Calcium and ER stress mediate hepatic apoptosis after burn injury

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Abstract

A hallmark of the disease state following severe burn injury is decreased liver function, which results in gross metabolic derangements that compromise patient survival. The underlying mechanisms leading to hepatocyte dysfunction after burn are essentially unknown. The aim of the present study was to determine the underlying mechanisms leading to hepatocyte dysfunction and apoptosis after burn. Rats were randomized to either control (no burn) or burn (60% total body surface area burn) and sacrificed at various time-points. Liver was either perfused to isolate primary rat hepatocytes, which were used for *in vitro* calcium imaging, or liver was harvested and processed for immunohistology, transmission electron microscopy, mitochondrial isolation, mass spectroscopy or Western blotting to determine the hepatic response to burn injury *in vivo*. We found that thermal injury leads to severely depleted endoplasmic reticulum (ER) calcium stores and consequent elevated cytosolic calcium concentrations in primary hepatocytes *in vitro*. Burn-induced ER calcium depletion caused depressed hepatocyte responsiveness to signalling molecules that regulate hepatic homeostasis, such as vasopressin and the purinergic agonist ATP. *In vivo*, thermal injury resulted in activation of the ER stress response and major alterations in mitochondrial structure and function – effects which may be mediated by increased calcium release by inositol 1,4,5-trisphosphate receptors. Our results reveal that thermal injury leads to dramatic hepatic disturbances in calcium homeostasis and resultant ER stress leading to mitochondrial abnormalities contributing to hepatic dysfunction and apoptosis after burn injury.

Keywords: thermal injury • liver • ER stress • unfolded protein response • apoptosis • calcium

Introduction

A burn injury represents one of the most severe forms of trauma and occurs in over two million people in the United States per year [1]. According to the World Health Organization (WHO), an estimated 330,000 deaths per year worldwide are related to thermal injury [2]. A severe burn represents a devastating injury affecting nearly every organ system and leading to significant morbidity and mortality [3]. Burn is an extreme and therefore a

useful model of human response to trauma or injury. Burn produces a profound hypermetabolic stress response characterized by increased glucose production, lipolysis and protein catabolism [3–5]. The hypermetabolic stress response is driven by the inflammatory response, which encompasses hormones, cytokines and acute phase proteins [6–8]. Clinical studies have shown that a sustained or increased inflammatory and acute phase responses can be life threatening with the uncontrolled and prolonged action of pro-inflammatory cytokines and acute phase proteins contributing to multi-organ failure, hypermetabolism, morbidity and mortality [7–9].

The liver, with its metabolic, inflammatory, immune and acute phase functions, plays a pivotal role for patient survival and recovery by modulating multiple pathways [9–13]. In a recent study, Price and colleagues [14] examined the outcome of 290 burned

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patients who suffered from liver disease prior to the burn injury. They showed that liver disease increased the mortality risk from 6% (total population) to 27%. The increased risk held when they compared this group with a propensity score-matched group of patients without liver disease but with similar demographics and medical comorbidities. The authors concluded that liver impairment worsens the prognosis in patients with thermal injury [14] and liver integrity is essential for survival after burn. However, a burn injury causes liver injury which persists over a prolonged time [10, 11, 15]. Hepatic changes at the cellular level after burn injury included increased hepatocyte apoptosis and an overall decrease in the production of constitutive serum proteins such as albumin [10, 12, 13]. Increased apoptosis is a central component of organ dysfunction in many pathological states, including diseases affecting liver function [16–18]. Thus, it is likely that increased cell death contributes to compromised hepatic function after burn. However, the mechanisms by which burn injury induces acute and lasting changes in hepatic function are poorly understood. Elucidating the molecular events which lead to compromised hepatic function after burn is critical for developing therapeutic strategies for decreasing mortality and improving long-term outcome of these patients.

Experimental procedures

Animal model of burn injury

All animal procedures conformed to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats weighing 300–350 g were housed in wire bottom cages with a 12-hr light–dark cycle. All animals were acclimated to their environment for 7 days. Rats received water *ad libitum* for the entire study period. Sixty percent total body surface area (TBSA) burns were administered as previously described [19]. All animals received analgesia (Buprenex 0.05 mg/kg, i.m.) and general anaesthesia (Ketamine 40 mg/kg body weight and Xylazine 5 mg/kg body weight, both injected intraperitoneally) prior to the burn. After receiving the thermal injury, rats were immediately resuscitated with intraperitoneal Ringer's lactate (60 ml/kg intraperitoneally). Analgesia was administered every 12 hrs (or more often if discomfort was evident). Animals were sacrificed at 24, 48 or 120 hrs after burn.

Liver processing

Livers were harvested after laparotomy and either placed in liquid nitrogen (Western blotting), fixed in 4% paraformaldehyde for histology (apoptosis and proliferation), homogenized and fractionated (mitochondrial physiology) or primary hepatocytes were isolated

by hepatic perfusion (calcium imaging). Prior to sacrifice blood was obtained from each animal for analysis of serum enzymes.

Primary rat hepatocyte isolation

The protocol for hepatic perfusion was as follows: rats were anesthetized with 0.1 ml of 50 mg/ml pentobarbital per 100 g body weight injected intraperitoneally. After the rat was anesthetized, a laparotomy was performed, and the *vena porta* identified. Subsequently, a catheter was inserted into the portal vein and the catheter attached to a peristaltic pump that is primed with buffer A (Krebs-Ringer-HEPES Ca^{2+} , Mg^{2+} free, 0.5 mM ethylene glycol tetraacetic acid (EGTA), pH 7.4). The inferior *vena cava* was then severed and buffer A was perfused through the liver for 4 min. Perfusate was switched to buffer B (Krebs-Ringer-HEPES, 1 mM CaCl_2 , Mg^{2+} free, 14,000 U collagenase class II, pH 7.4) for 9 min. After collagenase digestion, the liver was removed and placed into chilled buffer C (Krebs-Ringer-HEPES, 2 mM CaCl_2 , Mg^{2+} free, 0.1% bovine serum albumin [BSA]). Connective tissue was then removed by filtration. Finally, the cells were plated in William's E media on collagen coated cover slips with 10% foetal bovine serum, 2 mM glutamine, 10 U/ml penicillin and 10 $\mu\text{g/ml}$ streptomycin. Cells were only used if cell viability was greater than 90% after isolation, determined by Trypan blue staining. After plating, hepatocytes were used in calcium imaging experiments 14 to 18 hrs after isolation to limit de-differentiation. The differentiation state of hepatocytes was confirmed by morphology and by determination of albumin synthesis (data not shown).

Hepatic serum enzymes

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined using Behring nephelometer (Behring, Deerfield, IL, USA).

Liver apoptosis

Terminal deoxyuridine nick end labelling (TUNEL) (Apoptag, Oncogene, Baltimore, MD, USA) staining to identify apoptotic hepatocytes *in situ* was performed as suggested by the manufacturer. For each time-point we used eight animals per group. Six sections of each liver block were obtained at 40- to 50- μm 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 were pooled. The data were then quantified as the percentage of apoptotic cells *per* hundred hepatocytes.

Western blotting

Western blotting was accomplished using standard techniques as described [20].

Antibodies

GRP78/Bip, inositol requiring enzyme-1 (IRE-1), C/EPB homologous protein (CHOP) and Calreticulin were purchased from Abcam, Inc., Cambridge, MA, USA; cytochrome C, cytochrome c oxidase (CytOx) and P-PERK were from Cell Signaling Tech, Inc., Danvers, MA, USA; protein disulfide isomerase (PDI) and Calnexin was from Assay Designs, Inc., Ann Arbor, MI, USA; oxidoreductase endoplasmic reticulum oxidoreduction (ERO)-1 was from Novus Biologicals, Inc., Littleton, CO, USA; IP₃R type 1, as previously described [21], endoplasmic reticulum (ER) calcium ATPase sarco-ER calcium ATPase (SERCA)-1 was from Affinity Bioreagents Inc., Golden, CO, USA.

Calcium imaging

Calcium measurements were performed on hepatocytes 14–18 hrs after plating on collagen-coated cover slips. Fura-2 (2.5 μ M) was loaded into hepatocytes at room temperature for 30 min. in imaging solution (107 mM NaCl, 7.2 mM KCl, 1.2 mM MgCl₂, 1 mM CaCl₂, 11.5 mM glucose, 0.1% BSA and 20 mM HEPES 7.2), and the cells were incubated for a further 30 min. in the same solution without Fura-2 prior to imaging. Fura-2 images were acquired on a Nikon TE2000 inverted microscope using a Nikon 60 $\times$ oil immersion SuperFluor objective with a 1.3 numerical aperture (Nikon, Melville, NY, USA). All imaging was performed at 25°C in imaging solution. Images were captured at 0.5 Hz with a Roper Scientific Coolsnap HQ camera (Roper, Greenville, SC, USA). Rapid filter changes for ratiometric imaging were computer controlled via a Sutter Lambda 10–2 filter wheel controller and MetaFluor data acquisition and analysis software. Raw data were acquired with MetaFluor and graphed in Sigma Plot. Fluorescent images were pseudo-coloured using the integrated mean density (IMD) display mode in MetaFluor for display purposes in Fig. 2A. The histogram in Fig. 2B was generated from the single cell cytosolic calcium concentration of 70 control and 70 burned hepatocytes pooled from three separate experiments. Each trace in Fig. 2C, E and G are averages calculated from 20–30 single cell measurements from one experiment. Quantified data in Fig. 2D, F and H are pooled from at least three separate experiments, comprising hundreds of single cell traces.

Subcellular fractionation

Subcellular fractionations were performed exactly as described previously [21, 22].

Mass spectroscopy

Identification of proteins by trypsin digestion and matrix assisted laser desorption/ionization-time of flight mass spectrometry

(MALDI-TOF) was performed by the Mass Spectrometry Core of the Biomolecular Resource Facility at the University of Texas Medical Branch.

Mitochondrial isolation and respiration

Liver tissue (400 mg) was minced on ice and transferred (10% w/v) to isolation buffer (250 mM sucrose, 10 mM HEPES, 0.5 mM EGTA, 0.1% BSA, pH 7.4). The sample was gently homogenized by three to four strokes with a Dounce homogenizer with a loose fitting pestle. The homogenate was centrifuged at $500 \times g$ for 5 min. at 4°C. The supernatant fraction was retained, whereas the pellet was washed with isolation buffer and centrifuged again. The combined supernatant fractions were centrifuged at $7800 \times g$ 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 $7800 \times g$ for 10 min. Oxygen consumption of isolated mitochondria was measured at 25°C using a model 782 oxygen meter system and model 1302 Microcathode oxygen electrode (Strathkelvin, Glasgow, UK).

Mitochondrial swelling

Isolated mitochondria (approximately 1 OD₅₂₀ absorbance unit) were resuspended in 2 ml 150 mM KCl, 25 mM NaHCO₃, 1 mM MgCl₂, 3 mM KH₂PO₄, 20 mM HEPES, 5 mM succinate, pH 7.4. Mitochondrial swelling was monitored by time resolved absorbance at OD₅₂₀ and subsequent swelling upon successive additions of 5 μ M CaCl₂. Control experiments included the addition of 5 μ M cyclosporine A to inhibit the mitochondrial permeability transition pore (PTP).

Statistical analysis

Unpaired Student's t-tests were used for statistical comparison between control and burned groups. Data are expressed as the mean $\pm$ S.E.M. and are pooled from at least three separate experiments. Significance was accepted at $P < 0.05$.

Results

All animals survived the 60% TBSA burn injury. Consistent with clinical findings, burned rats displayed elevated serum AST, ALT and decreased serum albumin, indicating compromised hepatic function (Fig. 1A–C). Significantly, thermal injury in these animals also led to caspase-3 activation and increased numbers of TUNEL-positive hepatocytes indicative of widespread diffuse hepatocyte apoptosis (Fig. 1D–F). Thus, we believe that this animal model of thermal injury accurately recapitulates the phenomena associated with the disease state in human patients after burn.

Fig. 1 Burn injury causes hepatic damage, dysfunction and hepatocyte apoptosis. Hepatic dysfunction of the rat burn model mimics the post-burn human disease state. **(A)** Serum aspartate aminotransferase (AST) 24 and 48 hrs after burn injury (each time-point represent eight animals per group). **(B)** Serum alanine aminotransferase (ALT) 24 and 48 hrs after burn injury (each time-point represent eight animals per group). **(C)** Serum albumin 24 and 48 hrs after burn injury (each time-point represent eight animals per group). **(D)** Caspase-3 activity in liver cytosolic fractions was determined by fluorometric Asp-Glu-Val-Asp cleavage assays, as described previously [22]. The data represent the average of three separate experiments. Each experiment was performed in triplicate. **(E)** TUNEL staining of a liver section before (control) and 24 hrs after burn injury (burn). Arrow indicates TUNEL positive cells **(F)** Quantified TUNEL-positive cells 24, 48 and 120 hrs after burn injury (each time-point represent eight animals per group). Time after injury in hours is indicated. C, control; B, burn. Data in this and subsequent figures are the mean $\pm$ S.E.M. * $P < 0.05$.

We hypothesized that altered calcium dynamics may mediate hepatocyte apoptosis after burn injury. In order to test this hypothesis, we isolated primary hepatocytes from thermally injured and sham treated animals and investigated alterations in intracellular calcium storage and release. Cytosolic-free calcium concentration was evaluated using the ratiometric calcium indicator dye fura-2. As shown in Fig. 2A and B, resting cytosolic calcium was significantly elevated in hepatocyte cultures isolated from burned animals. To evaluate whether this elevated cytosolic calcium was associated with depleted ER calcium stores, we utilized the SERCA inhibitor thapsigargin (TG). Treatment of cells with TG unmasks a passive calcium leak from the ER, and the amplitude of the peak of calcium release after addition of TG reflects the calcium loading state of the ER [23]. As expected, TG-sensitive calcium stores were significantly lower in burn *versus* sham treated animals (Fig. 2C and D), suggesting that the elevated calcium concentrations in the cytosol of hepatocytes isolated from burned animals arose from the ER stores. Depletion of ER calcium stores would be expected to result in altered hepatic response to signalling molecules important for hepatocyte homeostasis that are linked to intracellular calcium release such as hormones, growth factors and purinergic agonists. We made the assumption that hepatocytes from burned animals would have decreased responsiveness to agonists coupled to phospholipase C, which releases calcium from ER stores *via* the

production of the second messenger inositol 1,4,5-trisphosphate. The peptide hormone Arg-vasopressin (AVP) and the purinergic agonist ATP are known to release calcium from hepatocytes by coupling to phospholipase C [24]. Indeed, hepatocyte calcium responses to saturating doses of both AVP and ATP were dramatically suppressed in hepatocytes isolated from burned animals (Fig. 2E–H). Thus, burn injury induces significant defects in the ability of hepatocytes to respond to extracellular stimuli, which would severely compromise hepatic function.

We have previously shown that cytochrome c binding to the inositol 1,4,5-trisphosphate receptor (IP₃R) contributes to apoptotic calcium release in hepatocytes and other cell types [21, 22]. This association can be specifically monitored by examining the subcellular redistribution of cytochrome c from mitochondrial-enriched fractions to ER-enriched fractions [21, 22]. To examine if cytochrome c binding to IP₃R participates in burn-induced hepatic damage *in vivo*, we isolated mitochondrial, cytosol and ER-enriched fractions from livers of control and burned animals 24 hrs after injury. As shown in Fig. 3A, cytochrome c translocates from the mitochondrial fraction to the ER-enriched fraction 24 hrs after burn injury in three separate pairs of animals. This translocation is specifically mediated by cytochrome-c binding to IP₃R, as (i) genetic knock out of IP₃R eliminates this translocation [21] and (ii) dominant negative peptides which block this interaction

Fig. 2 Burn injury results in significant alterations of hepatocyte calcium homeostasis. **(A)** Representative fura-2 ratio images of cytosolic calcium in isolated hepatocytes from a control and an animal 24 hrs after burn injury. Ratio values are pseudo-coloured as per the scale bar. **(B)** Histogram of resting cytosolic calcium in control (black bars) and burned (red bars) hepatocytes ($n = 70$ for each condition). **(C)** Single cell imaging of depletion of endoplasmic reticulum calcium stores with thapsigargin (TG) in control hepatocytes (black) and hepatocytes isolated from burned animals (red). Each trace is an average of 20 hepatocytes from a single experiment. **(D)** Quantified TG-releasable stores in hepatocytes isolated from control (black) and burned (red) animals pooled from three separate experiments. Peak release is quantified as the change in ratio (ΔR) divided by the initial ratio (R). **(E)** Single cell imaging of hepatocyte responses to Arg-vasopressin (AVP) in hepatocytes isolated from control (black) and burned (red) animals. **(F)** Quantified responses to vasopressin in hepatocytes isolated from control (black) and burned (red) animals. **(G)** Single cell imaging of hepatocyte responses to extracellular ATP in hepatocytes isolated from control (black) and burned (red) animals. **(H)** Quantified responses to ATP in hepatocytes isolated from control (black) and burned (red) animals.

also eliminates this translocation in hepatocytes [22]. Although correlative, this finding strongly suggests that cytochrome C binding to IP3R after burn mediates hepatocyte apoptosis *in vivo*. Cytochrome c oxidase (CytOx) serves as a control for the distribution of the mitochondria. To further examine the molecular changes which occur in the liver after burn injury, we performed one-dimensional SDS-PAGE and MALDI-TOF to identify abundant proteins which display changes in expression levels in whole liver lysates 24 hrs after burn injury. As shown in Fig. 3B, serum albumin precursor levels are decreased, as predicted from decreased serum levels of albumin. Levels of the matrix mitochondrial urea cycle protein carbamoyl-phosphate synthetase-1 are also increased, which is expected due to the increased ammonia burden caused by muscle catabolism after burn injury. Interestingly, we found up-regulation of the ER luminal chaperone BiP/Grp78, a classic indicator of ER stress.

A major consequence of depleted ER luminal calcium (Fig. 2C and D) would be a decreased protein folding capacity of the ER, since critical molecular chaperones that participate in the folding, assembly and maturation of secretory proteins, such as calnexin

and calreticulin require elevated ER luminal calcium to function properly [25]. Accumulation of misfolded proteins in the ER lumen would in turn lead to activation of the ER stress response [26]. ER stress is sensed by the transmembrane proteins IRE-1 and PERK-like ER kinase (PERK), which undergo oligomerization and phosphorylation in response to the presence of misfolded ER luminal proteins [27]. To determine if ER stress is induced in the liver *in vivo* 24 hrs after burn injury, we prepared lysates from whole liver from two control and six injured animals. Consistent with our hypothesis that burn injury induces ER stress, we found increased phosphorylation of IRE-1 and PERK (Fig. 3C). As expected, activation of these molecules was associated with concomitant up-regulation of the calcium-dependent ER chaperones calnexin and calreticulin. We also saw modest increases in PDI, another ER chaperone induced by ER stress. Changes in PDI levels were most likely masked due to the high abundance of this protein under normal physiology. On the other hand, the levels of the oxidoreductase ERO-1 were compromised after burn injury, which would be expected to have deleterious effects on peptide disulfide bond formation in the ER lumen. Surprisingly, we also found significant

Fig. 3 Thermal injury induces hepatic endoplasmic reticulum (ER) stress response *in vivo*. **(A)** Cytochrome c and cytochrome c oxidase (CytOx) distribution in mitochondrial (mito), cytosolic (cyt) and ER fractions from three pairs of control and burned animals 24 hrs after injury. Cytochrome c appearance in the ER fraction is indicated by a red asterisk. **(B)** Proteins which display prominent alterations 24 hrs after burn injury. Bands are detected by Coomassie staining. Band identities were determined by trypsin digestion and MALDI-TOF. **(C)** Western blots of ER stress proteins and ER calcium handling/chaperone proteins and/or their phosphoprotein levels in liver samples isolated from two control and six burned animals 24 hrs after burn injury. All Western blots are from the same animals. *Non-specific band.

up-regulation of the ER calcium ATPase SERCA-1 and the ER-resident calcium channel IP₃R type 1. Up-regulation of SERCA-1 may be an adaptive response to ER calcium store depletion, while up-regulation of IP₃R is known to be associated with increased susceptibility to apoptotic cell death [28]. Many of these effects persisted up to 48 and 72 hrs after burn injury (not shown). Apoptosis induction in response to ER stress is mediated by the BH3 only Bcl-2 family member Bim [29]. We found significant induction of Bim_L, and to a lesser extent Bim_{EL} (Fig. 3C). Furthermore, we saw up-regulation of phospho Jun N terminal kinase (Fig. 3C), which is also associated with pro-apoptotic ER

stress signalling. Thus, these results indicate a significant induction of the hepatic ER stress response and pro-apoptotic signalling pathways *in vivo* after burn, which likely contributed to liver dysfunction. As these experiments were performed in whole liver lysates, it is possible that the observed changes occurred in non-parenchymal cell types. However, as most TUNEL-positive cells in liver sections are hepatocytes (Fig. 1E), we believe that this interpretation is unlikely.

Increases in cytosolic calcium are known to be associated with mitochondrial calcium overload, release of pro-apoptotic factors, decreased respiration and swelling [30]. The consequences of these

Fig. 4 Thermal injury induces hepatic mitochondrial dysfunction *in vivo*. **(A)** State 3 respiration in isolated mitochondria from animals 48 hrs after burn injury (red) or in control animals (black). **(B)** Calcium-induced swelling in mitochondria isolated 48 hrs after burn injury. Isolated mitochondria were challenged in bulk solution with successive additions of 5 μM calcium chloride as indicated. Mitochondrial swelling was monitored by measuring the optical density at 520 nm (see Experimental Procedures). Decreases in optical density indicate mitochondrial swelling. Cyclosporine A (cspA; 5 μM), a blocker of permeability transition pore (PTP) opening, was used to confirm that calcium-induced swelling was due to PTP opening. Data represent the pooled responses from three animals in each treatment group. Note the earlier onset and more rapid and complete swelling of mitochondria isolated from burned animals. **(C)** Transmission electron microscope images from livers of control animal. Note prominent mitochondrial cristae and the surrounding rough ER. M, mitochondria; RER, rough endoplasmic reticulum. **(D)** Transmission electron microscope images of liver sections from an animal 48 hrs after burn injury. Note prominent loss of mitochondrial electron density with lack of cristae. Focal dilation of rough ER is clearly apparent and indicated by arrows. Both micrographs were taken at the same magnification. Scale bars are 500 nm.

alterations include mitochondrial depolarization, decreased respiration and ATP synthesis and metabolic dysfunction, which ultimately result in apoptosis. We hypothesized that the observed increased cytosolic calcium in hepatocytes of burned animals would lead to compromised mitochondrial function. In order to assess mitochondrial structure and function following severe burn, we purified mitochondrial fractions from the livers of control and thermally injured animals. We first investigated state 3 respiration and susceptibility to calcium-induced swelling induced by opening of the PTP. As expected, state 3 mitochondrial respiration was significantly reduced in animals subjected to thermal injury (Fig. 4A). Mitochondria from injured animals were also more susceptible to calcium-induced swelling (Fig. 4B). To examine mitochondrial structure *in situ*, we performed transmission electron microscopy of liver sections from control and thermally injured animals. We observed a significant loss of mitochondrial electron density and cristae in liver

sections of burned animals (Fig. 4C and D). In addition, our electron microscopy analysis revealed focal dilation of the rough ER after burn injury (Fig. 4D, arrows). Thus, physiological changes witnessed in isolated mitochondria *in vitro* are associated with consistent morphological changes in mitochondrial and ER structures *in situ*.

Discussion

In this report, we show that burn injury leads to increased hepatocyte cytosolic calcium and subsequent depletion of ER calcium stores. What is the mechanism by which calcium is released from ER stores? Several lines of evidence suggest that these changes are mediated by increased IP₃R activity. First, we found that burn injury was associated with cytochrome c release and translocation

to ER-enriched fractions, strongly suggesting cytochrome c binding to IP₃R and increased calcium release activity (Fig. 3A) [21]. Second, the expression levels of one of the hepatic IP₃R channels, IP₃R-1, is up-regulated after burn injury (Fig. 3C). Up-regulation of IP₃R protein levels is associated with increased susceptibility to calcium store depletion and apoptotic cell death [28, 31–33]. Third, the calcium storage capacity is not likely to be compromised, as ER luminal calcium storage/chaperone proteins calnexin and calreticulin are up-regulated. Furthermore, the hepatic SERCA pump is also up-regulated, suggesting decreased calcium pump activity is not a likely mechanism. Finally, the alterations in mitochondrial physiology such as increased susceptibility to PTP, decreased respiration and the *in situ* morphological changes are consistent with apoptotic IP₃R-linked increases in mitochondrial calcium [21, 34–36]. It remains to be determined what upstream signals lead to increased IP₃R activity in hepatocytes after burn injury. One possibility is alterations in Fas death receptor signalling, as is seen in many other pathological states leading to liver apoptosis and subsequent dysfunction [37, 38]. Consistent with this hypothesis, Fas-dependent hepatocyte apoptosis requires IP₃R activity [22].

We found that depletion of ER calcium stores resulted in ER stress and mitochondrial dysfunction, both of which likely contribute to hepatocyte apoptosis and subsequent liver dysfunction. This finding is of therapeutic significance, because limiting the unfolded protein burden with 'chemical chaperones' may promote

hepatocyte survival [39]. Furthermore, the ongoing development of pharmacological agents which limit pro-apoptotic ER stress signalling pathways may have therapeutic benefits such as improving organ function and patient survival [40].

The results presented here reveal the novel finding that thermal injury of the skin leads to hepatocyte calcium derangements, ER stress, mitochondrial dysfunction and apoptosis. The findings provide a mechanistic platform for understanding of the molecular changes that occur in hepatic physiology in response to burn injury. Furthermore, they suggest that pathways associated with hepatocyte calcium homeostasis and ER stress may be adequate targets for the development of treatment regimes for severely burned patients.

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References

- Brigham PA, McLoughlin E. Burn incidence and medical care use in the United States: estimates, trends and data sources. *J Burn Care Rehabil.* 1996; 17: 95–107.
- World Health Organization (WHO). A graphical overview of the global burden of injuries. The injury chart book. Volume 29. Geneva: WHO; 2002.
- Herndon DN, Tompkins RG. Support of the metabolic response to burn injury. *Lancet.* 2004; 363: 1895–902.
- Herndon DN, Hart DW, Wolf SE, et al. Reversal of catabolism by beta-blockade after severe burns. *N Engl J Med.* 2001; 345: 1223–9.
- van den Berge G, Wouters P, Weekers F, et al. Intensive insulin therapy in critically ill patients. *New Engl J Med.* 2001; 345: 1359–67.
- Finnerty CC, Herndon DN, Przgora R, et al. Cytokine expression profile over time in severely burned pediatric patients. *Shock.* 2006; 26: 13–9.
- Tracey KJ, Fong Y, Hesse DG, et al. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature.* 1987; 330: 662–4.
- Tracey KJ, Lowry SF, Fahey TJ, 3rd, et al. Cachectin/tumor necrosis factor induces lethal shock and stress hormone responses in the dog. *Surg Gynecol Obstet.* 1987; 164: 415–22.
- Moshage H. Cytokines and the hepatic acute phase response. *J Pathol.* 1997; 181: 257–66.
- Jeschke MG, Low JF, Spies M, et al. Cell proliferation, apoptosis, NF-kappaB expression, enzyme, protein, and weight changes in livers of burned rats. *Am J Physiol Gastrointest Liver Physiol.* 2001; 280: G1314–20.
- Jeschke MG, Micak RP, Finnerty CC, et al. Changes in liver function and size after a severe thermal injury. *Shock.* 2007; 28: 172–7.
- Jeschke MG, Rensing H, Klein D, et al. Insulin prevents liver damage and preserves liver function in lipopolysaccharide-induced endotoxemic rats. *J Hepatol.* 2005; 42: 870–9.
- Klein D, Schubert T, Horch RE, et al. Insulin treatment improves hepatic morphology and function through modulation of hepatic signals after severe trauma. *Ann Surg.* 2004; 240: 340–9.
- Price LA, Thombs B, Chen CL, et al. Liver disease in burn injury: evidence from a national sample of 31,338 adult patients. *J Burns Wounds.* 2007; 7: e1.
- Jeschke MG, Barrow RE, Herndon DN. Extended hypermetabolic response of the liver in severely burned pediatric patients. *Arch Surg.* 2004; 139: 641–7.
- Lockshin RA, Zakeri Z. Cell death in health and disease. *J Cell Mol Med.* 2007; 11: 1214–24.
- Reed JC. Apoptosis-based therapies. *Nat Rev Drug Discov.* 2002; 1: 111–21.
- Yin XM, Ding WX. Death receptor activation-induced hepatocyte apoptosis and liver injury. *Curr Mol Med.* 2003; 3: 491–508.
- Herndon DN, Wilmore DW, Mason AD Jr. Development and analysis of a small animal model simulating the human postburn hypermetabolic response. *J Surg Res.* 1978; 25: 394–403.
- Boehning D, Joseph SK. Functional properties of recombinant type I and type III inositol 1, 4,5-trisphosphate receptor isoforms expressed in COS-7 cells. *J Biol Chem.* 2000; 275: 21492–9.
- Boehning D, Patterson RL, Sedaghat L, et al. Cytochrome c binds to inositol

- (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis. *Nat Cell Biol.* 2003; 5: 1051–61.
22. **Wozniak AL, Wang X, Stieren ES, et al.** Requirement of biphasic calcium release from the endoplasmic reticulum for Fas-mediated apoptosis. *J Cell Biol.* 2006; 175: 709–14.
23. **Thastrup O, Cullen PJ, Drobak BK, et al.** Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+} -ATPase. *Proc Natl Acad Sci USA.* 1990; 87: 2466–70.
24. **Kawanishi T, Blank LM, Harootunian AT, et al.** Ca^{2+} oscillations induced by hormonal stimulation of individual fura-2-loaded hepatocytes. *J Biol Chem.* 1989; 264: 12859–66.
25. **Burdakov D, Petersen OH, Verkhratsky A.** Intraluminal calcium as a primary regulator of endoplasmic reticulum function. *Cell Calcium.* 2005; 38: 303–10.
26. **Gorlach A, Klappa P, Kietzmann T.** The endoplasmic reticulum: folding, calcium homeostasis, signaling, and redox control. *Antioxid Redox Signal.* 2006; 8: 1391–418.
27. **Ron D, Walter P.** Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol.* 2007; 8: 519–29.
28. **Khan AA, Soloski MJ, Sharp AH, et al.** Lymphocyte apoptosis: mediation by increased type 3 inositol 1,4,5-trisphosphate receptor. *Science.* 1996; 273: 503–7.
29. **Puthalakath H, O'Reilly LA, Gunn P, et al.** ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell.* 2007; 129: 1337–49.
30. **Dong Z, Saikumar P, Weinberg JM, et al.** Calcium in cell injury and death. *Annu Rev Pathol.* 2006; 1: 405–34.
31. **Blackshaw S, Sawa A, Sharp AH, et al.** Type 3 inositol 1,4,5-trisphosphate receptor modulates cell death. *FASEB J.* 2000; 14: 1375–9.
32. **Li C, Fox CJ, Master SR, et al.** Bcl-X(L) affects Ca^{2+} homeostasis by altering expression of inositol 1,4,5-trisphosphate receptors. *Proc Natl Acad Sci USA.* 2002; 99: 9830–5.
33. **Jayaraman T, Marks AR.** T cells deficient in inositol 1,4,5-trisphosphate receptor are resistant to apoptosis. *Mol Cell Biol.* 1997; 17: 3005–12.
34. **Hajnóczky G, Csordas G, Madesh M, et al.** Control of apoptosis by IP(3) and ryanodine receptor driven calcium signals. *Cell Calcium.* 2000; 28: 349–63.
35. **Szalai G, Krishnamurthy R, Hajnóczky G.** Apoptosis driven by IP(3)-linked mitochondrial calcium signals. *EMBO J.* 1999; 18: 6349–61.
36. **Joseph SK, Hajnóczky G.** IP3 receptors in cell survival and apoptosis: Ca^{2+} release and beyond. *Apoptosis.* 2007; 12: 951–68.
37. **Higuchi H, Gores GJ.** Mechanisms of liver injury: an overview. *Curr Mol Med.* 2003; 3: 483–90.
38. **Dosreis GA, Borges VM, Zin WA.** The central role of Fas-ligand cell signaling in inflammatory lung diseases. *J Cell Mol Med.* 2004; 8: 285–93.
39. **Ozcan U, Yilmaz E, Ozcan L, et al.** Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science.* 2006; 313: 1137–40.
40. **Wiseman RL, Balch WE.** A new pharmacology—drugging stressed folding pathways. *Trends Mol Med.* 2005; 11: 347–50.