Effect of Estrogen on Mitochondrial Function and Intracellular Stress Markers in Rat Liver and Kidney following Trauma-Hemorrhagic Shock and Prolonged Hypotension

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Trauma-hemorrhage (T-H) is known to impair tissue perfusion, leading to tissue hypoxia, and thus affecting mitochondria, the organelles with the highest oxygen demand. In a model of T-H and prolonged hypotension without fluid resuscitation, administration of a small volume of 17β-estradiol (E2), but not vehicle, prolonged the survival of rats for 3 h, even in the absence of fluid resuscitation. The main finding of this study is that T-H followed by prolonged hypotension significantly affects mitochondrial function, endoplasmic reticulum (ER) stress markers and free iron levels, and that E2 ameliorated all these changes. All of these changes were observed in the liver but not in the kidney. The sensitivity of mitochondrial respiration to exogenous cytochrome c can reflect increased permeability of the outer mitochondrial membrane for cytochrome c. Increased levels of free iron are indicative of oxidative stress, but neither oxidative nor nitrosylative stress markers changed. The spliced isoform of XBP1 mRNA (an early marker of ER stress) and the expression of C/EBP homologous protein (CHOP) (a protein regulating ER stress-induced apoptosis) were elevated in T-H animals but remained unchanged if T-H rats received E2. Both the prevention of elevated sensitivity of mitochondrial respiration to cytochrome c and a decrease in ER stress by E2 maintain functional integrity of the liver and may help the organ during prolonged hypotension and following resuscitation. A decrease in free iron levels by E2 is more relevant for resuscitation, often accompanied by oxidative stress reaction. Thus, E2 appears to be a novel hormonal adjunct that prolongs permissive hypotension during lengthy transportation of the injured patient between the injury site and the hospital in both civilian and military injuries.

INTRODUCTION

Trauma-hemorrhage (T-H) followed by resuscitation is often fatal in civilian and military trauma. T-H affects various organs (for example, liver, kidney, lung, heart), but most frequently the liver (1–4). Recently it has been found that deleterious effects of T-H are influenced by sex hormones (5) and that estrogen (E2) improves immune and cardiovascular response parameters (6). However, the precise mechanism by

which sex hormones (i.e. estrogen) produce salutary effects has yet to be determined (7). T-H consists of two phases: the hypovolemic phase, characterized by systemic hypotension, which can lead to circulatory failure if not followed by resuscitation; and the subsequent resuscitation phase, which restores blood flow and oxygen delivery to the tissues.

The first hypotensive phase is accompanied by reduced delivery of oxygen

to the tissues. Little is known regarding the mechanisms operating in this phase. It has been shown that circulatory parameters can be improved by E2 during this phase. After major blood loss of 62% of the circulating blood volume, animals cannot maintain their blood pressure and will expire if fluid resuscitation is not provided (8). When a small volume of E2, and not vehicle, was administered after 62% blood loss, the animals not only demonstrated an acute survival during prolonged hypotension (3 h), but also prolonged survival after fluid resuscitation. However, the mechanism by which small volume E2 administration (0.4 mL/kg body weight [BW]) sustains prolonged permissive hypotension and improves survival remains unknown. We hypothesized that E2 influ-

ences vital biochemical processes as early as during prolonged permissive hypotension.

The second phase of T-H, resuscitation, elicits a highly predictable acute inflammatory response (9). This phase often is associated with oxidative stress, which can be alleviated by sex hormones (5). It has been shown that T-H followed by resuscitation induces lipid peroxidation and protein nitrosylation (10), both of which subside after administration of the testosterone receptor antagonist, flutamide (10). T-H followed by resuscitation heightens the expression of heme oxygenase-1 (HO-1), a protective enzyme, and the administration of E2 further increases its expression (11). In addition, it has been shown that E2 facilitates accumulation of iron in iron storage (12), suggesting that E2 increases iron storage capacity in the tissues, which in turn may decrease the prooxidative capacity of the intracellular free-iron pool. This was further supported by the fact that decreased hepatic E2 receptor levels increase lipid peroxidation (13). These experiments show that E2 has different beneficial effects on the physiological and biochemical processes after resuscitation.

Taken together, these findings suggest that the tissue-protective effects of E2 begin very early after T-H, during the hypotensive phase. During this phase of impaired circulation and oxygen delivery, mitochondrial function is mostly affected, since mitochondria are the main consumers of oxygen in cells to synthesize adenosine triphosphate (ATP). Studies have shown that T-H decreases hepatic ATP levels even after resuscitation (4,14,15), suggesting that T-H may cause mitochondrial dysfunction. In addition, T-H causes endoplasmic reticulum (ER) stress, which is characterized by activation of different signaling pathways, inositol requiring enzyme/X-box protein 1, and RNA-dependent protein kinase–like ER kinase (PERK) (16). ER stress triggers the initially protective unfolded protein response (UPR), however, persistent ER stress leads to induction of apoptosis,

and has been shown to occur in different models of T-H followed by reperfusion (17,18).

To target the possible early E2-mediated effects, we investigated which of the above described biochemical processes (oxidative/nitrosylative/ER stress, altered mitochondrial function) are operative during prolonged hypotension and whether E2 affects these parameters.

MATERIALS AND METHODS

Animal Model

Male Sprague-Dawley rats 6–8 wks old (Charles River, Wilmington, MA, USA) were anesthetized with isoflurane; both femoral arteries and a femoral vein were cannulated to monitor blood pressure, remove blood and administer fluid, respectively. Rats were then allowed to awaken and were rapidly bled to a mean blood pressure of 40 mmHg within 10 min. This pressure was maintained by further bleeding the rats in small volume until 62% of the circulating blood volume was removed (approximately 45 min from the onset of bleeding). Rats then received vehicle (cyclodextrin, 0.4 mL/kg BW) or cyclodextrin estrogen (water soluble; 1 mg/kg BW); no additional fluid was administered thereafter for 2 h. A time point of 2 h after removal of 62% of the circulating blood volume was selected for this study, since our previous study has shown that the majority of the group receiving a small volume of E2 after T-H and prolonged hypotension survived for 3 h, but the vehicle group did not (8). Therefore a 2-h period of hypotension allowed us to obtain tissue samples from a reasonable number of vehicle-treated animals. At 2 h, rats were killed and tissues harvested for analysis. Experiments were performed in adherence to the National Institutes of Health (NIH) *Guidelines for the Care and Use of Laboratory Animals*. The Institutional Animal Care and Use Committee of the University of Alabama at Birmingham (Birmingham, AL) approved this project.

Mitochondrial Function

Respiratory parameters of mitochondria were determined in rat liver homogenates containing mitochondria with a Clark-type oxygen electrode apparatus (Oroboros Ltd., Innsbruck, Austria) as described previously (19). Considering that a population of mitochondria can be lost during the isolation process, we performed these measurements in tissue homogenates without isolating mitochondria. Rat liver homogenates were incubated in a buffer consisting of 80 mmol/L KCl, 20 mmol/L Tris-HCl, 1 mmol/L diethylene triamine pentaacetic acid, 5 mmol/L KH_2PO_4 and 0.1% fatty acid-free BSA (pH 7.4, 25°C). State 2 respiration was stimulated either by the addition of 5 mmol/L glutamate plus 5 mmol/L malate or 10 mmol/L succinate. The transition to State 3 respiration was induced by addition of 125 μmol/L ADP. After conversion of all added adenosine diphosphate (ADP) to ATP, mitochondrial respiration returned to State 4. Increasing concentrations of carbonyl cyanide m-chlorophenylhydrazone (CCCP) then were added to the mitochondrial suspension until the maximum oxygen uptake rate was reached (normally in a range of 0.5–2.0 μmol/L) to determine the maximum electron transfer capacity. Finally 2.5 μmol/L of cytochrome c was added to test whether mitochondrial respiration could be accelerated, which is supportive for an increased permeability of the outer membrane.

Electron Paramagnetic Resonance (EPR) Measurements

Intracellular free iron was detected by EPR as described previously (20). Frozen tissues were homogenized in a buffer containing desferrioxamine B aspirated in standard 1-mL syringes and frozen in liquid nitrogen. Samples were pressed out of the syringes for EPR analysis. EPR spectra were recorded at liquid nitrogen temperature with an EPR spectrometer (MiniScope MS200, Magnettech, Berlin, Germany). EPR settings: microwave frequency 9.6 GHz; modulation frequency 100 kHz; modulation amplitude 10 G.

The magnitude of signals at g = 4.3 was determined.

Dot Blot Analysis of Protein Carbonyls

Total homogenates from liver (250 μg) were derivatized with 10 mmol/L dinitrophenyl hydrazine in 2 mol/L HCl (120 min, room temperature). The protein hydrazones were precipitated with trichloroacetic acid, washed with ethanol/ethylacetate (1:1), resuspended in loading buffer (vide supra) and blotted in duplicate. Total homogenates from liver were blotted in duplicate on a single Hybond-P membrane (Amersham/GE Healthcare, Little Chalfont, UK), using a Bio-Dot apparatus (Bio-Rad, Munich, Germany). Protein from each sample (250 μg) was diluted with loading buffer (225 mmol/L Tris, pH 6.8, 50 mg/mL SDS, 50% w/v glycerol) to give 500 μL, and denatured at 95°C for 3 min. One hundred μL were blotted per dot; another 100 μL per dot were blotted in duplicate on a second blot for Coomassie staining (20 min) as a loading control. The blots were blocked with buffer A (10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20 and 5% non-fat milk powder) for 1 h, then incubated sequentially with a polyclonal anti-dinitrophenyl antibody (1:500; Sigma, St. Louis, MO, USA) and a polyclonal rabbit anti-mouse horseradish peroxidase-linked antibody (1:10,000; Sigma) in buffer A for 12 h (4°C) and 2 h (25°C), respectively. After each antibody incubation, the blots were washed four times with buffer A lacking milk. Finally, blots were stained with enhanced chemiluminescence (ECL) substrate (Amersham/GE Healthcare) for 5 min and exposed to Hyperfilm MP (GE Healthcare, Life Sciences, Munich, Germany). The signals were quantified with Adobe Photoshop CS3 10.0 software (Adobe Systems Inc., San Jose, CA, USA) including background subtraction. Intensities were normalized to the Coomassie staining.

Western Blot Analysis (3-Nitro-Tyrosine)

SDS-PAGE of liver homogenates was performed according to Laemmli (18),

but under nonreducing conditions. Fifty μg of protein per sample were separated on 8% polyacrylamide gels in a Hoefer SE600 electrophoresis system (Hoefer Scientific Instruments, San Francisco, CA, USA; gel size 140 × 140 × 1.5 mm, with a reduced separation distance of 9 cm). Proteins were transferred onto PVDF membranes (Hybond-P, GE Healthcare Life Sciences, Munich, Germany) by semidry blotting in a Hoefer Semi-Phor TE70. Blots were probed with a monoclonal anti-nitrotyrosine antibody (Calbiochem, Darmstadt, Germany) and a polyclonal anti-mouse horseradish peroxidase linked antibody (GE Healthcare), both applied in 5% skim milk. Immunoreactive bands were detected by ECL (GE Healthcare) and resulting films scanned on a Sharp JX-330 flatbed scanner. Band volumes were evaluated with Quantity One V2.7 (PDI, Huntington Station, NY, USA), and immunostaining was normalized to general protein staining (identically prepared samples run on a parallel gel, stained with Coomassie Brilliant Blue R-250). As negative and positive controls, 50 μg BSA and 0.9 μg nitrated BSA, respectively, were blotted. BSA was nitrated with freshly made peroxynitrite (from $NaNO_2$ and H_2O_2) (17).

Determination of 2-Thiobarbituric Acid-Reactive Substances (TBARS)

Liver homogenates were prepared at 4°C from frozen tissue pieces (1:2, wt:vol) in 135 mmol/L KCl, 20 mmol/L Tris-HCl, 1 mmol/L desferal and 28 μmol/L 3,5-di-tert-butyl-4-hydroxy-toluene (BHT, final concentrations) using a Potter-Elvehjem homogenizer (electrically driven Teflon pestle, VWR International, Vienna, Austria). Homogenates were frozen and stored at –80°C. Determination of TBARS was adapted from a method described by Yagi (21,22). Samples were thawed at 25°C and precipitated (0.66–1.18 mg liver tissue protein) with 3.5 mol/L H_2SO_4 and 1% (wt/vol) phosphotungstic acid for 5 min at 22°C and centrifuged at 4°C (10 min, 2,000g). Pellets were resuspended once more in H_2SO_4 and phosphotungstic acid, precipitated and cen-

trifuged. The final sediments were suspended in 600 μl deionized water supplemented with BHT and desferal (32 μmol/L each). Samples were mixed with 150 μl TBA reagent (46.5 mmol/L TBA dissolved in 50% [vol/vol] acetic acid) and heated at 95°C for 60 min. After cooling on ice, TBARS were extracted into 750 μl n-butanol by vortexing for 30 s. After centrifugation (4°C, 10 min, 2,000g), 600 μL of the butanol phase were mixed with 1400 μl butanol (22°C) and measured on an F4500 Spectrofluorimeter (Hitachi, Ltd, Tokyo, Japan) at 700V PMT voltage, excitation 515 nm (5 nm slit width) and emission wavelengths 553 nm (20 nm slit width) using 1,1,3,3-tetramethoxypropane as standard.

Expression Analysis

RNA was extracted from liver specimens using TriReagent (Molecular Research Center Inc., Cincinnati, OH, USA). From total RNA, 1 μg was used for cDNA synthesis with Superscript (Invitrogen, Carlsbad, CA, USA) and anchored oligo-dT-primers (3.5 μmol/L final concentration). To check the generation of amplifiable cDNA in the reverse transcription, a conventional PCR step was performed using glycerine aldehyde dehydrogenase-specific primers, as described elsewhere (23). Analysis of gene expression was performed by means of real-time PCR using specific primer pairs as indicated in Table 1 spanning over exon/exon junctions, where possible. PCR was carried out on an iCycler iQ (Bio-Rad) using iTaq polymerase (0.625 U/reaction; Bio-Rad) and a final concentration of 200 μmol/L dNTP (each) and 3 mmol/L $MgCl_2$ with the provided reaction buffer (1×) in a final volume of 25 μL. Each reaction well was loaded with primers (500 mol/L each) and SYBR GREEN I (0.5×, Sigma, St. Louis, MO, USA). All sequences of interest were amplified in duplicate in parallel from one aliquot of cDNA on the same plate using the following PCR protocol: initial Taq-polymerase-activating step at 95°C for 3 min, 40 cycles with 20 s denaturation step at 94°C, 30 s annealing

Table 1. Primer sequences for gene expression analysis.

Target	Accession	Sense Primer	Antisense Primer	Source
TNF	X66539	tgc ctc agc ctc ttc tca tt	tgt ggg tga gga gca cat ag	(31)
iNOS	D14051	cac ctt gga gtt cac cca gt	tgt tgt agc gct gtg tgt ca	(32)
HO-1	NM_012580	cca gcc aca cag cac tac	gcg gtc tta gcc tct tct g	(33)
GRP78[a]	S63521	gtt ctg ctt gat gtg tgt cc	ttt ggt cat tgg tga tgg tg	(34)
XBP1[b]	BC079450	ctt gtg att gag aac cag gag	aag agg caa cag cgt cag	(19)
CHOP	NM_024134	ttg ggg gca cct ata tct ca	ctc ctt cag tcg ctg ttt cc	[c]
Cyc[d]	X52815	tat ctg cac tgc caa gac tga gtg	ctt ctt gct ggt ctt gcc att cc	(35)

[a]GRP78: endoplasmic reticulum (ER) stress marker.
[b]XBP1: early marker of ER stress.
[c]CHOP primer designed for this study; no outside source.
[d]Cyc: cyclophilin A.

step at 62°C, 40 s extension step at 72°C, and 15 s step at 85°C during which data were collected. Quantification of the template concentration was achieved using the built-in iCycler iQ Detection System Software, Version v3.1 (Bio-Rad, Hercules, CA, USA) by applying the ΔΔCt method. For normalization, the values of the duplicates specific for the different target sequences were averaged and calculated in relation to the mean of the values obtained from cyclophilin A, which showed no influence of the expression by the experimental conditions (data not shown). For quantitative determination of the spliced variant of XBP-1 mRNA, 10 μL from the PCR reaction product were separated on a 2% agarose gel, stained with ethidium bromide, and visualized by 300 nm UV transillumination. Density of both products, unspliced and spliced variants, was quantified via computer-assisted densitometric scanning using the public domain Scion Image program (PC version of "NIH Image" developed at the US National Institutes of Health) available online at http://www.scioncorp.com, and the ratio of the spliced-to-unspliced variant was determined in each sample.

Data Analysis and Statistics

Data analysis of quantitative parameters was performed using SPSS 11.0 for Windows (SPSS Inc, Chicago, IL, USA). To plot results, data from groups were calculated as medians indicating 25% and 75% quartiles. To analyze differences between shock and the respective sham groups, nonparametric Mann–Whitney test was performed; differences were considered significant if $P < 0.05$.

RESULTS

Liver

Mitochondria. E2 raised respiratory control values in sham animals respiring on glutamate and malate (G-M), but not on succinate. These changes were not seen in severe shock animals (Figure 1A, Figure 2A), suggesting that they were reversed during shock. T-H caused a significant increase in State 2 respiration in liver mitochondria respiring on either G-M (Figure 1B) or succinate (Figure 2B). This increase, however, was not influenced by E2 (Figure 1B, Figure 2B). Total capacity of respiratory chain to transport electrons (determined in the presence of CCCP) in severe shock animals increased significantly with G-M (Figure 1C) and with succinate (Figure 2C). E2 lowered this parameter, which, however, remained above control values. The maximal respiratory capacity was increased further with the addition of cytochrome c to succinate-respiring mitochondria isolated from T-H; this parameter was again decreased in T-H animals treated with E2 (Figure 2C).

Free iron. Levels of free iron were increased significantly in the liver of T-H animals. These changes were abolished in the presence of E2 (Figure 3A).

Oxidized proteins and lipid peroxidation. There was no significant change in levels of carbonylated or nitrosylated proteins (Figure 4). Level of TBARS remained unchanged (Figure 3B).

ER stress. The marker of ER stress, XBP1, shows that the spliced variant of XBP1 (XBP1[S], Figure 5A) increased at the end of the shock phase in T-H. Administration of E2 after T-H did not influence the levels of XBP1(S) in sham animals, but decreased the level of XBP1(S) in shock animals, abolishing the significant difference between sham and T-H + E2 groups (Figure 5B). A similar picture was observed for C/EBP homologous protein (CHOP) expression at mRNA level. The expression was elevated significantly in the T-H but not in the T-H + E2 group (Figure 5C). Another marker of ER stress, Grp78, was not changed (Figure 6A).

Inflammatory response. The markers of inflammatory response, tumor necrosis factor (TNF)-α and inducible nitric oxide synthase (iNOS) were not changed significantly at the mRNA level, although they showed a trend to increase in E2 groups (Figure 6B, C). There was no significant difference in the levels of HO-1, an enzyme, shown to have a protective capacity (Figure 6D).

Kidney

Neither changes caused by T-H nor effects of E2 were found in kidney. Therefore, these data are not shown in the Results section.

DISCUSSION

The main finding of this study is that T-H followed by prolonged hypotension significantly affects mitochondrial
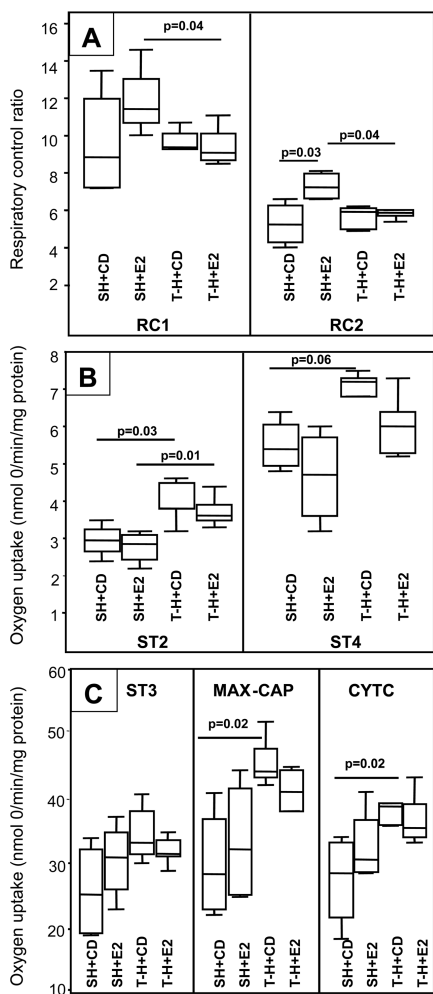
Figure 1. Effect of trauma-hemorrhage (T-H) on the functional parameters of mitochondria respiring on glutamate and malate. (A) Respiratory control; (B) State 2 and State 4 respiration rates; (C) State 3 respiration rate. RC: respiratory control; RC1: ratio of State 3 (ST3)-to-State 2 (ST2); RC2: ratio of ST3-to-State 4 (ST4); MAX-CAP: maximal respiration capacity (in the presence of carbonyl cyanide m-chlorophenylhydrazone (CCCP)); CYTC: respiration in the presence of both CCCP and cytochrone c; SH + CD: sham plus vehicle (cyclodextrin); SH + E2: sham plus estrogen; T-H + CD: T-H plus vehicle; T-H + E2: T-H plus estrogen.
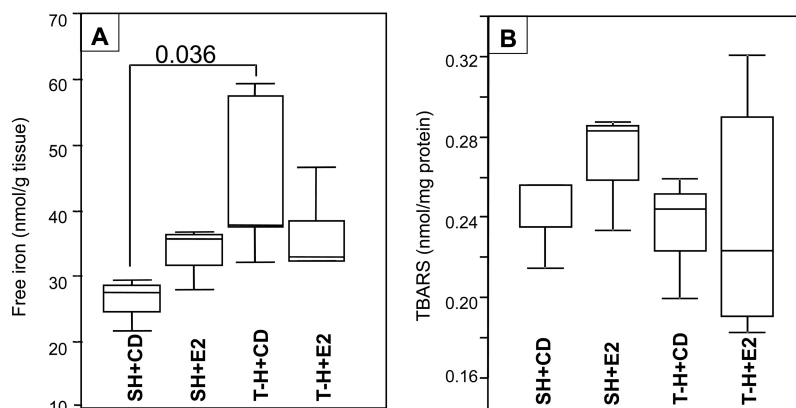
function, ER stress markers and free iron levels, and that E2 diminishes these changes. All of these effects appeared to be liver-specific, because they were not observed in the kidney. This suggests that liver is more susceptible
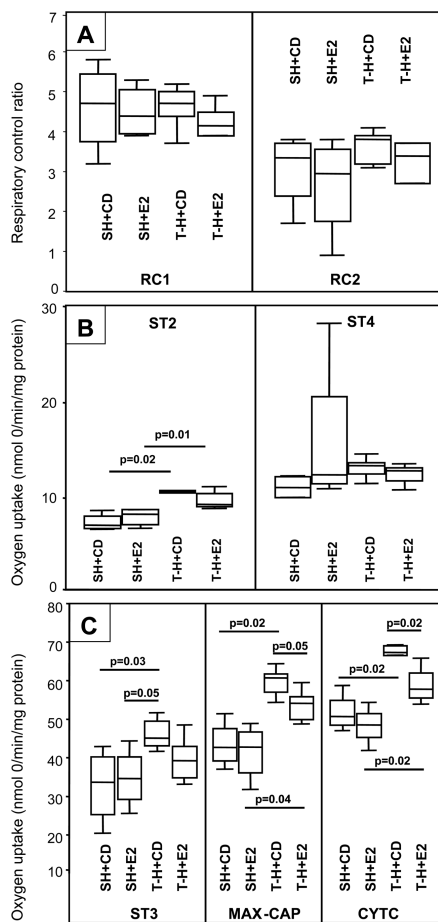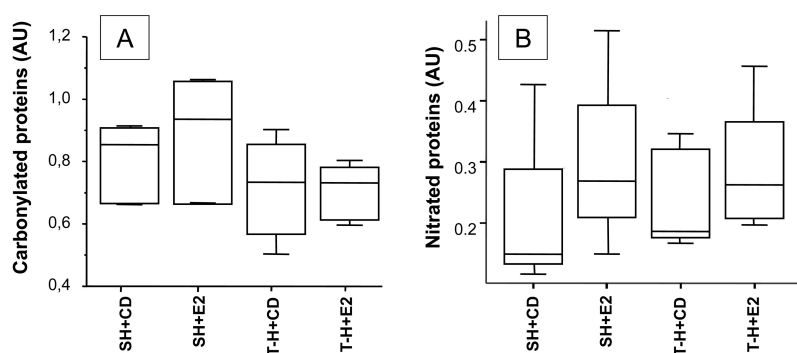

Figure 2. Effect of T-H on the functional parameters of mitochondria respiring on succinate. (A) Respiratory control; (B) State 2 and State 4 respiration rates; (C) State 3 respiration rate.

Figure 3. Effect of T-H and E2 on levels of free iron (A) and TBA-reactive substances (B) in liver.

to prolonged hypotension after T-H than kidney.

Determination of mitochondrial function in liver revealed that T-H increased the maximum electron transfer capacity of the mitochondrial respiratory chain and the sensitivity of mitochondrial succinate-dependent respiration to cytochrome c. Neither effect was detectable in animals that received E2. Accelerated electron transfer capacity may generate a higher membrane potential, facilitating ATP synthesis, but it also stimulates mitochondrial reactive oxygen species (ROS) production (24,25), which in turn can interfere with intracellular signaling cascades or induce oxidative stress. In addition, we found elevated levels of free iron in the livers of T-H animals. This was not observed in E2-treated animals. Accumulation of free iron is known to be a potent inducer of oxidative stress (26). The combination of elevated electron transfer capacity and increased free iron levels was not accompanied by either activation of lipid peroxidation or increased nitrosylation/carbonylation of proteins in our samples. However, after resuscitation, increased levels of oxidative stress markers were shown to occur in this model of T-H (10). Thus, it appears that similar to hypoxia or ischemia followed by reperfusion, oxidative stress may be triggered during the reoxygenation phase of resuscitation, indicating that oxidative stress development requires increased

Figure 4. Effect of T-H and E2 on levels of carbonylated (A) and nitrated (B) proteins in liver.

oxygen tension within the tissues. It is possible that decreased free iron levels and the presumed decreased generation of mitochondrial ROS mediated by E2 would give rise to less oxidative stress during reperfusion. As this tissue-protecting effect would become beneficial during resuscitation only, it cannot be the effect responsible for prolonged survival after the hypotensive phase.

Figure 5. Effect of T-H and E2 on XBP1 activation; representative PCR products of XBP1 (A); statistical presentation of XBP1 (B) and expression of C/EBP homologous protein (CHOP) (C).

Mitochondrial succinate-dependent respiration in T-H and prolonged hypotension animals were more sensitive to cytochrome c. Treatment with E2 diminished the effects of cytochrome c. Mitochondria are known to induce apoptosis via release of small proteins, such as cytochrome c and AIF, owing to increased permeability of the outer mitochondrial membrane (reviewed in [27]). Increased sensitivity to cytochrome c may be due to the increased permeability of the outer mitochondrial membrane for cytochrome c. This is supported indirectly by the fact that CHOP, a critical messenger inducing increased perme-

ability of outer mitochondrial membrane and apoptosis, was upregulated at the mRNA level in T-H, but not in animals subjected to T-H treated with E2. CHOP is a part of the so-called UPR, which is activated via splicing of XBP1 in response to ER stress.

T-H and resuscitation have been shown to induce ER stress (16,19). The GRP78 (also called BiP) is a chaperone and a key protein in ER stress that triggers UPR upon binding to three specific stress sensors, IRE1, PERK and activating transcription factor 6 (ATF6), and simultaneously to unfolded proteins. ER stress, which results in accumulation of unfolded proteins, induces redistribution of GRP78 between stress sensors and unfolded proteins in favor of unfolded proteins, thereby liberating the sensors, which activate UPR. One of the first products of UPR is the IRE1-mediated unconventional splicing of XBP1 mRNA, giving rise to an active transcription factor, which induces expression of UPR relevant genes, including *GRP78*. Upregulation of *GRP78* leads to increased folding capacity of the ER and is therefore a feedback mechanism that blocks UPR. However, if protein aggregation is persis-
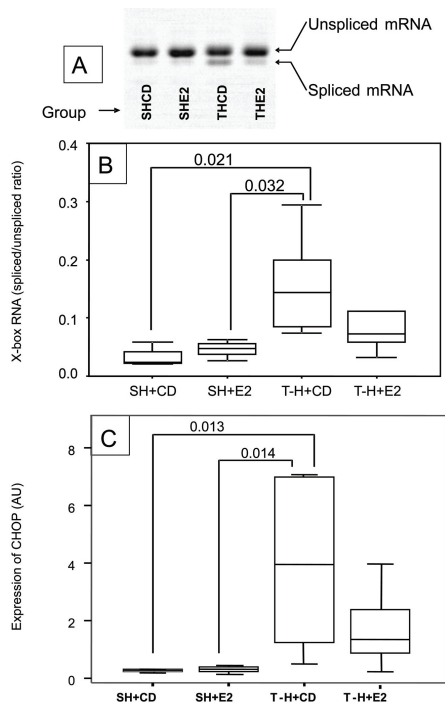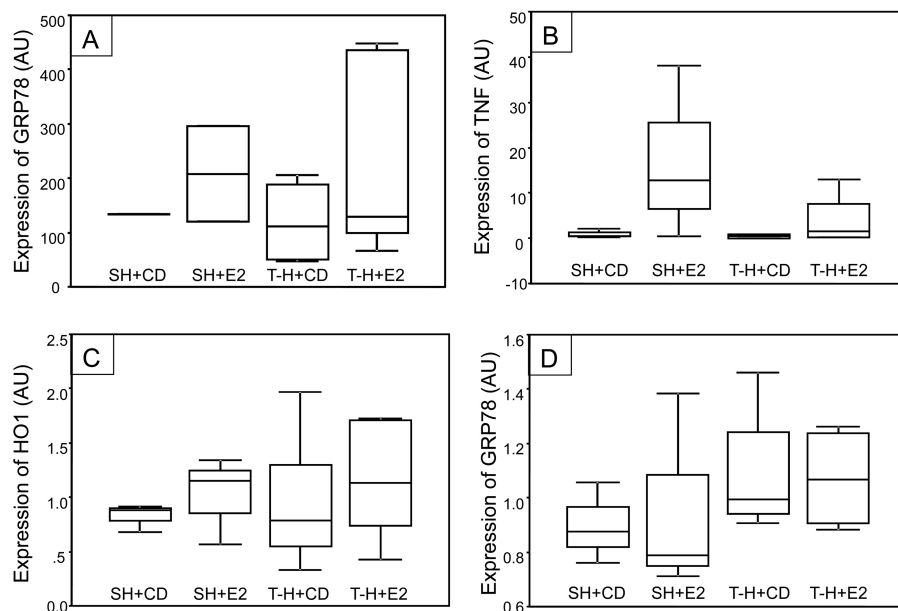
Figure 6. Effect of T-H and E2 on the expression of GRP78 (A); tumor necrosis factor (TNF)-α (B); inducible nitric oxide synthase (iNOS) (C); heme oxygenase (HO)-1 (D) in liver.

tent and stress cannot be resolved, UPR switches from a prosurvival to a proapoptotic signaling pathway, which is executed by upregulation of CHOP as mentioned above (reviewed in [28]).

Here, we show increased splicing of XBP1 indicating that ER stress already was activated during the prolonged hypotensive phase after T-H. However, GRP78 mRNA was not yet upregulated. In a different model of T-H, we have shown previously that splicing of XBP1 mRNA already was evident during reperfusion, while GRP78 mRNA was upregulated only after full reperfusion (19). Both markers remained elevated until 18 hours after shock and were associated with a proapoptotic phenotype, suggesting unresolved ER stress and dysfunction (18). Here we show that T-H–mediated splicing of XBP1 mRNA is sensitive to E2. In the presence of E2, we did not observe a significant increase in the spliced isoform of XBP1. Activation of UPR suggests ongoing ER stress resulting in accumulation of unfolded nonfunctional proteins. Functionality of the ER, however, also may be important during prolonged hypotension, because a number of secreted factors supporting blood parameters (for example, oncotic pressure, coagulation) are critical for maintaining blood circulation.

T-H affects mitochondrial function and free iron levels, and induces ER stress during the shock phase. These effects are all sensitive to E2; however, we do not know whether these effects occur independently or are linked to each other. Consequently, we do not know whether a common underlying mechanism could explain the different effects of E2, that is, the modulation of mitochondrial function, free iron levels and ER stress, or if this hormone has multiple intracellular targets. It has been suggested that E2 controls mitochondrial biogenesis and oxygen consumption via expression of mitochondrial transcription factor (29). The effect of E2 on free iron levels can be explained by the fact that E2 facilitates sequestration of iron in iron storage (12). There is, however, no data available on

the mechanism by which E2 influences ER stress, but an estrogen-specific interaction at the ER membrane has been demonstrated (30).

Our data suggest that the target(s) for E2 *during the hypotensive phase* are located before activation of UPR. It is possible that E2 preserves microperfusion and oxygen delivery. That would prevent ER stress and reduce mobilization of free iron from intracellular iron storages. The latter would have positive feedback to further preserving liver function and microcirculation. However, additional investigation of the processes occurring during prolonged hypotensive phase of T-H is required to clarify the underlying mechanisms of E2-mediated tissue protection.

CONCLUSION

The effects of E2 during prolonged hypotension can be divided into two groups, the first may have beneficial effects during hypotensive phase, and the second can exert beneficial effects mainly during resuscitation. The first group includes the prevention of elevated sensitivity of mitochondrial respiration to cytochrome c and a decrease in ER stress, both essential to support liver function during prolonged hypotension. Mitochondrial function is necessary to maintain the entire metabolism of cells, and the endoplasmic reticulum is the most important subcellular organelle responsible for specific liver functions. The second group, in our opinion, includes a decrease in free iron levels by E2, which is likely more important during and after the resuscitation/reoxygenation phase rather than during prolonged hypotension because oxidative stress is activated during reperfusion. However, this finding could explain the E2-mediated antioxidative effect, and, possibly, also could account for the decreased inflammatory reaction within tissues observed after resuscitation.

Nonetheless, since not only 3-hour survival but also long-term survival was improved by E2 during the prolonged hypotensive phase, even in the absence of

fluid resuscitation, it appears that this hormone may be a useful adjunct for trauma victims for whom transport time to the emergency room is prolonged, and also in combat casualty situations.

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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