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Cardiac mitochondrial damage and loss of ROS defense after burn injury: the beneficial effects of antioxidant therapy

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Abstract

Mechanisms of burn-related cardiac dysfunction may involve defects in mitochondria. This study determined 1) whether burn injury alters myocardial mitochondrial integrity and function; and 2) whether an antioxidant vitamin therapy prevented changes in cardiac mitochondrial function after burn. Sprague-Dawley rats were given a 3° burn over 40% total body surface area and fluid resuscitated. Antioxidant vitamins or vehicle were given to sham and burn rats. Mitochondrial and cytosolic fractions were prepared from heart tissues at several times postburn. In mitochondria, lipid peroxidation was measured to assess oxidative stress, mitochondrial outer membrane damage and cytochrome-*c* translocation were determined to estimate mitochondrial integrity, and activities of SOD and glutathione peroxidase were examined to evaluate mitochondrial antioxidant defense. Cardiac function was measured by Langendorff model in sham and burn rats given either vitamins or vehicle. Twenty-four hours postburn, mitochondrial outer membrane damage was progressively increased to ~50%, and cytosolic cytochrome-*c* gradually accumulated to approximately three times more than that measured in shams, indicating impaired mitochondrial integrity. Maximal decrease of mitochondrial SOD activity occurred 8 h postburn (~63.5% of shams), whereas maximal decrease in glutathione peroxidase activity persisted 2–24 h postburn (~60% of shams). In burn animals, lipid peroxidation in cardiac mitochondria increased 30–50%, suggesting burn-induced oxidative stress. Antioxidant vitamin therapy prevented burn-related loss of membrane integrity and antioxidant defense in myocardial mitochondria and prevented cardiac dysfunction. These data suggest that burn-mediated mitochondrial dysfunction and loss of reactive oxygen species defense may play a role in postburn cardiac dysfunction.

Keywords

burn trauma; cardiac function; mitochondria

IN THE HEART, MITOCHONDRIA comprise ~35% of cardiomyocyte volume. Abnormalities in mitochondrial structure and function have been found to occur in numerous myocardial injury and disease states (19). Previously, our laboratory demonstrated burn injury-induced cardiac dysfunction in several animal models and in humans (10, 27), but the molecular mechanisms of burn-related cardiac injury remain unclear. Recently, we identified apoptosis

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and increased mitochondrial calcium levels in cardiac myocytes challenged by burn serum (3, 17). Apoptosis has been associated with mitochondrial injury, such as opening of mitochondrial permeability transition pore and release of apoptotic factor cytochrome-c (15), since intramitochondrial Ca^{2+} homeostasis plays a significant role in the regulation of mitochondrial permeability transition pore (8). Our previous studies suggested that burn injury altered cardiac mitochondria.

A major source of mitochondrial injury is oxidative stress produced by reactive oxygen species (ROS), by-products of energy production in mitochondria. Various pathological conditions cause the imbalance between ROS generation and ROS scavenging, and accumulated ROS can alter the function of proteins, lipids, and DNA through structural modifications (2, 5). Numerous studies have confirmed that ROS alter multiple aspects of mitochondrial function; in contrast, mitochondria provide ROS defense mechanisms, including enzymatic anti-oxidants. Three major families of intracellular antioxidant enzymes, including glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD), have been identified in mitochondria (1, 20, 25). Those enzymes are involved in maintaining mitochondrial integrity, protecting cells from apoptosis, and improving organ function under disease conditions (13, 20).

In burn injury, oxidative stress has been well recognized and has been hypothesized to contribute to the development of distant organ injury or failure (12). In experimental and clinical studies, burn-associated oxidative stress has been confirmed by downregulation of antioxidant activities and oxidative modifications of proteins and lipids in multiple organs (9, 26). Antioxidant vitamin therapy has been shown to promote postburn recovery of organ function and to attenuate burn-related immunosuppression (4, 11). Our previous studies confirmed that pre- and postburn administration of an antioxidant regimen provided significant cardiac protection by attenuating myocardial inflammation and NF- κ B activation (11). However, whether burn injury alters antioxidant defense and produces oxidative stress in myocardial mitochondria has not been determined, nor has the role of mitochondrial oxidative stress in postburn cardiac dysfunction been studied.

In the present study, we determined whether a cutaneous burn injury damaged cardiac mitochondria, produced oxidative stress, and impaired mitochondrial defense against ROS. To further investigate whether these factors contributed to postburn myocardial contractile defects, we next applied antioxidant vitamin therapy to burn-injured animals and examined status of myocardial mitochondria and cardiac function at multiple postburn time points. We hypothesized that burn injury promoted cardiac mitochondrial damage, subsequently contributing to the impaired heart function. This study was also directed to identify potential targets of therapeutic interventions that may improve cardiac function in injury and disease.

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

Rats were deeply anesthetized with isoflurane and secured in a constructed template device, as described previously (17). 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% of the 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⁻¹·% burn⁻¹ lactated Ringer solution, with one-half of this calculated volume given intraperitoneally immediately after completion of the burn injury, and the remaining volume was 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. Hearts were collected 2, 4, 8, 12, 24, 48, and 72 h after injury (*N* = 3–6 at each time point) for in vitro assessment of mitochondrial properties. Sham-burned rats were handled in an identical manner, including fluid replacement and buprenorphine administration, but given no burn injury, and these rats served as appropriate controls.

Antioxidant vitamin therapy and experimental groups

Adult rats were given a vitamin regimen 2 days before burn and continued for 24 h after burn injury. Vitamins were given in 0.5 ml water by oral gavage twice each day. Daily dosages included 417 units vitamin A, 3.7 mg zinc, 24 mg vitamin C, and 20 units vitamin E. A subgroup of sham burn rats also received the vitamin treatment to provide appropriate antioxidant vitamin-treated sham burns. Additional burn and sham burn rats were given water (vehicle) without vitamins to provide additional control groups. In the first group of studies, the time course of burn-related injury to mitochondria, alterations in mitochondrial ROS scavenging capacity, and burn-related mitochondrial lipid peroxidation were studied in sham burns (*N* = 5) and in subgroups of burns killed either 2, 4, 8, 24, 48, or 72 h after burn injury (*N* = 4–5 rats per time period). Since maximal burn-related changes in mitochondrial function occurred over the first 24 h after burn injury, additional groups of sham burn and burned animals were randomized to receive either oral vehicle (water) or oral antioxidant vitamins, as described above, and subgroups of vitamin-treated burns and vehicle-treated burns were killed 2, 4, 8, or 24 h postburn (*N* = 5 rats per group per time period); mitochondrial function was studied. In the next group of studies, the effect of antioxidant vitamin treatment on myocardial performance was studied in the following experimental groups: 1) vehicle-treated shams; 2) antioxidant vitamin-treated shams; 3) vehicle-treated burns studied 8 h postburn; 4) vehicle-treated burns studied 24 h postburn; 5) antioxidant-treated burns studied 8 h postburn; and 6) antioxidant-treated burns studied 24 h postburn. All experimental groups included to evaluate ventricular performance had seven to nine rats per group per time period.

Isolation of cytosol and mitochondrial fractions from heart tissues

The cytosol and mitochondrial fractions were prepared by differential centrifugation, according to the previously described procedure of Du et al. (6) with modifications. Heart tissues were homogenized in three volume of homogenizing buffer [20 mM HEPES-KOH (pH 7.5), 250 mM sucrose, 10 mM KOH, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.1 mM PMSF] and followed by centrifugation at 600 *g* for 5 min to remove the unbroken tissue debris, cells, and cell nuclei. The supernatants were collected and centrifuged at 10,000 *g* for 30 min to pellet mitochondria. The mitochondria pellets were resuspended in resuspension buffer [200 mM mannitol, 10 mM HEPES-KOH (pH 7.5), 70 mM sucrose, and 1 mM EGTA]. The resulting supernatants were further centrifuged at 100,000 *g* for 30 min to eliminate contamination by the membrane fraction. All procedures were performed at 4°C. The protein concentrations of cytosol and mitochondrial fractions were quantified using Bio-Rad DC RC protein assay kit (BioRad, Hercules, CA).

Measurement of cytochrome-*c* oxidase activity and assessment of outer mitochondrial membrane damage

Both mitochondrial cytochrome-*c* oxidase activity and outer membrane integrity were evaluated using cytochrome-*c* oxidase assay (Sigma, St. Louis, MO). Experimental procedures were performed according to the manufacturer's protocol; 20 µg freshly isolated mitochondrial fraction were 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 the 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 is related to oxidation of ferrocytochrome-*c* by cytochrome-*c* oxidase and was recorded using a kinetic program (5-s delay; 10-s interval; 6 repeated readings). Cytochrome-*c* oxidase activities were calculated and normalized for the amount of protein per reaction, and results were expressed as units per milligram mitochondrial protein. Mitochondrial outer membrane integrity was assessed by measuring cytochrome-*c* oxidase activity of mitochondria in the presence or absence of the detergent, *n*-dodecyl β-D-maltoside. The mitochondrial outer membrane damage was assessed from the ratio between cytochrome-*c* oxidase activity without and with detergent.

Measurement of SOD activity in mitochondria

SOD activity was measured based on a method originally described by Nebot and colleagues (22), using SOD assay kit (Calbiochem, San Diego, CA). Approximately 100 µg mitochondrial protein were used for each reaction, and all assays were performed in duplicate. According to the vendor's protocol, sample was first incubated with 1-methyl-2-vinylpyridinium in assay buffer at 37°C for 1 min to eliminate interference. Immediately after adding substrate [5,6,6α,11β-tetrahydro-3,9,10-trihydroxybenzo(c)fluorine, the oxidation of which is regulated by SOD], absorbance at 525 nm was recorded using a kinetic program: 10-s interval and six readings. The SOD activity was determined from the ratio of the auto-oxidation 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 GPx assay kit (Calbiochem, San Diego, CA). Mitochondrial extracts were at first diluted with assay buffer, and ~50 µg protein were used per reaction; all assays were performed in duplicate. The sample was added to a solution containing 1 mM GSH, 0.4 U/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 was recorded at absorbance at 340 nm (A_{340}) using a kinetic program: 30-s interval and six readings. The GPx activity was determined by the rate of decrease in A_{340} [1 mU/ml GPx = (A_{340}/min)/0.0062]. Results were normalized by protein amount per reaction and expressed as microunits per milligram mitochondrial protein.

Measurement of CAT activity in mitochondria

CAT activity was measured according to previously developed methods (21) using Amplex Red CAT assay kit (Molecular Probes, Eugene, OR). The mitochondrial extracts were diluted with reaction buffer (0.1 M Tris·HCl, pH 7.5) to ~0.5 mg/ml. Diluted mitochondrial sample (25 µl) or standard was added to 40 µM H_2O_2 for 30 min at room temperature. A fluorogenic probe that reacts with unutilized H_2O_2 (50 µl 100 µM *N*-acetyl-3,7-dihydroxyphenoxazine, Amplex Red) was added to the mitochondrial sample and incubated at 37°C for an additional 30 min. The amount of end product (resorufin) is recorded spectrophotometrically at 560 nm. CAT activities were calculated and normalized for protein. All measurements were performed in duplicate, and results were expressed as units per milligram mitochondrial protein.

Measurement of lipid peroxidation in mitochondria

Lipid peroxidation was assessed by levels of malondialdehyde (MDA), a marker of lipid oxidation using lipid peroxidation assay kit (Calbiochem, San Diego, CA) (7). To prevent sample oxidation, all mitochondrial extracts were normalized to 1–1.5 mg/ml in resuspension buffer with 5 mM butylated hydroxyl toluene. Standards were prepared, and experimental procedures were performed, according to the manufacturer's protocol. For each reaction, 200-µl sample/standard were added to 650-µl chromogenic reagent (provided by the manufacturer) and 150 µl of 12 N HCl. After reaction 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.

Western blots

Protein samples of mitochondrial or cytosolic fractions were mixed with Laemmli's loading buffer, boiled for 5 min, subjected to 15% SDS-PAGE gels, and transferred to nitrocellulose 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: anti-cytochrome-*c* and anti-GAPDH (Chemicon, Temecula, CA), anti-cytochrome-*c* oxidase

(Molecular Probes, Eugene, OR), anti-ANT (Santa Cruz Biotechnology, Santa Cruz, CA), anti-MnSOD (manganese-dependent SOD) (Stressgen Bioreagents, Victoria, British Columbia, Canada), anti-GPx4 (Genetex, San Antonio, TX), and anti-GPx1 (Abcam, Cambridge, MA). The membranes were then rinsed and incubated with corresponding horseradish peroxidase-conjugated anti-mouse IgG (Roche, Indianapolis, IN) or anti-rabbit IgG (Stressgen Bioreagents). Antibody dilutions and incubation time were according to the manufacturer's instructions. Membranes were then rinsed, and bound antibodies were detected by using enhanced chemiluminescence (Amersham, Piscataway, NJ).

Measures of cardiac contraction and relaxation (Langendorff model)

For studies of cardiac contraction and relaxation, awake animals were anticoagulated with sodium heparin (1,000 units, Elkins-Sinn, Cherry Hill, NJ) 8 or 24 h after burn (or sham burn) and decapitated with a guillotine. The heart was rapidly removed and placed in ice-cold (4°C) Krebs-Henseleit bicarbonate buffered solution (118 mmol/l NaCl, 4.7 mmol/l KCl, 21 mmol/l NaHCO₃, 2.5 mmol/l CaCl₂, 1.2 mmol/l MgSO₄, 1.2 mmol/l KH₂PO₄, and 11 mmol/l glucose). All solutions were prepared on the day of experimental performance and bubbled with 95% O₂ and 5% CO₂ (pH 7.4; PO₂, 550 Torr; PCO₂, 38 Torr). A 17-gauge cannula placed in the ascending aorta was connected to a buffer-filled reservoir for perfusion of the coronary circulation at a constant flow rate of 5 ml/min. Hearts were suspended in a temperature-controlled chamber maintained at 38 ± 0.5°C, and a constant flow pump (Holter, model 911, Critikon) was used to maintain perfusion of the coronary arteries by retrograde perfusion of the aortic stump cannula. Coronary perfusion pressure was measured, and effluent was collected to confirm coronary flow rate. Contractile function was assessed by measuring intraventricular pressure with a saline-filled latex balloon placed in the left ventricular (LV) chamber. LV pressure (LVP) was measured with a Statham pressure transducer (model P23ID, Gould Instruments) attached to the balloon cannula; the rates of LVP rise (+dP/dt) and fall (−dP/dt) were obtained using an electronic differentiator (model 7P20C, Grass Instruments) and were recorded (model 7DWL8P, Grass Recording Instruments, Quincy, MA).

A Frank-Starling relationship for each heart was determined by plotting LV developed pressure (peak systolic pressure minus LV end-diastolic pressure) and ±dP/dt responses vs. increases in preload (LV volume). Because heart rate varied after burn injury, hearts were paced through an electrode attached to the right atrium (3–4 Hz, 2–10 W for 4-ms duration; Grass Stimulator, Grass Instruments). Hearts were paced at twice the minimum capture voltage; thus in vitro heart rates were similar in all experimental groups, and differences in cardiac performance could not be attributed to burn-related differences in heart rate. In addition, ventricular performance was assessed in all hearts as perfusate Ca²⁺ concentration was incrementally increased from 1 to 8 mmol/l.

Statistical analysis

All values are expressed as means ± SE. ANOVA was used to assess an overall difference among the groups for each of the variables. Levene's test for equality of variance was used to suggest the multiple-comparison procedure to be used; once equality of variance among the four groups was suggested, multiple-comparison procedures were performed. Student

Newman-Keuls was used for examining differences in myocardial performance. One-way ANOVA and a two-sided post hoc Dunnett *t*-test were used to compare the control group with each of the other groups for mitochondrial outer membrane damage, lipid peroxidation, GPx, cytochrome-*c*, and MDA levels. Probability values < 0.05 were considered statistically significant (analysis was performed using SPSS for Windows, version 7.5.1).

RESULTS

Burn injury impaired mitochondrial integrity in the heart

Leak of mitochondrial protein cytochrome-*c* into cytosol provides a measure of mitochondrial injury. We examined levels of cytochrome-*c* in cytosolic and mitochondrial fractions isolated from heart tissue collected at several time points after burn injury (2, 4, 8, 12, 24, 48, and 72 h). As shown in Fig. 1A, Western blot analysis demonstrated a progressive increase of cytosolic cytochrome-*c* during the first 24 h after burn. Twenty-four hours postburn, cytosolic cytochrome-*c* levels were more than three times higher than levels measured in sham burn rats. Cytosolic cytochrome-*c* levels returned toward sham levels 48 and 72 h postburn. In contrast, expression of the cytosolic marker protein GAPDH was unchanged during the entire postburn time course; furthermore, we could only detect cytochrome-*c* but not GAPDH in the mitochondrial fraction, indicating the complete separation of cytosol from mitochondria.

In addition, we examined levels of cytochrome-*c* in the mitochondrial fractions and detected a significant decrease in mitochondrial cytochrome-*c* 24 h after burn (Fig. 1B). Expression of mitochondrial matrix protein ANT (used as a control) remained unchanged. This experiment demonstrated burn-related redistribution of cytochrome-*c* between cytosol and mitochondria, indicating that burn injury induced cardiac mitochondrial damage.

Using an alternative approach to evaluate mitochondrial integrity, we examined mitochondrial outer membrane damage as determined by cytochrome-*c* oxidase activity in mitochondrial fractions, with and without detergent *n*-dodecyl β -D-maltoside. As described in the MATERIALS AND METHODS section, the ratio of cytochrome-*c* oxidase activity measured in the presence/absence of detergent represents the percentage of mitochondrial outer membrane damage. As shown in Fig. 2, cardiac mitochondrial outer membrane damage was significantly increased 2–24 h after burn injury.

Burn injury altered mitochondrial defense against ROS in the heart

To determine whether burn trauma altered ROS defense in cardiac mitochondria, we compared the enzymatic activities of SOD, GPx, and CAT in the mitochondrial fractions from sham burn and burned rats. We found that both SOD and GPx activities were significantly decreased by burn, whereas the CAT activity remained unchanged (Fig. 3). In this regard, SOD activity was reduced 2–24 h postburn, and the maximal defect was apparent 8 h postburn when SOD activity was 63.5% of that measured in sham burns. GPx activity fell to 60% of sham burn levels 2 h postburn, and this difference persisted through 48 h postburn.

The mitochondrial-specific SOD isoform is MnSOD, and mitochondrial GPx isoforms include GPx1 and GPx4 (14). We examined the expression levels of these antioxidant enzymes in the cardiac mitochondria fractions by Western blot. As shown in Fig. 3B, no significant changes were evident in burned animals compared with those measured in sham burns. CAT expression was not detected (data not shown), probably due to its low mitochondrial expression, while CAT activity in mitochondria was measurable in our studies.

Increased lipid oxidation in heart mitochondria from burn rats

Because burn injury damaged cardiac mitochondrial ROS defense, we speculated that burn may provoke oxidative damage to cardiac mitochondria. To address this issue, we examined the level of lipid peroxidation in cardiac mitochondria isolated from sham burn and burned rats. As shown in Fig. 4, concentrations of MDA, a marker for lipid peroxidation, were significantly elevated in the cardiac mitochondria prepared from burned rats compared with values measured in shams. Increased MDA levels persisted throughout the postburn study period (72 h), suggesting prolonged oxidative damage in heart mitochondria.

Cytochrome-c oxidase activity in hearts of burned animals

Since cytochrome-*c* oxidase is an important enzyme involved in the mitochondrial respiratory complex, which controls energy metabolism (16), we examined the activity and expression of cytochrome-*c* oxidase in the cardiac mitochondrial fractions from sham burn and burned rats. As shown in Fig. 5, there was no change in these parameters throughout the postburn period, suggesting that burn injury did not alter mitochondrial respiratory function.

Antioxidant vitamin therapy maintained cardiac mitochondrial integrity during burn injury

We further investigated whether administration of antioxidant vitamins protected cardiac mitochondria from burn injury. Antioxidant vitamins or vehicle were given to sham and burn rats, as described in experimental groups. As shown in Fig. 6A, cytosolic cytochrome-*c* was significantly increased 4 h and progressively rose over 24 h postburn in animals treated with vehicle. In contrast, cytochrome-*c* remained at sham burn levels in burned animals treated with antioxidant vitamins. Similarly, burn injury increased mitochondrial outer membrane damage in rats treated with vehicle alone, but this damage was diminished in burn animals given antioxidant vitamins (Fig. 6B), suggesting that antioxidant vitamin therapy protects cardiac mitochondria integrity.

Antioxidant vitamin therapy prevented burn-stimulated downregulation of ROS defense and oxidative damage in cardiac mitochondria

We examined SOD and GPx activities in myocardial mitochondria of burned rats treated with either antioxidant vitamins or vehicle. As expected, burn injury severely impaired both SOD and GPx activities in rats treated with vehicle alone. However, antioxidant vitamin therapy promoted significant recovery of SOD activity 4, 8, and 24 h postburn, while GPx activity was improved 8 and 24 h postburn (Fig. 7A). In addition, by Western blots, we compared levels of MnSOD, GPx1, and GPx4 in cardiac mitochondria in groups of burns

treated with antioxidant vitamins and in burns treated with vehicle, but no significant alterations were detected (data not shown).

To assess whether antioxidant vitamin therapy can alter burn-induced oxidative damage in cardiac mitochondria, we examined mitochondrial lipid peroxidation as determined by MDA levels. As shown in Fig. 7B, treatment with antioxidant vitamins in burn injury prevented burn-induced lipid peroxidation in mitochondria.

Antioxidant vitamin therapy improved postburn heart function

We examined the hemodynamic function in vivo and in vitro LV function in sham burns as well as in burn rats killed either 8 or 24 h after burn injury. Subgroups of vehicle-treated burns and antioxidant vitamin-treated burns were included. These time points were selected based on previous studies showing that modest myocardial contractile defects are evident as early as 8 h postburn, while burn-related contractile deficits are maximal 24 h after burn.

Mean arterial blood pressure was lower 24 h after burn than that measured in sham burns, despite aggressive fluid resuscitation. Burn rats given antioxidant vitamins produced mean arterial blood pressure values similar to those measured in sham burns treated with antioxidant vitamins. Hematocrit and hemoglobin values fell significantly 24 h postburn, regardless of vitamin therapy, and hemodilution was attributed to the aggressive fluid resuscitation. In addition, decreased serum ionized calcium levels were observed in all burn-injured animals, regardless of antioxidant vitamin therapy (Table 1).

Compared with sham burns, LVP and $\pm dP/dt_{\max}$ responses measured during stabilization of the heart (perfused at a constant heart rate, constant LV end-diastolic volume, and constant coronary flow rate, Table 2) were lower in vehicle-treated burns but significantly improved by antioxidant vitamin therapy in burns. LVP and $\pm dP/dt_{\max}$ responses to incremental increases in preload (LV volume) were significantly lower 24 h after vehicle-treated burn, while recovery of cardiac contraction and relaxation was evident with antioxidant vitamin therapy in burns. Coronary perfusion pressure, coronary vascular resistance, time to maximum $-dP/dt$, and in vitro heart rate were not different among groups (data not shown). Cardiac parameters measured 8 h postburn tended to be lower than values measured in sham burns (Table 2), but did not achieve statistical significance. In addition, antioxidant vitamin therapy in sham burn rats did not alter hemodynamic or metabolic function compared with that measured in vehicle-treated shams. Similarly, LV responses were nearly identical in vehicle-treated shams and antioxidant vitamin-treated shams.

DISCUSSION

Although significant evidence has accumulated supporting the impact of mitochondrial abnormalities in heart disease, little is known about the role of mitochondria in burn-mediated cardiac dysfunction. Using a burned animal model, this is the first study, to our knowledge, confirming that burn trauma produces mitochondrial damage in the heart, as demonstrated by mitochondrial outer membrane damage and mitochondrial release of cytochrome-*c*. In addition, we showed that burn injury downregulated the activities of antioxidant enzymes SOD and GPx in cardiac mitochondria, and burn-mediated oxidative

damage to mitochondria was confirmed by increased lipid peroxidation. Our study of heart function 8 and 24 h after burn injury indicated that the deterioration of mitochondrial function preceded the appearance of cardiac dysfunction after burn trauma. Antioxidant vitamin therapy in burned animals prevented the deregulation of cardiac mitochondria and subsequently improved heart function, suggesting that burn-induced cardiac dysfunction may be mediated, in part, by myocardial mitochondrial injury and dysfunction.

Our results showed that burn injury caused significant impairment of antioxidant defense in the cardiac mitochondria. Imbalance between ROS production and scavenging leads to ROS mediated injury. Previous NMR spectroscopy findings of unchanged myocardial ATP content after burn suggested that the injury does not alter mitochondrial respiratory function in the heart (31). In the present study, we observed no changes in the activity and expression of cytochrome-*c* oxidase, an enzyme in the mitochondrial respiratory complex, in hearts from both burn and sham burn rats. These results suggested that burn injury may have little effect on ROS production in cardiac mitochondria. However, in the heart of burn-injured animals, we observed rapid downregulation of mitochondrial SOD and GPx activities, accompanied by a significant rise in lipid peroxidation, suggesting that burn injury increases mitochondrial oxidative damage by decreasing ROS scavenging in cardiac mitochondria. We also showed that the expression levels of these mitochondrial expressed enzymes, MnSOD, GPx1, and GPx4, were not changed in myocardium. Antioxidant vitamin therapy preserved postburn SOD and GPx activities, with no vitamin-related alteration in enzyme expression, suggesting that mitochondrial SOD and GPx activities are regulated by factors other than enzyme expression. This hypothesis is supported by numerous other studies indicating posttranslational modulation for both SOD and GPx; for example, tyrosine nitration of MnSOD leads to its inactivation during heart and kidney allograft rejection (18, 23).

Our data showed that burn injury promoted lipid oxidation in cardiac mitochondria, a consequence of overproduction of hydroxyl radicals, and we attributed this phenomenon to the burn-mediated defects in antioxidant activities. Studies by others have proposed that downregulation of antioxidant activities in mitochondria increases the susceptibility of mitochondria to oxidative damage. In mice with either reduced expression of mitochondrial SOD or completely lacking SOD, oxidative damage to mitochondrial proteins, lipids, and DNA has been shown to occur (20, 28). GPx is one of the primary enzymatic defense systems against hydrogen peroxide and lipid hydroperoxide. Our data suggested that GPx may not be the sole regulator of lipid oxidation in mitochondria. Antioxidant vitamin therapy in burns suppressed increased lipid peroxidation in cardiac mitochondria; however, recovery of GPx activity was observed 8 and 24 h, but not 2 and 4 h, after burn injury. These data indicate that other enzymes may also contribute to the increased mitochondrial MDA after injury. Recently, peroxiredoxin family proteins (Prxs) have been identified as antioxidant enzymes, which protect against hydrogen peroxide, peroxynitrite, and a wide range of organic hydroper-oxides (29, 30). In our burn injury model, we detected the expression of two Prx isoforms, PrxIII and PrxV, in cardiac mitochondria (data not shown); Prxs may be additional anti-oxidant enzymes involved in the regulation of lipid peroxidation in cardiac mitochondria during burn trauma. However, assays for Prxs activities have not been commercialized. The current method involves large-scale recombinant protein production in yeast, which is experimentally difficult under our laboratory setting.

Release of cytochrome-*c* from mitochondria is initiated by ROS-mediated peroxidation of cardiolipin, a phospholipid component in mitochondrial inner membrane (24). Our studies did not directly link the burn-induced lipid peroxidation level as a causative factor of cytochrome-*c* translocation. However, our postburn time course studies confirmed that the maximal decrease in antioxidant activities and increase in lipid peroxidation occurred before either cytochrome-*c* translocation or outer membrane damage in cardiac mitochondria. Furthermore, antioxidant vitamin therapy improved mitochondrial antioxidant activities, suppressed lipid oxidation, and maintained mitochondrial integrity in the heart after burn injury. The data suggest that the oxidative damage in mitochondria is, in part, responsible for the burn-related loss of mitochondria integrity in the heart.

Taken together, our results suggest that mitochondrial damage and defects in mitochondrial ROS defense may be causative rather than consequential of burn-related cardiac dysfunction. In the hearts of burn-injured animals, we observed that mitochondrial release of cytochrome-*c*, damage of the mitochondrial outer membrane, increase of lipid peroxidation, and decrease of mitochondrial ROS defense occurred at earlier times during the postburn period (2–8 h postburn) and preceded the development of cardiac contractile dysfunction (minimal dysfunction 8 h after burn injury and maximal dysfunction 24 h postburn). Therefore, alterations in myocardial mitochondria cannot be a consequence of burn-related cardiac dysfunction. Our findings that antioxidant vitamin therapy prevented loss of mitochondrial integrity and improved mitochondrial antioxidant activities in the myocardium, paralleled by recovery of heart function, further suggest that mitochondrial function may play a pivotal role in burn-mediated cardiac dysfunction.

In the present study, we have identified a potential relationship between cardiac mitochondrial injury and postburn heart function; we also confirmed the beneficial effects of antioxidant vitamins in maintaining mitochondrial integrity and function in myocardium after burn and propose that alterations in cardiac mitochondria are causative factors of burn-related cardiac dysfunction. However, direct evidence to support our hypothesis is still lacking. In the future, we are interested in applying subcellular targeted interventions to investigate whether targeting specific aspects of mitochondrial function and specific mitochondrial molecules can alter burn-mediated heart dysfunction. These studies may lead to more efficient and more sensitive therapeutic strategies to improve cardiac function in injury and disease.

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Fig. 1.

Burn-induced cytochrome-*c* release from mitochondria to cytosol in the heart. Protein samples were extracted from the hearts of sham burns (Con) and burn rats at multiple time points postburn, as indicated. *A*: cytochrome-*c* and cytosolic marker GAPDH in cytosolic fractions. *Lanes 1–8*: cytosolic fractions; *lane 9*: mitochondria (mito) fraction from Con. *B*: cytochrome-*c* and mitochondrial marker ANT in mitochondrial fractions. Western blots were analyzed by densitometry. All values are means $\pm$ SE. *Significant difference from Con at $P < 0.05$ ($n = 3$).

Fig. 2.

Burn increased mitochondrial outer damage in the heart. Mitochondrial outer membrane damage was measured in cardiac mitochondrial preparations from Con and burned rats killed at different time points postburn. All values are means $\pm$ SE. *Significant difference from Con at $P < 0.05$ ($n = 3$).

Fig. 3.

A: activities of SOD (*A1*), glutathione peroxidase (GPx; *A2*), and catalase (CAT; *A3*) in cardiac mitochondria from Con and burn rats. All measured activities were normalized by the amount of mitochondrial protein per reaction. **B:** expression of manganese SOD (MnSOD; *B1*) and GPx1 (*B2*) and GPx4 (*B3*) in cardiac mitochondrial fractions from Con and burn rats. Western blots were analyzed by densitometry. All values are means $\pm$ SE. *Significant difference from Con at $P < 0.05$ ($n = 3$).

Fig. 4.

Burn increased malondialdehyde (MDA) levels in cardiac mitochondria. MDA concentrations were measured in cardiac mitochondrial preparations from Con and burned animals at different postburn time points. All measurements were normalized for the amount of protein per reaction and are expressed as nanomoles MDA per milligram mitochondrial protein. All values are means $\pm$ SE. *Significant difference from Con at $P < 0.05$ ($n = 3$).

Fig. 5.

A: mitochondrial cytochrome-*c* oxidase activity in the heart from Con and burned rats. All values were normalized with the amount of protein per sample and expressed as units per milligram mitochondrial protein ($n = 3$). *B*: mitochondrial expression of cytochrome-*c* oxidase in the hearts from Con and burn rats. Western blots were analyzed by densitometry ($n = 3$).

Fig. 6.

Antioxidant vitamins protected cardiac mitochondrial integrity from burn injury. *A*: expression of cytochrome-*c* and GAPDH in cytosolic fractions from Con and burn rats treated with antioxidant vitamins or with Con vehicle. Western blots were quantified by densitometry. *B*: levels of mitochondrial outer membrane damage in hearts of Con and burn rats treated with antioxidant vitamins or with Con vehicle. All values are means $\pm$ SE. *Significant difference compared with respective Con; †significant difference with antioxidant vitamins vs. with Con vehicle ($P < 0.05$, $n = 3$).

Fig. 7.

Antioxidant vitamins prevented cardiac mitochondrial downregulation of ROS defense and oxidative damage induced by burn injury. *A*: SOD (*A1*) and GPx (*A2*) activities in mitochondrial fractions from the hearts of Con and burn rats treated with antioxidant vitamins or Con vehicle. *B*: mitochondrial MDA concentrations in the hearts of Con and burn rats treated with antioxidant vitamins or Con vehicle. All values are means $\pm$ SE. *Significant difference compared with respective Con; †significant difference with antioxidant vitamins vs. with Con vehicle ($P < 0.05$, $n = 3$).

Table 1

Antioxidant vitamin treatment in burned rats

	Sham + Vehicle	Sham + Antioxidants	8-h Burn + Vehicle	8-h Burn + Antioxidants	24-h Burn + Vehicle	24-h Burn + Antioxidants
MAP, mmHg	125±3	149±5	116±14 [*]	136±4	93±3 [*]	126±2 [†]
HR, beats/min	461±22	484±24	500±12	433±22	433±21	500±10.9
pH	7.43±0.2	7.46±0.02	7.45±0.02	7.42±0.01	7.46±0.01	7.47±0.01
PCO ₂ , Torr	32.5±1.1	33.2±2.0	24.4±2.2	25.2±1.5	34.8±2.2	28.6±1.9
PO ₂ , Torr	109.6±5.9	92.1±7.8	115.6±10.4	119±4.7	91.5±4.9	88.3±6.9
HCO ₃ ⁻ , mmHg	21.4±0.9	23.3 ±0.7	17.2±0.5	15.9±1.1	24.1±1.0	20.6±1.0
Lactate, mmol	2.20±0.3	2.2±0.6	1.63±0.4	2.53±0.25	3.3±0.4 [*]	3.45 ±0.6 [*]
Base excess, mmol	1.64±0.4	1.48±0.2	-3.90±0.59 [*]	-5.27±0.07 [*]	-2.17±0.68 [*]	-0.68±0.5 [*]
Hct	4.3±3	46±1	43±1	41±1	27±5 [*]	30±1 [*]
Ca ²⁺ , mM	1.1±0.2	0.96±0.14	0.93±0.04	0.67±0.04 [*]	0.78±0.07 [*]	0.86±0.1 [*]
Na ⁺ , mM	141±0	144±1	139±0	139±1	138±1	147±0
O ₂ saturation	98.0±0.2	98.5±0.6	99.4±0.7	99.6±0.2	96.8±0.7	96.9±1.5
O ₂ content	19.8±1.5	18.8±0.8	21.5±0.6	22.2±0.6	16.9±0.4	12.7±0.6

All values are means ± SE. MAP, mean arterial pressure; HR, heart rate; HCO₃⁻, bicarbonate; Ca²⁺, serum calcium; Na⁺, serum sodium.

^{*} Difference compared with respective controls: $P < 0.05$.

[†] Significant difference in antioxidant vitamin-treated rats compared with vehicle-treated (i.e., 24-h burn + antioxidant vitamins vs. 24-h burn + vehicle): $P < 0.05$.

Table 2

Cardiac responses to burn injury in the presence/absence of antioxidant vitamin therapy

	Sham + Vehicle	Sham + Antioxidants	8-h Burn + Vehicle	8-h Burn + Antioxidants	24-h Burn + Vehicle	24-h Burn + Antioxidants
LVP, mmHg	88.6±2.9	87.9±2.1	80.3±5.5	86.9±3.9	64.3 ±5.0 *	81.7±1.9 †
+dP/dt _{max} , mmHg/s	2,190±30	2,050±47	1,818±112	1,940±58	1,321±122 *	1,880±44 †
-dP/dt _{max} , mmHg/s	1,775±71	1,716±51	1,471±151	1,600±53	999±96 *	1,570±46 †
dP40, mmHg/s	1,856±25	1,855±42	1,578±138	1,786±63	1,226 ±108 *	1,692±47 †
TPP, ms	91.4±4.6	99.4±2.7	81.6±1.6	92.3±2.1	88.0±3.1	103.5±2.5 †
RT90, ms	89±4.1	97.9±3.9	81.1±2.2	92.7±2.7	82.3±2.1 *	93.4±2.4 †
Time to +dP/dt _{max} , ms	57±4.4	62.3±2.3	52.9±1.4	52.7±0.9	49.8±0.9 *	64.3±2.1 †

All values are means ± SE. LVP, left ventricular pressure; +dP/dt_{max}, maximum rate of LVP rise; -dP/dt_{max}, maximum rate of LVP fall; dP40, developed pressure of 40 mmHg; TPP, time to peak pressure; RT90, time to 90% relaxation.

* Significant change compared with respective controls;

† significant difference in antioxidant vitamin-treated rats compared with vehicle-treated (i.e., 24-h burn + antioxidant vitamins vs. 24-h burn + vehicle): $P < 0.05$.