Severe Burn–Induced Endoplasmic Reticulum Stress and Hepatic Damage in Mice

*Juquan Song,[1,4] Celeste C Finnerty,[1,4] David N Herndon,[1,4] Darren Boehning,[1,3] and Marc G Jeschke[1,2,4]**

[1]Shriners Hospitals for Children and Departments of [2]Biochemistry and Molecular Biology, [3]Neuroscience and Cell Biology, and [4]Surgery, University of Texas Medical Branch, Galveston, Texas, United States of America

Severe burn injury results in liver dysfunction and damage, with subsequent metabolic derangements contributing to patient morbidity and mortality. On a cellular level, significant postburn hepatocyte apoptosis occurs and likely contributes to liver dysfunction. However, the underlying mechanisms of hepatocyte apoptosis are poorly understood. The endoplasmic reticulum (ER) stress response/unfolded protein response (UPR) pathway can lead to hepatocyte apoptosis under conditions of liver dysfunction. Thus, we hypothesized that ER stress/UPR may mediate hepatic dysfunction in response to burn injury. We investigated the temporal activation of hepatic ER stress in mice after a severe burn injury. Mice received a scald burn over 35% of their body surface and were killed at 1, 7, 14, and 21 d postburn. We found that severe burn induces hepatocyte apoptosis as indicated by increased caspase-3 activity ($P < 0.05$). Serum albumin levels decreased postburn and remained lowered for up to 21 d, indicating that constitutive secretory protein synthesis was reduced. Significantly, upregulation of the ER stress markers glucose-related protein 78 (GRP78)/BIP, protein disulfide isomerase (PDI), p–protein kinase R–like endoplasmic reticulum kinase (p-PERK), and inositol-requiring enzyme 1α (IRE-1α) were found beginning 1 d postburn ($P < 0.05$) and persisted up to 21 d postburn ($P < 0.05$). Hepatic ER stress induced by burn injury was associated with compensatory upregulation of the calcium chaperone/storage proteins calnexin and calreticulin ($P < 0.05$), suggesting that ER calcium store depletion was the primary trigger for induction of the ER stress response. In summary, thermal injury in mice causes long-term adaptive and deleterious hepatic function alterations characterized by significant upregulation of the ER stress response.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2009.00048

INTRODUCTION

Severe burn injury causes myriad metabolic alterations, including hyperglycemia, lipolysis, and protein catabolism (1). These changes can induce multiorgan failure and sepsis leading to significant morbidity and mortality (2,3).

By modulating metabolic and immune responses, the liver plays a pivotal role in mediating survival and recovery of burn patients (4–6). Preexisting liver disease is directly associated with adverse clinical outcomes following burn injury. Price *et al*. (7) showed that liver disease prior to burn injury increased mortality risk from 6% to 27%, indicating that liver impairment worsens the prognosis in patients with thermal injury. Severe burn also directly induces hepatic dysfunction and damage, delaying recovery (8). Therefore, ways to improve recovery and survival of patients with severe burns may be developed through better understanding of the mechanisms that mediate the pathophysiologic changes in the liver following thermal injury.

The endoplasmic reticulum (ER) is the site of secretory protein synthesis (9). The matured forms of secreted proteins exit the ER after a number of posttranslational modifications such as *N*-glycosylation, disulfide bond formation, lipidation, hydroxylation, and oligomerization. Unfolded/misfolded proteins in the ER lumen are either retained to complete the folding process or targeted for degradation (9,10). If the unfolded protein burden exceeds a certain threshold, there is activation of unfolded protein response (UPR). There are three ER-resident molecular sensors that mediate the UPR: inositol-requiring enzyme 1 (IRE-1), protein kinase R–like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6). Under conditions of ER stress, the ER luminal domains of these sensors dissociate from the intraluminal chaperone glucose-related protein 78 (GRP78)/BIP, leading to their activation and the initiation of downstream signaling processes collectively termed the ER stress response. UPR minimizes the unfolded protein burden in the ER and ultimately preserves cellular integrity. The UPR is clinically important in cancer, diabetes mellitus, and human genetic diseases (11–13). However, little is known regarding the role of the UPR in mediating metabolic disturbances induced by burn injury.

Address correspondence and reprint requests to *Marc G Jeschke, Department of Surgery, University of Texas Medical Branch, Shriners Hospitals for Children, 815 Market Street, Galveston, TX, 77550. Phone: 409-392-4978; Fax: 409-770-6919; E-mail: majeschk@utmb.edu or mcjeschke@hotmail.com.*
Submitted June 3, 2009; Accepted for publication June 8, 2009; Epub (www.molmed.org) ahead of print June 19, 2009.

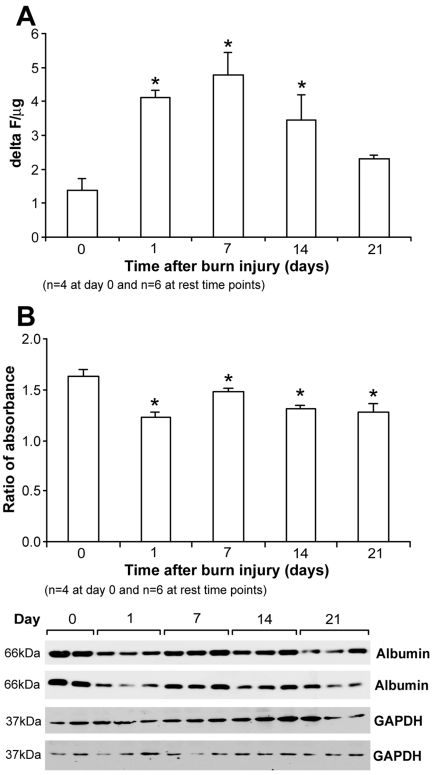

Figure 1. Burn injury causes hepatic damage and liver dysfunction. (A) Caspase-3 activity in liver cytosolic fractions after burn injury, as determined by caspase-3 activity assay. (B) The expression of liver-secreted albumin after burn injury. Time after injury (in d) is indicated. Data represent the mean ± SEM. *$P < 0.05$, value versus control for each time point after burn injury pooled from four control and six burned animals at each time point.

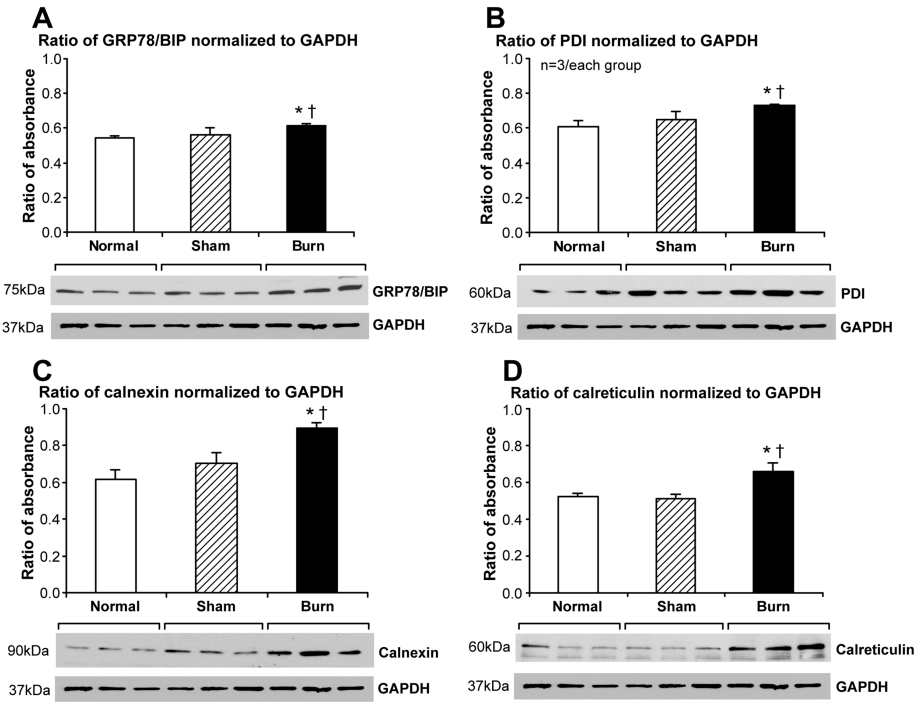

Figure 2. ER chaperone levels associated with the UPR are increased 24 h after a burn injury in mice. The expression of GRP78/BIP, PDI, calnexin, and calreticulin among the normal (n = 3), sham (n = 3), and burned (n = 3) groups normalized to GAPDH. Data are the mean ± SEM. *$P < 0.05$, burned versus normal. †$P < 0.05$, burned versus sham pooled from three animals at each time point.

Severe burn impairs hepatocyte structure and function, eventually leading to cell death (14). Burn-induced hepatocyte cell death occurs through both apoptotic and necrotic pathways (15). Hepatic damage after burn injury is associated with increased hepatocyte apoptosis and functional impairments such as decreased production of constitutive serum proteins such as albumin (14). However, the molecular mechanisms responsible for compromised hepatic function after burn injury are still unclear. We hypothesize that ER stress and UPR activation may play a prominent role in adaptive hepatic responses and subsequent hepatocyte apoptosis after burn injury. The investigation we report here shows, by use of a well-established mouse burn model, that ER stress/UPR is associated with hepatic functional impairment and dysfunction in mice in response to burn injury. Surprisingly, hepatic ER stress persisted for up to 3 weeks postburn, indicating dramatic and long-lasting changes in hepatic function. Furthermore, in a separate study, we found that this model recapitulates the clinically observed inflammatory response over similar time courses (16). Thus, this model should prove to be useful in further studies investigating ER stress and burn injury in transgenic animals.

MATERIALS AND METHODS

Animal Model of Burn Injury

Adult male C57BL/6 mice (6 to 8 wks old) were purchased from Harlan (Houston, TX, USA) and allowed to acclimate for 1 wk prior to the experiment. Mice were housed in a temperature-controlled cubicle with a 12-h light/dark cycle with laboratory chow and water *ad libitum*. All animal procedures were performed in adherence to the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston.

A well-established method was applied to induce a full-thickness scald burn (17). Mice were anesthetized with 2.5% isoflurane. The dorsal and lateral surfaces were shaved and 1 mL of 0.9% saline was injected subcutaneously along the spinal column to protect the spinal cord during the burn. Mice were placed on their backs and transferred to a mold with an opening providing a 35% total body surface area burn. Mice were then immersed in 97°C water for 10 sec. Lactated Ringer's solution (2 mL) was then administered intraperitoneally for resuscitation, and 0.1 mg/mL of buprenorphine was administered subcu-

taneously for analgesia. Mice were housed in separate cages for the duration of the experiment and pair fed. Sham animals were anesthetized and immersed in warm water (25°C). The normal control mice were not anesthetized and immersed in water. Mice were killed at discrete time points by decapitation under isoflurane anesthesia. Liver tissue was snap-frozen in liquid nitrogen and stored at –80°C.

Antibodies and Reagents

Antibodies against murine GRP78/BIP, IRE-1, p-IRE-1, CHOP, and calreticulin were purchased from Abcam Inc. (Cambridge, MA, USA); p-PERK antibody was purchased from Cell Signaling Tech Inc. (Danvers, MA, USA); ATF6 antibody was purchased from LifeSpan Biosciences Inc. (Seattle, WA, USA); and Calnexin and protein disulfide isomerase (PDI) were purchased from Assay Designs Inc. (Ann Arbor, MI, USA). Caspase-3 substrate Z-DEVD-R110 was purchased from American Peptide Company Inc. (Sunnyvale, CA, USA). SuperSignal West Pico Chemiluminescent Substrate was purchased from Thermo Scientific Inc. (Rockford, IL, USA).

Western Blotting

Approximately 100 mg of frozen liver tissue was homogenized 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, 1 × Complete protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN, USA), and a phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The homogenate was centrifuged at 20,000*g* for 30 min at 4°C and the pellet discarded. Then we analyzed 20 μg of each protein sample by SDS-PAGE and Western blotting. Band intensities were quantified with the GeneSnap/GeneTools software (Syngene, Frederick, MD, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

Caspase-3 Activity

Caspase-3 activity was measured as described previously (18). Briefly, 100 mg of liver tissue was homogenized in buffer A (8.25% sucrose, 10 mmol/L Tris-HCl, pH 7.5, 0.25 mmol/L EGTA, and protease inhibitor cocktail). The homogenate was then subjected to differential centrifugation to isolate a mitochondrial-enriched 10,000*g* pellet (P2), a cytosolic 100,000*g* supernatant (S3), and an ER-enriched 100,000*g* enriched pellet (P3). We then incubated 20 μg of the cytosolic (S3) fraction with 5 μmol/L of Z-DEVD-R110 in reaction buffer containing 5 mmol/L PIPES, pH 7.4, 1 mmol/L EDTA, 0.05% Triton, and 5 mmol/L DTT for 30 min at room temperature. Caspase-3 activity was quantified as the change in fluorescence per min per microgram protein.

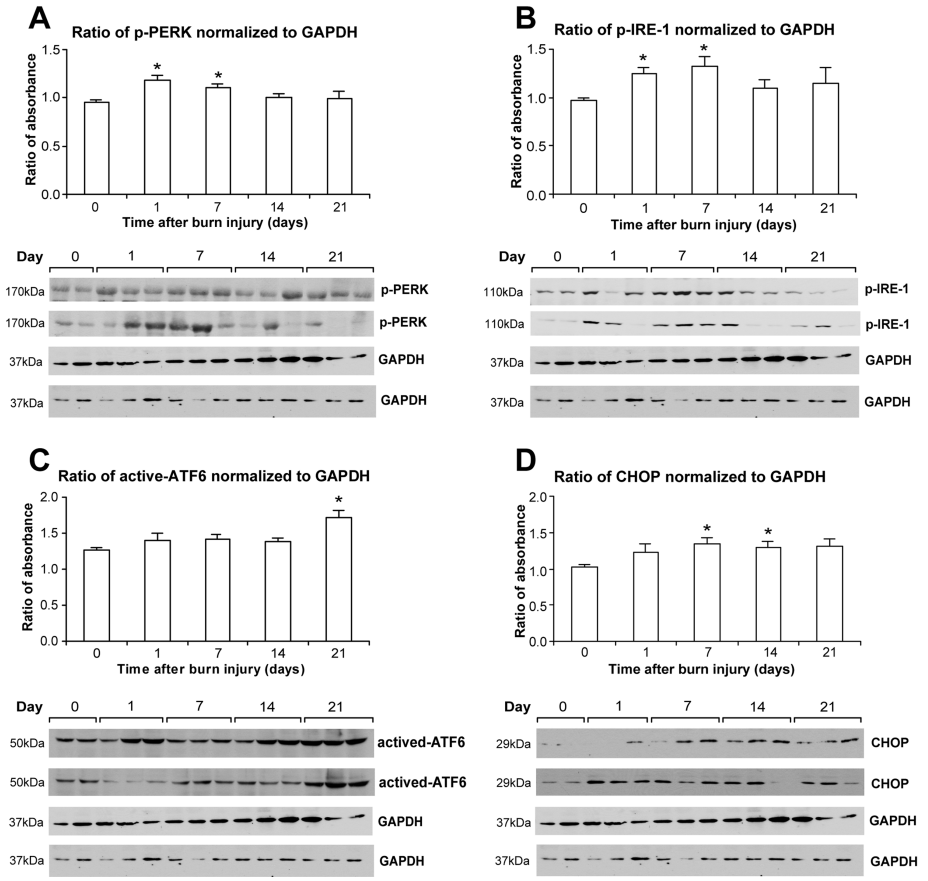

Figure 3. Time course of activation of the canonical ER stress pathway after burn injury. Protein levels of (A) p-PERK, (B) p-IRE-1, (C) cleaved ATF6, and (D) CHOP during the postburn time course. Protein levels are normalized to GAPDH. Data represent the mean ± SEM. **P* < 0.05, value versus control for each time point after burn injury pooled from four control and six burned animals at each time point.

Statistical Analysis

Statistical analysis was performed using an unpaired Student *t* test. Data are presented as mean ± SEM. Significance was accepted at $P < 0.05$.

RESULTS

Hepatic Damage and Liver Dysfunction in Mice Persists up to 21 d Postburn

Caspase-3 is a key mediator of the execution phase of apoptosis. Within 1 d of burn injury, the activity of caspase-3 in the cytosolic fraction increased two–three-fold compared with activity in the nonburn group ($P < 0.05$) (Figure 1A). Postburn, caspase-3 activity

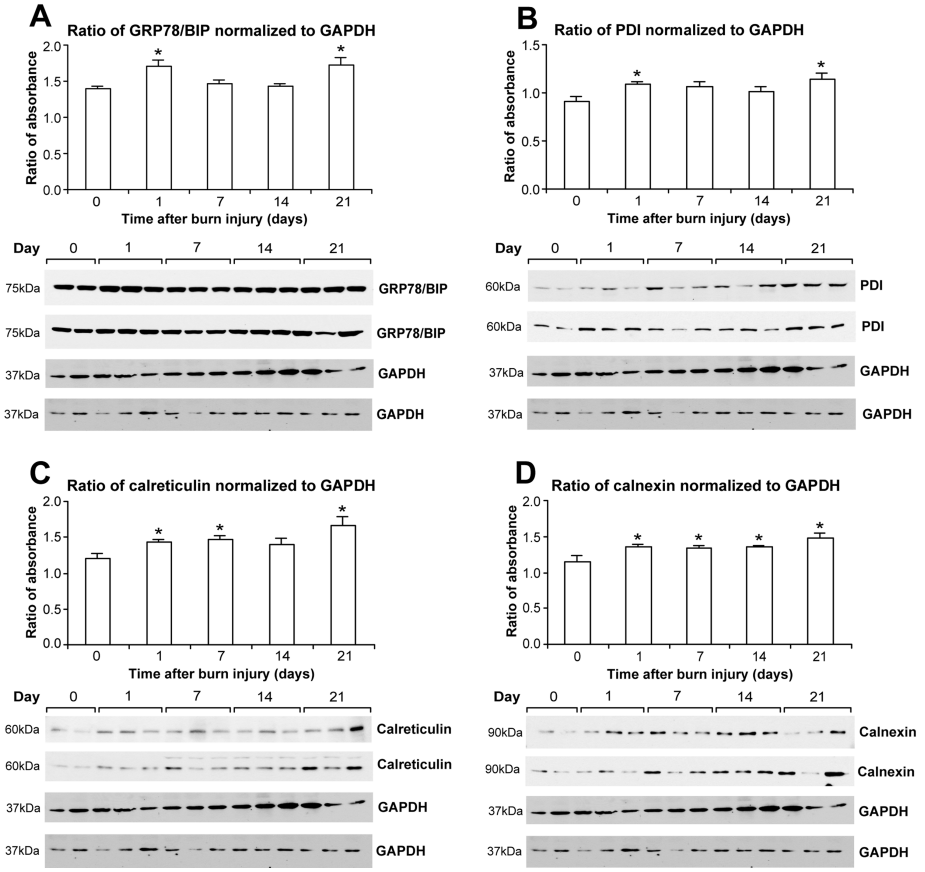

Figure 4. ER chaperone levels are increased up to 21 d after burn injury. Protein expressions of (A) GRP78/BIP, (B) PDI, (C) calreticulin, and (D) calnexin during the postburn time course. Protein levels are normalized to GAPDH. Data represent the mean ± SEM. *$P <$ 0.05, value versus control for each time point after burn injury pooled from four control and six burned animals at each time point.

remained elevated through d 14 and returned to normal by d 21.

Serum albumin produced by the liver maintains osmotic pressure of the blood compartment and carries molecules with low water solubility. Expression of albumin from liver tissue decreased within 1 d after burn injury and remained low 21 d later ($P < 0.05$) (Figure 1B), indicating that severe burn impaired murine liver function by decreasing the synthesis of secretory hepatic proteins.

Severe Burn Increases ER Chaperone Levels Associated with ER Stress 24 h after Burn Injury

Severe burn injury markedly increased GRP78/BIP, PDI, calnexin, and calreticulin compared with either the control or sham group, indicating that severe burn injury induces hepatic ER stress in mice ($P < 0.05$) (Figure 2A–D). There were no significant differences in protein expression between the normal control and sham groups, confirming that ER stress was induced by the burn and not by the anesthesia or other manipulations.

Burn-Induced Hepatic ER Stress Persists for up to 21 d Postburn

We found that the expression of phosphorylated PERK increased within 1 d postburn and then dropped to normal levels by d 14 (Figure 3A). Phospho-IRE-1 was increased at 1 d postburn, exhibiting a peak at 7 d postburn ($P < 0.05$) (Figure 3B). The levels of active (cleaved) ATF-6 increased significantly at the late time point of 21 d postburn ($P < 0.05$) (Figure 3C). Downstream ER stress signaling was also elevated, as evidenced by CHOP upregulation at 7 and 14 d postburn ($P < 0.05$) (Figure 3D).

The ER luminal molecular chaperone GRP78/BIP was significantly increased at 1 and 21 d postburn ($P < 0.05$) (Figure 4A). The expression of PDI also presented a similar change following burn injury (Figure 4B). The expression of ER luminal chaperones calnexin and calreticulin were significantly increased at 1 d postburn in mice, and remained elevated 21 d later ($P < 0.05$) (Figure 4C, D), suggesting that ER stress was activated by depletion of intracellular calcium stores. These findings indicate that burn injury in mice caused long-term activation of the ER stress response, hepatocyte apoptosis, and hepatic dysfunction lasting for 21 d.

DISCUSSION

We have previously shown in a rat model that intracellular calcium homeostasis is a major activator of postburn ER stress and mitochondrial dysfunction, both of which are likely contributors to hepatocyte apoptosis and subsequent liver dysfunction (19). In this study, we determined that severe burn activated transmembrane ER stress sensors in a temporally distinct manner. Significantly, we found that the UPR signaling pathway was activated up to 21 days postburn, a finding that indicates prolonged activation of the ER stress response. Furthermore, in a separate study, we found significant increases in serum cytokines/chemokines over a similar time course (16). Preliminary data from our translational research study indicated that ER stress persists for months in severely burned pediatric patients (unpublished data), suggesting that this mouse model closely mimics the clinically observed physiologic response to burn injury in humans.

In the UPR pathway, three major effectors modulate the downstream effects of

ER stress. IRE1, PERK, and ATF6 activation proceed independently in ER-stressed cells and lead to numerous downstream adaptive responses (20). Activation of PERK leads to phosphorylation of the eukaryotic translation-initiation factor 2-α, thereby inhibiting protein translation and resulting in cell-cycle arrest. Phosphorylation of IRE-1 activates X-box binding protein mRNA to upregulate UPR stress response genes by directly binding to stress element promoters (21). ATF6 is a basic leucine zipper transcription factor, which when cleaved translocates to the nucleus and binds to the stress-element promoters of genes upregulated in the UPR (22). The activation of IRE1, PERK, and ATF6 can proceed independently in response to ER stress. We found that IRE-1 and PERK were activated at early time points postburn (1–7 days), whereas ATF-6 was elevated only at the last postburn time point (21 days). Based on our animal model, we speculate that the temporal activation of each of these components may have significant implications for the pathogenesis of liver dysfunction after burn injury. In future studies we will address the relative contributions of each of these arms of the ER stress pathway. Finally, the mouse model developed in this study will provide powerful tools for using transgenic animals to decipher the precise molecular pathways mediating the postburn response.

In preliminary studies, we have found that a severe burn induces ER stress and is associated with insulin resistance in pediatric burn patients (unpublished data). Limiting the unfolded protein burden with "chemical chaperones" may promote hepatocyte survival (23). Ongoing development of pharmacologic agents that limit proapoptotic ER stress-signaling pathways may offer therapeutic benefits by improving organ function and patient survival (24). Our finding that burn causes ER stress and UPR activation may present a pivotal new direction in burn research, facilitating the development of novel therapeutic strategies and improved clinical outcomes for those with severe burn injuries.

ACKNOWLEDGMENTS

This work was supported by grants from Shriners Hospitals for Children (8460, 8640, 8660, 8740, and 8760) and the National Institutes of Health (GM56687, GM081685, NIH T32-GM08256, NIH P50-GM60338).

We thank Xinmin Wang from the Department of Neuroscience and Cell Biology, University of Texas Medical Branch, for his technical support. We also thank Eileen Figueroa and Steve Schuenke from the Department of Surgery for their assistance in preparing this manuscript.

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.

REFERENCES

1. Herndon DN, Hart DW, Wolf SE, Chinkes DL, Wolfe RR. (2001) Reversal of catabolism by beta-blockade after severe burns. *N. Engl. J. Med.* 345:1223–9.
2. Pereira C, Murphy K, Herndon D. (2004) Outcome measures in burn care: is mortality dead? *Burns* 30:761–71.
3. Brusselaers N, Monstrey SJ, Vandijck DM, Blot SI. (2007) Prediction of morbidity and mortality on admission to a burn unit. *Plast. Reconstr. Surg.* 120:360–1; author reply 361.
4. Jeschke MG, Micak RP, Finnerty CC, Herndon DN. (2007) Changes in liver function and size after a severe thermal injury. *Shock* 28:172–7.
5. Thomas S, Wolf SE, Chinkes DL, Herndon DN. (2004) Recovery from the hepatic acute phase response in the severely burned and the effects of long-term growth hormone treatment. *Burns* 30:675–9.
6. Nguyen TT, Gilpin DA, Meyer NA, Herndon DN. (1996) Current treatment of severely burned patients. *Ann. Surg.* 223:14–25.
7. Price LA, Thombs B, Chen CL, Milner SM. (2007) Liver disease in burn injury: evidence from a national sample of 31,338 adult patients. *J. Burns Wounds* 7: e1.
8. Kulaksiz H, Heuberger D, Engler S, Stiehl A. (2008) Poor outcome in progressive sclerosing cholangitis after septic shock. *Endoscopy* 40:214–8.
9. Ellgaard L, Molinari M, Helenius A. (1999) Setting the standards: quality control in the secretory pathway. *Science* 286:1882–8.
10. Ellgaard L, Helenius A. (2001) ER quality control: towards an understanding at the molecular level. *Curr. Opin. Cell Biol.* 13:431–7.
11. Koumenis C. (2006) ER stress, hypoxia tolerance and tumor progression. *Curr Mol. Med.* 6:55–69.
12. Lipson KL, Fonseca SG, Urano F. (2006) Endoplasmic reticulum stress-induced apoptosis and auto-immunity in diabetes. *Curr Mol. Med.* 6:71–7.
13. Matus S, et al. (2008) The stress rheostat: an interplay between the unfolded protein response (UPR) and autophagy in neurodegeneration. *Curr. Mol. Med.* 8:157–72.
14. Klein D, Schubert T, Horch RE, Jauch KW, Jeschke MG. (2004) Insulin treatment improves hepatic morphology and function through modulation of hepatic signals after severe trauma. *Ann. Surg.* 240:340–9.
15. Malhi H, Gores GJ, Lemasters JJ. (2006) Apoptosis and necrosis in the liver: a tale of two deaths? *Hepatology* 43: S31–44.
16. Finnerty CC, Przkora R, Herndon DN, Jeschke MG. (2009) Cytokine expression profile over time in burned mice. *Cytokine* 45:20–5.
17. Toliver-Kinsky TE, Cui W, Murphey ED, Lin C, Sherwood ER. (2005) Enhancement of dendritic cell production by fms-like tyrosine kinase-3 ligand increases the resistance of mice to a burn wound infection. *J. Immunol.* 174:404–10.
18. Boehning D, van Rossum DB, Patterson RL, Snyder SH. (2005) A peptide inhibitor of cytochrome c/inositol 1,4,5-trisphosphate receptor binding blocks intrinsic and extrinsic cell death pathways. *Proc. Natl. Acad. Sci. U. S. A.* 102:1466–71.
19. Jeschke MG, *et al*. (2009) Calcium and Er stress mediate hepatic apoptosis after burn injury. *J. Cell Mol. Med.* 2009, Jan 14. [Epub ahead of print].
20. Ron D, Walter P. (2007) Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol. Cell. Biol.* 8:519–29.
21. McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ. (2001) Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Mol. Cell. Biol.* 21:1249–59.
22. Molinari M, Galli C, Piccaluga V, Pieren M, Paganetti P. (2002) Sequential assistance of molecular chaperones and transient formation of covalent complexes during protein degradation from the ER. *J. Cell Biol.* 158:247–57.
23. Ozcan U, *et al*. (2006) Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* 313:1137–40.
24. Wiseman RL, Balch WE. (2005) A new pharmacology—drugging stressed folding pathways. *Trends Mol. Med.* 11:347–50.