Estrogen-provided cardiac protection following burn trauma is mediated through a reduction in mitochondria-derived DAMPs

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Submitted 10 June 2013; accepted in final form 16 January 2014

Yao X, Wigginton JG, Maass DL, Ma L, Carlson D, Wolf SE, Minei JP, Zang QS. Estrogen-provided cardiac protection following burn trauma is mediated through a reduction in mitochondria-derived DAMPs. Am J Physiol Heart Circ Physiol 306: H882–H894, 2014. First published January 24, 2014; doi:10.1152/ajpheart.00475.2013.—Mitochondria-derived danger-associated molecular patterns (DAMPs) play important roles in sterile inflammation after acute injuries. This study was designed to test the hypothesis that 17β-estradiol protects the heart via suppressing myocardial mitochondrial DAMPs after burn injury using an animal model. Sprague-Dawley rats were given a third-degree scald burn comprising 40% total body surface area (TBSA). 17β-Estradiol, 0.5 mg/kg, or control vehicle was administered subcutaneously 15 min following burn. The heart was harvested 24 h postburn. Estradiol showed significant inhibition on the productivity of H2O2 and oxidation of lipid molecules in the mitochondria. Estradiol increased mitochondrial antioxidant defense via enhancing the activities and expression of superoxide dismutase (SOD) and glutathione peroxidase (GPx). Estradiol also protected mitochondrial respiratory function and structural integrity. In parallel, estradiol remarkably decreased burn-induced release of mitochondrial cytochrome c and mitochondrial DNA (mtDNA) into cytoplasm. Further, estradiol inhibited myocardial apoptosis, shown by its suppression on DNA laddering and downregulation of caspase 1 and caspase 3. Estradiol’s anti-inflammatory effect was demonstrated by reduction in systemic and cardiac cytokines (TNF-α, IL-1β, and IL-6), decrease in NF-κB activation, and attenuation of the expression of inflammasome component ASC in the heart of burned rats. Estradiol-provided cardiac protection was shown by reduction in myocardial mitochondrial cytochrome c and mitochondrial DNA (mtDNA) into cytoplasm. Further, estradiol inhibited myocardial apoptosis, shown by its suppression on DNA laddering and downregulation of caspase 1 and caspase 3. Estradiol’s anti-inflammatory effect was demonstrated by reduction in systemic and cardiac cytokines (TNF-α, IL-1β, and IL-6), decrease in NF-κB activation, and attenuation of the expression of inflammasome component ASC in the heart of burned rats. Estradiol-provided cardiac protection was shown by reduction in myocardial mitochondrial cytochrome c and mitochondrial DNA (mtDNA) into cytoplasm.

estrogen; mitochondrial damage; DAMPs; myocardial inflammation; heart failure; burn

Recent advances in critical care techniques for burn trauma have led to a significant decrease in mortality across all age groups. However, based on the most recent estimations of the American Burn Association (http://www.ameriburn.org) and the Burn Foundation (http://www.burnfoundation.org), hospital administrations due to burn injuries are still close to half million, resulting in more than 45,000 hospitalizations, per year in the US (61). Burns and fire remain a significant threat to individuals as the third leading cause of fatal home injuries (73). Currently, intensive research from both preclinical and clinical sides has been focused on the development of novel therapeutic interventions for burn patients.

Burn has been shown to induce cardiac dysfunction within hours of burn injury, resulting in decreased cardiac output and left ventricular pressure (LVP) (74). Our lab established a rodent burn injury animal model to study this pathological condition (50). Our studies have shown that burn-induced cardiac dysfunction is associated with systemic and myocardial inflammation (5, 54), abnormal iron homeostasis (4, 55, 63, 76, 83), and metabolism disorders (86, 94). These findings in the rodent model are consistent with clinical observations (41, 45, 51, 59). Recently, we have further demonstrated that burn induced several aspects of mitochondrial damage in the heart such as impairments of mitochondrial membrane integrity and oxidative damage (89). Challenge of primary cardiomyocytes with serum isolated from burn animals produced improper Ca2+ homeostasis in mitochondria (56).

Abnormalities in mitochondrial structure and function have been found to occur in various myocardial injury and cardiac disease states (57). A major source of mitochondrial damage comes from oxidative stress. As the intracellular powerhouse of energy production, mitochondria also generate reactive oxygen species (mtROS) as by-products during mitochondrial metabolism. However, imbalanced accumulation of mtROS results in mitochondrial damage by altering the function of proteins, lipids, and DNA through structural modifications in various pathological conditions (6, 18). A growing body of evidence indicates that, during cell death and organ injuries, certain molecules escaped from impaired mitochondria may function as danger-associated molecular patterns (DAMPs) to stimulate inflammatory (46, 48, 82, 92), apoptotic (33), and autophagic (75, 85) responses. The list of mitochondria-derived DAMPs includes mtROS, cytochrome c, and mtDNA that incite cardiac apotosis and inflammation.

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dysfunction in the liver after hemorrhagic shock (47) and also decreased the activity of a multitude of pro-apoptotic factors under various pathological conditions (29, 35, 77). One focus of the current research in our laboratory is to evaluate whether estrogen agonists can be used as a cardiac protective intervention during severe burn injury. In this report, using a burn injury model in rats, we examined the therapeutic effects of a single pharmacological dose of 17β-estradiol on burn-induced production of mitochondria-derived DAMPs and myocardial injury.

MATERIALS AND METHODS

Experimental Model

Adult Sprague-Dawley male rats (320–350 g) were used in the present study. Animals obtained from Harlan Laboratories (Houston, TX) were conditioned in-house for 5–6 days after arrival with commercial rat chow and tap water available at will. All experiments performed in this study were reviewed and approved by The University of Texas Southwestern Medical Center’s Institutional Review Board for the care and handling of laboratory animals and conformed to all guidelines for animal care as outlined by the American Physiology Society and the National Institutes of Health.

Burn Procedure and Administration of 17β-Estradiol

Rats were deeply anesthetized with isoflurane and secured in a constructed template device as described previously (56). The skin exposed through the template was immersed in 100°C water for 12 s on the back and upper sides of the body to produce full-thickness cutaneous burns over 40% total body surface area. This burn technique produced complete destruction of the underlying neural tissue. After immersion, the rats were immediately dried and each animal was placed in an individual cage. All burned animals received standard fluid resuscitation consisting of 4 ml/kg per percentage burn Lactated Ringer’s solution with one-half of this calculated volume given intraperitoneally immediately after completion of the burn injury and the remaining volume given 8 h postburn. All burned rats were given analgesics for pain control (buprenorphine 0.5–1.0 mg/kg) every 8 h after burn injury. Sham-burned rats were handled in an identical manner including fluid replacement and buprenorphine administration but were given no burn injury. In the burned animals, a single dose of 0.5 mg/kg 17β-estradiol or an equivalent volume of control vehicle (corn oil) was given subcutaneously 15 min following burn injury (n = 8 rats/group).

Preparation of Blood Serum, Tissue Lysates, and Cellular Fractions

When animals were killed, blood was collected using BD vacutainer rapid serum tubes (RST) (BD Diagnostics, Franklin Lakes, NJ) followed by immediate centrifugation at 3,000 g for 15 min at 4°C to isolate serum. Serum preparations were allocated and stored at −80°C until used. Tissues were harvested, washed in PBS, snap-clamp frozen, and kept at −80°C. Tissue lysates were prepared using CelLyticMT reagent (Sigma-Aldrich, St. Louis, MO, catalog nos. C3228), and mitochondrial and cytosolic fractions were separated by differential centrifugation using mitochondria isolation kit for tissue (Thermo Fisher Scientific, Rockford, IL, catalog no. PI-89801) according to manufacturer’s protocols. When nuclear fractions were prepared, cardiac tissue was homogenized in buffer I [10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 1 mM DTT and protease inhibitors] and centrifuged at 10,000 g for 30 min. The pellets were resuspended in buffer II [20 mM HEPES (pH 7.9), 1.5 mM MgCl2, 0.42 M NaCl, 0.2 mM EDTA, 25% (vol/vol) glycerol, 1 mM DTT, and protease inhibitors], centrifuged at 20,000 g for 10 min, and the resulting supernatant fractions were collected. Protein concentrations in all preparations were quantified using Bio-Rad DC RC protein assay kit (Bio-Rad, Hercules, CA, catalog no. 500–0122).

Measurements of Mitochondrial Respiratory Activities

Activities of mitochondrial complex I–III were measured using enzyme assay kits according to manufacturer’s protocols (Abcam, Cambridge, MA, catalog nos. ab109721 and ab109905). Mitochondrial pellets were resuspended in PBS supplemented with 10% detergent provided in the kits. Protein concentrations of these mitochondrial lysates were estimated and 25 μg (for complex I) or 100 μg (for complex II + III) mitochondrial protein was used per reaction. Enzyme activities were measured spectrophotometrically in duplicate and expressed as changes of absorbance per minute per milligram protein.

![Fig. 1. 17β-Estradiol reduced cardiac mitochondrial reactive oxygen species (mROS) stress after burn. 17β-Estradiol or control vehicle was given to rats 15 min following burn. Mitochondrial and cytosol fractions were prepared from the heart tissue harvested 24 h postinjury. A: mitochondrial generation of H2O2 was measured in the presence of respiration substrates (succinate or malate + glutamate) by an Amplex Red fluorescence assay. B: MDA concentrations were measured and normalized by the amount of mitochondrial protein per reaction. All values are means ± SE. Statistical significances are labeled as * for a difference between sham and burn, and ¶ for a difference between estradiol- and control vehicle-treated groups (n = 8, P < 0.05). C: confirmation of mitochondrial isolation. Cytosolic (Cyto) and mitochondrial (Mito) fractions prepared from the rat heart tissue and analyzed by Western blot using antibodies against marker proteins adenine nucleotide translocase (ANT) or GAPDH. Data shown are representative of at least 4–6 animals per group.](http://ajpheart.physiology.org/)
Measurement of Mitochondrial $\text{H}_2\text{O}_2$

Mitochondrial $\text{H}_2\text{O}_2$ release was determined according to previous publications (14, 24, 62). Briefly, 40 $\mu$g fresh mitochondrial preparations were set to react with 5 $\mu$M Amplex Red and 0.1 U/ml horseradish peroxidase (HRP) (Invitrogen, Grand Island, NY, catalog no. A12222) in 200 $\mu$L reaction buffer (in mM: 125 KCl, 10 MOPS, 2 MgSO$_4$, 2 KH$_2$PO$_4$, 10 NaCl, 1 EGTA, 0.7 CaCl$_2$, pH 7.2). Superoxide dismutase (SOD) (Sigma-Aldrich, St. Louis, Mo; catalog no. S8409) was added at 50 U/ml to convert all superoxide into $\text{H}_2\text{O}_2$. Mitochondrial respiration substrates (2 mM malate or 20 mM glutamate or 5 mM succinate) were added to start the reaction. During 30 min incubation in the dark at 37°C, HRP catalyzed $\text{H}_2\text{O}_2$-dependent oxidation of Amplex Red. The end product Resorufin Red was measured by fluorescent reading at Ex/Em 570/620 (PHERAstar, BMG LABTECH, Cary, NC). Similarly, cytosolic $\text{H}_2\text{O}_2$ levels were measured according to a published method (7) with minor changes. One hundred micrograms of cytosolic fraction was added to 50 $\mu$M Amplex Red, 0.1 U/ml HRP, and 50 U/ml SOD in a total of 100 $\mu$L reaction (in 50 mM NaH$_2$PO$_4$, pH 7.4). Fluorescent reading was obtained following 15 min incubation in the dark at 37°C. All measurements were performed in at least duplicate. $\text{H}_2\text{O}_2$ concentrations (in $\mu$M) were calculated according to individual standard curves in these assays.

Measurement of Lipid Peroxidation in Mitochondria

Lipid peroxidation was determined by concentration of malondialdehyde (MDA), a marker of lipid oxidation using a lipid peroxidation assay kit (Enzo Life Sciences, Plymouth Meeting, PA, catalog no. BML-AK170-0001). To prevent sample oxidation, concentrations of all mitochondrial extracts were adjusted to 1–1.5 mg/ml in a resuspension buffer containing 5 mM butylated hydroxyl toluene (BHT). Standards were prepared and experimental procedures were performed according to manufacturer’s protocol. For each reaction, 200 $\mu$L of sample or standard was added to 650 $\mu$L chromogenic reagent (provided by the manufacturer) and 150 $\mu$L 12 N HCl. After incubation at 45°C for 60 min, the samples were cooled at 4°C and centrifuged at 10,000 g for 5 min. The supernatants were collected and absorbance at 586 nm was recorded. MDA concentration was calculated using a standard curve. All measurements were performed in duplicate.

Measurement of SOD Activity in Mitochondria

SOD activity was measured based on a method originally described by Nebot and colleagues (65), using SOD assay kit (Calbiochem, San Diego, CA, catalog no. 574601). Approximately 100 $\mu$g mitochondrial protein was used for each reaction, and all assays were performed according to the manufacturer’s protocol. Results were quantified by densitometry. All values are means ± SE. Statistical significances are labeled as * for a difference between sham and burn, and ¶ for a difference between estradiol- and control vehicle-treated groups ($n = 8$, $P < 0.05$).
formed in duplicate. According to manufacturer’s protocol, samples were first incubated with 1-methyl-2-vinylpyridinium in assay buffer at 37°C for 1 min to eliminate interference. Immediately after adding substrate (5,6,6/9251,11/9252-tetrahydro-3,9,10-trihydroxybenzo[c]fluorine, the oxidation of which is regulated by superoxide dismutase), absorbance at 525 nm was recorded using a kinetic program: 10-s interval; 6 readings. The SOD activity was determined from the ratio of the autoxidation rates measured in the presence and in the absence of SOD. Results were normalized by protein amount per reaction and expressed as units per milligram mitochondrial protein.

Measurement of GPx Activity in Mitochondria

GPx activity was measured using glutathione peroxidase assay kit (Calbiochem, San Diego, CA, catalog no. 353919). Mitochondrial extracts were at first diluted with assay buffer, and ~50 μg protein was used per reaction; all assays were performed in duplicate. The sample was added to a solution containing 1 mM glutathione (GSH), ≥0.4 unit/ml glutathione reductase, and 0.2 mM NADPH. The reaction was initiated by adding substrate tert-butyl hydroperoxide (final concentration 0.22 mM) and reduction in absorbance was recorded at 340 nm using a kinetic program: 30-s interval, 6 readings. The GPx activity was determined by the rate of decrease in absorbance [1 mU/ml GPx = (∆absorbance/min)/0.0062]. Results were normalized by protein amount per reaction and expressed as microunits per milligram mitochondrial protein.

Fig. 3. 17β-Estradiol attenuated cytochrome c release from cardiac mitochondria after burn. 17β-Estradiol or control vehicle was given to rats 15 min following burn. Cytosol fractions were prepared from the heart tissue harvested 24 h postinjury. Equal amount of protein samples were subjected to Western blot using antibodies against cytochrome c and cytosolic marker GAPDH. Results were analyzed by densitometry. All values are means ± SE. Statistical significances are labeled as * for a difference between sham and burn, and ¶ for a difference between estradiol- and control vehicle-treated groups (n = 8, P < 0.05).

Fig. 4. 17β-Estradiol decreased mitochondrial DNA (mtDNA) release from cardiac mitochondria after burn. 17β-Estradiol or control vehicle was given to rats 15 min following burn. Cytosol fractions were prepared from the heart tissue harvested 24 h postinjury. Total DNA was isolated from equal amount of cytosol samples. TaqMan exogenous internal positive control (IPC) DNA was spiked into all samples prior to DNA isolation as a positive control. Real-time PCR assays were performed using primers against rat mtDNA NADH, cytochrome b (Cyt B), cytochrome c oxidase subunit III (COXIII), or IPC. Results were expressed as a ratio of a target mtDNA to IPC. Values are means ± SE. Statistical significances are labeled as * for a difference between sham and burn, and ¶ for a difference between estradiol- and control vehicle-treated groups (n = 8, P < 0.05).

Fig. 5. 17β-Estradiol prevented cardiac mitochondrial functional deficiency and structural impairment after burn. 17β-Estradiol or control vehicle was given to rats 15 min following burn. Mitochondrial fractions were prepared from the heart tissue harvested 24 h postinjury, and samples were subjected to the measurements of complex I (A) and complex II/III (B) activities. Results were expressed as changes of absorbance per minute per milligram protein. Percentages of mitochondrial outer membrane damage were quantified in these mitochonrdia preparations (C). All values are means ± SE. Statistical significances are labeled as * for a difference between sham and burn, and ¶ for a difference between estradiol- and control vehicle-treated groups (n = 8, P < 0.05).
Measurement of Mitochondrial Outer Membrane Damage

Mitochondrial outer membrane integrity was evaluated using a cytochrome c oxidase assay (Sigma-Aldrich, catalog no. CYTOCOX1). Experimental procedures were performed according to the manufacturer’s protocol; 20 μg freshly isolated mitochondrial fraction was used for each reaction and duplicate reactions were conducted for each assay. For measurement of total mitochondrial cytochrome c oxidase activity, the mitochondria fraction was diluted in enzyme dilution buffer (10 mM Tris-HCl, pH 7.0 containing 250 mM sucrose) with 1 mM n-dodecyl β-D-maltoside and incubated on ice for 30 min. The reaction was initiated by adding freshly prepared ferrocytochrome c substrate solution (0.22 mM) to the sample. The decrease in absorbance at 550 nm, which is related to oxidation of ferrocytochrome c by cytochrome c oxidase, was recorded using a kinetic program (5-s delay; 10-s interval; 6 repeated readings). Cytochrome c oxidase activity was calculated and normalized for the amount of protein per reaction and results were expressed as units per milligram mitochondrial protein. Mitochondrial outer membrane damage was assessed from the ratio between the cytochrome c oxidase activities without and with detergent.

Detection of Free mtDNA Fragments in Cytoplasm

Cytosolic fractions were prepared from the heart tissue and total DNA was isolated using DNA extraction kit (Qiagen, Valencia, CA, catalog no. 69506). TaqMan exogenous internal positive control (IPC) DNA (Life Technologies, Carlsbad, CA, catalog no. 4308323) was spiked into all samples prior to DNA isolation as a positive control. TaqMan real-time PCR assays were performed using primers against rat mtDNA gene NADH, cytochrome b, cytochrome c oxidase subunit III, or IPC according to manufacturer’s protocol. Samples were tested in at least triplicates. Results were expressed as a ratio of target gene to IPC (all PCR reagents and gene assays were from Life Technologies, Carlsbad, CA).

Measurements of Cytokines and Troponin Levels by Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-10 in serum and in heart tissue lysates were examined using ELISA kits (Biosource, Camarillo, CA, catalog nos. MBS701635, MBS162519, MBS813479, and MBS815295). Results in the tissue lysates were normalized by protein amount and the results in serum were normalized by volume. Serum troponin-I levels were also determined by a commercial ELISA kit (Life Diagnostics, West Chester, PA). All measurements were obtained by following manufacturer’s protocols. Samples were tested at least in duplicates.

Western blot Analysis

Prepared SDS-PAGE protein samples were subjected to 15% SDS-PAGE gels, and transferred to PVDF membranes. Membranes were blocked with 5% nonfat milk-PBS at room temperature for 1 h and subsequently probed with one of the following antibodies according to experiments: cytochrome c (Abcam, Cambridge, MA, catalog no. ab110325), GAPDH (Millipore, Billerica, MA, catalog no. MAB374), c-Jun (Millipore, catalog no. 09-754), ANT (Santa Cruz Biotechnology, Santa Cruz, CA, catalog no. sc-9299), MnSOD (Santa Cruz Biotechnology, catalog no. sc-30080), GPx (Santa Cruz Biotechnology, catalog no. sc-133160), and NF-κB p65 (Cell Signaling, Danvers, MA, catalog no. 3033S). The membranes were then rinsed and incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA, catalog nos. 170-6515 and 170-6516). Antibody dilutions and incubation time were according to manufacturer’s instructions. Membranes were then rinsed and blotted with ECL solution (Bio-Rad, Hercules, CA, catalog nos. 170-6500 and 170-6501) and exposed to X-ray film (Kodak, Rochester, NY, catalog no. MR10). The bands were quantified using a densitometer (Bio-Rad, Hercules, CA, catalog no. GS-700). The results were normalized to GAPDH and the ratio of target protein to GAPDH was calculated.

Fig. 6. 17β-Estradiol inhibited cardiac apoptosis after burn. 17β-Estradiol or control vehicle was given to rats 15 min following burn, and hearts were harvested 24 h postinjury. A: heart tissue sections were applied to TUNEL assay (green). Positive control samples were pretreated with DNase I to activate DNA fragmentation, whereas the negative controls contained inhibited apoptotic reactions. In parallel, tissue sections were immune-stained with caspase 1 (B) and caspase 3 (C). All samples in A–C were costained with F-actin probe (red) and DAPI (blue). In B and C, negative controls were stained with FITC-conjugated anti-rabbit IgG. The original magnification is 63-fold, and fluorescence intensity was quantified and analyzed statistically. All values are means ± SE. Statistical significances are labeled as * for a difference between sham and burn, and ¶ for a difference between estradiol- and control vehicle-treated groups (n = 8, P < 0.05).
bound antibodies were detected by using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific; Rockford, IL, catalog no. 34077).

**Histology Analysis**

Fresh heart tissues were fixed by 10% neutral buffered formalin for 6 h and embedded with paraffin. Samples were sectioned at 5–7 μm, processed, and examined by the following approaches.

**Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay.** Cardiac apoptosis was assessed by an assay kit of fluorometric TUNEL system (Promega, Madison, WI, catalog no. G3250) according to manufacturer’s protocol. Briefly, tissue sections were then deparaffinized, rehydrated, permeabilized, fixed, and labeled with TdT reaction mixture for 1 h at 37°C. After the reactions were terminated, the sections were stained with a red fluorescence-labeled F-actin probe (CytoPainter F-actin staining kit, Abcam, Cambridge, MA, catalog no. ab112127). At last, each slide was sealed with one drop of ProLong Gold antifade reagent with DAPI (Life Technologies, Grand Island, NY, catalog no. P36931) for nuclear counterstain. The slides were then examined under Leica TSC SP5 confocal laser scanning microscope at 63× magnification.

**Caspase 1, caspase 3, and ASC.** Tissue sections were blocked with 10% goat serum in PBS and then stained with a rabbit polyclonal antibody against active caspase 1 (Bioss, Woburn, MA, catalog no. bs-0169R), caspase 3 (Abcam, Cambridge, MA, catalog no. ab3623), or ACS (Novus Biologicals, Littleton, CO, catalog no. NB1-78977) for 1 h at room temperature. In some experiments, a red fluorescence-labeled F-actin probe (CytoPainter F-actin staining kit, Abcam, Cambridge, MA, catalog no. ab112127) was used for costaining. At last, all slides were sealed DAPI and examined as described above.

**Hematoxylin and eosin (H and E) stain.** Tissue sections were deparaffinized, rehydrated, and incubated with hematoxylin histological staining reagent (Dako, Carpinteria, CA, catalog no. S3302) for 4 min followed by eosin secondary counterstain solution (Leica Microsystems, Buffalo Grove, IL, catalog no. 3801600) for 1 min. Slides were then cleared by xylene, mounted with mounting medium (Thermo Fisher Scientific, Rockford, IL, catalog no. 4111), and examined under Zeiss Axio Observer epifluorescence microscope at 40× magnification.

**Echocardiography**

Echocardiograms were performed in sedated rats in a random and blind manner to assess systolic function according to a previously published method (84). Mice were anesthetized with 5% isoflurane with 2.5 l/min O2 for 20 s, or until unconscious, followed by 2% isoflurane and O2 for an average of 12–15 min. Hair was removed from the thorax and upper abdomen by using Nair hair remover and gauze. Echocardiography was then performed using a Hewlett-Packard Sonos 5500 and an S12 Ultraband 5.0–12.0 (model 21380) pediatric neonatal transducer (Agilent Technologies, Andover, MA). For each animal, M-mode images were obtained at baseline and at specific time periods after burn injury. Left ventricular end-diastolic diameter (EDD) and left ventricular end-systolic diameter (ESD) were measured. Fractional shortening (FS) was calculated according to formulas $\text{FS}\% = (\text{EDD} - \text{ESD})/\text{EDD} \times 100\%$. All results were obtained at stable heart rates and measurements were averaged over five consecutive cardiac cycles.

**Statistical Analysis**

All data were expressed as means ± SE of at least three independent experiments using 4–8 animals/group. Student t-tests were used to assess the difference between the sham and sepsis groups and the groups with or without drug treatment. Probability values <0.05 were considered statistically significant (analyses were performed using SPSS for Windows, version 7.5.1).

**RESULTS**

In this study, using a rat burn injury model, a pharmacological dose of 17β-estradiol preparation was administered subcutaneously 15 min postinjury. By radioimmunoassay, bioavailability of estradiol was confirmed in the blood serum 24 h postadministration: 8 ng/ml in estradiol-treated group vs. 20 pg/ml in placebo group (29). Therapeutic effects of estradiol in the heart were examined as described in the following.

**Estradiol Suppresses Production of Mitochondrial-Derived DAMPs in the Heart After Burn**

**mtROS.** We first examined whether administration of estradiol alters mitochondrial oxidative stress and antioxidant defense in the heart after burn injury. As shown in Fig. 1A, the production of mitochondrial H2O2, the most stable form of ROS, was measured in the heart tissue using a standard Amplex

![Fig. 7. 17β-Estradiol suppressed cytokine production after burn. 17β-Estradiol or control vehicle was given to rats 15 min following burn. Blood was collected and heart tissues were harvested 24 h postinjury. Levels of cytokine TNF-α, IL-1β, IL-6, and IL-10 were measured in blood serum (A) and in heart tissue lysates (B). All values are means ± SE. Statistical significances are labeled as * for a difference between sham and burn, and ¶ for a difference between estradiol- and control vehicle-treated groups (n = 8, P < 0.05).](http://ajpheart.physiology.org/Downloaded from http://ajpheart.physiology.org)
Red assay (14, 24, 62). Compared with the sham controls, burn injury triggered a significant increase in \( \text{H}_2\text{O}_2 \) productivity in mitochondria: a near 80% increase in the presence of respiration substrates succinate or malate plus glutamate. However, intervention by \( 17\beta \)-estradiol decreased mitochondrial \( \text{H}_2\text{O}_2 \) generation to the levels similar to those of shams.

Since excessive mtROS causes oxidation of macromolecules in mitochondria (8, 15, 70), we alternatively measured levels of lipid peroxidation in the mitochondrial fractions to confirm estradiol-mediated effect on mtROS. As shown in Fig. 1B, concentrations of malondialdehyde (MDA), a marker for lipid peroxidation, were elevated ~30% in mitochondria from the hearts of burned rats compared with values measured in shams. However, this increase was effectively suppressed in response to treatment with estradiol.

In the experiments described above, we confirmed the quality of mitochondrial preparations, as shown in Fig. 1C. In the subcellular fractions from the heart tissue, separation of mitochondria from cytosol was complete, indicated by Western blot using antibodies against mitochondria-specific marker adenine nucleotide translocase (ANT) and cytoplasm-specific marker glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

We further evaluated whether estradiol affects mitochondrial antioxidant defense. A major component of the mitochondrial antioxidant defense mechanism is the expression of mitochondrial antioxidant enzymes, including manganese dependent superoxide dismutase (Mn-SOD) and glutathione peroxidase (GPx) (37, 89). At first, we compared SOD enzymatic activity in mitochondrial fractions from the hearts of sham and burned rats treated with estradiol or placebo. We detected that burn injury reduced mitochondrial SOD activity by ~30%, and this reduction was relieved by treatment with estradiol (Fig. 2A). We then examined estradiol’s effects on tissue and mitochondrial levels of MnSOD. As shown in Fig. 2B, we found that the total expression of MnSOD was not affected by burn but was increased ~20% in response to estradiol. Interestingly, burn produced a significant, nearly 40%, decrease in mitochondrial MnSOD content, suggesting that burn injury causes an impairment of transporting MnSOD protein to mitochondria in the heart. Restoration of cardiac mitochondrial MnSOD content was achieved in burned animals receiving estradiol.

Similarly, we examined burn and estradiol-mediated effects on mitochondrial GPx in the heart. As shown in Fig. 2C, burn severely reduced the enzymatic activity of GPx in mitochondria by more than 50%. However, the injury did not cause any detectable changes in either mitochondrial- or tissue-level GPx (Fig. 2D), suggesting that burn may decrease mitochondrial GPx activity via posttranslational modification. Further, as shown in Fig. 2, C and D, treatment by estradiol produced significant recovery of cardiac mitochondrial GPx activity in the burned animals and increased both total expression and mitochondrial content of GPx.

**Cytochrome c.** We previously showed that burn injury damages cardiac mitochondrial membrane integrity, leaking mitochondrial protein cytochrome c into cytoplasm (89). We therefore examined whether estradiol provides any prevention for the occurrence of this phenomena. As shown in Fig. 3, we examined cytosolic levels of cytochrome c in the heart of sham or burn rats treated with estradiol or placebo. The Western blot analysis demonstrated an over twofold increase in cytosolic cytochrome c in response to burn. In parallel, in the estradiol-treated group, there was no significant difference between sham and burn rats. This result was normalized by the expression of the cytosolic marker protein GAPDH, which was unchanged in response to burn or estradiol.

**mtDNA fragments.** By published real-time PCR (RPRC) analysis (22, 92), we quantified levels of mtDNA leaked to cytoplasm in the hearts of sham and burned rats given estradiol or placebo controls. As results summarized in Fig. 4, RPRC targeting mtDNA-specific gene sequences of NADH dehydrogenase, cytochrome b (Cyt B), and cytochrome c oxidase subunit III (COX III) all revealed that a significant amount of mtDNA was released into myocardial cytosol in response to burn injury. Treating the burned subjects with estradiol effectively reduced the levels of cytosolic mtDNA in the heart.
Estradiol Protects Mitochondrial Function and Structure in the Heart After Burn

OXPHOS activities. To evaluate estradiol-mediated effects on cardiac mitochondrial function, we compared activities of mitochondrial oxidative phosphorylation (OXPHOS) complexes in the hearts of sham and burned rats given estradiol or placebo. We found that the activities of complex I and II-III were severely impaired after burn, decreasing ~30% and ~60%, respectively (Fig. 5, A and B). However, this functional deficiency was recovered in estradiol-treated burned rats, indicating that postburn intervention by estradiol protects mitochondrial metabolic function in the heart.

Membrane integrity. In the hearts of sham and burned rats treated with estradiol or placebo control, we examined mitochondrial outer membrane damage. As described in our previous publications (88–90) and in the present study in MATERIALS AND METHODS, the ratio between cytochrome c oxidase activities in the mitochondria with presence/absence of detergent n-dodecyl β-D-maltoside represents the percentage of mitochondrial outer membrane damage. As shown in Fig. 5C, cardiac mitochondrial outer membrane damage was increased more than twofold in response to burn, but this increase was blocked in burned rats receiving estradiol treatment.

Estradiol Inhibits Myocardial Apoptosis After Burn

To evaluate whether estradiol provides an effect on myocardial apoptosis after burn, we applied the assay of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to detect DNA fragmentation in the heart tissue. As shown in Fig. 6A, we detected a striking ~100-fold increase in DNA fragmentation in the heart of burn injured rats, compared with their sham controls. Giving estradiol in burned animals provided an al-
most complete inhibition in this apoptotic response. To confirm this result, we further examined the expression levels of apoptosis factors, caspase 1 and caspase 3, in the heart using immunohistochemistry. Costaining heart tissue sections with anti-caspase 1 and fluorescent probes targeting cell marker filamentous F-actin and nucleus (4',6-diamidino-2-phenylindole, DAPI), indicated that burn-induced overexpression of caspase 1 protein was suppressed by estradiol treatment (Fig. 6B). Similar results were obtained when the heart tissue sections were stained with anti-caspase 3 (Fig. 6C). Taken together, our data suggest that estradiol provides an effective control over burn-induced apoptosis in the heart.

Estradiol Inhibits Myocardial Inflammation After Burn

**Cytokines.** To determine the effects of estradiol on burn-induced inflammatory responses, we examined cytokine concentrations in the blood and in the heart tissue of sham and burned rats received estradiol or placebo. As shown in Fig. 7, both systemic and myocardial cytokines, IL-1β, IL-6, IL-10, and TNF-α, increased significantly after burn, and treatment by estradiol suppressed this elevation.

**NF-κB pathway.** As NF-κB is a crucial mediator of inflammation, we next examined whether estradiol alters the activation status of NF-κB in the heart after burn. Activation of NF-κB is associated with its translocation from the cytosol to the nucleus (3). Thus, NF-κB levels in cytosolic and nuclear fractions from the heart tissue were examined by Western blot; cytosolic marker GAPDH and nuclear marker c-Jun were used as controls. As shown in Fig. 8, in response to burn injury, there was a significant decrease of NF-κB p65 in cytosol and an increase in the nucleus, indicating the activation of NF-κB in myocardium. However, in 17β-estradiol-treated burned animals, NF-κB p65 was mainly retained in the cytoplasm. Therefore, 17β-estradiol has a blockage effect on the activation of NF-κB in the heart.
Inflammasome pathway. Signal transduction pathways through inflammasomes exert critical transcriptional regulation of certain immune response genes such as IL-1β (1, 27). We examined whether estradiol has an effect on the expression of inflammasome adaptor protein ASC (apoptosis-associated speck-like protein containing a COOH-terminal caspase recruitment domain) in the heart. As shown in Fig. 9, by immunohistochemistry analysis, we detected an over 10-fold increase in ASC expression in the burned rats compared with shams. Intriguingly, giving estradiol suppressed this burn-associated upregulation of ASC in the heart, suggesting that estradiol inhibits myocardial inflammasome levels after burn.

Estradiol Decreases Myocardial Damage and Improves Cardiac Function After Burn

Previous investigation found that burn injury causes substantial elevation of serum troponin-I (cTn-I) (64), one of the most sensitive indicators of myocardial injury (28). To evaluate whether estradiol provides a cardiac protective effect, we compared the levels of serum cTn-I in the sham and burned rats given with estradiol or placebo. As shown in Fig. 10A, the serum level troponin-I increased nearly 10-fold in burned rats compared with sham controls. However, intervention by estradiol suppressed this response in burned animals to nearly the same level as in shams, suggesting estradiol’s effect on attenuation of myocardial damage after burn.

In addition, we analyzed histopathology of the heart tissue by hematoxylin and eosin staining. Consistent with published results (91, 93), we found that burn caused significant inflammatory infiltration and disorganization of cardiomyocytes (Fig. 10B). These changes were not detected in burned rats receiving estradiol, further indicating estradiol-mediated protection on the myocardium.

To analyze cardiac performance, we performed echocardiogram analysis on sham and burned rats receiving estradiol or placebo. As summarized in Fig. 10C, significant differences between the pre- and postburn measurements of left ventricular end-diastolic diameter (EDD) and left ventricular end-systolic diameter (ESD) showed in rats 12 and 24 h after injury. At these two time points, burn substantially reduced fractional shortening (FS%) from 87.96 ± 2.7% to 75.32 ± 5.8% and 68.67 ± 2.32%, respectively, indicating cardiac dysfunction. Statistically significant changes were not detectable between the sham and burned rats receiving estradiol, showing that the treatment improves cardiac performance after burn.

DISCUSSION

In this report, we performed a preclinical evaluation of estrogen agonist, 17β-estradiol, in burn trauma using a rat model. Our data show that a single dose of postinjury administration of estradiol reduced the release of mitochondrial-derived DAMPs, such as mtROS (Fig. 1), cytochrome c (Fig. 3), and mtDNA (Fig. 4) in the myocardium after burn. This intervention increased mitochondrial antioxidant defense (Fig. 2) and preserved cardiac mitochondrial respiratory function and membrane integrity (Fig. 5). In parallel, 17β-estradiol inhibited burn-induced cardiac apoptosis (Fig. 6) and attenuated systemic and myocardial cytokine production (Fig. 7). In the heart, the mechanism of estradiol’s anti-inflammatory action involved downregulation of NF-κB activation (Fig. 8) and ASC expression (Fig. 9). Estradiol also provided cardiac protection in the burn-injury model, as demonstrated by its suppression of cardiac injury marker troponin-I, amelioration of morphology and improvement of contractility (Fig. 10). Based on these results, we propose that burn injury triggers the release of DAMPs molecules from damaged mitochondria in the myocardium, resulting in downstream inflammation, apoptosis, and subsequent heart failure. A therapeutic intervention by 17β-estradiol following burn protects the heart via a control over the production of mitochondrial DAMPs (Fig. 11).

In the heart, mitochondria comprise ~30% of myocardial volume (43). Therefore, DAMPs molecules generated from damaged mitochondria play a significant role during cardiac pathogenesis. In the current study, we observed that estradiol protected cardiac mitochondria from burn-mediated injurious effects, and the most beneficial consequence of this protection is the reduction in mitochondria-derived DAMPs. Our results are consistent with previous reports of estrogen-mediated protection of mitochondrial function in other disease models. For example, estrogen was able to inhibit the downregulation of mitochondrial metabolism in heart, liver, and kidney in response to trauma-hemorrhage injury (34, 35, 47). Similar estrogen protection in brain mitochondria was observed in the models of Alzheimer’s disease (66) and aging (40). The proposed mechanisms of estradiol-mediated mitochondrial protection involve estrogen receptors (ERs) (31, 72), upregulation of the gene expressions of mitochondrial functional proteins (34, 35), and/or the antioxidant feature of estradiol itself (21). Although the exact pathways of how estradiol protects functional mitochondria and regulates the production of mitochondrial DAMPs in the heart during burn still remains to be illustrated, our present data suggest a possible mechanism that involves the upregulation of mitochondrial antioxidant enzymes. We observed that burn decreased the activities of MnSOD and GPx, which might be the result of impaired mitochondrial transporting of MnSOD and certain unknown posttranslational modification(s) of GPx (Fig. 2). Intervention by estradiol effectively restored MnSOD and GPx function in the mitochondria and increased expression of these two enzymes in the heart tissue (Fig. 2).

Burn-associated cardiac apoptosis was identified in animal models (53) and in cultured cardiomyocytes (11). It has been hypothesized that apoptosis of cardiomyocytes may contribute

![Fig. 11. A proposed mechanism of 17β-estradiol-mediated cardiac protection in burn trauma.](http://ajpheart.physiology.org/doi/abs/10.1152/ajpheart.00475.2013)
to postburn cardiac mechanical dysfunction. In this study, we demonstrated estradiol’s antiapoptotic effect in myocardium of the burned animals (Fig. 6). Similar estradiol effect was previously reported in several other disease models, such as ischemia-reperfusion injury (13), hypertension (26), and LPS-challenged cardiomyocytes (52). Because mitochondria-derived DAMP molecules, such as mtROS and cytochrome c, are known to promote apoptosis (10, 81), our data suggest that estradiol-mediated suppression on apoptosis may be achieved through its reduction in mitochondria DAMPs.

Burn-induced excessive inflammation is a major causative factor for distant multiorgan dysfunction (20, 25). In blood, systemic inflammatory responses are activated through signaling pathway, in which NF-κB is negatively regulated by the IκB kinase (IKK) signalosome pathway (38, 68), and/or through inflammasome pathway, in which inflammatory caspases 1 and 5 are controlled by the inflammasomes (16, 49, 58). This signal transduction paradigm is very likely applicable to the tissue-level inflammation in response to burn injury. Indeed, studies in this report showed that burn stimulated significant increases in the activation of NF-κB and the expression of inflammasome component ASC in the heart (Figs. 8 and 9), in co-occurrence with the overproduction of cytokines (Fig. 7). Importantly, these burn-induced inflammatory responses were greatly reduced in animals receiving estradiol therapy (Figs. 5–7). However, it is worthy to point out that cytokine levels in burned rats given estradiol were still higher than those in the sham controls (Fig. 7), indicating that estradiol did not completely suppress burn-induced cytokines in the presented experimental setting. We speculate that the reduction of cytokines by estradiol in the burned animals may be progressive or dose-dependent, and a complete inhibition could be reached at a later time point or at a higher estradiol dosage. Further, among the cytokines examined, in the heart, estradiol provided the most repression on TNF-α (over 90%) but the least on IL-6 (~32%). However, in the blood, estradiol provided the most repression on IL-6 (over 75%) but the least on IL-1β (~45%). This difference suggests that estradiol-provided anti-inflammatory effects may depend on tissue- or cell-specific mechanisms.

Cardiac protection by estradiol has been reported in animal models of hemorrhage shock (2, 36, 42) and ischemic-reperfusion injury (23, 67). In this report, we found that an acute postburn administration of estradiol significantly reduced serum troponin I, protected myocardial morphology, and improved cardiac contractility in the burn-injured animals (Fig. 10). Our data suggest that estradiol is effective to alleviate burn-induced cardiac dysfunction.

In summary, this study was built to evaluate the acute effects of estradiol administration on burn injury. We observed that, following severe burn injury, an early, single dose of estrogen dramatically reduced the production of mitochondrial DAMPs in the heart, decreased apoptosis and inflammation, and protected myocardium in a rodent model. In the same model, our group previously observed that estradiol suppresses brain inflammation and apoptosis (29). In both of these studies, rats were allowed to survive 24 h postburn for the purpose to examine the pure, acute effects of estradiol in burn injury. In future studies, the survival time of the burn-injured animals may need to be increased to further investigate estradiol’s effects on postburn complications that were frequently observed clinically. The data summarized in the current report suggest that 17β-estradiol may become an effective therapeutic regimen to alleviate multiorgan failure in burn trauma.

ACKNOWLEDGMENTS

We thank J. M. DiMaio, M.D., at University of Texas Southwestern (UTSW) for sharing the echocardiography equipment.

GRANTS

This project was supported in part by American Heart Association South Central Affiliate Beginning-in-Aid Grant 09BGIA2220114 to Q. S. Zang, and by UTSW Department of Surgery (internal funding to Q. S. Zang and J. G. Wigginton).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

CARCINOCRITICAL PROTECTION BY ESTRADIOL IN BURN TRAUMA


