Role of cytosolic vs. mitochondrial Ca\(^{2+}\) accumulation in burn injury-related myocardial inflammation and function

David L. Maass, Jean White, Billy Sanders, and Jureta W. Horton

Department of Surgery, University of Texas Southwestern Medical Center, Dallas, Texas

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Maass, David L., Jean White, Billy Sanders, and Jureta W. Horton. Role of cytosolic vs. mitochondrial Ca\(^{2+}\) accumulation in burn injury-related myocardial inflammation and function. Am J Physiol Heart Circ Physiol 288: H744–H751, 2005. First published September 23, 2004; doi:10.1152/ajpheart.00367.2004.—This study was designed to examine the role of mitochondrial Ca\(^{2+}\) homeostasis in burn-related myocardial inflammation. We hypothesized that mitochondrial Ca\(^{2+}\) is a primary modulator of cardiomyocyte TNF-\(\alpha\), IL-1\(\beta\), and IL-6 responses to injury and infection. Ventricular myocytes were prepared by Langendorff perfusion of hearts from adult rats subjected to sham burn or burn injury over 40% of total body surface area to produce enzymatic (collagenase) digestion. Isolated cardiomyocytes were suspended in MEM, cell number was determined, and aliquots of myocytes from each experimental group were loaded with fura 2-AM (2 \(\mu\)g/ml) for 45 min at room temperature to measure total cellular Ca\(^{2+}\), 2 min at 30°C followed by incubation at 37°C for 2 h to eliminate cytosolic fluorescent, and 20 min at 37°C in MnCl\(_2\) (200 \(\mu\)M) containing buffer to quench cytosolic fura 2-AM signal. In vitro studies included preparation of myocytes from control hearts and challenge of myocytes with LPS or burn trauma increased cytosolic Ca\(^{2+}\), cardiomyocytes (4, 34, 35). Our interest in mitochondrial Ca\(^{2+}\) overload exerts detrimental effects on cellular function, accelerating apoptosis and decreasing energy availability to support cellular function. Studies by others have suggested that cellular Ca\(^{2+}\) accumulation, secretion of proinflammatory cytokines such as TNF-\(\alpha\), and a rise in caspase-3 activity act in concert to promote apoptosis of several cell types, including cardiomyocytes (4, 34, 35). Our interest in mitochondrial Ca\(^{2+}\) handling arose from our previous finding that burn injury increases cardiomyocyte cytosolic Ca\(^{2+}\); increases cardiomyocyte secretion of inflammatory cytokines, including TNF-\(\alpha\) and IL-1\(\beta\); and promotes cardiomyocyte apoptosis (16, 23, 24, 43, 44).

Until recently, the ability to examine mitochondrial Ca\(^{2+}\) transport in intact living cells was limited by the technical approaches available. In the early 1990s, Rizzuto et al. (34) pioneered techniques for direct measurement of mitochondrial Ca\(^{2+}\) concentration. The recent availability of fluorescent dyes and fluorescent imaging techniques has provided a means of exploring simultaneous changes in cytosolic and organelle (mitochondrial) Ca\(^{2+}\). These techniques include controlling the cellular loading of esterified fluorescent dyes to promote preferential accumulation in the organelle of interest; other techniques selectively quench the fluorescent signal from cytosolic dye, optimizing measurements of mitochondrial fluorescence (32). The purpose of the present study was to apply new technical approaches to achieve selective loading of fluorescent indicators in mitochondria (14, 25), allowing us to examine the contribution of mitochondrial Ca\(^{2+}\) accumulation to myocardial inflammation and contractile function.

Mitochondria play a pivotal role in cellular metabolic activity, regulating homeostasis by providing ATP to support numerous cell functions. Mitochondria also regulate several enzymes of the tricarboxylic acid cycle, and recent interest in mitochondrial physiology has focused on the finding that this subcellular organelle regulates several aspects of programmed cell death (27, 28, 38), a homeostatic mechanism that eliminates injured or dysfunctional cells and is initiated by oxidant injury, heat shock, cytokines, or ischemia-reperfusion (8, 15, 39, 41). Altered mitochondrial function and morphology, described in a number of injury and disease states, have been attributed to generation of reactive oxygen species (10, 12, 13, 21). Oxidant-mediated mitochondrial injury and dysfunction have been supported further by the finding that exposure of isolated mitochondria to free radical-generating solutions or ischemic conditions disrupted matrix- and membrane-bound enzymes, altered mitochondrial permeability transition pore and mitochondrial membrane permeability, and produced mitochondrial swelling and NAD\(^{+}\)/NADH leakage (2, 3, 5, 9, 26, 29, 33, 40).

Recent studies suggested that although mitochondria provide a Ca\(^{2+}\)-buffering compartment that allows subcellular sequestration of Ca\(^{2+}\) under pathological conditions (6), mitochondrial Ca\(^{2+}\) overload exerts detrimental effects on cellular function, accelerating apoptosis and decreasing energy availability to support cellular function. Studies by others have suggested that cellular Ca\(^{2+}\) accumulation, secretion of proinflammatory cytokines such as TNF-\(\alpha\), and a rise in caspase-3 activity act in concert to promote apoptosis of several cell types, including cardiomyocytes (4, 34, 35). Our interest in mitochondrial Ca\(^{2+}\) handling arose from our previous finding that burn injury increases cardiomyocyte cytosolic Ca\(^{2+}\); increases cardiomyocyte secretion of inflammatory cytokines, including TNF-\(\alpha\) and IL-1\(\beta\); and promotes cardiomyocyte apoptosis (16, 23, 24, 43, 44).

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MATERIALS AND METHODS

Experimental model. Adult male Sprague-Dawley rats (320–350 g; Harlan Laboratories, Houston, TX) were conditioned in-house for 5–6 days after arrival; commercial rat chow and tap water were available at will. All studies were reviewed and approved by the University of Texas Southwestern Medical Center Institutional Review Board for the care and handling of laboratory animals and conformed to all guidelines for animal care as outlined by the American Physiological Society and the National Institutes of Health.

Burn procedure. Rats were deeply anesthetized (isoflurane) and secured in a constructed template device, and the surface of the skin exposed through the aperture in the template was immersed in 100°C water for 10 s on the back and upper sides. Use of the template produced a well-circumscribed burned area, avoided injury to the abdominal organs, and accomplished full-thickness dermal burns over 40% of the total body surface area (TBSA). We previously showed that exposure to this water temperature in adult rats destroys all underlying nerves and avoids injury to underlying organs. Sham-burn rats were subjected to identical preparation, except they were immersed in room-temperature water to serve as controls. Immediately after immersion, rats were dried, returned to individual cages, and given fluid (lactated Ringer solution, 4 mL·kg⁻¹·% burn⁻¹ ip, with one-half of the calculated volume given immediately after burn and the remaining volume given 8 h after burn). The total volume of Ringer solution given over the first 24 h after burn was 50–56 mL. Buprenorphine (0.5 mg/kg) was given every 8 h during the postburn period. Burned rats did not display discomfort or pain, moved freely about the cage, and consumed food and water within 20 min after recovering from anesthesia.

Cardiomyocyte isolation. At 24 h after sham or burn injury, rats received heparin (2,000 U ip) and were decapitated 20–30 min after systemic heparinization. Hearts were harvested and placed in a petri dish containing room-temperature heart medium [113 mM NaCl, 4.7 mM KCl, 0.6 mM KH₂PO₄, 0.6 mM Na₂HPO₄, 1.2 mM MgSO₄, 12 mM NaHCO₃, 10 mM KHCO₃, 20 mM t-glucose, 0.5× MEM amino acids (50×, GIBCO-BRL), 10× MEM HEPES, 30 mM taurine, 2.0 mM carnitine, and 2.0 mM creatine], which was bubbled constantly with 95% O₂–5% CO₂. Hearts were cannulated via the aorta and perfused with heart medium at a rate of 12 mL/min for a total of 5 min in a nonrecirculating mode. Enzymatic digestion was initiated by perfusing the heart with digestion solution that contained 34.5 mL of heart medium + 50 mg of collagenase II (catalog no. 4177, lot no. M083771, Worthington), 50 mg of BSA (fraction V; catalog no. 11018-025, GIBCO-BRL), 0.5 mL of trypsin (2.5%, 10×; catalog no. 15090-046, GIBCO-BRL), 100 μM CaCl₂, and 40 mM 2,3-butanedione monoxime. Enzymatic digestion was accomplished by recirculating this solution through the heart at a flow rate of 12 mL/min for 20 min. All solutions perfusing the heart were maintained at a constant temperature of 37°C. At the end of the enzymatic digestion, the ventricles were removed and mechanically dissociated in 6 mL of enzymatic digestion solution containing a 6-mL aliquot of 2× BDM-BSA solution [3 mg BSA (fraction V) and 150 mL of BDM stock (40 mM)]. After mechanical dissociation with fine forceps, the tissue homogenate was filtered through a mesh filter into a conical tube. The cells adhering to the filter were collected by washing with an additional 6-mL aliquot of 2× BDM-BSA solution (100 mL of BDM stock (40 mM)), 100 mL of heart medium, and 2 g of BSA (fraction V). Cells were then allowed to pellet in the conical tube for 10 min. The supernatant was removed, and the pellet was resuspended in 10 mL of 1× BDM-BSA. The cells were washed and pelleted further in BDM-BSA buffer with 100, 200, and then 500 μM Ca²⁺ to a final concentration of 1,000 μM. After the final pelleting step, the supernatant was removed, and the pellet was resuspended in MEM, prepared by addition of 10.8 g of 1× MEM (catalog no. M-1018, Sigma), 11.9 mM NaHCO₃, 10 mM HEPES, and 10 mL of 100× penicillin-streptomycin (catalog no. 1540-122, GIBCO-BRL) with 950 mL of MilliQ water; total volume was adjusted to 1 liter. At the time of MEM preparation, the medium was bubbled with 95% O₂–5% CO₂ for 15 min, and pH was adjusted to 7.1 with 1 M NaOH. The solution was then filter sterilized and stored at 4°C until use. At the final concentration of Ca²⁺, the cardiomyocyte cell number was calculated and myocyte viability was determined (16, 44, 47).

Cytosolic Ca²⁺ measurements. Aliquots of cells were loaded with fura 2-AM for 45 min at room temperature in the dark. Myocytes were then suspended in 1.0 mM Ca²⁺-containing MEM and washed to remove extracellular dye; myocytes were placed on a glass slide on the stage of a Nikon inverted microscope. The microscope was interfaced with Grooney optics for epi-illumination, a triocular head, phase optics, and a ×30 phase-contrast objective and mechanical stage. Excitation illumination source (300-W compact xenon arc illuminator) was equipped with a power supply. In addition, this InCyt Im2 fluorescence imaging system (Intracellular Imaging, Cincinnati, OH) included an imaging workstation and Intel Pentium Pro 200-MHz-based personal computer. The computer-controlled filter changer allowed alternation between the 340- and 380-nm excitation wavelengths. Images were captured by a monochrome charge-coupled device camera equipped with a television relay lens. InCyt Im2 Image software allowed measurement of intracellular Ca²⁺ concentrations from the ratio of the two fluorescent signals generated from the two excitation wavelengths (340 and 380 nm); background was removed by the InCyt Im2 software. The calibration procedure included measuring fluorescence ratio with buffers containing different concentrations of Ca²⁺. At each wavelength, the fluorescence emissions were collected for 1-min intervals, and the time between data collection was 1–2 min (18, 19). Because quiescent or noncontracting myocytes were used in these studies, the Ca²⁺ levels measured reflect diastolic levels.

Mitochondrial Ca²⁺ measurements. Two distinct loading conditions were used to optimize mitochondrial Ca²⁺ loading. The first approach eliminated cytosolic fluorescence by use of the Mn²⁺ quench method (7, 25, 32). After isolated cardiomyocytes were loaded with fura 2-AM as described above, the myocytes were incubated for 20 min in buffer containing 200 μM MnCl₂. Previous studies showed that Mn²⁺ that entered the cells was bound to fura 2-AM, quenching the cytosolic fluorescence (32). The Mn²⁺-containing buffer (MEM) was then replaced with Mn²⁺-free buffer, and mitochondrial Ca²⁺ levels were measured using the InCyt Im2 fluorescence imaging system described above. The ability of Mn²⁺ to quench cytosolic fluorescence in rat cardiomyocytes was consistent with previous reports by Miyata et al. (32).

Mitochondrial Ca²⁺ levels were also measured after heat treatment of cells, a technique shown previously to eliminate cytosolic fluorescence (7, 25). With this experimental protocol, cardiomyocytes were loaded with fura 2-AM as described above and heat treated by further incubation at 37°C for 1.5 h. The cells were then resuspended in fresh buffer, and the remaining cellular (mitochondrial) fluorescence was measured as described above.

In vitro challenge of primary cardiomyocytes. To determine whether changes in mitochondrial Ca²⁺ paralleled changes in cytosolic Ca²⁺, two additional experimental approaches were used. Cardiomyocytes isolated from control or naïve rats were challenged with LPS (25 μg/5 × 10⁴ cardiomyocytes for 18 h) or burn serum (BS; collected 24 h after a full-thickness burn injury over 40% of TBSA in adult rats and added to cardiomyocyte buffer 10% by volume for 18 h). Additional aliquots of cardiomyocytes were pretreated with ruthenium red (RR; 50 μM for 60 min; Sigma) before the LPS or BS challenge described above. After in vitro challenge of cardiomyocytes with LPS or BS (in the presence or absence of RR), aliquots of cardiomyocytes were loaded with fura 2-AM and cytosolic and organellae Ca²⁺ levels were measured; supernatants were collected to measure secreted cytokines (TNF-α, IL-1β, and IL-6 by rat ELISA; Endogen, Woburn, MA).

Cytokine secretion by cardiomyocytes. Myocytes were pipetted into microtiter plates at 5 × 10⁴ cells/well (12-well cell-culture cluster,
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Corning, NY) for 18 h (CO2 incubator at 37°C). Supernatants were collected to measure myocyte-secreted TNF-α, IL-1β, IL-6, and IL-10 (rat ELISA; Endogen). We previously examined the contribution of contaminating cells (nonmyocytes) in our cardiomocyte preparations using flow cytometry, cell staining (hematoxylin and eosin), and light microscopy. We confirmed that <2% of the total cell number in a myocyte preparation consisted of noncardiomyocytes. Because our preparations are 98% cardiomyocytes, we concluded that a majority of the inflammatory cytokines measured in the cardiomocyte supernatant were indeed cardiomocyte derived (18).

Isolated coronary-perfused hearts. Hearts were removed from control Sprague-Dawley rats and placed in ice-cold (4°C) Krebs-Henseleit bicarbonate-buffered solution (in mM: 118 NaCl, 4.7 KCl, 21 NaHCO3, 1.25 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, and 11 glucose). All solutions were prepared each day with demineralized, deionized water and bubbled with 95% O2-5% CO2 (pH 7.4, 550 mmHg PO2, 38 mmHg arterial PCO2). A 17-gauge cannula, placed in the ascending aorta, was connected via glass tubing to a buffer-filled reservoir to allow perfusion of the coronary circulation at a constant flow rate. Hearts were suspended in a temperature-controlled chamber maintained at 38°C, and a constant-flow pump (model 7335-30, Ismatec, Cole-Parmer Instrument, Chicago, IL) 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. Contractile function was assessed by measuring intraventricular pressure with a distilled water-filled latex balloon attached to a polyethylene tube and threaded through the apex of the left ventricular (LV) chamber. After stabilization of each heart for 15–20 min, burst perfusion was added to the perfusate (10% by volume) in the presence or absence of RR (6 mmol/l). The heart was perfused in a recirculating mode for the designated period of time. Peak systolic LV pressure and LV end-diastolic pressure were measured, and the rates of LV pressure rise (±dP/dt) and fall (−dP/dt) were obtained using an electronic differentiator (model 7P20C, Grass Instruments, Quincy, MA) and recorded. LV developed pressure was calculated by subtracting end-diastolic pressure from peak systolic pressure. Data from the Grass recorder was input to a Dell Pentium computer, and a Grass PolyVIEW Data Acquisition System was used to convert acquired data to digital form.

RR as an inhibitor of mitochondrial Ca2+. RR-containing buffer was prepared as described by Faulk and colleagues (11). Briefly, RR powder was dissolved in Krebs-Henseleit bicarbonate buffer to achieve a final concentration of 6 mmol/l and filtered. In hearts designated for pretreatment with RR, perfusion (Langendorff) was initiated with standard buffer and continued for 10–15 min to achieve stabilization of LV pressure and ±dP/dtmax; perfusion was continued for an additional 45 min in a recirculating manner using the RR-containing perfusate. In hearts designated for BS challenge, RR-containing perfusate was administered for ≥20 min before addition of BS to buffer; perfusion was continued for an additional 45–60 min, and all indexes of contraction and relaxation were then measured.

To examine the effects of inhibiting RR on cardiomocyte cytosolic/mitochondrial Ca2+ levels, RR was dissolved in MEM as described above (50 μM); isolated myocytes were incubated in RR-containing medium for ≥60 min before LPS challenge or the addition of BS to buffer. Incubation of myocytes was continued for an additional 18 h as described in Cytokine secretion by cardiomocytes.

Statistical analysis. Values are 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. If equality of variance among the groups was suggested, Bonferroni’s multiple comparison procedures were performed; if inequality of variance was suggested by Levene’s test, Tamhane’s multiple comparisons (which do not assume equal variance in each group) were performed. P < 0.05 was considered statistically significant.

RESULTS

Measurements of cardiomocyte Ca2+ after in vivo burn injury. Our initial studies were directed to determine that technical manipulation of esterified fluorescent dye loading accomplished preferential accumulation of the dye in the mitochondria. Cells were incubated with MnCl2 to quench the fluorescent signal from the cytosolic compartment, producing only mitochondria-related fluorescence. Cells stored at room temperature (for a period equivalent to cells subjected to heat treatment or the Mn2+ quench technique) retained 95% of their initial fluorescence, producing 87 ± 14 nM cytosolic Ca2+ (Fig. 1). Heat treatment of the cardiomocytes or the Mn2+ quench technique eliminated cytosolic fluorescence, allowing measurement of mitochondrial Ca2+ (20 ± 1 and 26 ± 1 nM, respectively); these values are consistent with previous reports of mitochondrial Ca2+ concentrations (32).

After confirming that the heat treatment and the Mn2+ chloride quench techniques provided consistent measures of mitochondrial Ca2+ fluorescence, we measured cytosolic and mitochondrial Ca2+ in cardiomocytes prepared from hearts harvested 24 h after sham burn injury or burn injury over 40% TBSA. At 24 h after burn injury, cytosolic Ca2+ levels increased from 90 ± 3 to 293 ± 6 nM (P < 0.05); similarly, burn injury was associated with a significant rise in mitochondrial Ca2+ from 24 ± 1 to 75 ± 2 nM (P < 0.05; Fig. 2).

Effects of LPS or BS challenge on myocyte cytosolic and mitochondrial Ca2+. Subsequent studies used cardiomocytes isolated from naïve hearts; the use of primary myocyte cultures eliminated neurohumoral and endocrine responses that occur in vivo after burn injury and allowed us to examine cell-specific responses. BS or LPS challenge of naïve myocytes increased cytosolic, as well as mitochondrial, Ca2+ (Fig. 3). BS challenge in cardiomycocytes for 18 h (10% by volume and prepared from adult rats with burn over 40% TBSA with serum

![Fig. 1. Total myocyte Ca2+ immediately after fura 2 loading (control) or after incubation for 1.5 h in buffer alone at room temperature (untreated). Heat treatment or addition of Mn2+ to myocyte buffer quenched cytosolic fluorescence, allowing measurement of mitochondrial Ca2+. Values are means ± SE for 6 hearts/group, with 6 aliquots of myocytes prepared per heart for each experimental condition.](http://ajpheart.physiology.org/Downloadedfrom/10.220.33.5)
collected 24 h after burn) increased cytosolic Ca\(^{2+}\) by 88% (from 86 ± 12 nM in myocytes incubated in medium alone for 18 h to 169 ± 6 nM, \(P < 0.05\)); the rise in cytosolic Ca\(^{2+}\) with BS challenge was paralleled by a 79% increase in mitochondrial Ca\(^{2+}\) from 21 ± 2 to 43 ± 2 nM (\(P < 0.05\)). Compared with cytosolic Ca\(^{2+}\) levels measured in cardiomyocytes incubated in medium alone for 18 h (87 ± 14 nM), LPS challenge for 18 h produced a 112% increase in cytosolic Ca\(^{2+}\) (185 ± 15 nM, \(P < 0.05\)) and an 87% increase in mitochondrial Ca\(^{2+}\) (from 23 ± 1 nM in the absence of LPS to 43 ± 2 nM after 18 h of LPS challenge, \(P < 0.05\)). Incubating cardiomyocytes in RR-containing medium (50 μM) had no effect on mitochondrial or cytosolic Ca\(^{2+}\) in the absence of LPS or BS challenge. However, pretreatment of cardiomyocytes with RR for 60 min prevented mitochondrial Ca\(^{2+}\) loading that was associated with LPS or BS challenge. In contrast, pretreatment of cardiomyocytes with RR did not alter the LPS- or BS-related increase in cytosolic Ca\(^{2+}\) (Fig. 3).

**Cytokine response.** BS or LPS challenge in isolated cardiomyocytes promoted secretion of TNF-α, IL-1β, and IL-6 (Fig. 4). Pretreating the cardiomyocytes with RR prevented the cardiomyocyte cytokine secretion that was associated with either in vitro challenge.

**Myocardial contractile performance.** Because we showed previously that BS challenge in isolated hearts impaired ventricular contraction and relaxation (unpublished data), we examined the effects of inhibiting mitochondrial Ca\(^{2+}\) loading on BS-related changes in myocardial contractile performance. Adding BS (10% by volume) to the perfusate of isolated control hearts (and recirculation of the BS-containing perfusate for 45–60 min) produced significant myocardial contractile depression (\(P < 0.05\); Fig. 5). LV developed pressure and ±dP/dt responses to incremental increases in LV volume were significantly less in hearts treated with BS than in those perfused in an identical manner for an identical time with perfusate alone. In contrast, addition of sham serum (10% by volume) to cardiac perfusate for 45–60 min had no effect on ventricular function (data not shown). The addition of RR to isolated perfused hearts in the absence of BS had no effect on LV pressure or ±dP/dt responses. However, pretreatment of isolated hearts with RR for 15–20 min before BS challenge significantly attenuated BS-related contraction and relaxation defects.

BS challenge in isolated hearts also impaired ventricular performance-coronary flow relations; LV pressure and ±dP/dt values measured as coronary flow rate was incrementally increased from 3 to 12 ml/min were consistently lower in burn plasma-treated hearts than in hearts perfused with buffer alone. Pretreating isolated hearts with RR prevented BS-related im-

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**Fig. 2.** Myocyte cytosolic Ca\(^{2+}\) levels, measured 24 h after burn over 40% total body surface area (TBSA), rose significantly above those measured in control myocytes. Burn injury promoted a rise in mitochondrial Ca\(^{2+}\) over that measured in control myocytes. Values are means ± SE; 6 burns and 6 sham burns were used for myocyte preparations. *Significantly different from respective control, \(P < 0.05\).

**Fig. 3.** Cardiomyocytes isolated from control rats \((n = 6)\) were incubated for 18 h in 1) medium alone to provide an appropriate control, 2) medium containing ruthenium red (RR, control + RR), 3) medium + burn serum (BS, 10% by volume), 4) burn serum + RR, 5) medium containing 25 μg of LPS, or 6) medium containing 25 μg of LPS + RR. BS or LPS promoted myocyte Ca\(^{2+}\) loading. RR prevented BS- or LPS-related mitochondrial Ca\(^{2+}\) loading (\([\text{Ca}^{2+}]_{\text{mt}}\) (top) but not cytosolic Ca\(^{2+}\) accumulation (\([\text{Ca}^{2+}]_{\text{c}}\) (bottom)). Values are means ± SE. Mitochondrial Ca\(^{2+}\) levels were determined using the Mn\(^{2+}\) quenching technique. *Significantly different from medium alone, \(P < 0.05\). Significantly different from BS or LPS alone, \(P < 0.05\).
Fig. 4. BS or LPS challenge in naïve cardiomyocytes promoted significant TNF-α, IL-1β, and IL-6 secretion. Pretreatment with RR attenuated BS- or LPS-mediated inflammatory cytokine response. Hearts were harvested from 6 rats to prepare myocytes. Values are means ± SE. *Significantly different from medium alone, \( P < 0.05 \). \( \) Significantly different from BS or LPS alone, \( P < 0.05 \).

Fig. 5. Ventricular function [left ventricular (LV) pressure (LVP) and maximum rate of rise or fall of LVP (±dP/dt max)] was studied as preload, or LV volume was incrementally increased. LVP and ±dP/dt max were significantly lower at all levels of preload in hearts perfused with medium containing BS. Pretreating hearts with RR ablated contractile dysfunction associated with BS challenge in control hearts. Values are means ± SE of 8 hearts in each group. *Significantly different from control, \( P < 0.05 \).
DISCUSSION

In this study, we showed that mitochondrial Ca\(^{2+}\) can be measured in a reproducible manner using the Mn\(^{2+}\) quench technique or after suitable heat treatment of cardiomyocytes. We further showed that in vivo burn injury or in vitro challenges such as LPS or BS promoted a rise in cardiomyocyte cytosolic Ca\(^{2+}\) that was paralleled by mitochondrial Ca\(^{2+}\) accumulation, confirming that the mitochondrial membrane does not provide a barrier to Ca\(^{2+}\) (11). Our finding that RR inhibited cardiomyocyte mitochondrial Ca\(^{2+}\) accumulation is consistent with a previous report by Wan-Yi and colleagues (42) that intravenous administration of RR in rats with burn over 30% of TBSA attenuated burn-related myocyte injury and mitochondrial damage and preserved myocardial high-energy phosphate stores; they also report that RR prevented postburn myocardial Ca\(^{2+}\) accumulation. However, our present study extended that previous work, confirming that RR-related alteration in burn-mediated mitochondrial Ca\(^{2+}\) accumulation prevented cardiomyocyte secretion of proinflammatory cytokines TNF-\(\alpha\), IL-1\(\beta\), and IL-6. These data further suggest that Ca\(^{2+}\) accumulation by this organelle provides one upstream signal for myocardial inflammatory responses to injury and disease. Finally, RR provided significant cardioprotection against BS-mediated myocardial contraction and relaxation defects, suggesting that mitochondrial Ca\(^{2+}\) accumulation modulates myocardial proinflammatory cytokine responses, which, in turn, contribute to the myocardial contractile dysfunction. Although studies by Wan-Yi and colleagues confirmed that RR reduced cardiomyocyte Ca\(^{2+}\) accumulation and preserved mitochondrial respiratory function after burn injury, the in vivo administration of RR in their study likely altered mitochondrial function and Ca\(^{2+}\) handling in several organs and in several cell types. Our use of RR in isolated cardiomyocytes and isolated perfused hearts confirmed the cardio-specific effects of this polysaccharide dye in an environment free of neurohormonal responses to injury that occur in the intact subject.

Our interest in mitochondrial Ca\(^{2+}\) accumulation arose from previous reports that this intracellular organelle plays a pivotal role in the overall response of the organ to injury or stress. Mitochondria provide a Ca\(^{2+}\)-buffering compartment, allowing subcellular sequestration of Ca\(^{2+}\) under pathological conditions (37). Mitochondrial Ca\(^{2+}\) levels have been thought to regulate ATP synthesis, and a rise in mitochondrial Ca\(^{2+}\) levels has been shown to accelerate ATP production (30). A significant rise in cytosolic Ca\(^{2+}\) levels triggers Ca\(^{2+}\) sequestration by the mitochondria, providing a protective mechanism against a toxic rise in cytosolic Ca\(^{2+}\) levels. However, a persistent increase in cytosolic Ca\(^{2+}\) and persistent mitochondrial Ca\(^{2+}\) accumulation lead to excessive Ca\(^{2+}\) cycling by the mitochondria, alterations in membrane potential, a gradual reduction in mitochondrial synthesis of ATP and activation/inactivation of Ca\(^{2+}\)-sensitive metabolic enzymes (48). Thus altered mitochondrial Ca\(^{2+}\) homeostasis can exert detrimental effects on cardiac function.

Fig. 6. BS challenge (10% by volume) in hearts isolated from control rats altered coronary flow-ventricular performance relations. Pretreating hearts with RR ablated the impaired LVP and \(\pm\frac{dP}{dt}\) responses associated with BS challenge. Values are means \(\pm\) SE of 8 hearts in each group. *Significantly different from control, \(P < 0.05\).
cellular function, impairing oxidative phosphorylation and decreasing energy available to support cellular function.

Altered mitochondrial function has been described in a number of injury and disease states. For example, cardiac injury produced by ischemia-reperfusion has been linked to mitochondrial dysfunction, as indicated by altered cellular respiratory rate and morphological changes indicated by mitochondrial swelling (31). The role of mitochondrial Ca$^{2+}$ homeostasis in maintaining myocardial contractile function and ventricular compliance has been suggested by the finding that Ca$^{2+}$-antagonists restore injury-related changes in mitochondrial Ca$^{2+}$ handling, and recovery of mitochondrial Ca$^{2+}$ homeostasis improves LV contractile function (20, 36, 46).

Although we have shown that Ca$^{2+}$-antagonists given after major burn injury prevent burn-related rise in cytosolic Ca$^{2+}$ and improve myocardial contractile performance (44), the effects of the Ca$^{2+}$-antagonists on mitochondrial Ca$^{2+}$ were not examined in our previous study and remain to be resolved (44).

Studies from our laboratory confirmed that burn trauma alone, sepsis alone, or burn injury complicated by sepsis promotes cardiomyocyte cytosolic Ca$^{2+}$ accumulation (17–19, 45) and cardiomyocyte apoptosis (22). Because it is clearly recognized that the mitochondria initiate programmed cell death, we focused our present studies on mitochondrial Ca$^{2+}$ handling in a model of burn injury using in vitro models designed to simulate cellular derangements that are characteristic of burn trauma or sepsis. Our previous studies showed that LPS or BS challenge in primary cardiomyocyte cultures promotes myocyte Na$^{+}$ and Ca$^{2+}$ loading, increases proinflammatory cytokine secretion by this cell population, promotes myocyte apoptosis, andalters cardiomyocyte contractile performance (4, 16, 18, 24).

LPS- or BS-related mitochondrial Ca$^{2+}$ accumulation was abrogated by RR pretreatment, supporting a previous suggestion that this hexavalent dye blocks Ca$^{2+}$ influx via the mitochondrial uniporter (1, 11). In our studies, addition of RR to myocyte incubation medium or cardiac perfusate decreased cardiomyocyte secretion of proinflammatory cytokines and ameliorated burn serum-related LV contraction and relaxation defects. These data are consistent with the report by Benzi and Lerch (1), who described that RR inhibited myocardial Ca$^{2+}$ uptake, which, in turn, enhanced postischemia-related contractile performance. Collectively, these data suggest that increases in cardiomyocyte cytosolic Ca$^{2+}$ modulate mitochondrial Ca$^{2+}$ cycling, and mitochondrial Ca$^{2+}$ accumulation is one mechanism of burn- or LPS-related myocardial inflammation and dysfunction. The mechanisms by which a rise in mitochondrial Ca$^{2+}$ modulates secretion of proinflammatory cytokines may include activation of mitochondria-related caspases. The inflammatory caspases-1 and -11 have been shown to be upregulated in burn and burn sepsis and, in turn, promote a robust TNF-α and IL-1β response (unpublished data). Although the precise mechanisms by which sepsis or a nonseptic injury such as burn trauma alters myocardial cellular function remain to be elucidated, data presented here strongly support the hypothesis that mitochondrial Ca$^{2+}$ accumulation is one mechanism that underlies myocardial inflammatory cytokine responses and, in turn, contributes to myocardial injury and contractile dysfunction.

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