

# XBP-1s Is Linked to Suppressed Gluconeogenesis in the Ebb Phase of Burn Injury

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The first 24 h following burn injury is known as the ebb phase and is characterized by a depressed metabolic rate. While the postburn ebb phase has been well described, the molecular mechanisms underlying this response are poorly understood. The endoplasmic reticulum (ER) regulates metabolic rate by maintaining glucose homeostasis through the hepatic ER stress response. We have shown that burn injury leads to ER stress in the liver during the first 24 h following thermal injury. However, whether ER stress is linked to the metabolic responses during the ebb phase of burn injury is poorly understood. Here, we show in an animal model that burn induces activation of activating transcription factor 6 (ATF6) and inositol requiring enzyme-1 (IRE-1) and this leads to increased expression of spliced X-box binding protein-1 (*XBP-1s*) messenger ribonucleic acid (mRNA) during the ebb phase. This is associated with increased expression of *XBP-1* target genes and downregulation of the key gluconeogenic enzyme glucose-6-phosphatase (*G6Pase*). We conclude that upregulation of the ER stress response after burn injury is linked to attenuated gluconeogenesis and sustained glucose tolerance in the postburn ebb phase.

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## INTRODUCTION

Maintaining blood glucose levels in the narrow range of 60 to 140 mg/dL tightly regulates glucose metabolism in healthy individuals regardless of nutritional state (1). However, severe traumas such as burn injury perturb glucose homeostasis by increasing abnormal energy substrate production and utilization (2). Lactate production from the burn wound (3), release of gluconeogenic amino acids from catabolic skeletal muscle (4) and increased production of the stress hormones glucagon (5), catecholamines (6) and cortisol (7)

impinge on the liver to increase gluconeogenesis after burn injury. In patients, unrestrained gluconeogenesis results in increased hepatic glucose production after burn injury (8).

Hepatic gluconeogenesis is regulated largely at the transcriptional level by the key enzymes phosphoenolpyruvate carboxykinase (*PEPCK*) (9) and glucose-6-phosphatase *G6Pase* (10). Gene expression of *PEPCK* and *G6Pase* enzymes is regulated primarily by transcription factors such as cyclic adenosine monophosphate (cAMP) response element (CRE)-binding protein (CREB) (11) and

Forkhead box protein O1 (FoxO1) (12,13), in addition to coactivators such as peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$  (*PGC-1 $\alpha$* ) (14) that are abundantly expressed in the liver. Gluconeogenesis is regulated in a temporal manner with CREB acting acutely (<8 hours) and FoxO1 acting long term (18–24 hours) (15).

The endoplasmic reticulum (ER) senses changes in nutrient supply by linking metabolic cues to cellular signaling mechanisms (16). An example of this signaling mechanism is initiation of the mammalian ER stress response pathway (17). The ER stress response is mediated through three proximal sensors which include protein kinase RNA-activated (PKR)-like endoplasmic reticulum kinase (PERK), ATF6 and IRE-1 (18).

ATF6 is activated by proteolytic cleavage in the Golgi apparatus. The active cleaved p50 fragment of ATF6 subsequently translocates the nucleus where it is able to upregulate genes responsible

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for increasing the folding capacity of the ER such as XBP-1 (19). Subsequently, IRE-1 splices the mRNA of XBP-1, which leads to production of the spliced XBP-1 protein (XBP-1s) (20). XBP-1s has been shown to attenuate hepatic gluconeogenesis by inhibiting the nuclear translocation of FoxO1 (21), while the p50 fragment of ATF6 attenuates hepatic gluconeogenesis by competing with CREB for the CREB-regulated transcription coactivator 2 (CRIC2) (22).

The first 24 h following burn injury is known as the ebb phase and is characterized by decreased metabolic rate and intravascular volume, poor tissue perfusion and low cardiac output (23,24). Furthermore, in an animal burn model, we have shown that burn injury leads to an increase in hepatic ER stress within the first 24 h after thermal injury (25). However, how ER stress mechanistically contributes to metabolic alterations in the ebb phase of burn injury is essentially unknown. We considered the possibility that induction of the ER stress response is mechanistically linked to decreased metabolic rate during the ebb phase of burn injury. In the current study, we show that ER stress-induced upregulation of *XBP-1s* is linked to attenuated gluconeogenesis and sustained glucose tolerance in the ebb phase postburn injury.

## MATERIALS AND METHODS

### Animal Model of Burn Injury

The Animal Care Committee of Sunnybrook Research Institute approved all animal experiments. The *Guide for the Care and Use of Laboratory Animals* used by the National Institutes of Health (NIH) were met (26). Male Sprague Dawley rats (Taconic, Hudson, NY, USA), 250 to 300 g, were allowed to acclimate for 1 wk before conducting experiments. Rats were housed in an institutional animal care facility and received regular rodent chow and water *ad libitum* throughout the studies. A total of  $n = 9$  animals were in each of the sham and burn groups; 18 animals total were used.

A well-established method was used to induce a full-thickness scald burn (27). Animals were anesthetized with general anesthesia (ketamine [Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada] 40 mg/kg body weight and xylazine [Bayer Healthcare, Toronto, ON, Canada] 5 mg/kg body weight, both injected intraperitoneally [IP]). A 60% total body surface area (TBSA) burn was administered by placing animals in a mold that exposed a defined area of shaved skin on the dorsum of the trunk and the abdomen. The mold was lowered into 96° to 98°C water, scalding the back for 10 s and the abdomen for 1.5 s. This method delivers a full-thickness cutaneous burn. After burn injury, rats were resuscitated with Ringer lactate (Baxter Corporation, Mississauga, ON, Canada), 30  $\mu$ L/g to prevent volume depletion. Animals were observed, administered analgesia (buprenorphine [Schering-Plough/Merck, Whitehouse Station, NJ, USA] 0.01 mg/kg body weight, injected subcutaneously) and housed in individual cages. Sham animals underwent the same procedure without the burn injury. Food consumption and overall morbidity were monitored three times daily. Rats were exsanguinated under isoflurane for euthanization after 24 h. All animals survived to the time analysis.

Plasma was collected by incubating the blood for 30 min on ice with 0.5 mol/L ethylenediaminetetraacetic acid (EDTA) and centrifuging at 4°C, 15 min at 537g. The liver was perfused with 1 $\times$  phosphate-buffered saline (PBS) until blanched.

### Determination of Blood Glucose Levels and Plasma Insulin

Blood glucose values were determined using OneTouch Ultra test strips and automatic glucometer (LifeScan, Burlington, ON, Canada). Plasma insulin levels were determined in duplicate using an Insulin ELISA (enzyme-linked immunosorbent assay) kit according to the manufacturer's specifications (Alpco Diagnostics, Salem, NH, USA).

### Intraperitoneal Glucose Tolerance Test

Glucose tolerance tests were performed by glucose injection (IP; 2 g of 20% D-glucose solution in PBS per kg body weight) after a 4-h fast (28). A glucose tolerance curve was generated and the area under the curve (AUC) was calculated. The mean AUC per group was plotted and analyzed.

### Liver-Specific cAMP Levels

Liver-specific cAMP levels (23 mg of tissue) were determined in duplicate using the acetylated version of the cAMP ELISA kit according to the manufacturer's specifications (Cell Biolabs Inc, San Diego, CA, USA).

### Plasma Corticosterone and Glucagon Levels

Plasma corticosterone and glucagon levels were determined in duplicate using the corticosterone and glucagon ELISA kits according to manufacturer's specifications (Alpco Diagnostics).

### Western Blot Analysis

Approximately 100 mg of frozen liver tissue was homogenized in lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.8, 1% [w/v] Triton X-100, 50 mmol/L EDTA, 0.5 mmol/L phenylmethanesulfonyl fluoride, 100  $\mu$ mol/L NaF, 1 $\times$  cComplete protease inhibitor mixture [Calbiochem Biochemicals, Billerica, MA, USA], and 100 $\times$  phosphatase inhibitor cocktail [Sigma-Aldrich, St. Louis, MO, USA]). The homogenate was centrifuged at 17,400g for 30 min at 4°C and the pellet discarded. Western blotting was performed with 50  $\mu$ g of protein. Band intensities were quantified with ImageJ software (NIH, Bethesda, MD, USA). The blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific Inc., Rockford, IL, USA).

Antibodies against  $\alpha/\beta$  tubulin and lactate dehydrogenase were purchased from Cell Signaling Technologies (Danvers, MA, USA). Binding immunoglobulin protein (BiP), phosphorylated inositol

**Table 1.** Primer sequences.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
Dnajb9	AGACACGCCAGGATGGTCCAGT	TGACGGTCTGCAGTGCTTGC
Pdia3	CTGGTCCC GGCCCTCCGATT	ACGTCTGAGGCGGAGGCGAG
XBP-1U	TCCGCAGCACTCAGACTACGT	ATGCCCAAAGGATATCAGACTC
XBP-1S	GAGTCCGCAGCAGGTG	CGTCAGAATCCATGGGAA
PEPCK	CTACCTCTGGCCAAGATTGGTA	GTTGCAGGCCCAGTTGTTGA
G6-Pase	CCCAGACTAGAGATCCTGACAGAAT	GCACAACGCTCTTTTCTTTTACC
PGC1- $\alpha$	AAAGGGCCAAGCAGAGAGA	GTAATCACACGGCGCTCTT
18s rRNA	GTAACCCGTGAACCCCAT	CCATCCAATCGGTAGTAGCG

requiring enzyme (pIRE-1), total IRE-1 and lamin B1 antibodies were purchased from Abcam (Cambridge, MA, USA). FoxO1 antibody was purchased from Santa Cruz Biotechnologies Inc (Santa Cruz, CA, USA). Vinculin antibody was purchased from Sigma-Aldrich. TO13/14 ATF6 antibody was a kind gift from Alan Volchuk (University of Toronto, Toronto, Canada).

#### Subcellular Fractionation

Subcellular fractionation using 0.5 g of liver tissue using a nuclear extraction kit (Affymetrix, Santa Clara, CA, USA) was performed according to the manufacturer's specifications.

#### RNA Isolation and Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted from liver tissue using both TRIzol reagent (Life Technologies, Burlington, Ontario, Canada) and RNeasy Kit (Qiagen, Valencia, CA, USA) according to manufacturers' specifications. To prepare cDNA, approximately 2  $\mu$ g of total RNA was reverse transcribed using oligo-dT primers and Superscript II Reverse Transcriptase (Life Technologies). Real-time RT-PCR was carried out in an Applied Biosystems Step One Plus Real-time PCR system. Samples were run in duplicate to control for experimental error. Analysis was performed using the Livak method (29). A complete primer list is given in Table 1.

#### Statistical Analysis

Statistical analysis was performed using a Student *t* test. Data are presented

as mean  $\pm$  SEM. Significance was accepted at  $P < 0.05$ .

## RESULTS

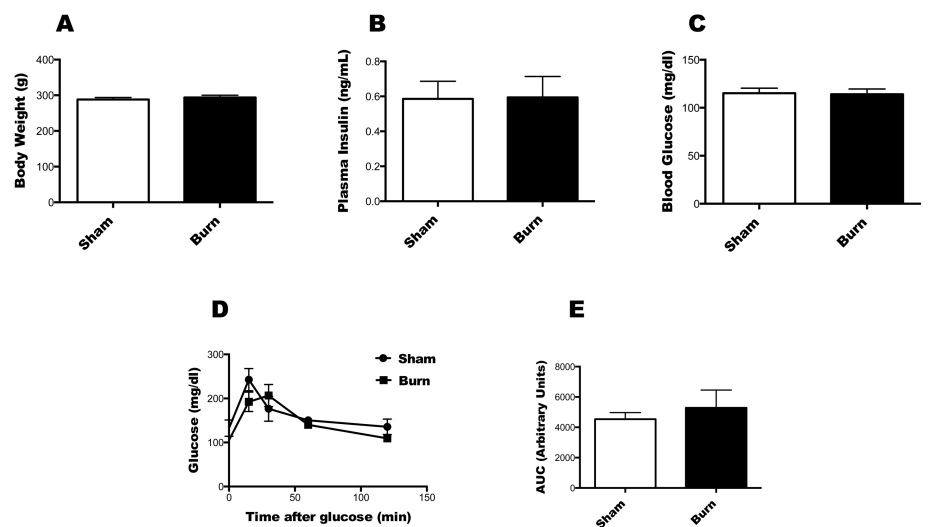
### Glucose Metabolism in the Ebb Phase of Burn Injury

To study the effects of burn injury during the ebb phase on glucose metabolism, a 60% TBSA thermal injury was administered to rats prior to 24-h euthanization. Rats were fed *ad libitum* to assess the direct effects of burn injury on hepatic gluconeogenesis independent of diet and nutritional state. Burned rats consumed less food the day of the burn (approximately 6 h after burn) but no difference in food consumption was ob-

served at 18 to 24 h (data not shown). When compared with sham-injured animals, thermally injured animals exhibited no change in weight, plasma insulin or blood glucose levels (Figures 1A–C). Glucose clearance was assessed after burn injury by administering glucose through IP injection after a 4 h fast in sham ( $n = 4$ ) and burn rats ( $n = 4$ ). Blood glucose levels were elevated markedly in both groups but returned to normal within 100 min (Figure 1D). Quantitative analysis of the AUC of blood glucose profiles show there is no difference in glucose clearance between sham and burn animals (Figure 1E). Together, this data indicates that thermally injured animals sustain glucose tolerance in the ebb phase of burn injury.

### Gluconeogenic Gene Expression Is Attenuated despite Increased Stress Hormone Production

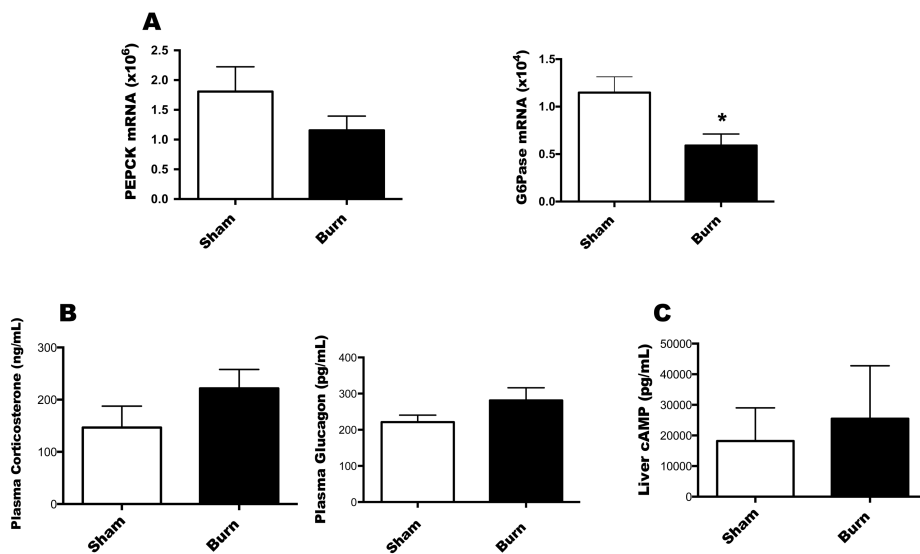
Hepatic gluconeogenesis is increased in rats during the flow phase of burn injury without a net increase in glucose output (4). However, it is unknown if this occurs in the ebb phase of burn injury. To that extent, we analyzed the



**Figure 1.** Glucose homeostasis is unaltered in the ebb phase of burn injury. Total body weight (A), plasma insulin levels (B) and blood glucose levels (C) were measured in *ad libitum* fed sham and burn rats. (D) Intraperitoneal glucose tolerance tests (PGTT) were performed on 4 h fasted sham ( $n = 4$ ) and burn ( $n = 4$ ) rats. (E) Area under the curve (AUC) of PGTT tests. Data are shown as means  $\pm$  SEM.

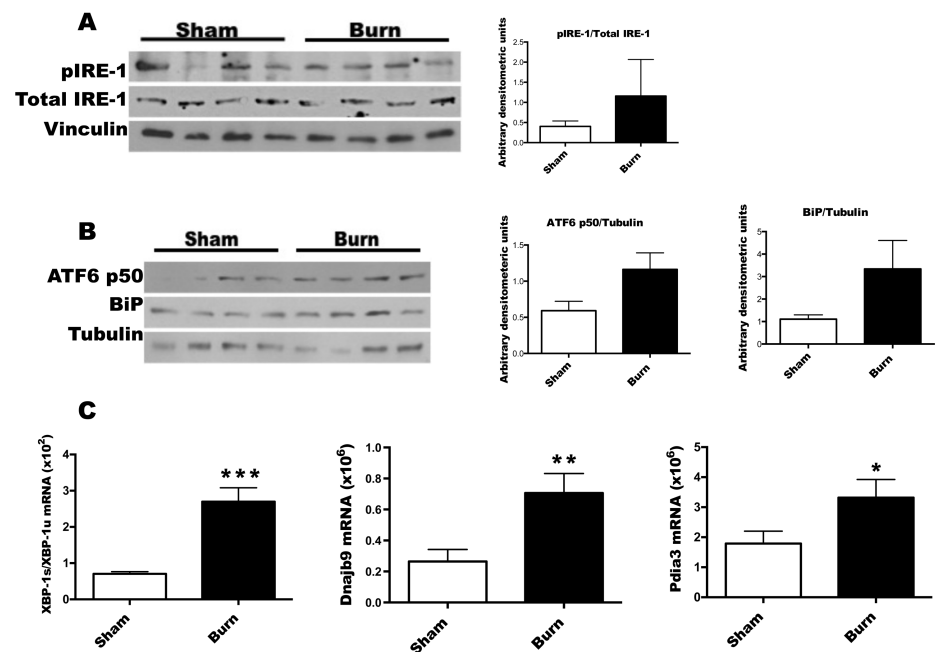
### XBP-1 Splicing Is Increased Postburn Injury

Hepatic gluconeogenesis can be attenuated *via* the IRE-1 (32) and ATF6 (22) branches of the ER stress pathway. In agreement with our previous studies, we found increased phosphorylation of hepatic IRE-1, increased cleavage of ATF6, and increased levels of the ER chaperone BiP after burn injury (Figures 3A, B). XBP-1s expression is increased downstream of ATF6 activation and splicing by phosphorylated IRE-1 (19). Thus, to examine the functional consequences of ATF6/IRE1 activation, we analyzed mRNA levels of *XBP-1s* and *XBP-1s* target genes. When compared with sham animals, thermally injured animals had a significant increase in liver-specific *XBP-1s* mRNA levels ( $P < 0.001$ , Figure 3C). Expression of *XBP-1s* target genes *Dnajb9* and *Pdia3* were increased significantly ( $P < 0.01$ ,  $P < 0.05$ , respectively) in ther-



**Figure 2.** Hepatic gluconeogenesis is attenuated after burn injury. (A) Real-time RT PCR of hepatic gluconeogenic gene expression of *PEPCK* and *G6Pase* were measured in sham and burn rats. \* $P < 0.05$  value for sham versus burn. (B) Plasma corticosterone and glucagon levels, and (C) liver specific cAMP levels ( $n = 8$  sham;  $n = 9$  burn) were measured in duplicate in sham and burn rats. Data are shown as mean  $\pm$  SEM.

mRNA levels of two key enzymes involved in hepatic gluconeogenesis, *G6Pase* and *PEPCK*. When compared with sham animals, thermally injured animals exhibited a significant decrease in *G6Pase* mRNA expression ( $P < 0.05$ , Figure 2A), and *PEPCK* mRNA also trended downwards but did not reach significance. Stress hormones glucagon and cortisol are elevated significantly after burn injury and contribute to increased gluconeogenesis (30). Therefore, we examined whether the decrease in gluconeogenesis was due to decreased stress hormone levels. Plasma glucagon and corticosterone were not significantly different 24 h after burn injury, but trended upwards (Figure 2B). cAMP, a secondary messenger activated downstream of glucagon, is known to increase the expression of both *PEPCK* and *G6Pase* (9,31). Liver derived cAMP levels were unchanged after burn injury (Figure 2C). This data suggests that the decrease in gluconeogenesis is not simply due to alterations in stress hormones released after burn injury.



**Figure 3.** The spliced form of the transcription factor X-box-binding protein-1 (*XBP-1s*) is increased in the liver in the ebb phase of burn injury. Phosphorylation of hepatic IRE-1 (A) and activation of cleaved p50 ATF6 and BiP (B) were analyzed in sham and burn rats. Similar results were obtained in three independent cohorts. (C) Real-time RT PCR gene expression of the ratio of *XBP-1s* to *XBP-1u* and *XBP-1s* target genes *Dnajb9* and *Pdia3* in the liver were measured in duplicate in sham and burn rats. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  value for sham versus burn. Data are shown as mean  $\pm$  SEM.

mally injured animals (Figure 3C). This data suggests upregulation of *XBP-1s* as a plausible mechanism by which hepatic gluconeogenesis is suppressed in the ebb phase of burn injury.

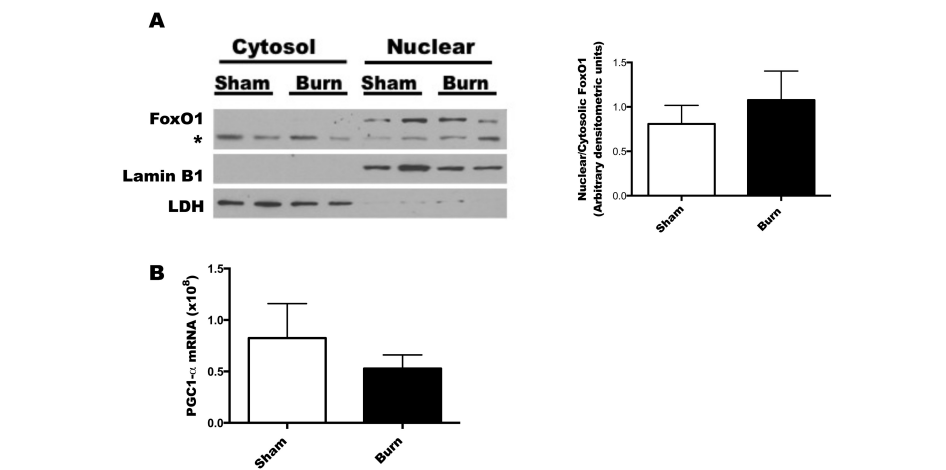
### FoxO1 Translocation after Burn Injury

The transcription factor FoxO1 plays a key role in regulating the expression of both *PEPCK* and *G6Pase* (33). Further, *XBP-1s* is hypothesized to attenuate gluconeogenesis by antagonizing the cellular translocation of FoxO1 to the nucleus (21). Thus, the cellular distribution of FoxO1 was analyzed after burn injury. Hepatic FoxO1 was present in both the cytosolic and nuclear fractions in sham and thermally injured animals (Figure 4A). Semiquantitative analysis of FoxO1 distribution indicates that FoxO1 localization to the nucleus is not significantly different following burn injury. The coactivator *PGC-1 $\alpha$*  has been shown to interact with FoxO1 to increase expression of gluconeogenic genes (34). Therefore, we analyzed hepatic *PGC-1 $\alpha$*  mRNA after burn injury. *PGC-1 $\alpha$*  expression was similarly unaffected following burn injury (Figure 4B). These results suggest that additional transcriptional regulators other than FoxO1 mediate upregulated *PEPCK* and *G6Pase* in the ebb phase of burn injury.

### DISCUSSION

The ebb phase of burn injury is characterized by a decrease in metabolic rate (23). However, the mechanisms underlying this response are poorly understood. Glucose intolerance as evidenced by burn-induced hyperglycemia is observed early in the first 2 to 8 h of the ebb phase in pediatric patients (35), as well as rat (36) and guinea pig models (37) of burn injury. However, in pediatric patients, blood glucose levels return to normal in the preceding 12 to 24 h (35). In agreement with the pediatric data, we found that blood glucose levels of thermally injured animals were equivalent to that of sham animals at 24 h.

Increases in the stress hormones glucagon, cortisol and catecholamines



**Figure 4.** Hepatic FoxO1 is distributed in both the cytosol and nucleus in the ebb phase of burn injury. (A) Distribution of hepatic FoxO1 determined by subcellular fractionation. Nuclear/cytosolic ratio of FoxO1 as determined by densitometry >1 indicates more nuclear distribution of FoxO1, whereas <1 indicates a more cytosolic distribution. Lamin B1 was used as a nuclear specific marker and LDH was used as a cytosolic-specific marker. Experiments were repeated in five independent cohorts. \*Nonspecific band. (B) Real-time RT PCR gene expression of the hepatic FoxO1 coactivator *PGC-1 $\alpha$* . Data are shown as mean  $\pm$  SEM.

have been shown to increase blood glucose levels via gluconeogenesis postburn injury (38). We show here that both corticosterone (rodent equivalent to cortisol) and glucagon levels are mildly (but not significantly) elevated. Consistent with this, our data does not show a concomitant increase in gluconeogenesis as evidenced by a significant decrease in *G6Pase* mRNA. We have shown previously that blood glucose levels as well as *G6Pase* mRNA expression are increased after 24 h (39,40). These differences can be attributed to the Ensure-based diet given to the rats in those studies. Diets high in protein have been shown to increase hepatic gluconeogenesis in the first 24 h (41). In agreement with the findings presented in this study, Vemula *et al.* (42) have found a significant decrease in the expression of both *G6Pase* and *PEPCK* in the liver 24 h after burn injury using a 20% TBSA rat burn model. The authors point to a shift in energy substrate utilization as the rationale for decreased gluconeogenic gene expression.

Recent studies have implicated the mammalian ER stress pathways in the regulation of hepatic gluconeogenesis

(22,32,43). We now show that ER stress is linked to attenuated gluconeogenesis (as manifested by decreased *G6Pase* mRNA) in the ebb phase of burn injury. Wang *et al.* (22) have shown that overexpression of the active p50 fragment of ATF6 could decrease blood glucose levels and fasting gluconeogenic gene expression in both lean and diabetic animals. We show that p50 ATF6 protein levels are increased after burn injury. It is plausible that increases in p50 ATF6 protein levels resulted in reciprocal attenuation of gluconeogenic gene expression. Consistent with this, we show that increased p50 ATF6 and concurrent phosphorylation of IRE-1 led to increased *XBP-1s* mRNA (19).

Increased expression of *XBP-1s* also has been shown to regulate glucose homeostasis (32). Indeed, we have shown that *XBP-1s* expression is increased significantly during the ebb phase of burn injury. The increased expression of *XBP-1s* correlates with the observed glucose tolerance. *XBP-1s* has been shown to attenuate gluconeogenesis by inhibiting the translocation of hepatic FoxO1 to the nucleus in diabetic mouse models (21). We, however, did not find com-

plete exclusion of FoxO1 from the nucleus after burn injury. Instead, we found that FoxO1 was evenly distributed in the cytosolic and nuclear compartments of the liver. Frescas *et al.* (44) have shown that nuclear localization of FoxO1 is necessary but not sufficient for the expression of gluconeogenic genes. Insulin-dependent phosphorylation of the kinase Akt has been shown to inhibit FoxO1 translocation to the nucleus thereby inhibiting gluconeogenesis (45). We have shown previously that insulin-dependent phosphorylation of Akt is inhibited significantly at 24 h after burn injury (39). Given that FoxO1 has partial nuclear localization, there appears to be another mechanism by which FoxO1-mediated gluconeogenesis is attenuated. Our data suggests that burn induced ER stress, specifically *XBP-1* splicing, plays a key role in regulating hepatic gluconeogenesis. Presently, we have not shown a direct role for *XBP-1s* in the inhibition of FoxO1-mediated gluconeogenesis in the ebb phase of burn injury. Future studies will focus on determining a direct link between *XBP-1s* expression and attenuated FoxO1 mediated gluconeogenesis.

## CONCLUSION

We conclude that upregulation of the ER stress response is linked to attenuated gluconeogenesis and sustained glucose tolerance in the postburn ebb phase. We hypothesize that *XBP-1s* is the central regulator which attenuates gluconeogenesis by modulating *G6Pase* levels. These findings point to the possible mechanism by which metabolic rate is decreased in the postburn ebb phase.

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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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