Selective decontamination of the digestive tract attenuated the myocardial inflammation and dysfunction that occur with burn injury

Jureta W. Horton, Jing Tan, D. Jean White, David L. Maass, and James A. Thomas. Selective decontamination of the digestive tract attenuated the myocardial inflammation and dysfunction that occur with burn injury. Am J Physiol Heart Circ Physiol 287: H2241–H2251, 2004. First published July 15, 2004; doi:10.1152/ajpheart.00390.2004.—This study examined the effects of oral antibiotics to selectively decontaminate the digestive tract (SDD) on postburn myocardial signaling, inflammation, and function. We hypothesized that antibiotic therapy to eliminate pathogens from the gastrointestinal (GI) tract would reduce myocardial inflammatory responses and improve postburn myocardial performance. Sprague-Dawley rats received polymyxin E (15 mg), tobramycin (6 mg), and 5-flucytosin (100 mg) by oral gavage twice daily for 3 days preburn and 24 h postburn. Experimental groups included 1) sham burn given vehicle (3 mL water), 2) sham plus SDD, 3) burn over 40% total body surface area (TBSA) plus SDD, and 4) burn over 40% TBSA given vehicle. All burns received lactated Ringer solution (4 mg/kg·h·%burn), myocardial signaling (PKC/ p38 MAPK/NF-κB) was studied 2, 4, and 24 h postburn; and cytokine secretion (systemic and myocyte secreted cytokines, ELISA) and cardiac function were examined 24 h postburn. Vehicle-treated burn injury increased myocardial PKC/ p38 MAPK expression, promoted NF-κB nuclear translocation, promoted TNF-α, IL-1β, IL-6, and IL-10 secretion, and impaired myocardial function. SDD attenuated burn-related proinflammatory myocardial signaling, cytokine secretion, and myocardial contractile defects. Our data suggest that burn-related loss of GI barrier function and translocation of microbial products serve as upstream mediators of postburn myocardial inflammatory signaling and dysfunction.

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AN OBLIGATE ROLE for signaling through the Toll/IL-1 pathway has been established in the myocardial contractile dysfunction that occurs after major burn injury (18, 27, 39). The Toll/IL-1 pathway acts in innate immune responses, regulating pathogen recognition in the afferent limb, whereas participation in the efferent limb includes regulation of proinflammatory and microbial activities (14, 15, 24, 30, 36, 37, 43). Although the precise extracellular mediators that trigger Toll/IL-1 signaling in the myocardium after a noninfectious insult such as burn injury remain unknown, Sambol and colleagues (31) described that mesenteric lymph duct ligation attenuated postburn myocardial contractile dysfunction. These data implicated gut-derived factors as upstream mediators of burn-related Toll/IL-1 signaling (32). Despite these findings, neither bacteria nor endotoxin are routinely identified in the systemic circulation after experimental or clinical burn injury (6, 32, 39). However, splanchnic hyperperfusion and bacterial translocation to the mesenteric lymph nodes, liver, and spleen have confirmed that burn injury alters several aspects of gastrointestinal barrier function (10, 12, 17, 21, 48). Whether bacteria entrapped by the reticuloendothelial organs play a role in downstream organ inflammatory cascades or dysfunction is unknown.

Aggressive and rapid fluid resuscitation from burn injury corrects perfusion deficits and restores splanchnic perfusion, and the use of topical antibiotics such as silver sulfadiazine limits postburn wound infection (3, 4, 34). Administration of oral, nonabsorbable antibiotics to eliminate gastrointestinal pathogens has not been used widely in clinical burn populations. Yet, over the last 20 years, 53 randomized trials that included 8,500 patients have evaluated oral antibiotic therapy in intensive care unit patients (29, 40). Studies from Europe and the United Kingdom have confirmed that oral antibiotics decrease morbidity and mortality in critically ill patients (35, 41, 42). Experimental studies have confirmed that oral antibiotic therapy prevents burn-related bacterial translocation, attenuates postburn immunosuppression, and improves postburn survival (45, 46); however, the effects of selective decontamination of the digestive tract (SDD) on the myocardial inflammation and dysfunction that occur after major burn injury have not been examined. Therefore, this present study examined the effects of pre- and postburn oral antibiotics with SDD on 1) myocardial signaling cascades mediated by Toll-like receptor 4 (TLR4) binding, 2) myocardial inflammatory cytokine secretion, and 3) myocardial contractile function. If gut-derived pathogens are the primary mechanism by which cutaneous burn injury initiates an inflammatory signaling cascade that culminates in myocardial inflammation and dysfunction, then SDD would be expected to attenuate postburn myocardial inflammation and dysfunction.

MATERIALS AND METHODS

Experimental Model

Adult Sprague-Dawley male rats (320–350 g) 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 studies 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 Physiological Society and the National Institutes of Health.

Catheter Placement and Burn Procedure

Rats were anesthetized lightly with isoflurane 18–20 h before the burn experiment, and body hair on the side, back, and neck was removed by 18 U.S.C. Section 1734 solely to indicate this fact. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
closely clipped. The neck region was treated with a surgical scrub, the left carotid artery was exposed, and a polyethylene catheter (PE-50) inserted into the artery was advanced retrogradely to the level of the left carotid artery was exposed, and a polyethylene catheter (PE-50) placed in the right external jugular vein was used to administer fluids and drugs. All catheters were filled with heparinized saline, exteriored, and secured at the nape of the neck. Eighteen hours after catheter placement, animals were deeply anesthetized (isoflurane). The neck region was treated with a surgical scrub, 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 well-circumscribed full-thickness dermal burns over 40% of the total body surface area (TBSA). Exposure to this water temperature in adult rats destroys all underlying nerves and avoids injury to underlying organs. Sham burn rats were subjected to an identical preparation except that they were immersed in room temperature water to serve as controls. Immediately after immersion, rats were dried and returned to individual cages, and each external jugular catheter was connected to a swivel device (BSP99 Syringe Pump, Braintree Scientific; Braintree, MA) for fluid administration. Body temperature was measured with a rectal temperature probe (model 44TA, YSL-Tele Thermometer), and the respiratory rate was monitored by counting respiratory movement. Systemic blood pressure was measured using a Gould-Statham pressure transducer (model IDP23, Gould Instruments; Oxnard, CA) connected to a Grass medical recorder (model 7D Polygraph, Grass Instruments; Quincy, MA). A Grass tachycardiograph (model 7PF4) was used to monitor heart rate. A Grass Poly VIEW Data Acquisition System was used to convert acquired data into digital form.

**Lactated Ringer Resuscitation and Antibiotic Therapy**

Fluid resuscitation consisted of lactated Ringer solution (4 ml·kg⁻¹·%burn⁻¹), with one-half of the calculated volume given during the first 8 h postburn and the remaining volume given over the next 16 h postburn. The total volume of Ringer solution given over the first 24 h postburn 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 15 min after recovering from isoflurane anesthesia. In the sham burn animals, the external jugular vein was cannulated, and lactated Ringer solution was given to maintain cather patency (0.2 ml·kg⁻¹·h⁻¹); sham burns also received identical regimens of analgesics (buprenorphine) throughout the study period.

Oral antibiotic therapy for selective decontamination of the digestive tract included polymyxin E (15 mg), tobramycin (6 mg), and 5-flucytosin (100 mg); the antibiotics were mixed in 3 ml of water and were given by oral gavage twice daily for 3 days before burn injury and for 24 h after injury. The animals that served as controls were given the same volume of water by oral gavage at the same time intervals described for antibiotic therapy. Therefore, the following experimental groups were studied: sham burned rats given water by oral gavage (group 1); sham burns given antibiotic therapy by oral gavage as described above (group 2); rats given burn injury over 40% TBSA that were fluid resuscitated and given vehicle by oral gavage (group 3); and rats given burn injury over 40% TBSA that received fluid resuscitation and oral antibiotic therapy as described above (group 4). Twenty-six rats were included in each of the four experimental groups. Four rats from each group were killed either 2, 4, or 24 h after burn injury (4 rats/group × 3 time periods = 12 rats/group) to examine myocardial phosphorylated p38 MAPK and phosphorylated PKCε (p-PKCε) measured with anti-phosphorylated p38 and anti-p-PKCε antibodies as well as NF-κB activation. Nine rats per group were killed 24 h postburn to examine myocardial function (Langendorff), whereas 6 rats/group were used to prepare cardiomycocytes (myocyte cytokine secretion and cellular calcium and sodium measured 24 h postburn; 8 = 6 = 14 rats/group).

**Western Blot Analysis**

Hearts collected 2, 4, or 24 h postburn were snap frozen and stored at −80°C until analysis. Frozen myocardial tissue was homogenized in a glass–glass homogenizer in 200 μl of ice-cold lysis buffer (10 mM HEPES (pH 7.4), 2 mM EDTA (pH 8), 0.1% CHAPS, protease inhibitors, and phosphatase inhibitors (1 mM PMEF, 0.1 mM sodium vanadate, and 5.0 mM NaN3)). The homogenized samples were allowed to sit on ice for 20 min and were then centrifuged at 10,000 rpm for 10 min at 4°C. The protein concentration was determined by the Bio-Rad protein assay (Bio-Rad; Hercules, CA).

Equal amounts of protein (40 μg) were used to measure the kinase of interest (total p38 MAPK or total PKCε). Protein samples were separated on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membranes (Millipore; Bedford, MA). The membranes were blocked by a 1-h incubation in a Tris-buffered saline (TBS)-TWEEN (TBS-T) solution (20 mM Tris (pH 7.6), 135 mM NaCl, and 0.1% Tween) containing 3% BSA and 1% nonfat dry milk. Western blots were developed for total protein, striped, and developed for the phosphorylated forms using specific antibodies for the kinase of interest (phosphorylated-p38 antibody or p-PKCε antibody, Santa Cruz Biotechnology; Santa Cruz, CA) added to the membranes at a dilution of 1:200 and incubated for 1 h at room temperature. After the primary antibody incubation, the membrane was washed three times with TBS-T. Horseradish peroxidase-conjugated secondary antibody was then added to the membrane (1:1,000, Bio-Rad) and incubated for 1 h at room temperature. The membrane was again washed three times with TBS-T and one time with TBS. The bound antibodies were visualized by enhanced chemiluminescence (Amer sham; Piscataway, NJ) (2). The density of protein signals from each group was expressed as relative integrated intensity compared with values measured in the group of normal hearts.

**NF-κB Nuclear Translocation**

**Nuclear protein extraction.** A modified procedure was used to isolate nuclear protein. All steps were performed on ice. Hearts were thawed in the presence of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, and 0.1% NP40; 200 μl) and protease inhibitors plus 0.5 mM DTT. Tissue was then homogenized by hand using a glass–glass dounce homogenizer. The cells were allowed to incubate for 20 min on ice and then centrifuged at 12,000 rpm for 5 min at 4°C. The supernatants (cytosolic fraction) were removed and frozen at −80°C. The remaining pellet was then resuspended in 200 μl of

**Table 1. Hemodynamic and metabolic responses to burn and burn treatment with SDD**

<table>
<thead>
<tr>
<th></th>
<th>Sham + Vehicle</th>
<th>Sham + SDD</th>
<th>Burn + Vehicle</th>
<th>Burn + SDD</th>
</tr>
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<tbody>
<tr>
<td>MAP, mmHg</td>
<td>150 ± 5</td>
<td>165 ± 5</td>
<td>93 ± 3*</td>
<td>159 ± 4</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>523 ± 12</td>
<td>514 ± 14</td>
<td>505 ± 21</td>
<td>513 ± 16</td>
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<tr>
<td>pH</td>
<td>7.47 ± 0.04</td>
<td>7.48 ± 0.02</td>
<td>7.46 ± 0.01</td>
<td>7.50 ± 0.07</td>
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<tr>
<td>Base excess, mmol/l</td>
<td>1.64 ± 0.4</td>
<td>0.13 ± 0.3</td>
<td>−2.6 ± 0.3*</td>
<td>−0.5 ± 0.3</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>2.20 ± 0.3</td>
<td>2.22 ± 0.3</td>
<td>4.14 ± 0.31*</td>
<td>2.29 ± 0.13</td>
</tr>
<tr>
<td>HCO3−, meq/l</td>
<td>20 ± 17</td>
<td>21 ± 1</td>
<td>23 ± 1</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>PO43−, mmol/l</td>
<td>24 ± 1</td>
<td>28 ± 1</td>
<td>28 ± 1</td>
<td>28 ± 1</td>
</tr>
<tr>
<td>PO2, mmol/l</td>
<td>116 ± 5</td>
<td>94 ± 4</td>
<td>108 ± 3</td>
<td>117 ± 5</td>
</tr>
<tr>
<td>Hct, %</td>
<td>43 ± 3</td>
<td>42 ± 1</td>
<td>37 ± 2</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>O2 saturation, %</td>
<td>99.8 ± 0.9</td>
<td>99.0 ± 0.3</td>
<td>96.0 ± 0.2</td>
<td>99.8 ± 0.5</td>
</tr>
<tr>
<td>O2 content, %</td>
<td>21.5 ± 12.6</td>
<td>19.7 ± 0.6</td>
<td>16.4 ± 0.6*</td>
<td>19.1 ± 0.8</td>
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<tr>
<td>Body temperature, °C</td>
<td>38.8 ± 0.1</td>
<td>38.9 ± 0.1</td>
<td>38.8 ± 0.1</td>
<td>38.7 ± 0.2</td>
</tr>
<tr>
<td>Ca2+, mmol/l</td>
<td>1.29 ± 0.1</td>
<td>1.26 ± 0.04</td>
<td>0.82 ± 0.02*</td>
<td>1.04 ± 0.02</td>
</tr>
<tr>
<td>Na+, mmol/l</td>
<td>141.2 ± 0.6</td>
<td>147.4 ± 0.3</td>
<td>144.8 ± 0.7</td>
<td>141.8 ± 0.8</td>
</tr>
</tbody>
</table>

All values are means ± SE. SDD, selective decontamination of the digestive tract; MAP, mean arterial pressure; HR, heart rate; Hct, hematocrit; Ca²⁺, serum ionized calcium; Na⁺, serum ionized sodium. *Significant burn-related difference compared with the respective control group at P < 0.05.
ice-cold buffer C [20 mM HEPES (pH 7.9), 420 mM NaCl, 1.8 mM MgCl₂, 0.2 mM EDTA (pH 8), and 25% glycerol] and protease inhibitors as described above plus 0.5 mM DTT. The pellets were completely resuspended by pipetting and were incubated for 20 min on ice with gentle resuspension of the pellets every couple of minutes. After incubation, the pellets were centrifuged for 5 min at 14,000 rpm at 4°C. The supernatant was collected (nuclear fraction) and frozen at −80°C. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad). Nuclear and cytosolic extracts were stored at −80°C.

Electrophoretic mobility shift assay. NF-κB binding activity was examined by electrophoretic mobility shift assay (EMSA). Double-stranded oligonucleotides corresponding to the consensus NF-κB binding site of the κ light chain enhancer (5'-AGTTGAGGGGACTT-TCCAGGC-3') were purchased from Promega Biotech (Madison, WI). In a total volume of 10 μl, 3.5 pmol of oligonucleotide, 10 units of T4 polynucleotide kinase in 1× forward buffer (GIBCO-BRL; Gaithersburg, MD), and 30 μCi of [γ-32P]ATP (DuPont-New England Nuclear; Boston, MA) were incubated at 37°C for 60 min. The reaction was stopped by the addition of 1 μl of 0.5 mM EDTA. The volume was brought up to 50 μl with the addition of STE buffer [10 mM Tris HCl (pH 7.5), 10 mM NaCl, and 1 mM EDTA]. Labeled probe was separated from unbound ATP using ProbeQuant G-50 Micro Columns from Pharmacia Biotech. The activity of the labeled probe was determined, and the probe was stored at −20°C.

Twenty micrograms of nuclear proteins were incubated in 1× gel shift buffer [100 mM HEPES (pH 7.6), 250 mM KCl, 5 mM DTT, 5 mM EDTA, and 25% glycerol]. In addition, these were incubated with polydeoxyinosinic-deoxycytidylic acid and 2 μl of probe containing 400,000 and 500,000 counts/min. The final reaction volume of 20 μl was achieved with water. The reactions were incubated at room temperature for 1 h, and 2 μl of 10× loading buffer was then added [10× loading buffer consisted of 30% glycerol, 0.25% xylene cyanol, 0.05% bromophenol blue, 0.05% methyl blue, and 40 mM Tris HCl (pH 8.0)].
and 0.25% bromophenol blue (BPB)]. The samples were then separated on an 8% polyacrylamide gel in 0.5× TBE (25 mM Tris-HCl, 25 mM boric acid, and 0.5 mM EDTA). Finally, the gels were dried and exposed to X-ray film. Competition analyses were performed by including a 50 M excess of unlabeled double-stranded DNA oligonucleotides in the binding reaction. Nonspecific competitor DNA contained an activator protein-1 (AP-1) binding element. The results from each group were expressed as relative integrated intensity and compared with values measured in a group of normal hearts (control).

Western blot analysis of p50/p65 NF-κB dimers. Forty micrograms of protein extracts, of either cytoplasmic or nuclear, harvested as stated in the EMSA assay, were combined with 2 μl of 2× sample loading buffer [0.063 M Tris-HCl (pH 6.8), 2% SDS, 5% mercaptoethanol, 20% glycerol, and 0.2% BPB]. The samples were boiled for 5 min and resolved on a 12.5% polyacrylamide gel. The gels were then transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked for 1 h at room temperature in TBS-T [120 mM Tris (pH 7.6), 0.9% NaCl, 0.05% Tween 20, and 5% dried milk]. Blocked membranes were then incubated overnight at 4°C with monoclonal NF-κB p65 antibody or p50 antibody (Santa Cruz Biotechnology; 1:100 dilution) in TBS-T-5% milk. After incubation, membranes were washed for 10 min at room temperature in TBS-T, followed by three 10-min washes in TBS-T. After the washes, membranes were incubated for 1 h in secondary antibody (Bio-Rad) diluted 1:1,000 in TBS-T. Membranes were then washed as described above and exposed to a mixture of luminol plus hydrogen peroxide under alkaline conditions (SuperSignal West Pico, Pierce Endogen; Rockford, IL) for 5 min. The resulting chemiluminescent reaction was detected by X-ray film. Experiments were repeated three times for each experimental sample (6).

Cardiomyocyte Isolation

Twenty-four hours after burn injury, animals from each experimental group (N = 5–6 rats/group) were heparinized, blood samples were collected, and rats were decapitated; 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 d-glucose, 0.5× minimum essential medium (MEM) amino acids (50×, GIBCO-BRL no. 11130-051), 10 mM 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 nonrecirculating mode. Enzymatic digestion was initiated by perfusing the heart with digestion solution, which contained 34.5 ml of the

Fig. 2. Equal amounts of protein were used for Western blot analysis examining myocardial phosphorylated p38 (p-p38) MAPK using an anti-p-p38 antibody. Similarly, equal amounts of protein were used to determine total p38 MAPK using Western blot analysis. Burn injury promoted an increase in p-p38 MAPK in the heart (A), 4 (B), and 24 h postburn (C). SDD attenuated the burn-related increase in myocardial p-p38 MAPK. Denstometric analysis of several blots from each group at each time period is summarized (bottom) with each Western blot (top). p38 MAPK phosphorylation was quantified by digital image analysis. All values are means ± SE. *S significant difference from sham burn (control) at P < 0.05; †significant difference between SDD-treated burns and burns given vehicle at P < 0.05.
heart medium described above plus 50 mg of collagenase II (Worth-
ington 4177, lot no. MOB3771), 50 mg BSA (endotoxin free, fraction
V, GIBCO-BRL no. 11018-025), 0.5 ml trypsin (2.5%, 10X, GIBCO-
BRL 15090-046), 100 M CaCl2, and 40 mM 2,3-butanedione mono-
oxime (BDM). Enzymatic digestion was accomplished by recirculat-
ing 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 2X BDM-
BSA solution [3 mg BSA (fraction V) to 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 addi-
tional 10 ml aliquot of 1X BDM-BSA solution [prepared by combin-
ing 100 ml of BDM stock, 40 mM; 100 ml of heart medium described
above, and 2 mg 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 1X BDM-BSA. The cells
were washed and pelleted further in BDM-BSA buffer with increasing
increments of calcium (100, 200, and 500 nM) to a final concentration
of 1,000 nM). After the final pelleting step, the supernatant was
removed, and the pellet was resuspended in MEM [prepared by
adding 10.8 g of MEM (Sigma M-1018), 11.9 mM NaHCO3, 10
M HEPES, and 10 ml penicillin-streptomycin (100X, GIBCO-BRL no.
1540-122) with 950 ml MilliQ water]; the 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 the 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 calcium, the cardiomyo-
cyte cell number was calculated, and myocyte viability was deter-
mined (20).

Cytokine Secretion by Cardiomyocytes

Myocytes were pipetted into microtiter plates at 5 X 104
cell·ml⁻¹·well⁻¹ (12-well cell culture cluster, Corning; 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; Woburn, MA). We previously examined the con-
tribution of contaminating cells (nonmyocytes) in our cardiomycocyte
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 was noncardiomyocytes. Because
our preparations are 98% cardiomyocytes, we concluded that a ma-
jority of the inflammatory cytokines measured in the cardiomycocyte
supernatant was indeed cardiomyocyte derived (19).

Intracellular Ca²⁺ and Na⁺ Concentration Measurements

Separate aliquots of cells were loaded with either fura-2 AM for 45
min or sodium-binding benzofurazan isophthalate (SBFI) for 1 h at
room temperature in the dark. Myocytes were then suspended in 1.0

Fig. 3. NF-κB nuclear translocation was determined by electrophoretic mobility shift assay (EMSA). Burn trauma in vehicle-
treated rats promoted NF-κB nuclear migration 2, 4, and 24 h postburn. SDD administration attenuated burn-related NF-κB
activation. A representative EMSA is included, and densitometric analysis of multiple gel shifts was performed for each group at
each time period. All values are means ± SE. *Significant difference from sham burn (control) at P < 0.05; †significant difference
between SDD-treated burns and burns given vehicle at P < 0.05.
mM calcium-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 tricircular head, phase optics, 30X phase-contrast objective, and mechanical stage. The 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 (CCD) camera equipped with a television relay lens. InCyt Im2 Image software allowed measurement of intracellular Ca²⁺ and Na⁺ concentrations ([Ca²⁺]ᵢ and [Na⁺]ᵢ, respectively) from the ratio of the two fluorescence signals generated from the two excitation wavelengths (340 and 380 nm); background was removed by the InCyt IM2 software. The calibration procedure included measuring the fluorescence ratio with buffers containing different concentrations of either calcium or sodium. At each wavelength, the buffers containing different concentrations of either calcium or sodium were used in these studies, the calcium levels measured remained constant 24 h after burn trauma with sodium heparin (200 units, Elkins-Sinn; Cherry Hill, NJ) and cervically dislocated. The heart was rapidly removed and placed in a petri dish containing ice-cold (4°C) Krebs-Henseleit bicarbonate buffered solution containing (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; P O₂, 500 mmHg; P CO₂, 38 mmHg). A cannula placed in the ascending aorta was 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 ± 0.5°C, and a constant flow pump (model 7335-30, ismaTec, Cole-Palmer Instrument; Chicago, IL) was used to maintain perfusion of the coronary artery (in ml/min) 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 water-filled latex balloon attached to a polyethylene tube and threaded through the apex of the left ventricular chamber. Peak systolic left ventricular pressure was measured with a Statham pressure transducer (model P23 ID, Gould Instruments) attached to the balloon cannula, and the rates of left ventricular rise (+dP/dt) and fall (−dP/dt) were obtained using an electronic differentiator (model P23 ID, Gould Instruments) and recorded (model 7DWL8P, Grass). Left ventricular developed pressure (LVP) was calculated from peak systolic left ventricular pressure and left ventricular end-diastolic pressure. Data from the Grass recorder were input to a Dell Pentium computer, and a Grass Poly VIEW Data Acquisition system was used to convert the acquired data into digital form.

Isolated Coronary Perfused Hearts

To examine cardiac contraction and relaxation, rats were anticoagulated 24 h after burn trauma with sodium heparin (200 units, Elkins-Sinn; Cherry Hill, NJ) and cervically dislocated. The heart was rapidly removed and placed in a petri dish containing ice-cold (4°C) Krebs-Henseleit bicarbonate buffered solution containing (in mM)

<table>
<thead>
<tr>
<th>Buffer Component</th>
<th>Concentration (mM)</th>
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<tr>
<td>NaCl</td>
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</tr>
<tr>
<td>KCl</td>
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<tr>
<td>NaHCO₃</td>
<td>21</td>
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<tr>
<td>NaHPO₄</td>
<td>11</td>
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To examine cardiac contraction and relaxation, rats were anticoagulated 24 h after burn trauma with sodium heparin (200 units, Elkins-Sinn; Cherry Hill, NJ) and cervically dislocated. The heart was rapidly removed and placed in a petri dish containing ice-cold (4°C) Krebs-Henseleit bicarbonate buffered solution containing (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; P O₂, 500 mmHg; P CO₂, 38 mmHg). A cannula placed in the ascending aorta was 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 ± 0.5°C, and a constant flow pump (model 7335-30, ismaTec, Cole-Palmer Instrument; Chicago, IL) was used to maintain perfusion of the coronary artery (in ml/min) 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 water-filled latex balloon attached to a polyethylene tube and threaded through the apex of the left ventricular chamber. Peak systolic left ventricular pressure was measured with a Statham pressure transducer (model P23 ID, Gould Instruments) attached to the balloon cannula, and the rates of left ventricular rise (+dP/dt) and fall (−dP/dt) were obtained using an electronic differentiator (model P23 ID, Gould Instruments) and recorded (model 7DWL8P, Grass). Left ventricular developed pressure (LVP) was calculated from peak systolic left ventricular pressure and left ventricular end-diastolic pressure. Data from the Grass recorder were input to a Dell Pentium computer, and a Grass Poly VIEW Data Acquisition system was used to convert the acquired data into digital form.

**Fig. 4.** Translocation of p50 and p65 from the cytosol to the nucleus was evident after burn injury in vehicle-treated rats. SDD inhibited translocation of these NF-κB dimmers. A: nuclear protein; B: cytosplasmic protein. Top, representative Western blot; bottom, densitometric analysis of multiple Western blots in each group at each time period. Burn-related translocation of the NF-κB dimer components was altered by SDD treatment before burn injury. All values are means ± SE. *Significant difference from sham burn (control) at P < 0.05; †significant difference between SDD-treated burns and burns given vehicle at P < 0.05.

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lower circulating TNF-α, IL-1β, and IL-6 levels compared with values measured in vehicle-treated burn rats \((P < 0.05)\).

**Burn-Related Alterations in Myocardial Signaling**

To determine whether burn injury altered myocardial inflammatory signaling, hearts were collected at several times postburn from each experimental group; specifically, we determined whether burn injury increased myocardial expression of p-PKCε using an anti-p-PKCε antibody. Myocardial p-PKCε was increased \(2, 4, \) and \(24\) h postburn, whereas total protein content was unchanged. Figure 1 includes representative Western blots of p-PKCε measured \(2\) \((A), 4\) \((B), \) and \(24\) h postburn \((C)\). Densitometric analysis of the four to five blots of p-PKCε per time period per group are summarized \((bottom)\) with the Western blots \((top)\). p-PKCε was quantified by digital image analysis and normalized to total PKCε (in densitometric units; Fig. 1). SDD treatment attenuated burn-related increases in p-PKCε in the heart.

We also examined phosphorylated p38 MAPK in subsets of hearts from all experimental groups. Western blot analysis was performed using anti-phospho-p38 MAPK antibodies to detect active or phosphorylated forms of p38 MAPK after the total p38 MAPK was measured. The representative Western blots shown in Fig. 2 are paralleled by densitometric analysis of the four to five blots that examined phosphorylated p38 MAPK by digital image analysis normalized to total p38 MAPK in each group at each time period. Expression of phosphorylated p38...
MAPK was increased significantly 2 h after burn in vehicle-treated animals (Fig. 2A); phosphorylated p38 MAPK measured 4 h postburn remained above that measured in time-matched sham burns (Fig. 2B), whereas phosphorylated-p38 MAPK measured 24 h after burn injury was not different from that measured in sham burned hearts (Fig. 2C). SDD treatment attenuated the burn-related increase in myocardial phosphorylated p38 MAPK 2 and 4 h after burn injury (Fig. 2).

NF-κB Activation

Burn trauma in vehicle-treated rats promoted NF-κB nuclear translocation 2 h postburn compared with that measured in time-matched sham burns (Fig. 3). Specific binding of NF-κB was confirmed by cold competition assay with 40- and 100-fold excess of unlabeled NF-κB oligonucleotide. As shown in Fig. 3, cold competition assay with 40-fold excess unlabeled NF-κB had no effect; similarly, 40-fold excess AP-1 oligonucleotide had no effect (data not shown). As also shown in Fig. 3, SDD administration attenuated the burn-mediated NF-κB activation at all time periods studied. Because multiple independent gel-shifts were performed on several animals from each experimental group at each time postburn (N = 4–5 animals per group per time period), the gel shifts were examined by densitometric analysis, and the data are summarized in Fig. 3, bottom.

The NF-κB heterodimer is composed of p50 and p65 subunits, and Western blots were performed to confirm NF-κB activation by translocation of p50 and p65 subunits to the nucleus. Representative Western blots of p65 are shown in Fig. 4, top, whereas Fig. 4, bottom, summarizes the densitometric analysis of multiple Western blots for p50 in each group at each time period (N = 4–5 rats per group per time period). Translocation of p50 and p65 from the cytosol to the nucleus was confirmed in vehicle-treated burns, and these changes were attenuated by SDD treatment in burn injury.

Cardiomyocyte Secretion of Inflammatory Cytokines

To determine whether SDD before burn injury altered myocardial inflammatory cytokine responses, cardiomyocytes were prepared 24 h postburn from subsets of animals from each of the four experimental groups. Myocytes prepared from vehicle-treated burn rats secreted significantly more TNF-α (Fig. 5, top), IL-1β (Fig. 5, bottom), IL-6 (Fig. 6, top), and IL-10 (Fig. 6, bottom) compared with cytokines secreted by myocytes prepared from vehicle-treated sham rats (P < 0.05). These data confirm our previous reports showing that burn trauma promotes a robust myocardial inflammatory response (7, 19, 20, 25). In contrast, cardiomyocytes prepared from burned rats given oral antibiotic therapy secreted significantly less cytokines compared with cytokine levels secreted by myocytes prepared from vehicle-treated burn rats (Figs. 5 and 6, P < 0.05).

Cardiomyocyte Sodium/Calcium Responses to Burn Injury and Antibiotic Therapy

Oral antibiotic therapy in sham burns did not alter either intracellular calcium (Fig. 7, top) or sodium (Fig. 7, bottom). Burn injury in the absence of SDD produced significant cardiomyocyte accumulation of sodium and calcium compared with cation levels measured in cardiomyocytes prepared from the respective sham burned groups (P < 0.05). In contrast, SDD treatment attenuated burn-related cardiomyocyte accumulation of sodium and calcium.

Cardiac Contractile Function

We then determined whether burn-related alterations in myocardial signaling and cytokine responses were associated with differences in cardiac function, and we examined whether SDD altered burn-related myocardial defects. As seen in Fig. 8, hearts from sham burned animals had similar systolic (+dP/dtmax) and diastolic (−dP/dtmax) responses to either increases in left ventricular volume (A) or increases in perfuse calcium (B) regardless of SDD administration in group 2 sham rats. Hearts from vehicle-treated burn rats had significant decreases in systolic performance as indicated by lower LVP as well as a reduced rate of maximal pressure generation compared with

Fig. 8. Burn injury in the absence of SDD significantly reduced left ventricular developed pressure (LVP; left), +dP/dtmax (middle), and −dP/dtmax (right) responses to either increases in left ventricular volume (A) or increases in perfuse calcium (B) compared with values generated by hearts from sham-burned animals. SDD administration in burned animals attenuated burn-related myocardial contractile defects. All values are means ± SE. *Significant difference among groups at P < 0.05.

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ORAL ANTIBIOTICS PREVENT BURN-RELATED MYOCARDIAL DYSFUNCTION

A

B

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hearts from sham burned animals (P < 0.05). These hearts also had impaired diastolic function as indicated by decreased ventricular relaxation rates (−dP/dt_{max}) compared with values measured in sham burned animals. These deficits were evident from the responses to either increases in preload (Fig. 8A) or to increases in perfusate calcium concentration (Fig. 8B). Systolic and diastolic function in SDD-treated burn rats was similar to that seen in sham burn rats, confirming that antibiotic therapy directed to neutralize pathogens from the digestive tract confers resistance to burn-related myocardial dysfunction.

DISCUSSION

We had hypothesized that gut-derived mediators initiate an inflammatory signaling cascade in the myocardium that culminates in myocardial contractile dysfunction. To test this hypothesis, we examined several aspects of a myocardial inflammatory signaling cascade shown to occur after major burn injury (2, 7, 20, 38, 39). Specifically, we examined burn-related changes in myocardial p-PKCε and phosphorylated p38 MAPK because phosphorylated forms have been described as activated kinases. Furthermore, these kinases function downstream from the Toll/IL-1 receptor complex, a signaling complex shown to play a pivotal role in the recognition of bacterial products such as endotoxin and shown to be present in the heart. In this present study, increases in myocardial p-PKCε and phosphorylated p38 MAPK and nuclear translocation of NF-κB were paralleled by increased cardiomyocyte secretion of proinflammatory cytokines and profound myocardial contractile defects. In our study, an oral antibiotic therapy, shown previously to decontaminate the digestive tract (45, 46), attenuated burn-related inflammatory signaling cascades, attenuated cardiomyocyte secretion of inflammatory cytokines, and improved myocardial contractile function. These data suggest that gut derived pathogens/microbial products initiate inflammatory signaling cascades in downstream organs such as the heart, contributing to postburn organ injury and dysfunction.

During the last 20 years, we have developed excellent model systems for examining the pathophysiology of postburn myocardial abnormalities, and the use of these models has defined the adverse myocardial inflammatory responses that contribute to myocardial mechanical defects. Although we have identified a number of proinflammatory mediators (including TNF-α and IL-β) that contribute to the overall pathogenesis of postburn myocardial injury and dysfunction (7, 19, 25, 26), and we have identified a number of the intracellular signaling pathways by which cardiomyocytes respond specifically to cutaneous burn injury, we have not, to date, identified the mechanisms by which cutaneous burn injury provides a stimulus specifically to the cardiomyocyte, triggering myocardial inflammation. We have shown previously that splanchic hypoperfusion occurs after burn trauma, contributing to the loss of intestinal barrier function and promoting translocation of bacteria to the mesenteric lymph nodes, liver, and spleen. However, in that previous study, blood cultures remained negative after burn injury (20). Our time-course studies have shown that splanchic hypoperfusion is a relatively early event in the postburn period and is corrected by aggressive fluid resuscitation to replace isotonic fluid loss. Other studies have shown that cardiodepressant mediators that appear in the mesenteric lymph after burn injury contribute to postburn myocardial depression (9, 11, 31, 47). Whether mediators contained in mesenteric lymph exert their cardiodepressive effects via the CD14/Toll/IL-1 signaling pathway remains unclear (1, 32, 38, 39). However, it is clear that the heart has an intrinsic infection-sensing system; components of the Toll/IL-1 pathway (myD88, IRAK, and TRAF6) are expressed in the heart, and receptors that activate the Toll/IL-1 pathway including TLR2, TLR4, and IL-1 receptor 1 have been identified in cardiac tissue (5, 13, 22, 43). Furthermore, our recent studies have provided compelling evidence that Toll/IL-1 signaling mediates myocardial contractility responses to either sepsis or burn trauma (38, 39) Thus TLR4, while playing a pivotal role in immune recognition of gram negative infection, is also the primary sensor of myocardial depressant activities in a noninfectious injury such as burn trauma.

SDD included oral administration of nonabsorbable antibiotics to eliminate pathogens from the digestive tract. Over the last 20 years, 25 clinical trials have evaluated SDD in European intensive care units (8, 16, 23, 33), and yet SDD has had limited experimental or clinical application in North America. Clinical application of SDD in 31 patients with burn >30% TBSA confirmed a reduced incidence of respiratory tract infection, bacteremia, and mortality while the length of hospital stay was unaltered (28). Experimental studies of SDD in thermal injury have described that oral antibiotic therapy in rats with burn over 40% full thickness scald injury, prevented burn-related immunosuppression, and reduced postburn mortality (45, 46). A review by Van Saene and colleagues (40) in 1991 described that SDD therapy is not widely used because of “... praxis of opinion over evidence.” This review further proposed that the unfair and unfavorable criticism of SDD suggests the need for well-designed prospective studies in North American patient populations. Recent interest has focused on newer antibiotics and bacteriophage therapies, and there appears to be little, if any, interest in the use of older antibiotics that are inexpensive and no longer under patent. Despite underutilization of this therapeutic strategy, SDD reduces morbidity and mortality. The data presented herein suggest that the protective effects of SDD are related to a downregulation of inflammatory signaling cascades and decreased secretion of inflammatory cytokines. Our data further suggest that SDD provides an attractive and inexpensive therapeutic regimen in patient populations at increased risk for infectious complications and increased mortality.

REFERENCES


