE-1 and phospho-JNK (Figure 2C-F). These effects were signifi-

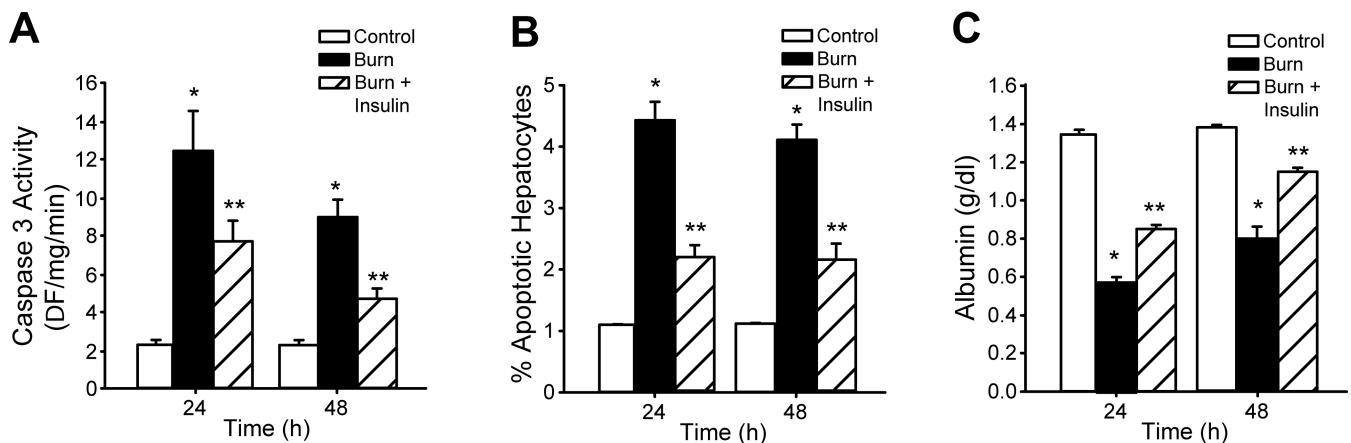


Figure 1. Insulin decreases hepatic apoptosis and increases albumin production after burn injury. (A) Caspase-3 activity in liver lysates from control (white bars), burn (black bars), and burn + insulin (hatch bars) animals 24 and 48 h after burn injury. (B) Percent of apoptotic hepatocytes as determined by TUNEL staining in control, burn and burn + insulin animals 24 and 48 h after burn injury. (C) Serum albumin levels in control, burn, and burn + insulin animals 24 and 48 h after burn injury. * $P < 0.05$ relative to control. ** $P < 0.05$ relative to burn.

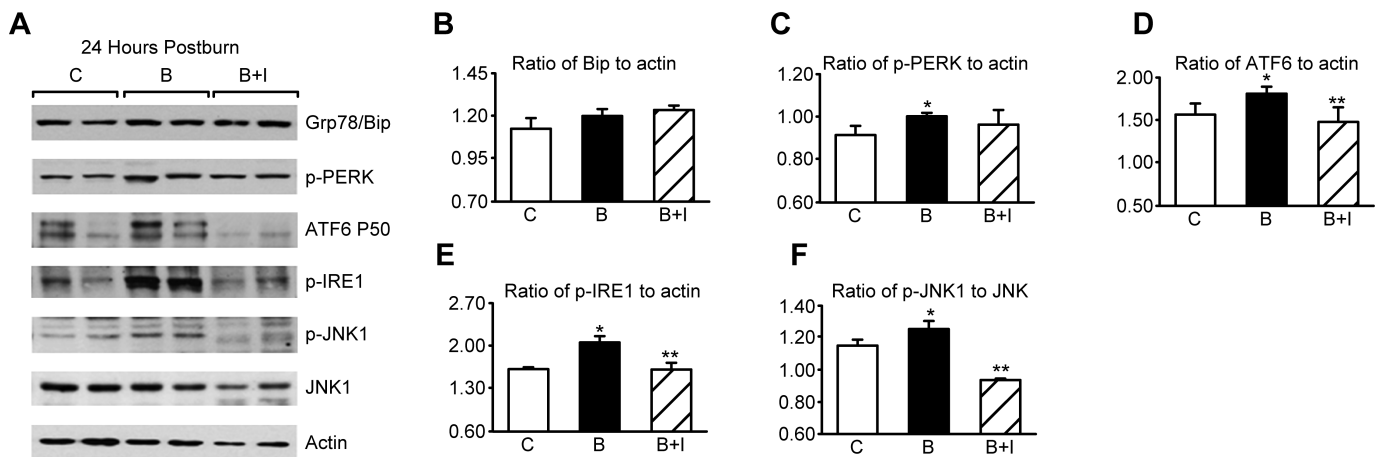


Figure 2. Insulin decreases hepatic ER stress 24 h after burn injury. (A) Representative Western blots from control (C), burn (B), and burn + insulin (B + I) liver lysates 24 h after burn injury. Two separate animals from each group are shown. (B) Densitometric ratio of Grp78/Bip to actin. (C) Ratio of phospho (active) PERK to actin. (D) Ratio of cleaved (active) ATF6 to actin. (E) Ratio of phospho (active) IRE1 to actin. (F) Ratio of phospho (active) JNK to total JNK. * $P < 0.05$ relative to control. ** $P < 0.05$ relative to burn.

cantly inhibited in all cases by insulin administration. At 48 h postburn, similar changes persisted, and we also found significant upregulation of the ER-resident chaperone Grp78/Bip, which is a transcriptional target of ATF6 (Figure 3A–F).

Burn has been shown to cause profound calcium alterations in the ER by depleting ER stores and increasing cytosolic calcium (11). Increased cytosolic calcium has been shown to induce mitochondrial swelling, decrease respiratory

capacity and release of mitochondrial apoptotic factors such as cytochrome c (20–23). Previously we have shown that burn injury causes mitochondrial swelling and decreased state-3 respiration (11). Consistent with our previous studies, by using transmission electron microscopy we found marked alterations in mitochondrial morphology after burn injury (Figure 4A–C). Mitochondrial swelling, loss of cristae (double asterisks; Figure 4B) and loss of matrix electron density (single asterisk, Figure 4B) were

prominent in hepatocytes from burned animals. Furthermore, burn injury was associated with fragmentation of the tubular ER membrane structure (arrows; Figure 4B). Importantly, insulin administration reversed these ultrastructural changes in the mitochondria (Figure 4C). To examine mitochondrial function, we measured state-3 respiration in isolated hepatic mitochondria. We found that burn injury caused a marked impairment in hepatic mitochondria state-3 respiration, and this could be reversed signifi-

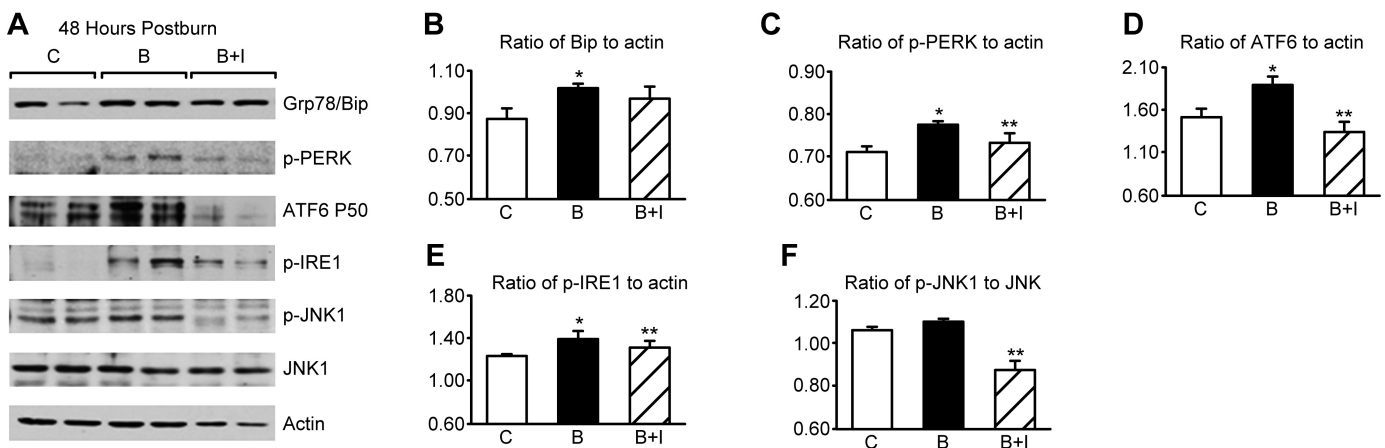


Figure 3. Insulin decreases hepatic ER stress 48 h after burn injury (A) Representative Western blots from control (C), burn (B), and burn + insulin (B + I) liver lysates 48 h after burn injury. Two separate animals from each group are shown. (B) Densitometric ratio of Grp78/Bip to actin. (C) Ratio of phospho (active) PERK to actin. (D) Ratio of cleaved (active) ATF6 to actin. (E) Ratio of phospho (active) IRE1 to actin. (F) Ratio of phospho (active) JNK to total JNK. * $P < 0.05$ relative to control. ** $P < 0.05$ relative to burn.

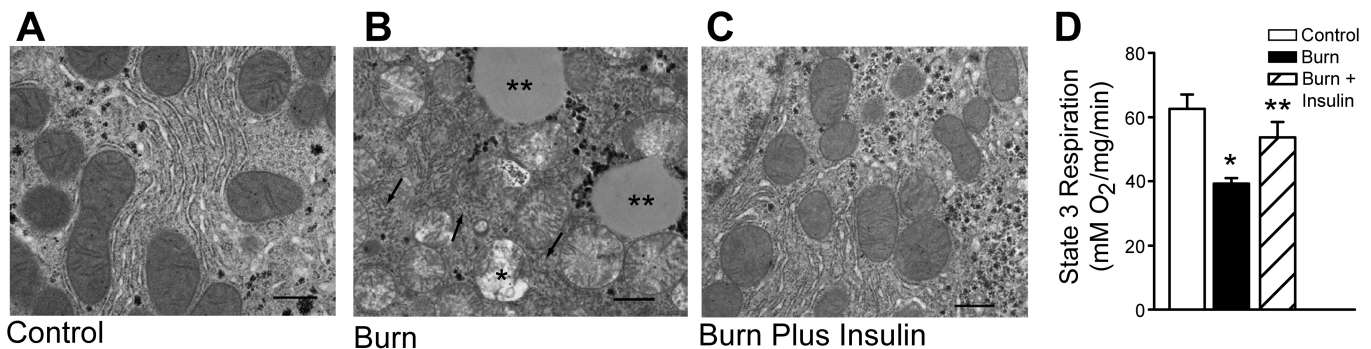


Figure 4. Insulin reverses mitochondrial damage and associated respiratory dysfunction after burn injury. (A) Transmission electron microscopy of hepatocytes from normal liver, (B) hepatocytes from burned animals and (C) hepatocytes from burn-plus-insulin rats. A single asterisk highlights a mitochondrion with significant loss of matrix electron density. Two mitochondria with loss of cristae are highlighted with double asterisks. Arrows indicate fragmented ER. (D) State-3 respiration in isolated mitochondria from control, burn and burn + insulin rats. **P* < 0.05 relative to control. ***P* < 0.05 relative to burn.

cantly by insulin administration (Figure 4D).

Burn injury induces the acute phase response and subsequent production of inflammatory mediators such as IL-6, CINC-1 and MCP-1. We found that burn increases the production of these cytokines 5- to 20-fold compared with control 24 and 48 h after burn injury (Figure 5). Importantly, insulin administration reduces IL-6, MCP-1, and CINC-1 significantly to almost normal levels, indicating that insulin suppresses the inflammatory response potently postburn.

DISCUSSION

The liver with a wide range of central physiologic, metabolic and immune

functions is central for survival in critically ill patients or after a severe injury such as a burn injury (7,9,12). We have shown previously that a burn leads to marked alterations in liver integrity and function associated with gross alterations in ER calcium, with increased cytosolic calcium concentrations and hepatic apoptosis and ER stress/UPR that impact postburn morbidity and mortality (7,9,11,19,24). We now hypothesize that attenuation of hepatic apoptosis and ER stress will be associated with alleviated hypermetabolism and subsequent improved postburn outcome. To test this hypothesis we used an agent that is safe and widely used in the clinical setting, insulin (13,14,16). We found that insulin

administration postburn attenuated hepatic ER stress, which was associated with an improved mitochondrial function, decreased apoptosis and decreased inflammation.

Intensive insulin therapy is beneficial in terms of postburn morbidity (16,25). In a recent prospective randomized trial, we found that intensive insulin therapy decreased the incidence of infections and sepsis significantly, along with dampened acute phase and inflammatory responses (15,16). We also showed that intensive insulin improved hepatic and renal function (15–17). The mechanisms by which insulin causes these effects are not determined, but based on this study and several other studies (5,25,26), all of

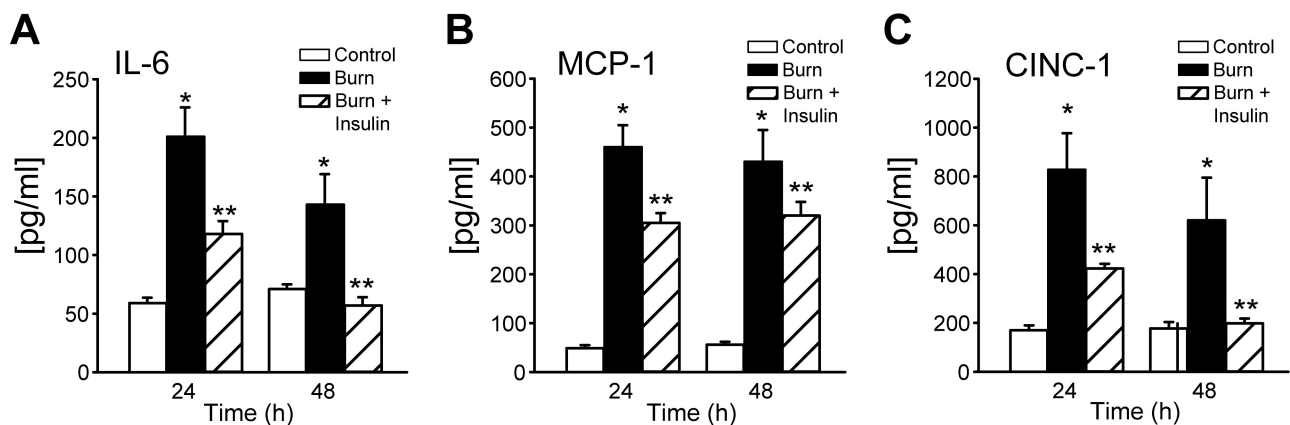


Figure 5. Insulin decreases levels of inflammatory markers after burn injury. (A) IL-6, (B) MCP-1 and (C) CINC-1 levels in control, burn and burn + insulin rats 24 and 48 h after burn injury. **P* < 0.05 relative to control. ***P* < 0.05 relative to burn.

which showed that insulin had anti-inflammatory effects, improved organ function, and decreased incidence of infection and sepsis, we suggest that insulin exerts anti-inflammatory effects and improves organ function by alleviating ER stress/UPR and exerting pro-mitogenic effects in various organs, such as liver, heart and kidney (5,15,16). In the present study, we did not test the hypothesis that an alleviated ER stress/UPR leads to an improved survival after a severe injury, but we speculate, based on a recent study in a burn-sepsis model in which it was demonstrated clearly that insulin improves survival, that a reduced ER stress response is associated with improved morbidity and mortality (26).

The ER, a membranous organelle that functions in the synthesis and processing of secretory and membrane proteins, is critical in the cellular stress response (27). Certain pathological stress conditions disrupt ER homeostasis and lead to accumulation of unfolded or misfolded proteins in the ER lumen (27–29). The ER stress response limits the unfolded protein burden in the ER lumen by inhibiting translation and inducing the nuclear transcription of additional chaperone proteins. If the unfolded protein burden cannot be reversed, apoptotic cell death ensues. To cope with this stress, cells activate a signal transduction system linking the ER lumen with the cytoplasm and nucleus, called the unfolded protein response (UPR) (28,29). ER stress is detected by transmembrane proteins that monitor the load of unfolded proteins in the ER lumen, and transmit this signal to the cytosol (27). Two of these proteins, inositol-requiring enzyme-1 and PKR-like ER kinase, undergo oligomerization and phosphorylation in response to increased ER stress (27). Work in our laboratory recently has demonstrated increased phosphorylation of IRE-1 and PERK in rodents and humans after burn injury, indicating postburn activation of ER-stress signaling pathways.

ER stress and UPR not only have been shown to affect inflammatory and stress responses, but also various metabolic re-

sponses (27,30,31). Specifically, several recent studies linked ER stress/UPR and JNK signaling to insulin resistance and hyperglycemia (11,12,19,32–35). Upon binding to the α -subunit on the extracellular portion of its receptor, insulin induces autophosphorylation of the β -subunit, leading to conformational changes and phosphorylation of insulin receptor substrate (IRS)-1 at a critical tyrosine residue, which in turn leads to activation of the phosphatidylinositol-3 kinase (PI3K)/Akt pathway (33,34). Recent work now suggests that stress-induced insulin resistance may in part be due to phosphorylation-based negative feedback, which may uncouple the insulin receptor or insulin receptor-associated proteins from their downstream signaling pathways, altering insulin action (34). Specifically, phosphorylation of IRS-1 at serine residues by various kinases may preclude its tyrosine phosphorylation by the insulin receptor tyrosine kinase, thus inhibiting insulin receptor trafficking (36).

We did not evaluate the effect of insulin administration on insulin resistance in the present study. The reason being that the study approach would have been to give the animals a peritoneal glucose tolerance test (PGTT) and therefore have the variable of PGTT stress altering our primary outcomes. Therefore, a PGTT was not within our outcome measures. However, in a recent clinical trial, we found that insulin administration improves insulin resistance (16) thus indicating that insulin not only alters glucose levels but also improves insulin resistance. We found in the present study that insulin decreased JNK activation. Activated JNK phosphorylates the IRS-1 kinase, which blocks IRS-1 tyrosine phosphorylation by the insulin receptor, leading to impaired insulin receptor signaling (32–34). We propose that this is one of the underlying mechanisms by which burn injury induces insulin resistance and hyperglycemia. Insulin decreased JNK activation, and it is therefore possible that insulin administration improves insulin resistance through in-

hibiting the JNK pathway (and thus allowing IRS-1 activation by the insulin receptor). That a burn in fact induces major insulin resistance not only has been shown clinically, but also in a rodent burn study which was very similar to the present study (19). The authors found that burn induces marked hepatic insulin resistance 1 to 3 d postburn associated with IR, PI3K, Akt and JNK signaling (19). Therefore, we suggest that insulin has a dual effect, decreasing glucose levels by increasing cellular uptake and by restoring insulin receptor signaling.

To examine mitochondrial and ER structure *in situ*, we performed transmission electron microscopy of liver sections from control, burn and burn-plus-insulin treated animals. We observed a significant loss of mitochondrial electron density and cristae in liver sections of burned animals. In addition, our electron microscopy analysis revealed fragmentation of the rough ER after burn injury. Thus, physiological changes observed in isolated mitochondria *in vitro* (that is, decreased state-3 respiration) are associated with consistent morphological changes in mitochondrial and ER structures *in situ*. Furthermore, we found that insulin treatment reversed these changes and improved hepatic mitochondrial and ER structure. These results clearly indicate that insulin is beneficial by improving not only hepatic function, but also by alleviating hepatocyte damage.

It is currently not clear whether inflammation induces ER stress and apoptosis or whether ER stress and apoptosis induce the inflammatory response. In the present study, we found that burn induced key inflammatory mediators. Insulin administration reduced IL-6, MCP-1, and CINC-1 significantly to almost normal levels when compared with burn, indicating that insulin leads to a decreased inflammatory response. As insulin also attenuated ER stress/UPR, we cannot address the question whether decreased inflammation causally leads to an alleviated ER stress response or *vice versa*. However, it appears that both responses are linked, and that insulin has

antiinflammatory effects and alleviates the hepatic stress response.

In the present study, we found that insulin decreased hepatocyte apoptosis after a burn injury. Insulin administration further decreased ER stress/UPR, and improved hepatic mitochondrial respiration. The underlying mechanisms by which insulin improves hepatocyte apoptosis are not known. We hypothesize that insulin activates PI3K/Akt leading to decreased IP₃R activation, which will restore intracellular calcium signaling and storage. Restored calcium signaling will reduce ER stress/UPR, which, in turn, will decrease JNK expression, which will decrease insulin resistance. Therefore, we conclude that insulin administration is a beneficial adjunct in critical care medicine to improve hepatic function and structure.

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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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