TLR4 inactivation and rBPI21 block burn-induced myocardial contractile dysfunction

JAMES A. THOMAS,1,2 MAY F. TSEN,1 D. JEAN WHITE,3 AND JURETA W. HORTON3

Departments of 1Pediatrics, 2Molecular Biology, and 3Surgery,
University of Texas Southwestern Medical Center, Dallas, Texas 75390

Received 30 December 2001; accepted in final form 7 June 2002

Thomas, James A., May F. Tsen, D. Jean White, and Jureta W. Horton. TLR4 inactivation and rBPI21 block burn-induced myocardial contractile dysfunction. Am J Physiol Heart Circ Physiol 283: H1645–H1655, 2002—Both large burns and severe gram-negative sepsis are associated with acute myocardial contractile dysfunction. Because others have reported that burn injury may be followed by transient endotoxemia, we hypothesized that bacterial endotoxin induces contractile impairment after burn trauma. We tested this hypothesis in two rodent models. In each model, postburn myocardial contractility was assessed using Langendorff preparations of excised hearts. In the first model, mice expressing either a mutant form of or no Toll-like receptor 4 (TLR4), a critical element of the mammalian endotoxin receptor, were resistant to postburn myocardial contractile dysfunction. In the second model, starting 30 min or 4 h after burn injury, rats were infused with recombinant bactericidal/permeability-increasing protein (rBPI21), a protein that binds and neutralizes endotoxin. Hearts from rBPI21-treated animals were completely protected from postburn contractile impairment. Because burn-induced contractile dysfunction can be prevented either by blocking signaling through the endotoxin receptor or by neutralizing circulating LPS, bacterial endotoxin may contribute to impaired myocardial contractility after burn injury.

burn injury; contractile function; signal transduction; transgenic animals; endotoxin; recombinant bactericidal/permeability-increasing protein

MAJOR BURN TRAUMA provokes cardiac injury and contractile dysfunction. Myocardial cellular disruption and hemodynamic alterations, including decreased cardiac output, shock, and left ventricular (LV) failure have been documented in burned patients (36, 44, 50, 63). Deficits in myocardial contraction and relaxation have also been described in rats, guinea pigs, rabbits, and sheep after major burn injury (1, 6, 21, 22, 24, 26, 27, 35, 64). The contractile deficits are transient, appearing 2 h and resolving by 72 h after the burn, and occur despite fluid resuscitation to maintain adequate preload (21).

The molecular basis of burn-induced cardiac dysfunction is complex and incompletely understood. The delay between thermal injury and onset of impaired contractility suggests a multistep process, involving long- and short-range signaling and new gene expression. Intervention studies implicate proinflammatory molecules as major contributors to impaired contractility. Initial studies highlighted the role of oxygen-derived free radicals and leukocyte-derived products as mediators of contractile dysfunction (25–27). Additional investigations have underscored the importance of different proinflammatory mediators in this cardiac response. For example, TNF-α inhibition prevents postburn myocardial dysfunction (17). Where each of these agents operates in the cascade of events that starts with tissue damage at the burn site and culminates in impaired contraction and relaxation of the heart, however, needs to be established.

Endotoxin and septic shock also cause acute cardiac contractile dysfunction. Administration of LPS to either human beings or experimental animals leads to impaired contractility that is independent of preload and afterload (2, 38, 52). As with burn injury, this effect is both transient and reversible, with no apparent long-term consequences. Furthermore, interventions that block LPS-triggered intracellular signals protect against myocardial dysfunction. Inhibition of NF-kB, a critical transcription factor in the host response to infection and injury, as well as genetic deletion of IL-1 receptor-associated kinase, a kinase in the Toll/IL-1 signal transduction pathway, disrupt intracellular signaling responses to LPS and attenuate LPS-induced myocardial dysfunction (Refs. 9, 19, and 48, and unpublished results). Similarly, patients with septic shock exhibit defective systolic and diastolic ventricular function (12, 39, 40), but cardiac function returns to presepsis baseline in survivors.

Gut-derived endotoxin may play a role in several postburn complications, including early death, multiple organ failure, systemic inflammatory response syndrome, and increased susceptibility to infection (8, 13, 16, 30, 66, 67). Many of these studies, however, have been complicated by the inability to document endotoxemia consistently after burn injury. Endotoxin has been detected in the first hour after burn injury in both

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.
patients (11, 60, 62) and experimental animals (8, 57, 67). In general, postburn endotoxemia has been seen after large burns accompanied by inadequate fluid resuscitation or in a burn injury model, with aggressive fluid resuscitation, complicated by sepsis (49). In contrast, other investigators have been unable to demonstrate significant endotoxemia after burn injury, particularly in animals or humans with small to moderate burns [<25% total body surface area (TBSA)] and after adequate fluid resuscitation. Most studies have sampled systemic venous blood for evidence of endotoxin translocation for an endogenous site of gram-negative colonization, even though higher LPS concentrations might be expected in the portal venous or mesenteric lymphatic compartments. In fact, systemic endotoxemia may represent a spillover phenomenon or a failure to contain LPS within the portal circulation. Moreover, quantitation of circulating endotoxin is technically challenging, requiring meticulous collection and measurement. Thus, whereas the failure to detect endotoxin after burn injury may indicate its absence from the circulation, it may also result from sampling a suboptimal compartment or insensitive assay technique.

Because of the similarity between LPS- and septic shock-induced myocardial depression and the contractile dysfunction seen after major burn injury, we hypothesized that endotoxin plays a role in postburn cardiac depression. The inherent difficulties in directly detecting LPS after burn injury, however, forced us to adopt a novel strategy to test this hypothesis. With the use of two experimental approaches, one genetic and one pharmacological, that render animals insensitive to the effects of endotoxin, thereby bypassing the need to document endotoxemia, we could determine whether this molecule contributed to postburn contractile dysfunction. In the first set of experiments, mutant mice that are unresponsive to endotoxin received a large burn injury, and their hearts were assayed for burn-induced contractile dysfunction. In the second study we examined the myocardial responses of burned rats treated with the endotoxin inhibitor recombinant bacterial/permeability-increasing protein (rBPI21). Results from these studies suggest that LPS may play a significant role in burn-triggered myocardial contractile dysfunction.

MATERIALS AND METHODS

**Experimental animals.** Two animal species were used for these experiments. Mice were used to determine the involvement of the principal LPS sensor, Toll-like receptor 4 (TLR4), in postburn contractile dysfunction. The availability of different TLR4 mutant and comparable wild-type (WT) strains make the mouse an ideal species for this type of genetic experiment. The need to maintain a patent intravenous catheter to administer a continuous infusion over a prolonged period dictated the choice of rats as experimental subjects for the LPS inhibitor experiments. Previous studies in this and other laboratories have shown nearly identical cardiac responses of both mice and rats to severe burn injury (61, 65), suggesting that similar mechanisms of burn-induced myocardial depression may be operative in the two species.

All animals were used in compliance with the guidelines established by the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern (UTSW) Medical Center and performed in accordance with National Institutes of Health guidelines for the use of laboratory animals. C3H/HeJ (TLR4 mutant; Jackson Laboratories; Bar Harbor, ME) and WT C3H/HeN mice (Harlan; Indianapolis, IN) of both sexes were between 9 and 10 wk of age at the time of the experiment. C57BL/10ScNcr (ScN; TLR4 null) mice were purchased (National Cancer Institute; Frederick, MD) and C57BL/10ScSn (ScSn) mice were bred in specific pathogen-free facilities in our animal colony at UTSW Medical Center. Sprague-Dawley rats (Harlan) weighed between 300 and 350 g when studied.

**Genotyping of TLR4-deficient mice.** ScSn (TLR4 WT) and ScN (TLR4 deficient) mice were genotyped using PCR. The ScSn strain is the progenitor for C57/10ScCr (ScCr), which is both LPS and IL-12 unresponsive (7, 59). The ScCr mouse possesses a 70-kb deletion that encompasses much of the TLR4 gene, resulting in no expression of TLR4 protein (41, 72). Mouse genomograms were generated using genomic DNA from tail biopsies and primers that either flank the deletion or amplify WT sequence absent from the TLR4-deficient strains. The CR delete primer pair (sense strand sequence: 5'-GCA AGT TTT CCA TTT CTA CAT TCT C-3'; antisense strand sequence: 5'-CTT CCA TTT CCA ATA GGT AG-3') spans the deleted region in ScCr mice and generates a 140-bp amplicon. The 80K primer pair (sense strand sequence: 5'-ATG ATG ATC ACC ACA G-3'; antisense strand sequence: 5'-TTT CCA TTT CGT CGC TAT AG-3') anneals to the sequence deleted in ScCr mice and generates a 390-bp amplicon. Genomic DNA was prepared from tail biopsies as previously described (56). Five hundred nanograms of genomic DNA were amplified using the following cycling parameters for each primer pair: 94°C for 2 min once, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and finishing with 72°C for 5 min. PCR products were analyzed using horizontal gel electrophoresis through a 1.2% agarose-0.5% Tris-borate-EDTA gel stained with ethidium bromide.

**Mouse burn procedure.** Mice were deeply anesthetized (methoxyflurane), and their sides and back were carefully shaved from the base of the tail to the base of the neck. Animals were then assigned to the following sham burn or burn-injured groups: 1) sham-treated C3H/HeN (TLR4 WT), 2) sham-treated C3H/HeJ (TLR4 mutant), 3) burn-injured C3H/HeN, 4) burn-injured C3H/HeJ, 5) sham-treated ScSn (TLR4 WT), 6) sham-treated ScN (TLR4 null), 7) burned ScSn, and 8) burned ScN. Mice underwent a 40% TBSA burn injury by application of brass probes (2 x 3 cm with a 3 mm thickness) heated to 100°C in boiling water to the animals' side and back for 5 s. Mice were given lactated Ringer (LR) fluid resuscitation (4 ml/kg -1-percent burned surface area) intraperitoneally. All animals received analgesics (0.05 mg/kg im butorphanol) every 8 h after burn trauma (61). Animals were monitored closely for the first 8 h after burn trauma to determine adequate recovery from the anesthesia, animal responsiveness to external stimuli, the absence of pain, and the ability to consume food and water.

**Rat cannulation, burn procedure, and rBPI21 infusion.** Rats were anesthetized lightly with methoxyflurane 18–20 h before the burn injury. Body hair on the side, back, and neck was closely clipped, and the neck region was treated with a surgical scrub. The right external jugular vein was exposed, and a polyethylene (PE)-50 catheter was inserted for the administration of fluids and rBPI21. The catheter was filled with heparinized saline and exteriorized at the nape of the neck with silk sutures via a subcutaneous tunnel.
The following day, rats were then assigned to sham burn or burn groups. The rats were deeply anesthetized (methoxyflurane), secured in a template device, and then received a 40% TBSA burn injury by immersion in 100°C water for 10 s to achieve a full-thickness burn. Animals designated for the sham burn group received identical regimens of anesthesia and handling but no burn injury was given. Sham-injured animals underwent the following treatments: 30 min after sham burn: 1) no treatment, 2) treatment with vehicle [6 ml of 5 mM sodium citrate and 150 mM NaCl (pH 5), with 0.2% poloxamer 188 and 0.002% polysorbate 80] alone, or 3) treatment with rBPI21, (XOMA; Berkeley, CA, reconstituted in vehicle per manufacturer’s instructions) as a continuous infusion (1 mg·kg⁻¹·h⁻¹) until the hearts were removed for Langendorff preparations 24 h after sham burn. Burn-injured rats were subjected to the following interventions: 1) fluid resuscitation with LR solution (4 ml·kg⁻¹·percent burned surface area⁻¹), with one-half administered in the first 8 h and the second half given in the ensuing 16 h; 2) fluid resuscitation (4 ml·kg⁻¹·percent burned surface area⁻¹) + vehicle; 3) fluid resuscitation + rBPI21, as a continuous infusion (1 mg·kg⁻¹·h⁻¹) beginning 30 min after injury (early BPI); or 5) fluid resuscitation + rBPI21, as a continuous infusion beginning 4 h after injury (delayed BPI). This last group was included to simulate the clinical scenario of the delayed arrival of burn-injured patients to a regional burn center. rBPI21 was kindly provided by XOMA for these experiments.

All rats received analgesic (0.05 mg/kg im buprenorphine) every 8 h after burn trauma (61). Animals were monitored closely for the first 8 h after burn trauma to determine adequate recovery from the anesthesia, animal responsiveness to external stimuli, the absence of pain, and the ability to consume food and water.

**Langendorff-perfused hearts.** To examine cardiac contractile function, awake mice from all experimental groups were anticoagulated with heparin sodium (100 units, Elkins-Sinn; Cherry Hill, NJ) and killed by cervical dislocation 24 h after injury. Rats were heparinized with 1,000 units and decapitated. Hearts were rapidly removed and placed in ice-cold (4°C) Krebs-Henseleit bicarbonate-buffered solution containing (in mM) 118 NaCl, 4.7 KCl, 21 NaHCO₃, 2.5 CaCl₂ in mice (1.25 CaCl₂ in rats), 1.2 MgSO₄, 1.2 KH₂PO₄, and 11 glucose). All solutions were prepared with demineralized, deionized ultrapure water (water purification system, Hydro Picosystem, Hydro Services and Supply; Durham, NC). This solution was then filtered (Millipore filter system, 0.22 μm) and bubbled with 95% O₂-5% CO₂ (pH 7.4; Po₂, 555 mmHg; Pco₂, 38 mmHg). All instrumentation in contact with the hearts was routinely acid washed to render it pyrogen free. PE-50 Intramedic tubing was placed in the ascending aorta in mice and a 17-gauge cannula was used in rats 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 38.6 ± 0.5°C, and the coronary arteries were perfused by retrograde flow through the aortic stump cannula using a constant-flow pump (model TIA, Ismatec, Cole Palmer; Vernon Hills, IL). Hearts from sham- and burn-injured animals were perfused in pairs with the same perfusion apparatus with aliquots of the same batch of Krebs-Henseleit solution, guaranteeing identical perfusion conditions for hearts from animals receiving different injuries. Contractile function was assessed in mice by measuring intraventricular pressure with Intramedic tubing (PE-50) threaded into the LV. In rats, contractile function was assessed by measuring intraventricular pressure with a distilled H₂O-filled latex balloon attached to a PE tube and threaded into the LV through the apex of the heart. LV pressure (LVP) was measured with a Statham pressure transducer (model P23 ID, Gould Instruments; Oxnard, CA) attached to the cannula, and the rates of LVP rise (+dP/dt) and fall (–dP/dt) were obtained using an electronic differentiator (model 7P20C, Grass Instruments; Quincy, MA), recorded (model 7DWL8P, Grass Recording Instruments), and transferred to a Dell Pentium computer. Starling relationships were determined by plotting LV systolic pressure and ±dP/dtmax values against increases in LV volume (rats only). Incremental increases in either coronary flow or perfusate Ca²⁺ concentration have been shown to improve ventricular performance. Thus, in our studies, hearts isolated from mice, as well as rats, were studied by increasing coronary flow and by examining ventricular function-flow rate relationships. Similarly, LVP and ±dP/dtmax responses to increases in perfusate calcium were also plotted for both rats and mice. Hearts were paced through an electrode attached to the right atrium (4.8–5.0 A for 1-ms duration, Grass Stimulator).

**Measurement of LPS in burn serum.** After burn injury, rats were deeply anesthetized and exsanguinated by cardiac puncture. Serum was fractionated by centrifugation in endotoxin-free blood collection tubes (supplied by XOMA). Endotoxin assays were conducted using Plasma-QCL assay kits (BioWhittaker; Walkersville, MD) as described by the manufacturer. Before analysis, plasma samples were first diluted 1:20 with 10 mM MgCl₂ and then heated at 70°C for 15 min. Aliquots of each sample were also spiked to contain a known amount of endotoxin before dilution and heating to assess potential sample-dependent inhibition or enhancement. Escherichia coli 055:B4 LPS (BioWhittaker) was used as the control endotoxin.

**Statistical analysis.** LVP, ±dP/dt, and –dP/dt were each analyzed as a function of treatment group and either coronary flow rate or calcium level. ANOVA followed by the Student-Newman-Keuls procedure were performed to determine differences among treatment groups. Finally, Satterthwaite’s approximation was used to determine the degrees of freedom for the studentized range critical values at the 0.05 level of significance.

**RESULTS**

**LPS-hyporesponsive mice do not develop burn-induced myocardial contractile dysfunction.** If endotoxin contributes to burn-triggered cardiac contractile dysfunction, endotoxin-resistant mice should exhibit normal cardiac contractility after burn injury. Several LPS-resistant mouse strains have been described. The molecular basis for nonresponsiveness has been determined for two of these strains, and both involve Tlr4 mutations. Poltorak and colleagues (41) have demonstrated that C3H/HeJ mice carry a point mutation in Tlr4 that results in expression of a TLR4 protein incapable of responding to LPS.

The cardiac responses to burn trauma were first compared in the WT C3H/HeN and endotoxin-hyporesponsive C3H/HeJ (TLR4 mutant) mice. Hearts were harvested 24 h after burn trauma (or sham burn), and contractile function was assessed using a modified Langendorff isolated perfusion preparation. As seen in Fig. 1A, hearts from burn-injured C3H/HeN mice, which express WT TLR4, exhibited substantial systolic...
and diastolic contractile dysfunction 24 h after injury. LV developed pressure and the rate of pressure development in response to increasing coronary flow were significantly depressed compared with sham-burn WT hearts. Additionally, myocardial relaxation was impaired in burned C3H/HeN mice compared with sham-treated animals. This myocardial dysfunction is consistent with cardiac depression previously described in burned mice (61). In contrast, hearts from endotoxin-resistant C3H/HeJ mice exhibited no impairment in LV pressure generation and maximal rate of LV pressure fall (−dP/dt max) assayed diastolic performance. A: response of WT and TLR4 mutant hearts to increasing coronary flow rates. B: response of hearts to increasing extracellular Ca²⁺ concentration. Data are expressed as means ± SE and are the results of 1 representative trial. The experiment was repeated once with nearly identical results.

The preceding results indicate that one mutant Tlr4 allele blocks the development of burn-triggered myocardial dysfunction. This effect could occur because WT TLR4 protein is required for the impaired contractility after burn injury. Alternatively, the resistance to dysfunction could signify that the mutant TLR4 protein interferes with the function of another molecule or pathway normally responsible for the cardiac response to burns. To determine whether TLR4 is required for burn-induced contractile depression, the response of mice with a second Tlr4 allele was examined. ScCr
mice are homozygous for a mutant Tlr4 that prevents expression of any TLR4 protein and are LPS unresponsive (41, 43). The ScCr strain, however, also has a second mutation, in the gene encoding the IL-12 receptor β-subunit (42), that might affect the animal’s response to burn injury. The ScCr line arose from the ScSn strain, which is also LPS unresponsive but lacks the IL-12 receptor gene mutation (A. Poltorak, personal communication). Although it seemed likely a priori that the parental strain harbored the same Tlr4 mutation as the ScCr mice, the ScN genotype has not, to our knowledge, been reported. Therefore, before testing their contractile responses to burn injury, ScN mice were examined for the same Tlr4 mutation present in ScCr mice: a 74-kb deletion that results in a null Tlr4 allele (43). With the use of primer pairs that amplify a part of the region deleted in ScCr mice and that flank the deletion, genomic DNA from unrelated WT (C57BL/6), congenic control (ScSn), ScCr, ScN, and F1 progeny (first-generation offspring) from a WT × ScN intercross were compared for different Tlr4 alleles. As seen in Fig. 2, lanes 2 and 3, both WT and control (ScSn) mice have the WT Tlr4 allele. ScN mice, on the other hand, carry the same large genomic deletion present in ScCr mice, as demonstrated by the amplification of a 140-bp fragment that spans the deletion breakpoint (Fig. 2, lanes 5 and 6). Finally, F1 progeny resulting from mating B6 and Scn mice are heterozygous for WT and null Tlr4 alleles (Fig. 2, lane 4). Thus Scn mice share the same Tlr4 mutation as the ScCr animals derived from them.

The contractile responses to burn injury were then compared in WT ScSn and TLR4-deficient Scn mice. Animals received a thermal or sham injury as before; 24 h after injury, the hearts were removed, and contractile function was assessed using the modified Langeland procedure. Sham injury induced no change in contractility in mice of either genotype (Fig. 3A). Ternally injured TLR4 WT ScSn animals demonstrated impaired systolic and diastolic function in response to increasing coronary flow (Fig. 3A). In contrast, TLR4-deficient Scn mice were resistant to burn-induced contractile function, exhibiting maximal LVP, +dP/dt, and −dP/dt responses to increasing coronary flow that mimicked those observed in sham-burned mice (Fig. 3A). The pattern of myocardial responsiveness to increasing perfusate calcium concentration was the same as seen with increasing coronary flow. WT ScSn mice exhibited significant contractile impairment after burn injury, whereas the response of TLR4-deficient Scn mice was no different from unburned animals (Fig. 3B). The combined results of these experiments with C3H/HeJ and Scn mice indicate that TLR4 is required for myocardial contractile dysfunction after burn injury.

**Endotoxin inhibitor blocks burn-induced contractile dysfunction.** Although TLR4 is required for normal LPS responsiveness, other microbial macromolecules can also signal through this pathway, including lipoteichoic acid (53), a heat-sensitive cell-associated mycobacterial factor (34), and chlamydial components (47). Thus the failure of TLR4 mutant mice to develop burn-induced myocardial dysfunction could be due to resistance to a variety of molecular determinants. If LPS is responsible for this phenomenon in WT animals, specific pharmacological inhibition of this molecule should block postburn contractile depression. BPI, a protein originally isolated from the azurophilic granules of neutrophils, binds and neutralizes endotoxin (33, 37) and has been used safely in clinical trials for different types of shock (10, 18, 32). To determine whether endotoxin contributes to burn-triggered cardiac dysfunction, rBPI21 was administered after burn trauma, and myocardial contractile function was examined.

First, the effect of rBPI21 or vehicle administration on contractility in sham-injured animals was investigated. As seen in Fig. 4, neither infusion with vehicle alone nor rBPI21 given to sham-burned rats had a discernible effect on the cardiac contractility. The LV developed pressure response of vehicle- or rBPI21-treated hearts to increasing LV volume, coronary flow, or perfusate Ca^2+ concentration was indistinguishable from untreated sham-burned hearts (Fig. 4). Similarly, treatment with either vehicle or rBPI21 had no effect on ±dP/dt_{max} (data not shown). Thus neither vehicle nor rBPI21 affects cardiac contractile function in unburned rats.

---

**Fig. 2.** Scn mice lack TLR4. Genomic DNA was prepared from tail biopsies from C57BL6 (B6), ScSn, ScN, and ScCr mice as well as from a mouse resulting from a cross between B6 and Scn mice (F1 B6 × Scn). The DNA was amplified with primers that flank the deletion in chromosome 4 in ScCr mice that renders them TLR4 deficient (CR delete, expected amplicon size 140 bp) and primers that detect the WT Tlr4 allele (80K, expected amplicon size 390 bp). Reaction products were fractionated on 1.2% agarose-0.5× TBE gel. MW, molecular weight marker.
The effect of rBPI21 administration on burn-induced contractile dysfunction was then examined. Cannulated rats underwent burn and 30 min after injury fluid resuscitation with LR solution was initiated. Burned animals were then divided into the following treatment groups: 1) fluid resuscitation with LR solution alone, 2) resuscitation with LR solution plus vehicle, 3) LR solution plus rBPI21 (1 mg·kg\(^{-1}\)·h\(^{-1}\)) administered starting 30 min after burn injury, and 4) LR plus rBPI21 (1 mg·kg\(^{-1}\)·h\(^{-1}\)) starting 4 h after burn. The rates of infusion and final volume of fluid infused for the 24-h period were identical in all animals. Twenty-four hours after injury, the hearts were removed, and contractile responses to increases in preload, coronary flow, and extracellular Ca\(^{2+}\) concentration were assessed using a modified Langendorff perfusion preparation. The hearts from burned animals receiving fluid resuscitation alone or LR solution plus vehicle exhibited marked impairment in LVP, +dP/dt\(_{max}\), and −dP/dt\(_{max}\) response to increasing LV end-diastolic volume (Fig. 5A), coronary flow (Fig. 5B), and calcium concentration (Fig. 5C). In contrast, hearts from burned animals that received rBPI21 30 min after injury demonstrated neither systolic nor diastolic contractile dysfunction in response to any inotropic stimulus (Fig. 5). In fact, contractile function in hearts from rBPI21-treated burned rats was indistinguishable from hearts from sham-injured rats, indicating that early rBPI21 treatment completely blocks burn-induced myocardial dysfunction.

The preceding results using rBPI21, coupled with the finding that TLR4 mutant mice are resistant to burn-
triggered myocardial dysfunction, suggest that LPS contributes to this contractile depression. Because previous unsuccessful attempts to document circulating endotoxemia in aggressively fluid-resuscitated, uninfected animals may have been due to insensitive assay methodology, we attempted to document the presence of endotoxin in the serum of burned animals. In a parallel set of experiments, rats underwent burn injury as described. Four hours after injury, the animals were exsanguinated via cardiac puncture, and their serum assayed for endotoxin. The serum from a total of 10 burned animals was examined. Endotoxin was undetectable in all samples. Thus either endotoxin is excluded from the systemic circulation in resuscitated animals after burn injury or it circulates in a form that renders it undetectable to a standard, highly sensitive detection protocol.

In the final group of burn-injured rats, rBPI21 treatment was withheld for 4 h after injury. The interval between thermal trauma and rBPI21 administration simulated the delay associated with transferring some burn patients to a regional burn center for definitive care. Hearts from burned animals receiving rBPI21 4 h after injury showed no discernible impairment in contractile responses to either increases in LV volume, coronary flow rate, or perfusate calcium concentrations, retaining systolic and diastolic responses similar to hearts from unburned animals (Fig. 5, A–C). Thus delaying rBPI21 treatment for up to 4 h after burn injury still prevents contractile dysfunction.

**DISCUSSION**

The results of these studies indicate that endotoxin may contribute to postburn myocardial contractile dysfunction. Functional inactivation or deletion of the principal signaling LPS receptor TLR4 (as opposed to the principal binding receptor, CD14) prevents depressed cardiac contractility. Moreover, specific pharmacological inhibition of LPS activity preserves normal contractile function after major burn injury. The combination of obligate TLR4 function in impaired contractility and preservation of normal contractile function in rBPI21-treated burn-injured animals suggests that LPS participates in postburn cardiac dysfunction. On the other hand, these findings do not exclude the possibility that other determinant, which signals through TLR4 and can be inhibited by rBPI21, leads to contractile dysfunction.

If LPS is responsible for burn-induced cardiac dysfunction, what is its origin? It would seem, at least a priori, that the gastrointestinal tract is the most likely source. Both the ileum and colon contain large numbers of gram-negative organisms at higher densities than other body sites, including the skin and oropharynx. Bacterial translocation to extraintestinal sites (such as the mesenteric lymph nodes, spleen, and liver) can occur after burn injury (8, 29, 57). Furthermore, other shock states, such as hemorrhagic shock, can also lead to endotoxemia, bacterial translocation (3, 20, 45, 46), and myocardial dysfunction (5, 14, 51). Thus if the gastrointestinal tract were the source of endotoxin after burn injury, it would suggest that shock caused by different injuries induce myocardial dysfunction through a common mechanism.

It is also possible that burn-induced myocardial dysfunction results from a substance other than LPS. This activity would have to satisfy the dual requirements of signaling through TLR4 and being inhibitable by rBPI21. This substance could be of microbial origin, like LPS. Other known macromolecules, such as lipotechoic acid from gram-positive organisms, signal through TLR4 (53), but the activity of this bacterial product is not neutralized by rBPI21 (31). It may nonetheless be
Fig. 5. rBPI21 prevents burn-triggered myocardial depression. Sprague-Dawley rats underwent sham or burn injury and were then divided into 5 treatment groups: sham injury (n = 6), burn plus fluid resuscitation with lactated Ringer solution alone (n = 5), burn plus fluid resuscitation and vehicle (burn + vehicle, n = 3), burn plus fluid resuscitation and rBPI21 (1 mg·kg⁻¹·h⁻¹ continuous infusion) reconstituted in vehicle beginning 30 min after injury (burn + BPI 30 min), n = 9], and burn plus immediate fluid resuscitation followed by rBPI21 in vehicle beginning 4 h after injury (burn + BPI 4 h), n = 5). Twenty-four hours after injury, hearts were removed, and the contractile responses to LV volume (A), increasing coronary flow (B), and increasing perfusate Ca²⁺ concentration (C) were assessed using a modified Langendorff preparation as described. Data are expressed as means ± SE and are the averages of all animals studied in each group.
possible to determine the origin of such molecules by testing them in a selective, microbe-free system.

A third possible explanation also exists: burn injury generates an endogenous TLR4-activating, rBPI21-inhibitable signal. There is, in fact, no reason why mammals might not synthesize substances (e.g., glycolipids) that resemble endotoxin in one or more ways, including the ability to trigger an innate immune response and precipitate cardiac dysfunction. This substance must simply be sequestered from the sensory apparatus (e.g., behind a membrane) under normal, uninjured conditions if the immune system is to avoid constitutive activation by or develop tolerance to the stimulus.

The present experiments used two different species of animals. The genetic studies employed mice because two different mutant alleles for the LPS sensor, TLR4, were available for study. Rats were used in the rBPI21 inhibition experiments, in contrast, because of the need to administer rBPI21 as a continuous infusion through a secure intravenous line for the 24-h study period. The need arose from the fact that rBPI21 has an extremely short half-life in serum and the most reliable dosing and pharmacokinetic data developed during preclinical and clinical trials were based on continuous intravenous infusions of the drug. Moreover, attempts to establish a continuous intravenous infusion in mice met with unacceptably high malfunction rates that precluded reliable delivery of the correct rBPI21 dose. While rats and mice do not respond identically to equivalent stimuli, they exhibit similar cardiovascular responses to burn injury and endotoxin challenges (19, 58, 61).

One provocative result from the inhibitor studies is that rBPI21 still protects the heart from burn-induced contractile dysfunction even when administration is delayed 4 h after injury. How might this observation shed light on the mechanism of burn-triggered myocardial depression? Two possible explanations could account for this effect of delayed rBPI21 treatment. First, it could indicate that a rBPI21-inhibitable cardiac depressant signal may be released more than 4 h after burn injury. rBPI21 treatment by 4 h would exert a prophylactic effect, neutralizing this activity as it appeared and thereby preventing impaired contractility. Alternatively, contractile dysfunction could result from a threshold effect. According to this view, large burns could trigger one or more prodepressant signals, possibly as early as 30 min after injury (Refs. 8 and 19 and unpublished results). These signals would accumulate in the postburn period, surpassing a certain threshold beyond which contractile function would begin to deteriorate. rBPI21 administration 4 h after injury might prevent sufficient accumulation of one or more of the signals required for contractile dysfunction, forestalling its appearance. Experiments varying the timing of rBPI21 administration should distinguish between these possibilities. In either case, this represents the second example in which an agent applied several hours after injury protects against contractile dysfunction. Hypertonic saline-Dextran (HSD) solutions also prevent cytokine production and preserve contractile function when administration is delayed after burn injury (23, 28).

The authors thank Robert Munford for thoughtful manuscript review and David L. Maass, Deborah Carlson, and Steve Carroll for assistance with endotoxin assays of burn serum. This work was supported in part National Institute of General Medical Sciences Grant 5-P50-GM-21681-37 (to J. W. Horton).

REFERENCES

2. Adams HR, Baxter CR, and Parker JL. Reduction of intrinsic contractile reserves of the left ventricle by Escherichia coli en-


43. Poltorak A, Smirnova I, Clisch R, and Beutler B.

Reynolds EM, Ryan DP, Sheridan RL, and Doody DP.

44. Poltorak A, Merlin T, Nielsen PJ, Sandra O, Smirnova I, Rhodes RS, DePalma RG, and Robinson AV.


61. White DJ, Maass D, Giroir BP, and Horton JW. Development of an acute burn model in adult mice for studies of cardiac function and cardiomyocyte cellular function. Shock 16: 122–129.


