

Hypertonic saline-dextran suppresses burn-related cytokine secretion by cardiomyocytes

JURETA W. HORTON, DAVID L. MAASS, JEAN WHITE, AND BILLY SANDERS

Department of Surgery, The University of Texas Southwestern
Medical Center, Dallas, Texas 75390-9160

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Horton, Jureta W., David L. Maass, Jean White, and Billy Sanders. Hypertonic saline-dextran suppresses burn-related cytokine secretion by cardiomyocytes. *Am J Physiol Heart Circ Physiol* 280: H1591–H1601, 2001.—Whereas hypertonic saline-dextran (HSD, 7.5% NaCl in 6% D70) improves cardiac contractile function after burn trauma, the mechanisms of HSD-related cardioprotection remain unclear. We recently showed that cardiomyocytes secrete tumor necrosis factor- α (TNF- α), a response that was enhanced by burn trauma. This study addressed the question: does HSD modulate cardiac contraction/relaxation by altering cardiomyocyte TNF- α secretion? Wistar-Furth rats (325 g) were given a burn injury over 40% of the total body surface area and were then randomized to receive a bolus of either isotonic saline or HSD (4 ml/kg, $n = 14$ rats/group). Sham burn rats were given either isotonic saline or HSD ($n = 14$ rats/group) to provide appropriate controls for the two burn groups. Hearts were isolated 24 h postburn for either Langendorff perfusion ($n = 8$ hearts/group) or to prepare cardiomyocytes ($n = 6$ hearts/group). Myocytes were stimulated with lipopolysaccharide (LPS) (0, 10, 25, or 50 μ g for 18 h) to measure cytokine secretion. Burn trauma increased myocyte TNF- α and interleukin-1 β and -6 secretion, exacerbated cytokine response to LPS stimulus, and impaired cardiac contraction. HSD treatment of burns decreased cardiomyocyte cytokine secretion, decreased responsiveness to LPS challenge with regard to cytokine secretion, and improved ventricular function. These data suggest that HSD mediates cardioprotection after burn trauma, in part, by downregulating cardiomyocyte secretion of inflammatory cytokines.

cardiac contractile function; rat model of burn trauma; tumor necrosis factor- α ; Langendorff perfusion

CONSIDERABLE CONTROVERSY has arisen regarding prehospital administration of isotonic fluids to resuscitate the severely injured patient. Problems cited have included difficulties in administering sufficient volumes of crystalloid during transport to stabilize arterial blood pressure and cardiac output, whereas other investigators have suggested that rapid and aggressive crystalloid resuscitation may alter clotting mechanisms and exacerbate blood loss in patients with uncontrolled hemorrhage (3, 10, 28, 30, 32, 36, 42, 46). Whereas large-volume crystalloid resuscitation has been used

successfully during the last 30 years to resuscitate severely injured patients with few reports of complications (29, 42), the search for alternative regimens of fluid resuscitation continues. Holcroft and colleagues (14) described the successful resuscitation of severely injured patients with hypertonic sodium chloride-6% Dextran 70 (HSD). The use of HSD has been shown to be a particularly effective resuscitation strategy in experimental models of bile-induced pancreatitis, hemorrhagic shock, burn trauma, and septic shock (5, 9, 12, 14, 17, 20, 26, 27, 41, 47, 48). Small-volume HSD (4 ml/kg) has been shown to support circulation of the injured patient during transport, to improve blood pressure and cardiac output in clinical and experimental shock, to improve cardiac contractile performance in burn trauma, and to improve survival in lethal models of hemorrhagic shock. The beneficial effects of HSD have been attributed to reduced total fluid requirements, improved cardiac contractility, reduced diuresis by vasopressin-induced water reabsorption, and improved immunologic responses to stress-related injury (5, 9, 12, 14, 20, 26, 27, 41, 47, 48).

Recent attention has focused on the role of HSD as an effective modulator of cell immune function after trauma. Several studies have shown that, whereas crystalloid resuscitation exacerbated shock-mediated cellular adhesion and numerous aspects of the shock-related inflammatory cascade, there was a remarkable absence of exaggerated inflammatory responses with HSD resuscitation (43). Recent focus on the immunologic consequences of HSD resuscitation of trauma led us to compare the effects of standard lactated Ringer solution resuscitation from burn trauma with reduced-volume lactated Ringer solution resuscitation plus HSD on postburn cardiac contractile performance and the cardiac synthesis of inflammatory cytokines. Tumor necrosis factor- α (TNF- α) is clearly recognized as an inflammatory mediator synthesized by cells within the reticuloendothelial system. However, recent studies by our laboratory (11, 45) and by others (24, 35, 40) have shown that stress-related injury, including burn trauma, hemorrhage, endotoxin challenge, and sepsis promote TNF- α synthesis within several peripheral

Address for reprint requests and other correspondence: J. W. Horton, Dept. of Surgery, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9160 (E-mail: JURETA.HORTON@utsouthwestern.edu).

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tissues, particularly the heart. The contribution of local TNF- α synthesis to organ injury has remained speculative, but strategies that inhibited TNF- α release or neutralized TNF- α have been shown to provide significant cardioprotection and to improve outcome after several types of ischemia and trauma (11, 24). Therefore, this present study was designed to examine the hypothesis that HSD given as an early pharmacological intervention after burn trauma provides significant cardiac protection by altering cardiac cytokine secretion.

METHODS AND MATERIALS

Experimental model. Adult Wistar-Furth rats (320–350 g) were used in the present study. Animals were obtained from Harlan Laboratories (Houston, TX) and were conditioned in-house for 5–6 days with commercial rat chow and tap water available at will. All studies performed were conducted under a protocol 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 Physiological Society and the National Institutes of Health.

Catheter placement and burn procedure. Rats were anesthetized lightly with methoxyflurane 18–20 h before the burn experiment. Body hair on the side, back, and neck was closely clipped, and the neck region was treated with a surgical scrub. The left carotid artery was exposed, a polyethylene catheter (PE-50) was inserted into the artery, and the tip was advanced retrogradely to the level of the aortic arch. In addition, the right external jugular vein was exposed, and a PE-50 catheter was inserted for the administration of fluids and drugs. The catheters were filled with heparinized saline and exteriorized at the nape of the neck with silk sutures via a subcutaneous tunnel. After catheter placement, rats were housed within the laboratory in individual cages. Eighteen hours after catheter placement, animals were deeply anesthetized with methoxyflurane and secured in a constructed template device as previously described (1), and the surface of the skin area exposed through an aperture in the template was immersed in 100°C water for 12 s on the back and upper sides. Use of the template limited the burned area, avoided injury to the abdominal organs, and produced full-thickness dermal burns over 40% of the total body surface area. Exposure to this water temperature in adult rats has been shown previously by our laboratory to destroy all underlying nerves, to produce a well-circumscribed burn area, and to produce a transient ($<0.5^{\circ}\text{C}$) rise in internal body temperature for 30 s after exposure to the 100°C water and an absence of injury to underlying organs. Sham burn rats were subjected to identical preparation, except that they were immersed in room temperature water to serve as controls. After immersion, all rats were immediately dried and placed in individual cages to recover from the anesthesia. Burned rats did not display discomfort or pain, moved freely about the cage, and consumed food and water within 20 min after the burn procedure. Immediately after the animal was returned to a cage, the external jugular catheter was connected to a swivel device (model 923, Holter pump, Critikon; Tampa, FL) for fluid administration (lactated Ringer solution, 4 ml/kg per percent burn by the Parkland formula) during the postburn period (total volume of lactated Ringer solution given over the first 24-h postburn was 50–56 ml/rat). In the sham burn group, the external jugular vein was cannulated and lactated Ringer solution ($0.2\text{ ml}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) was given to maintain

catheter patency. Body temperature was measured with a rectal temperature probe (model 44TA, YSL-Tele Thermometer), and respiratory rate was monitored by counting respiratory movement. Systemic blood pressure was measured using a Gould-Statham pressure transducer (model P23 ID, Gould Instruments; Oxnard, CA) connected to a Grass medical recorder (model 7D, Polygraph, Grass Instruments; Quincy, MA). A tachycardiograph (model 7P4F, Grass Instruments) was used to monitor heart rate.

Experimental groups. Rats were randomly divided into sham and burn groups; in addition, sham burn rats were divided into two additional subgroups: one group was given a bolus of HSD (4 ml/kg body wt) to examine the cardiac effects of a hyperosmotic solution in the absence of burn trauma; the remaining sham burn animals received a bolus of isotonic saline (4 ml/kg body wt). Lactated Ringer solution was given in both sham groups to maintain catheter patency.

In rats given a full-thickness burn injury, fluid resuscitation was initiated with lactated Ringer solution 10 min after completing the burn trauma. The total volume of lactated Ringer solution was calculated as 4 ml/kg per percent burn, with one-half the calculated volume given over the first 8 h postburn and the remaining volume of lactated Ringer solution given over the next 16 h after burn (Parkland formula). Forty-five minutes after lactated Ringer solution resuscitation was initiated, burn rats were randomly divided to receive a bolus of either isotonic saline or HSD given as 4 ml/kg body wt. After the isotonic saline or HSD infusion was completed, lactated Ringer solution resuscitation was resumed. The initial fluid resuscitation was conducted to simulate lactated Ringer solution administration during patient transport in the Dallas/Fort Worth metropolitan area. The timing for administering the isotonic saline or HSD infusion simulated in-hospital administration of HSD as previously described in our clinical studies (38).

An aliquot of arterial blood was collected (via the carotid artery catheter) 24 h postburn for measurement of systemic cytokine levels (ELISA). Rats were then anticoagulated with heparin sodium (1,000 units, Elkin-Sinn; Cherry Hill, NJ) and decapitated, and hearts were harvested for in vitro studies. In this study, hearts harvested from each of four experimental groups described above were randomly selected to assess either ventricular function (Langendorff perfusion, $n = 8$ hearts/group) or were perfused in a Langendorff mode with collagenase-containing buffer to prepare cardiomyocytes ($n = 6$ hearts/group). These times were selected to examine the effects of HSD on burn-mediated cardiac contractile deficits and cardiomyocyte secretion of cytokines based on our previous studies showing that contractile dysfunction is maximal by 24 h postburn and persists over 36–40 h after burn injury.

Isolated coronary perfused hearts. The heart was rapidly removed and placed in ice-cold (4°C) Krebs-Henseleit bicarbonate-buffered solution of (in mM) 118 NaCl, 4.7 KCl, 21 NaHCO_3 , 2.5 CaCl_2 , 1.2 MgSO_4 , 1.2 KH_2PO_4 , and 11 glucose. All solutions were prepared each day with demineralized, deionized water and bubbled with 95% O_2 -5% CO_2 (pH, 7.4; PO_2 , 550 mmHg; PCO_2 , 38 mmHg). A 17-gauge cannula was placed in the ascending aorta and connected via glass tubing to a buffer-filled reservoir for perfusion of the coronary circulation at a constant flow rate. Hearts were suspended in a temperature-controlled chamber maintained at $38 \pm 0.5^{\circ}\text{C}$, and a constant flow pump (model 911, Holter, 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 attached to a polyethylene tube and threaded into the left ventricular chamber. Left ventricular developed pressure (LVDP) was measured with a Statham pressure transducer (model P23 ID, Gould Instruments) attached to the balloon cannula, and the rates of left ventricular pressure (LVP) rise (+dP/dt) and fall (-dP/dt) were obtained using an electronic differentiator (model 7P20C, Grass Recording Instruments) and recorded (model 7DWL8P, Grass Recording Instruments). Data from the Grass recorder were input to a Dell Pentium computer, and a Grass PolyVIEW Data Acquisition System was used to convert acquired data into digital form.

Cardiomyocyte isolation. All pipettes, plates, test tubes, and other equipment used for preparation and culture of cardiomyocytes were sterile. Culture media, cytokine solutions, and other solutions used for preparation and culture of the myocytes were endotoxin-free (determined by a chromogenic limulus amoebocyte lysate assay, data not shown). Hearts were removed through a medial sternotomy using sterile techniques; the isolated heart was immediately placed in ice-cold calcium-free Tyrode's solution of (in mM) 136 NaCl, 5.36 KCl, 0.57 MgCl₂, 0.33 NaH₂PO₄, 10 HEPES, and 10 glucose. The aorta was cannulated within 90 s, and the excised heart was perfused with Tyrode's solution using a Langendorff perfusion apparatus. The Tyrode's solution was equilibrated with 95% O₂-5% CO₂ during perfusion of the heart. Perfusion was maintained for 5 min, and ventricular drainage was ensured by placing a 22-gauge needle in each ventricle. Perfusion was then continued for an additional 10 min using a collagenase solution containing 80 ml of calcium-free Tyrode's solution, 40 mg collagenase A (0.05%, Boehringer Mannheim, Indianapolis, IN), and 0.4 mg of protease (Polysaccharide XIV, Sigma, St. Louis, MO) with continuous oxygenation. After this enzymatic digestion, the heart was removed from the cannula and the ventricular tissue was separated from the base of the heart in a petri dish containing Tyrode's solution with 100 μM calcium where gentle mincing increased cell dispersion over 5 min. The myocyte suspension was then filtered, and the cells were allowed to settle. The supernatant was removed, and the cells were resuspended in 50 ml of Tyrode's solution. The rinsing and settling step was repeated three times with 10 min between each step and with gentle swirling between each step to allow myocyte separation. The calcium concentration of the rinsing solution was gradually increased during these steps, with calcium concentrations of 100 μM, 200 μM, and finally 1.8 mM. Cell viability was measured (trypan blue dye exclusion); myocytes with a rodlike shape, clear-formed edges, and well-defined striations were prepared with a final cell number of 5×10^4 cells/ml (18, 25).

To further explore the effects of HSD resuscitation from burn injury on cardiomyocyte cytokine responsiveness, myocytes were isolated from all four experimental groups (sham plus isotonic saline, sham plus HSD, burns given lactated Ringer solution and isotonic saline, and burns given lactated Ringer solution and HSD) and suspended in Eagle's minimum essential medium containing 10% fetal bovine serum. Myocytes were placed using pipettes into microtiter plates (cell number of 5×10^4 myocytes per microtiter well) (12-well cell culture cluster, Corning; Corning, NY) and subsequently stimulated with either 0, 10, 25, or 50 μg/well of lipopolysaccharide (LPS) (lot 65H 4053, *Escherichia coli*, Difco Laboratories; Detroit, MI) for 18 h (CO₂ incubator at 37°C). Supernatants were collected to measure myocyte-secreted TNF-α, interleukin (IL)-1β, and IL-6 (TNF-α and IL-1β rat ELISA,

Endogen, Woburn, MA; IL-6 rat ELISA, BioSource, Camarillo, CA).

Intracellular calcium measurement. Because previous studies have suggested that HSD may alter cardiac contractile function by modulating intracellular calcium homeostasis, intracellular Ca²⁺ concentration ([Ca²⁺]_i) was measured at room temperature with constant low stirring in a Hitachi F-2000 fluorescence spectrophotometer (20). Fura 2-acetoxymethyl ester (AM)-loaded myocytes were suspended in calcium-free saline and placed in a 1-ml quartz cuvette; a magnetic stirring bar in the bottom of the cuvette maintained the cells in suspension. The spectrophotometer was equipped with a 150-W xenon lamp, an interference filter with a 20-nm bandpass was used to establish the excitation wavelengths (340/380 nm), and the emission light was collected through a 510-nm filter with a 10-nm bandpass at a response time of 0.5 s. The calibration procedure included measuring fluorescence ratios with different calcium concentration buffers. [Ca²⁺]_i was measured as a ratio (R) of two fluorescent signals (F₁ and F₂) generated from the two excitation wavelengths (340 nm and 380 nm). Autofluorescence of myocytes that had not been loaded with fura 2-AM (indicated in the formula as fluorescence background, F_{backg}) was subtracted from fluorescence measured in myocytes loaded with fura 2-AM (indicated in the formula as F_{1,cell} and F_{2,cell}) as described by the formula: $R = (F_{1,cell} - F_{1,backg}) / (F_{2,cell} - F_{2,backg})$ and applied to the following equation of Grynkiewicz et al. (13)

$$[Ca^{2+}]_i = K_d \cdot \beta [(R - R_{min}) / (R_{max} - R)]$$

where K_d represents the dissociation constant of the Ca: fura 2 complex (224 nM at 37°C) and β is the ratio of fluorescence signals measured in calcium-free and calcium-saturated cells measured at 380 nm (13, 18). R is the ratio of fluorescence measured in cardiomyocytes at 340 and 380 nm, and R_{min} and R_{max} are the minimal and maximal ratios measured in calcium-free and calcium-saturated cells, respectively.

Contribution of emigrated leukocytes. To determine the presence of contaminating leukocytes that may have contributed to cytokine secretion measured in our cardiomyocyte cultures, cells were sorted on a Becton-Dickinson FACScan cytometer. Optimal settings for forward and side scatter, which correlate with cell size and cell complexity, respectively, were adjusted to distinguish different cell populations (6). Cardiac myocytes are ~40 times the size of leukocytes (5–10 μm), allowing separation of these cell populations based on size. In addition to FACScan, myocyte preparations were examined by hematoxylin and eosin staining and light microscopy to determine presence of contaminating leukocytes.

To determine whether leukocyte contamination was a major factor in cardiomyocyte TNF-α secretion after burn trauma, preliminary studies described above estimated that our cardiomyocyte cultures could contain a maximum of 1,000 leukocytes per microtiter well. Therefore, polymorphonuclear neutrophils (PMNs) were isolated 24 h after either burn trauma or sham burn from citrate anticoagulated blood using techniques previously described by our lab (15). The neutrophils were suspended in phosphate buffer solution, counted with a hemacytometer, and plated in 48-well culture dishes to achieve a concentration of leukocytes per well that would equal the greatest number of leukocytes that could contaminate cardiomyocyte preparations as determined by fluorescence-activated cell sorter (FACS) analysis and light microscopy. Light microscopy showed that <2% of the total cell number in the myocyte preparations was leukocytes, resulting in a maximum of 1,000 leukocytes per microtiter well. The plated leukocytes were then stimulated with LPS

(0, 10, 25, and 50 $\mu\text{g}/\text{well}$) as described for cardiomyocytes for 18 h, supernatants were harvested, and TNF- α produced by noncardiomyocyte cells was measured by ELISA.

Statistical analysis. All values are expressed as means \pm SE. ANOVA was used to assess an overall difference among the groups for each of the variables. Levene's test for homogeneity of variance was used to suggest the multiple comparison procedure to be used. If equality of variance among the four groups was suggested, multiple comparison procedures were performed (Bonferroni); if inequality of variance was suggested by Levene's test, Tamhane multiple comparisons (which do not assume equal variance in each group) were performed. A P value < 0.05 was considered statistically significant (analysis was performed using SPSS for Windows, Version 7.5.1).

RESULTS

All animals survived the respective experimental protocols. HSD reduced heart rate in sham burns ($P < 0.05$) (Table 1), whereas there were no metabolic or hemodynamic effects of HSD in this experimental group measured 24 h postburn (Tables 1 and 2). In addition, left ventricular function curves calculated for HSD-treated shams were nearly identical to those measured in isotonic-treated shams (Fig. 1). Burn trauma was associated with a lower mean arterial blood pressure 24 h postinjury, regardless of HSD administration. In addition, hematocrit was lower in all burn rats at this time, likely due to the hemodilutional effects of volume resuscitation. The lower Pco_2 and HCO_3^- levels in all burns 24 h postinjury were paralleled by increased respiratory rates but unchanged arterial pH, suggesting respiratory compensation for metabolic acidosis (Table 1). Serum TNF- α and IL-1 β levels were elevated 24 h postburn in rats given standard lactated Ringer solution resuscitation (Table 1), but HSD administration after burn injury attenu-

Table 2. *Cardiodynamic effects of saline hypertonic dextran in sham and burned rats*

| | Sham + Isotonic Saline | Sham + HSD | Burn + Isotonic Saline | Burn + HSD |
|-------------------------------|------------------------------|-----------------|------------------------------|-----------------|
| LVP, mmHg | 89 \pm 4 | 93 \pm 3 | 74 \pm 4* | 83 \pm 6 |
| +dP/dt, mmHg/s | 1,980 \pm 159 | 2,050 \pm 111 | 1,396 \pm 148* | 1,963 \pm 100 |
| -dP/dt, mmHg/s | 1,630 \pm 145 | 1,710 \pm 119 | 1,230 \pm 159* | 1,630 \pm 111 |
| dP40, mmHg/s | 1,800 \pm 167 | 1,761 \pm 112 | 1,249 \pm 128* | 1,783 \pm 99 |
| TPP, ms | 91.4 \pm 4.6 | 84.7 \pm 2.5 | 87.2 \pm 1.4 | 85.8 \pm 2.7 |
| Time to 90% relaxation, ms | 85.0 \pm 6.3 | 83.3 \pm 5.0 | 76.0 \pm 2.5* | 82.8 \pm 2.6 |
| Time to max +dP/dt, ms | 57.0 \pm 4.4 | 55.0 \pm 1.6 | 49.8 \pm 0.9* | 52.5 \pm 2.1 |
| Time to max -dP/dt, ms | 51.6 \pm 2.1 | 51.1 \pm 1.4 | 50.2 \pm 1.3 | 50.9 \pm 1.0 |
| CPP, mmHg | 41.8 \pm 5.2 | 49.6 \pm 3.7 | 48.8 \pm 4.4 | 41.2 \pm 3.1 |
| CVR, mmHg/s | 8.36 \pm 1.0 | 9.91 \pm 0.7 | 9.62 \pm 0.91 | 8.27 \pm 0.60 |
| HR, beats/min | 248 \pm 6 | 247 \pm 4 | 250 \pm 8 | 256 \pm 5 |

All values are means \pm SE. LVP, left ventricular pressure; TPP, time to peak pressure; CPP, coronary perfusion pressure; CVR, coronary vascular resistance; dP/dt, rate of LVP change; dP40, rate of LVP rise at developed pressure of 40 mmHg. *Significant difference among groups at $P < 0.05$.

ated the levels of systemic inflammatory cytokines TNF- α and IL-1 β .

Cardiac contraction and relaxation deficits were evident in burns given a bolus of isotonic saline followed by standard lactated Ringer solution resuscitation as indicated by the lower LVP and maximum +dP/dt and minimum -dP/dt (Table 2). In contrast, LVP and maximum +dP/dt were higher in HSD-treated burns compared with values measured in lactated Ringer solution-resuscitated burn rats. Cardiac contractile performance was further examined by plotting LVP and maximum \pm dP/dt versus incremental increases in preload or left ventricular volume. Consistent with our previous studies in burned guinea pigs (11), cardiac contractile depression was evident in burn rats given standard lactated Ringer solution resuscitation as indicated by the downward and rightward shift of left ventricular function curves; these contractile deficits occurred despite aggressive lactated Ringer solution resuscitation over 24 h postburn. In contrast, an initial HSD infusion in the early postburn period significantly improved cardiac contraction and relaxation despite an identical total volume of fluid administered during the 24-h postburn. Similarly, measures of contractile performance (LVP and maximum \pm dP/dt) plotted versus incremental increases in either coronary flow rate (Fig. 2) or perfusate calcium (Fig. 3) were always higher in HSD-treated burns compared with values measured in burns given standard care lactated Ringer solution resuscitation. The HSD-mediated improvement in ventricular responses to either coronary flow rate or perfusate calcium, confirm our previous findings with HSD administration in a guinea pig model of burn trauma and suggest that early postburn administration of HSD provides cardiac contraction and relaxation benefits that persist for at least 24 h after injury and fluid resuscitation.

Table 1. *Hemodynamic and metabolic effects of hypertonic saline dextran in burn trauma*

| | Sham + Isotonic Saline | Sham + HSD | Burn + Isotonic Saline | Burn + HSD |
|----------------------------|------------------------------|-----------------|------------------------------|-----------------|
| pH | 7.45 \pm 0.2 | 7.49 \pm 0.1 | 7.48 \pm 0.03 | 7.45 \pm 0.9 |
| PCO_2 , mmHg | 29 \pm 1 | 29 \pm 1 | 26 \pm 4* | 26 \pm 1* |
| PO_2 , mmHg | 112 \pm 4 | 117 \pm 1 | 133 \pm 11 | 124 \pm 9 |
| Hct, % | 37.4 \pm 2.3 | 33.2 \pm 1.0 | 27.3 \pm 5.1* | 28.3 \pm 1.6* |
| HCO_3^- , mmol/l | 20.3 \pm 0.5 | 23.7 \pm 0.4 | 18.7 \pm 1.6* | 19.9 \pm 0.3* |
| O_2 sat, % | 99.7 \pm 0.3 | 99.6 \pm 0.5 | 99.9 \pm 0.1 | 99.6 \pm 0.4 |
| CtO_2 , vol% | 14.7 \pm 2.3 | 14.5 \pm 0.5 | 12.1 \pm 2.3 | 12.6 \pm 0.7 |
| Ca^{2+} , mM | 1.1 \pm 0.2 | 0.93 \pm 0.02 | 0.78 \pm 0.07 | 0.78 \pm 0.2 |
| MAP, mmHg | 125 \pm 3 | 121 \pm 1 | 96.8 \pm 6* | 108 \pm 4* |
| HR, beats/ min | 480 \pm 22 | 427 \pm 10* | 433 \pm 21* | 493 \pm 7.5 |
| BT, $^{\circ}\text{C}$ | 38.9 \pm 0.3 | 38.9 \pm 1.2 | 38.6 \pm 0.2 | 38.7 \pm 0.3 |
| TNF- α , pg/ ml† | 17.1 \pm 5.8 | 1.2 \pm 4.3* | 50.8 \pm 17.2* | 8.6 \pm 1.2 |
| IL-1 β , pg/ml† | 10 \pm 2 | 43 \pm 2 | 1,697 \pm 139* | 51 \pm 10* |

All values are means \pm SE. HSD, hypertonic saline-dextran; Hct, hematocrit, O_2 sat, oxygen saturation; CtO_2 , oxygen content; MAP, mean arterial pressure; HR, heart rate; BT, body temperature. *Significant difference among groups at $P < 0.05$; †cytokine levels measured in plasma.

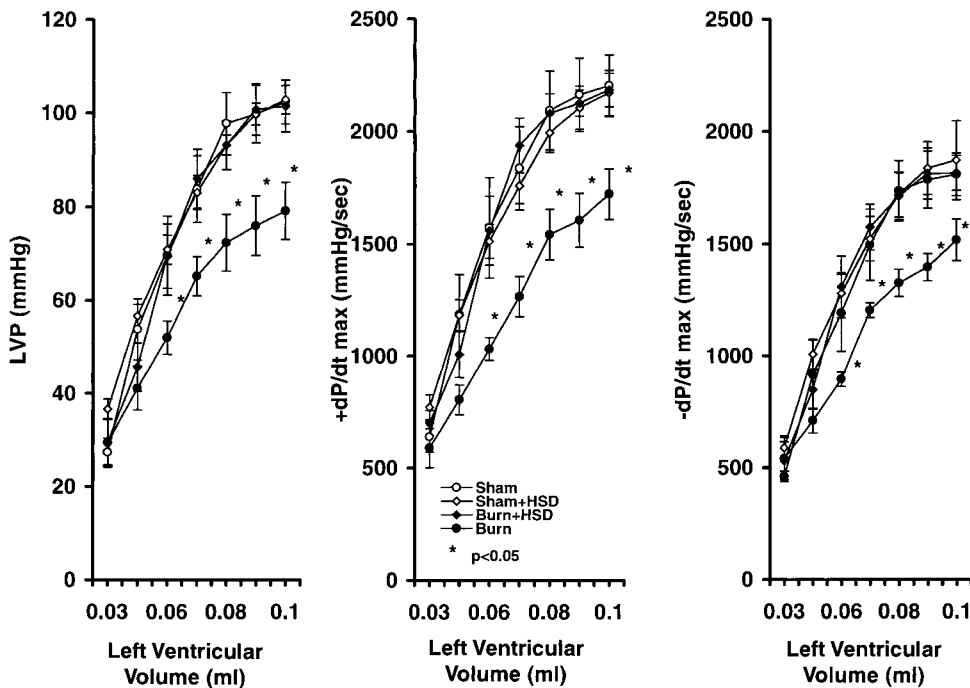


Fig. 1. Left ventricular developed pressure (LVP, peak systolic minus end-diastolic pressure) and maximal rate of LVP rise ($+dP/dt_{max}$) and fall ($-dP/dt_{min}$) responses to increases in preload (ventricular volume) ($n = 8$ animals/group). Groups include shams given either isotonic saline or hypertonic saline-dextran (HSD) (4 ml/kg). Similarly, burns were given a bolus of either HSD or isotonic saline and resuscitation was continued with lactated Ringer solution (4 ml/kg per percent burn). All values are means \pm SE. Statistical analysis included ANOVA and a multiple comparison procedure (Bonferroni). *Significant difference among groups at $P < 0.05$.

Figure 4 summarizes $TNF-\alpha$ (left), $IL-1\beta$ (middle), and $IL-6$ (right) concentrations measured in the supernatants of myocytes harvested from either shams, shams given HSD, burns given standard lactated Ringer solution resuscitation, and burns given HSD and lactated Ringer solution. Cardiomyocyte secretion of $TNF-\alpha$, $IL-1\beta$, and $IL-6$ was significantly higher ($P < 0.05$) in isotonic saline and lactated Ringer solution-resuscitated burns compared with values measured in sham burns. However, HSD administration after burn

trauma ablated this burn-enhanced $TNF-\alpha$ and interleukin secretion by cardiomyocytes.

In addition, cardiomyocytes (50,000 cells) from all experimental groups were challenged in vitro with either 0, 10, 25, or 50 μg /well of LPS for 18 h. As shown in Fig. 5, myocytes from sham burns responded to LPS challenge with a dose-related increase in $TNF-\alpha$ secretion ($P < 0.05$). However, myocytes prepared from isotonic saline-lactated Ringer-treated burns secreted significantly more $TNF-\alpha$ at each LPS dose compared

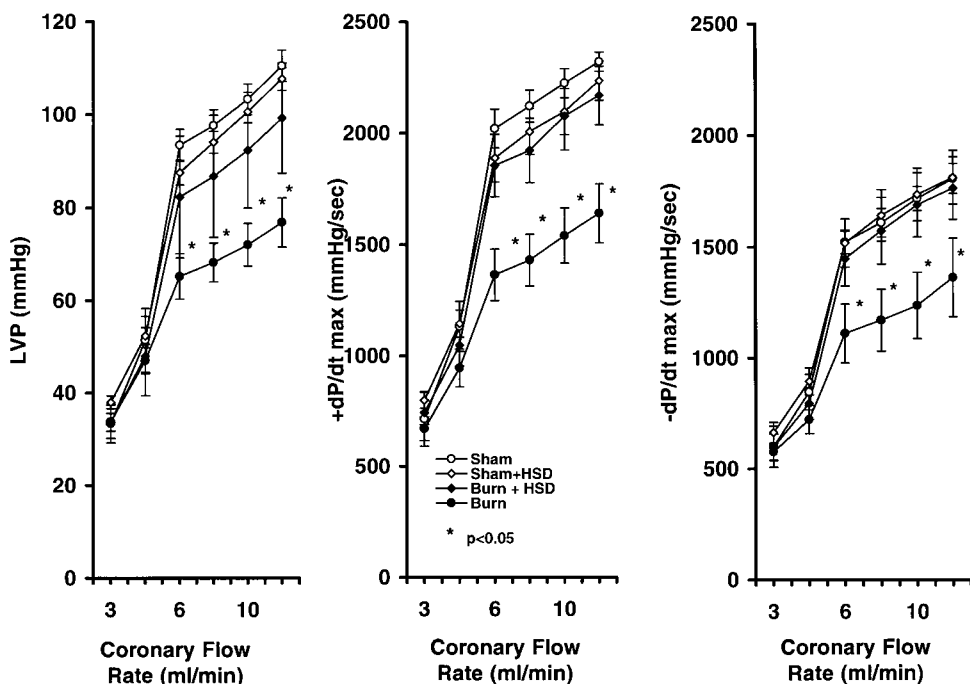
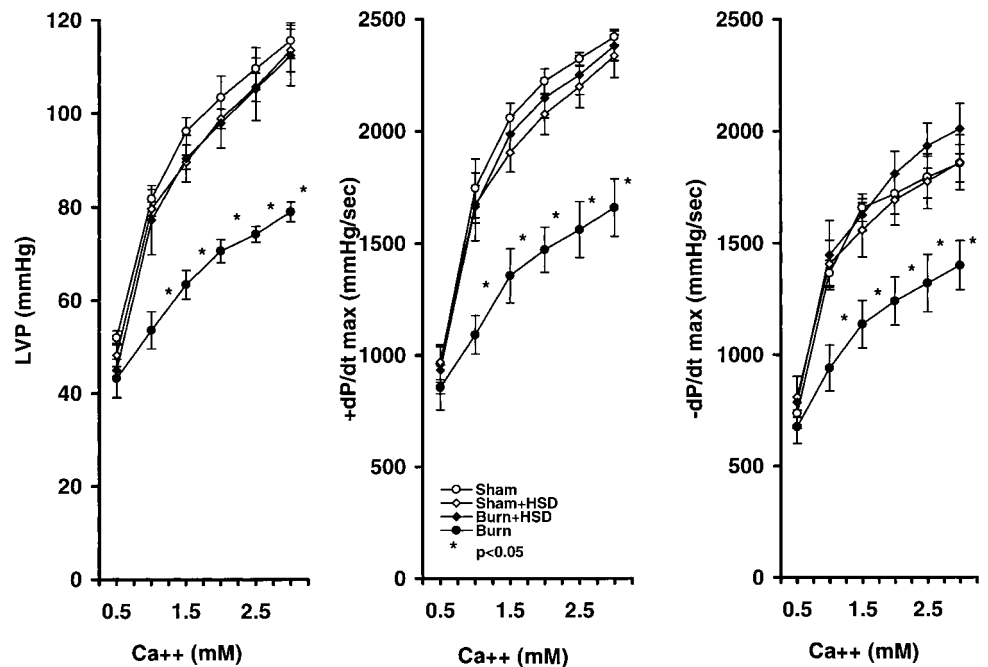


Fig. 2. Effects of increases in coronary flow rate on LVP and $\pm dP/dt_{max}$ in all experimental groups ($n = 8$ animals/group). Ventricular responses were significantly lower in burn group given isotonic saline followed by lactated Ringer solution compared with HSD and lactated Ringer-treated burns. All values are means \pm SE. Statistical analysis included ANOVA and a multiple comparison procedure (Bonferroni). *Significant difference among groups at $P < 0.05$.

Fig. 3. Increases in perfusate calcium levels improve left ventricular contraction and relaxation in all experimental groups ($n = 8$ animals/group). However, isotonic saline-treated burns had significantly reduced ventricular responses to perfusate Ca^{2+} compared with shams. HSD treatment of burn trauma significantly improved Ca^{2+} performance relationships. All values are means \pm SE. Statistical analysis included ANOVA and a multiple comparison procedure (Bonferroni). *Significant difference among groups at $P < 0.05$.



with responses measured in myocytes from sham burns ($P < 0.05$). In contrast, HSD treatment of burn trauma significantly attenuated the LPS-induced secretion of $\text{TNF-}\alpha$ by cardiomyocytes ($P < 0.05$); myocytes from HSD-treated rats (both burned and sham) secreted minimal $\text{TNF-}\alpha$ at all LPS doses.

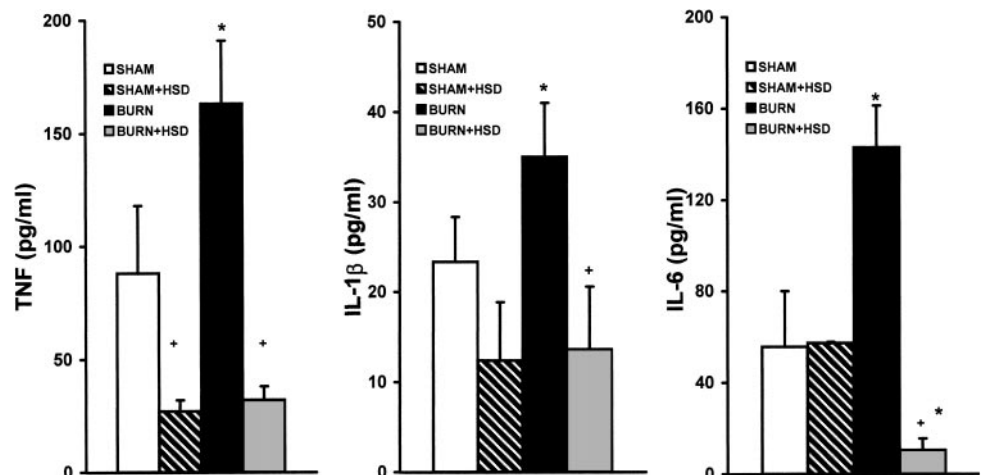
Similarly, myocytes from sham burns responded to *in vitro* LPS challenge with dose-dependent increases in $\text{IL-1}\beta$ and IL-6 secretion; however, burn trauma exacerbated cardiomyocyte secretion of $\text{IL-1}\beta$ (Fig. 6) and IL-6 (Fig. 7) with LPS stimulus. In contrast, the administration of HSD after burn trauma decreased myocyte secretion of $\text{IL-1}\beta$ and IL-6 in response to LPS challenge. Thus myocytes from burns resuscitated with standard care lactated Ringer solution secreted significantly more $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and IL-6 compared with levels measured in HSD-treated burns.

Examination of the cardiomyocyte preparation by FACSscan confirmed that $>93\%$ of the cells were myo-

cytes based on size. Examination of aliquots of these myocyte preparations by light microscopy determined that 93% of the cells were viable cardiomyocytes, 4% were dead cardiomyocytes, and $<2\%$ of the total cell population were neutrophils.

Figure 8 examines $\text{TNF-}\alpha$ secretory capacity of neutrophils (1×10^3 PMNs per microtiter well) stimulated with 0, 10, 25, and 50 μg LPS/well as described for the cardiomyocyte studies. This number of PMNs per well was based on the FACS analysis and light microscopy showing that $<2\%$ of our cell preparation was PMNs. These preliminary studies showed that the maximal number of PMNs that could contaminate our cardiomyocyte preparations was 1×10^3 cells per microtiter well. Our data confirmed that PMNs respond to LPS challenge by secreting $\text{TNF-}\alpha$ and this response was exacerbated by previous burn trauma (Fig. 8). However, the maximum $\text{TNF-}\alpha$ levels secreted by this PMN population could not account for the $\text{TNF-}\alpha$ levels mea-

Fig. 4. Cardiomyocyte secretion of tumor necrosis factor- α ($\text{TNF-}\alpha$) (left), interleukin ($\text{IL-1}\beta$) (middle), and IL-6 (right) in all experimental groups. Six hearts were used to prepare cardiomyocytes per group. All values are means \pm SE. *Significant difference in sham versus burn experimental groups at $P < 0.05$ using an unpaired Student's *t* test. (For example, isotonic saline-lactated Ringer burns were compared with isotonic-treated shams and HSD lactated Ringer-treated burns were compared with HSD-treated shams.) +Significant difference with HSD treatment versus isotonic saline-lactated Ringer solution treatment between either sham or burn groups at $P < 0.05$.



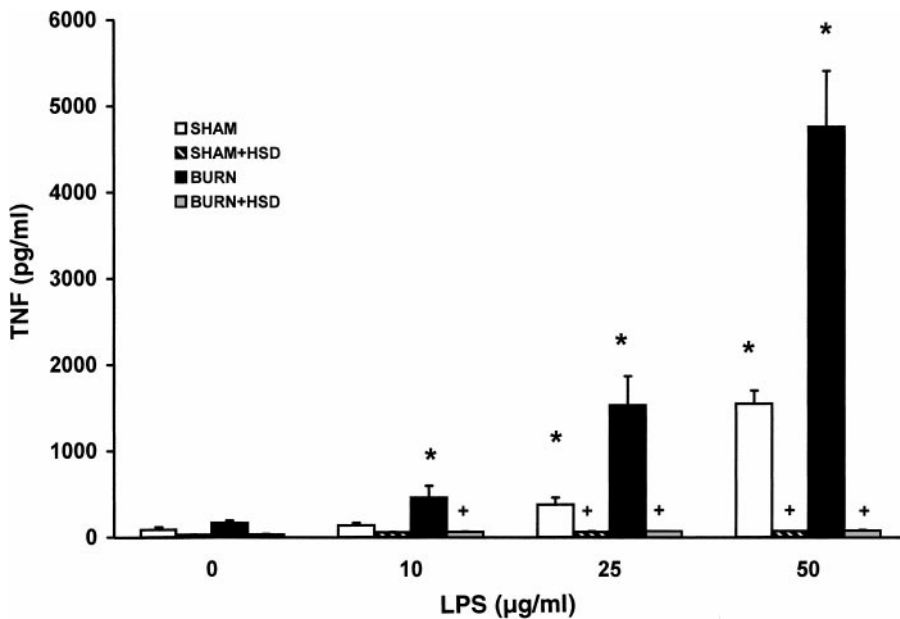


Fig. 5. TNF- α secretory responses of cardiomyocytes from all experimental groups to in vitro lipopolysaccharide (LPS) challenge. All values are means \pm SE. *Significant difference with LPS challenge compared with responses in the absence (0) of LPS at $P < 0.05$. + Within each in vitro experimental condition (at each LPS dose except zero) HSD reduced cardiomyocyte secretion of TNF- α by myocytes from both shams and burns (ANOVA and repeated measures).

sured in our myocyte preparation. For example, cardiomyocytes (5×10^4) prepared from burn rats secreted $4,880 \pm 300$ pg/ml TNF- α when challenged with LPS (50 μ g/well). In contrast, an identical LPS challenge (50 μ g/well) of leukocytes produced 244 ± 20 pg/ml of TNF. Thus contamination of our myocyte preps with PMNs would contribute to $<5\%$ of the total TNF- α secreted.

Finally, burn trauma promoted calcium accumulation in cardiomyocytes 24 h postburn (myocytes from sham burns, 93 ± 6 nM; myocytes from lactated Ringer solution-resuscitated burns, 293 ± 16 nM; $P < 0.05$). In contrast, HSD plus the administration of lactated Ringer solution significantly reduced the burn-related rise in cardiomyocyte calcium concentrations (123 ± 23

nM), whereas HSD in sham burns did not alter cardiomyocyte calcium levels (90 ± 12 nM).

DISCUSSION

Our studies confirm that major burn injury promotes cytokine secretion by cardiomyocytes, producing significant cardiac levels of TNF- α , IL-1 β , and IL-6. Our studies further confirmed that the LPS challenge of isolated cardiomyocytes evoked a robust cytokine secretory response, and a previous burn injury exacerbated this proinflammatory cytokine response of cardiomyocytes. It was of interest that cardiomyocytes from burns resuscitated with a bolus of isotonic saline plus lactated Ringer solution secreted significantly

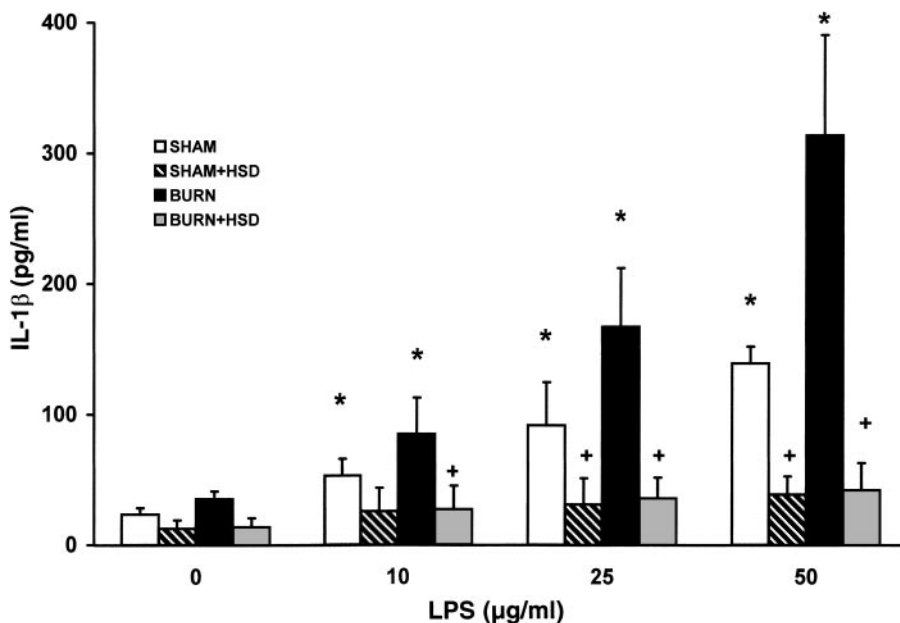
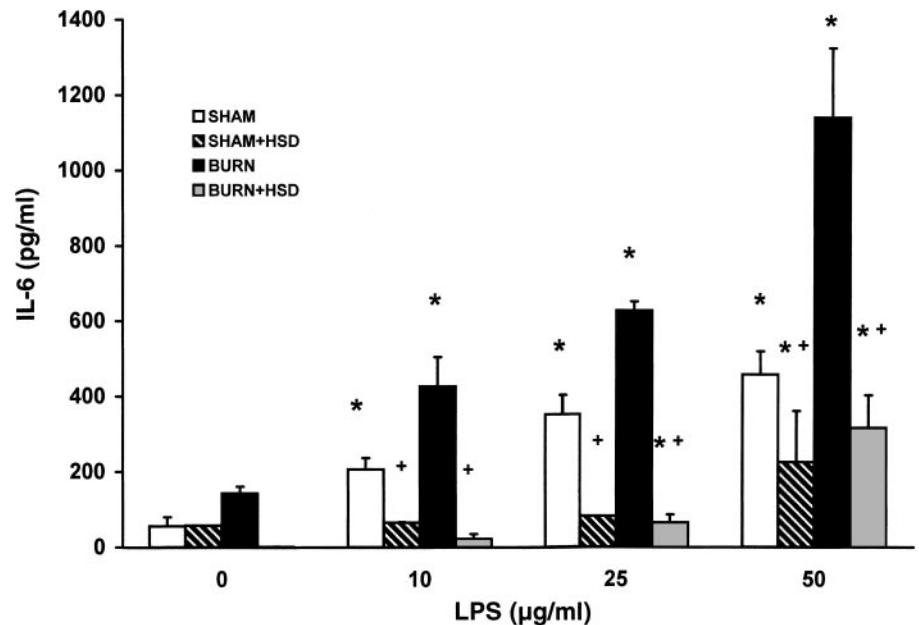


Fig. 6. IL-1 β secretory responses of cardiomyocytes to LPS challenge. All values are means \pm SE. *Significant difference with LPS challenge compared with responses in the absence (0) of LPS at $P < 0.05$. + Within each in vitro experimental condition (at each LPS dose except zero) HSD reduced cardiomyocyte secretion of IL-1 β by myocytes from both shams and burns (ANOVA and repeated measures).

Fig. 7. IL-6 secretory responses of cardiomyocytes to LPS challenge. All values are means \pm SE. *Significant difference with LPS challenge compared with responses in the absence (0) of LPS at $P < 0.05$. +Within each in vitro experimental condition (at each LPS dose except zero) HSD reduced cardiomyocyte secretion of IL-6 by myocytes from both shams and burns (ANOVA and repeated measures).



more TNF- α , IL-1 β , and IL-6 than sham cardiomyocytes at each LPS dose, suggesting that an initial injury such as burn trauma primes this cell population and exaggerates the inflammatory responses to a secondary stimulus such as LPS challenge. In our study, the burn-related increase in cardiomyocyte TNF- α , IL-1 β , and IL-6 secretion was paralleled by profound cardiac contractile dysfunction. These data suggest that local cardiac synthesis of inflammatory cytokines could contribute, in part, to the postburn cardiac contraction and relaxation deficits that occur after major burn trauma.

In our study, plasma levels of IL-1 β rose significantly in burns given standard isotonic saline followed by lactated Ringer resuscitation from burn trauma. Whereas plasma TNF- α levels tended to increase 24 h postburn in this experimental group, these changes did not achieve statistical significance. The finding of a higher myocardial TNF- α level compared with that

measured in the systemic circulation supports our hypothesis that cardiomyocyte secretion of TNF- α may produce myocardial cytokine levels that exceed those measured in the systemic circulation. Whereas circulating IL-1 β levels were higher than those measured in the cardiac compartment, it is likely that the high levels of TNF- α within the myocardium coupled with increased local synthesis of IL-1 β could contribute to the detrimental effect on cardiac myocyte function. It was of interest that HSD administration attenuated burn-related TNF- α , IL-1 β , and IL-6 secretion by cardiomyocytes, and these downregulated inflammatory cytokine responses were paralleled by a remarkable improvement in postburn cardiac contraction and relaxation.

For the past 30 years, there has been considerable debate regarding the advantages and disadvantages of crystalloid versus colloid resuscitation from trauma, and numerous studies have described the limitations and advantages of each resuscitation treatment (3, 10, 28–30, 32, 36, 42, 46). Because approximately one-third of the infused crystalloid solution remains within the vascular space, a large volume of crystalloid solution is required to adequately replace volume losses in the burn patient with significant fluid redistribution. The marked accumulation of crystalloid fluid in the extravascular compartment produces tissue edema, raising concerns that large-volume crystalloid infusion may compromise pulmonary function and promote the development of respiratory distress syndrome (28, 30, 31). Others have expressed concern that aggressive crystalloid resuscitation after trauma may promote myocardial edema. The resulting compression of coronary endothelial vessels could produce areas of relative myocardial ischemia, contributing to postburn cardiac dysfunction (7, 44). One advantage of colloids is the significant reduction in total fluid requirements, but concerns include the burn-related changes in endothe-

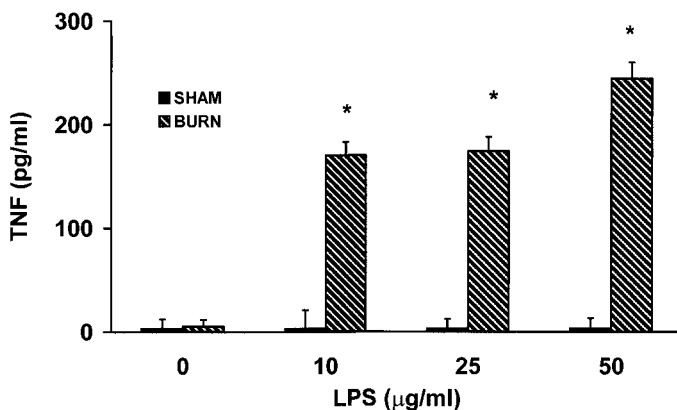


Fig. 8. TNF- α response of leukocytes (prepared from sham and burned rats) to in vitro LPS challenge. All values are means \pm SE. *Significant difference with LPS challenge compared with responses measured in absence of LPS at $P < 0.05$.

lial barrier function, inhibition of normal hemostasis, long-term retention of colloids within the body, and the occurrence of anaphylactoid reactions (2, 8, 34).

Whereas crystalloid resuscitation of burn trauma has been used successfully for the last 35 years with few complications, recent studies have raised additional questions about the effects of lactated Ringer solution on immunologic function after trauma. In this regard, Rhee and colleagues (43) showed that large-volume crystalloid resuscitation from hemorrhagic shock increased expression of the intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 and produced pulmonary edema and inflammatory cell infiltration compared with that seen with HSD resuscitation. It is well recognized that neutrophil activation and adherence and suppressed T-cell function occur after trauma including burn injury, and impaired immune defenses may contribute to posttrauma complications such as pneumonia and sepsis. Junger and colleagues (22, 23) showed that moderately hypertonic salt solutions enhanced T-cell proliferation. The hypertonic solution described by Junger contained salt concentrations corresponding to plasma sodium levels produced by trauma resuscitation with the 4 ml/kg dose of a 7.5% NaCl solution. This rescue of T cells from trauma-mediated suppression has been proposed to improve cellular immune function, providing immunologic protection against trauma-related complications such as sepsis and pneumonia. Whereas crystalloids have been routinely used for resuscitation of major burn injury, these recent studies suggest that the type of fluid used for resuscitation is not innocuous and may indeed exacerbate the burn-mediated inflammatory cascade.

These studies by Rhee and colleagues (43) and Junger and associates (22, 23) stimulated our interest in the effects of HSD resuscitation from burn trauma on the cardiac inflammatory responses. We previously showed that small-volume HSD, given within 6 h after a major burn injury in both clinical and experimental burns, significantly improved cardiac performance and reduced serum troponin I levels compared with values measured in subjects given standard crystalloid resuscitation (20, 21, 38). However, the mechanisms by which HSD reduced cardiomyocyte injury and improved cardiac contractile function has remained elusive.

The physiological effects of HSD have been attributed primarily to its effectiveness as a small-volume expander (9, 14, 20, 26). A previous study (26) suggests that each milliliter of hypertonic saline expands plasma volume by ~3 ml due to the osmotic effects of concentrated sodium chloride in retrieving water from the cellular space, whereas the dextran component retains fluid within the vascular space. Other studies (4, 26, 33, 37) have attributed the effectiveness of HSD resuscitation to hyperosmotic vasodilation of both the pulmonary and systemic microcirculation, improved venous return (Frank-Starling mechanism), or peripheral vasodilation and reduced afterload. Our previous study (16) confirmed a postburn decrease in coronary

blood flow despite aggressive crystalloid resuscitation, and we initially attributed the beneficial effects of HSD in burn trauma to improved coronary perfusion. In this regard, Mazonni and colleagues (33) showed that hypertonic solutions promoted vasodilation and decreased reperfusion-related injury. Whereas HSD may improve cardiac performance secondary to improving coronary perfusion, this present study focused on the effects of HSD resuscitation from burn trauma on the cardiomyocyte secretion of inflammatory cytokines.

Although the intracellular signaling mechanisms by which burn trauma promotes cardiomyocyte secretion of TNF- α , IL-1 β , and IL-6 remain unclear, recent studies from our laboratory have shown that burn trauma promotes free radical-mediated cell membrane peroxidation, neutrophil adherence and activation within the coronary microcirculation, and emigration of activated leukocytes into the myocardial tissue (15, 19). These findings raised a concern in our present study that emigrating neutrophils could contaminate our cardiomyocyte preparations and PMN-derived cytokines could contribute to the measured cytokine levels. In our study, even the maximal number of PMNs, calculated from FACS analysis of cardiomyocyte preparations, failed to account for the TNF- α levels measured in cardiomyocyte supernatants. These data minimized concerns regarding the potential contamination of cardiomyocytes by neutrophils and suggest that the secreted TNF- α levels were directly attributable to cardiomyocytes *per se*.

In this study, HSD administration after burn trauma prevented the rise in intracellular calcium concentration observed in burns given standard lactated Ringer resuscitation, a finding consistent with Junger's description (22, 23) of hypertonic-mediated suppression of calcium mobilization by T cells. It is likely that the decrease in inflammatory cytokine secretion observed in our study may be the direct result of normalization of $[Ca^{2+}]_i$ and stabilization of sarcolemma membrane potential. After crystalloid resuscitation of burn trauma, $[Ca^{2+}]_i$ was threefold higher in cardiac myocytes compared with that observed in cardiac myocytes from sham burns. Secretion of inflammatory cytokines is likely a calcium-dependent process. If the mechanism of LPS-stimulated secretion of cytokines is calcium independent, the presence of an elevated intracellular calcium level coupled with partially depolarized cell membrane potentials after burn trauma may increase synthesis via protein kinase C, increasing sensitivity for LPS-evoked secretion. We have recently shown (unpublished data) that burn trauma resuscitated with lactated Ringer solution promotes a partial depolarization of the sarcolemma transmembrane potential, and this depolarization was ablated with HSD treatment of burn trauma. These later data are consistent with HSD-mediated changes in transmembrane ion transport as described by Nakayama and colleagues (39). Therefore, it is likely that HSD alters cardiac cytokine secretion by several mechanisms including stabilization of the transmembrane potential and a decrease in transsarcolemma calcium

flux which, in turn, may play a critical role in down-regulating several intracellular kinases (protein kinase C and p38 mitogen-activated protein kinase) thought to play a critical role in signal transduction mechanisms that regulate transcription and/or translation of inflammatory cytokines (35).

In summary, our data suggest that HSD mediates cardioprotection in part after burn trauma by decreasing cardiomyocyte secretion of inflammatory cytokines. Whereas burn trauma increased secretion of inflammatory cytokines (TNF- α , IL-1 β , and IL-6) that may contribute to postburn cardiac dysfunction, the signal transduction mechanisms that regulate cytokine transcription and/or translation remain unknown in the setting of burn trauma. Our finding that HSD administration in the early postburn period downregulated cytokine secretion by cardiomyocytes was particularly important because locally produced cytokines within the myocardial tissue may not be buffered by circulating or soluble receptors that would have limited access to intramyocardially produced cytokines. Thus locally secreted TNF- α and interleukins may have detrimental consequences on cardiomyocyte membrane integrity, contributing to myocyte injury, and altering cardiac performance. However, it is likely that HSD improved cardiac performance by interrupting several aspects of the inflammatory cascade that culminated in increased TNF- α and interleukin secretion, perhaps by reducing burn-mediated oxygen free radical generation or decreasing neutrophil adherence and activation. From these considerations, the beneficial cellular effects of HSD on burn trauma warrant further study.

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