Staphylococcal α-Toxin Provokes Coronary Vasoconstriction and Loss in Myocardial Contractility in Perfused Rat Hearts

Role of Thromboxane Generation

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Background—Cardiac performance is severely depressed in septic shock. Endotoxin has been implicated as the causative agent in Gram-negative sepsis, but similar abnormalities are encountered in Gram-positive sepsis. We investigated the influence of the major exotoxin of Staphylococcus aureus, staphylococcal α-toxin, in isolated perfused rat hearts.

Methods and Results—α-Toxin 0.25 to 1 μg/mL caused a dose-dependent increase in coronary perfusion pressure that more than doubled. In parallel, we noted a decrease in left ventricular developed pressure and the maximum rate of left ventricular pressure rise (dP/dt max), dropping to a minimum of <60% of control. These changes were accompanied by a liberation of thromboxane A 2 and prostacyclin into the coronary effluent. The release of creatine kinase, lactate dehydrogenase, potassium, and lactate did not surpass control heart values, and leukotrienes were also not detected. Indomethacin, acetylsalicylic acid, and the thromboxane receptor antagonist daltroban fully blocked the α-toxin–induced coronary vasoconstrictor response and the decrease in left ventricular developed pressure and dP/dt max, whereas the lipoxygenase inhibitor nordihydroguaiaretic acid, the platelet activating factor antagonist WEB 2086, and the α-adrenergic antagonist phentolamine were entirely ineffective. Inhibition of nitric oxide synthase even enhanced the α-toxin–induced increase in coronary perfusion pressure and the loss in myocardial performance.

Conclusions—Purified staphylococcal α-toxin provokes coronary vasoconstriction and loss in myocardial contractility. The responses appear to be largely attributable to the generation of thromboxane and are even enhanced when the endogenous nitric oxide synthesis is blocked. Bacterial exotoxins, such as staphylococcal α-toxin, may thus be implicated in the loss of cardiac performance encountered in Gram-positive septic shock. (Circulation. 2000;101:78-85.)

Key Words: vasoconstriction ■ contractility ■ toxins

In addition to vascular hyporeactivity, progressive myocardial depression is a characteristic feature of cardiocirculatory changes of septic shock and contributes to the high mortality of this disease. Despite an elevated cardiac output, myocardial performance is deteriorated, as indicated by reduced left and right ventricular ejection fractions and dilatation of both ventricles.1–4 Circulating myocardial depressant agents have been implicated in these findings; endotoxin and secondarily induced cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1, are major culprits in this context.5–9 Both endotoxin per se and TNF-α are potent inducers of the inducible nitric oxide (NO) synthase in the myocardium, and excessive endogenous NO synthesis may depress myocardial contractile performance. Conversely, NO is known to play a basic role in the regulation of myocardial blood flow, and inhibition of NO synthesis caused myocardial ischemia in endotoxemic rats.10 This does not necessarily require a reduction of global myocardial perfusion, but microcirculatory disturbances leading to insufficient regional oxygen availability may be responsible for this phenomenon.10,11

There is growing evidence that the coronary circulation in sepsis is susceptible to a maldistribution of regional blood flow that may contribute to myocardial failure. Histological changes in the myocardium of septic sheep were compatible with focal ischemia on the microcirculatory rather than systemic level.12 In endotoxic dogs, disturbances of coronary microcirculation were associated with depressed contractility,13 and septic sheep were recently noted to be unable to sufficiently augment myocardial blood flow in response to increased O2 demands.14

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The typical pattern of cardiovascular dysfunction, however, is also encountered in Gram-positive septic shock. In a canine model of septic shock, intraperitoneal application of Escherichia coli or Staphylococcus aureus provoked myocardial depression, but, as anticipated, endotoxemia was discovered only with E. coli. The predominant toxin of S. aureus is the α-toxin, the prototype of pore-forming exotoxins from Gram-positive rods, and purified α-toxin provoked cardiovascular collapse in intact animals and suppressed tension development in isolated rat atria in vitro. Although the problem of quantification of exotoxins in biological samples is not fully resolved, because of the very rapid membrane incorporation of this agent, experimental data suggest that small numbers of exotoxins suffice to activate target cells. Following this line, we now investigated the impact of α-toxin on cardiac function in the absence of endotoxin, serum, and circulating inflammatory cells in isolated perfused rat hearts. We found that the α-toxin is a potent coronary vasconstrictor, and the pressure response is accompanied by a marked depression of myocardial contractility. Interestingly, both mediator analysis and pharmacological intervention strongly indicated that the abnormalities in cardiac performance can be attributed largely to the toxin-elicted thromboxane (Tx) formation. In view of the fact that α-toxin is a representative of a large family of pore-forming exotoxins originating from Gram-positive but also Gram-negative bacteria, the present findings suggest that these bacterial agents should be considered to be contributors to cardiac abnormalities in septic shock.

**Methods**

**Materials**

Purified α-toxin from S. aureus proven to be endotoxin-free was provided by S. Bhakdi, MD (Department of Medical Microbiology, Mainz, Germany). Salmonella abortus equii lipopolysaccharide (LPS) was obtained from C. Gallano, PhD (Max Planck Institute for Immunology, Freiburg, Germany). Indomethacin was purchased from ICN Biomedicals Inc and the TXA₂ receptor antagonist daltroban (BM 13.505) from Boehringer. The α-adrenergic antagonist phentolamine, the lipoygenase inhibitor nordihydroguaiaretic acid (NDGA), and acetylsalicylic acid were purchased from Sigma; the NO synthase inhibitor 4-[(N-monomethyl-L-arginine (L-NMMA)) from Calbiochem; and the platelet-activating factor antagonist WEB 2086 from Boehringer.

**Preparation and Isolated Heart Perfusion**

Male Wistar rats (Charles River, Sulzfeld, Germany) were heparinized (heparin 1000 IU/kg) and anesthetized (pentobarbitone 60 mg/kg) by intraperitoneal injection. The hearts were rapidly excised and immersed in ice-cold Krebs-Henseleit buffer solution (KHBS). After the organ weight had been determined, the hearts were attached to a Langendorff perfusion apparatus. The hearts were retrogradely perfused at a constant flow (10 mL min⁻¹ g⁻¹ heart) with a modified KHBS containing (in mmol/L) NaCl 125, KCl 4.3, KH₂PO₄ 1.1, MgCl₂·6H₂O 1.3, CaCl₂·2H₂O 2.4, NaHCO₃ 25, and glucose 13.32. The perfusate was gassed with carbogen (5% CO₂/95% O₂). The pH was 7.4±0.03. PO₂ 500±45 mm Hg and PCO₂ 35±5 mm Hg at 37°C. All hearts were initially rinsed with 150 mL KHBS in a nonrecirculating mode before switching to recirculation (total volume 50 mL).

For monitoring coronary perfusion pressure (CPP), the aortic cannula was connected to a pressure transducer (Braun). To measure left ventricular contractility, a latex balloon attached to a second pressure transducer was inserted into the left ventricular cavity. Left ventricular developed pressure (LVDP) was calculated as the difference between peak-systolic and end-diastolic pressure (8 to 12 mm Hg), and the maximum rate of left ventricular pressure rise (dP/dtmax) was computed by a differentiator (Schwarzar P 48, Picker). The hearts were paced at 320 to 360 bpm by a Stimulator P Type 201 (Hugo Sachs Elektronik). All physiological parameters were continuously recorded on a 12-channel polygraph (Schwarzar CU 12-N, Picker). At the end of each experiment, the heart weight was measured again, and the magnitude of edema formation was calculated as the difference between heart weight before and after perfusion.

**Experimental Protocols**

After the hearts had been equilibrated for 20 minutes, time was set to zero, and staphylococcal α-toxin was admixed to the perfusate at final concentrations of 0.25 (n=6), 0.5 (n=9), and 1 (n=6) µg/mL. Physiological variables were monitored for 120 minutes. Perfusate samples were taken twice before as well as 10, 20, 40, 60, 90, and 120 minutes after toxin application. For pharmacological intervention, either indomethacin 100 µmol/L (n=6), acetylsalicylic acid 500 µmol/L (n=4), daltroban 10 µmol/L (n=4), NDGA 5 µmol/L (n=3), WEB 2086 10 µmol/L (n=3), L-NMMA 25 µmol/L (n=4), or phenolamine 5 µmol/L (n=4) was preadmixed to the perfusate after the recirculating perfusion was begun. In these experiments, α-toxin was used at a concentration of 0.5 µg/mL. Control experiments included the perfusion with only buffer fluid (n=8) and with buffer fluid enriched with the respective pharmacological inhibitors (n=3). In a separate type of study, LPS 100 ng/mL was admixed to the recirculating medium for 1 hour, followed by administration of α-toxin 0.5 µg/mL (n=3) or sham application of exotoxin (n=3). Further studies addressing α-toxin-induced morphological changes used a 1-hour and a 2-hour perfusion period in the presence of 1 µg/mL exotoxin and in the absence of inhibitors (n=3). Additional experiments using 0.5 µg/mL α-toxin were terminated after 40 minutes (n=4) to determine the increase in heart weight at this time point.

**Histological Analysis of Tissue Injury and of Residual Cells in the Myocardium**

After the perfusion was stopped, the coronary vasculature was perfused with a fixative (paraformaldehyde 4% in PBS, pH 7.4). Thin slices of the left ventricular free wall were placed in the fixative at 4°C for 1.5 hours, followed by dehydration in a graded series of acetone solutions (4°C). Tissue blocks were embedded in Immu-no-bed (Polyscience Inc) at 4°C for 12 hours. Sections 5 µm thick were cut, transferred to coated slides, and stained with hematoxylin/eosin solution. The sections were examined at a magnification of ×400 or ×250 to determine the presence of adhering and infiltrating neutrophils, eosinophils, basophils, monocytes, lymphocytes, and platelets. The total number of cells was analyzed in 10 separate fields for each tissue section and expressed as cells/mm².

**Biochemical Assays**

TXA₂, prostacyclin (PGI₂), and TNF-α were measured by commercially available ELISAs (Cayman Chemical Co; Biosource). Leuko-trienes (LTs) (LTB₄, LTC₄, LTD₄, and LTE₄) were analyzed by use of previously described chromatographic techniques. Lactate dehydrogenase (LDH), creatine kinase (CK), lactate, and potassium (K⁺) were measured by routine techniques.

**Statistical Analysis**

All data are given as mean±SEM. Data were analyzed by 1-way ANOVA followed by Tukey’s honestly significant difference test when differences among groups were to be determined. A value of P<0.05 was considered to be significant.

**Results**

Whereas control hearts displayed stable values of CPP over the observation period, α-toxin caused a rapid, dose-
dependent increase in CPP (Figure 1). At the highest dose (1 μg/mL), CPP more than doubled within <20 minutes, and almost equally high plateau values were achieved with 0.5 μg/mL α-toxin. With corresponding dose- and time-dependence, myocardial contractility was depressed in response to α-toxin (Figures 2 and 3). Within 20 minutes, a decline of LVDP and dP/dt max to values <60% below baseline occurred in the presence of 1 μg/mL α-toxin, and 0.5 μg/mL resulted in a loss of contractility of ~15%. Even the myocardial depression caused by the lowest dose of toxin (0.25 μg/mL) differed from control. In addition, α-toxin provoked an increase in heart weight, which nearly reached its maximum after 40 minutes (Figure 4). These changes in heart physiology were accompanied by a release of TxB2 and 6-keto-PGF1α, the stable metabolites of TXA2 and prostacyclin, into the perfusate (Figure 5). Markers of myocardial cell necrosis (CK, LDH, K+1) and lactate accumulated in the perfusate to some minor extent, but no significant difference between control and α-toxin–challenged hearts was noted (Table). Cysteinyl-LTs (LTC4/LTD4/LTE4) and LTB4 were not detected in response to administration of α-toxin (data not shown).

In the presence of indomethacin, the α-toxin–induced increase in CPP was totally blocked (Figure 6), and the loss of myocardial performance was completely abolished (Figures 7 and 8). This was also true using acetylsalicylic acid (data not shown). Neither TxB2 nor 6-keto-PGF1α was detected under these conditions (Figure 5). Indomethacin did, however, not affect the α-toxin–induced increase in heart weight (Figure 4). The release of CK, LDH, K+, and lactate was not changed in the presence of the cyclooxygenase inhibitor (Table).

Similarly, the specific TXA2 receptor antagonist daltroban fully blocked the rise in CPP and the depression of contractility (Figures 6, 7, and 8), whereas the synthesis of TxB2 was not affected (Figure 5). However, there was some decrease in α-toxin–induced heart weight gain in the presence of daltroban (Figure 4). In contrast, neither the lipoxygenase inhibitor NDGA, the platelet-activating factor receptor antagonist WEB 2086 (data not shown), nor the α-adrenergic receptor antagonist phenolamine affected the α-toxin–elicited coronary vasoconstriction and the loss in myocardial performance (Figures 6, 7, and 8). In the presence of the NO synthase inhibitor L-NMMA, the α-toxin–elicited increase in CPP and the decrease in contractile performance were even markedly enhanced (Figures 6, 7, and 8).
Staphylococcal α-toxin did not provoke release of TNF-α into the perfusate. Administration of LPS did not provoke any significant changes in CPP, LVDP, or dP/dt max. Moreover, previous LPS administration did not influence the vasoconstriction and the loss in myocardial performance induced by a subsequent α-toxin challenge (data not shown).

Histological examination of control hearts did not show any morphological abnormality. This was also true for hearts exposed to α-toxin 1.0 μg/mL for 1 and 2 hours, except for some myofibrillar contraction bands indicating a minor morphologically detectable injury (Figure 9). Because leukocytes and platelets might adhere to the coronary endothelium before excision of the hearts and thus might contribute to the prostanoid synthesis, we analyzed the number of these cell types in isolated hearts before onset of toxin challenge. The number of each leukocyte type or thrombocytes ranged below 2 cells/mm², thus excluding any significant contribution to the overall mediator generation.

Discussion

Pore-forming proteinaceous exotoxins have been characterized for a large variety of clinically important Gram-positive and Gram-negative bacteria, including S aureus, Streptococcus pyogenes, Streptococcus pneumoniae, E coli, Proteus spp, and Pseudomonas aeruginosa. Transmembrane pore formation with subsequent electrolyte fluxes and secondary signaling events has been noted as a basic toxin mechanism. A large number of biological effects were found to be provoked by these agents; however, their impact on the coronary vasculature and cardiac performance is largely unknown.

The present study characterized staphylococcal α-toxin as a potent inducer of cardiac abnormalities, including a marked coronary vasoconstrictor response and a severe depression of myocardial contractility. Clearly, these changes were caused by the staphylococcal exotoxin and not by any contamination with LPS and LPS-related cytokine generation. First, no LPS is detectable in the purified toxin preparation. Second, sterile tubing was used throughout, and the recirculating buffer fluid was repeatedly proved to be endotoxin-free (LPS...
20 pg/mL, the detection limit of the limulus-based LPS assay used). Third, administration of large quantities of LPS in the absence of α-toxin did not reproduce the α-toxin–elicited changes. Moreover, preapplication of LPS did not enhance the exotoxin-induced changes. These data do not exclude a role of endotoxin in eliciting cardiac abnormalities in sepsis; however, under the present experimental conditions, no such effect was demonstrated in rat hearts.

Although the primary feature of staphylococcal α-toxin, its capability to induce circumscribed membrane lesions, might favor the assumption that the cardiac abnormalities are caused via overt cell damage, this is evidently not the case. Markers of cell injury (CK, LDH, K⁺) did not differ between toxin-free and toxin-perfused hearts, and microscopic studies after application of α-toxin showed no signs of endothelial or myocardial cell necrosis. Instead, the present data collectively indicate that the α-toxin–elicited cardiac abnormalities are largely attributable to toxin-induced thromboxane formation: (1) the formation of this vasoconstrictor agent was enhanced in toxin-treated hearts; (2) the cyclooxygenase inhibitors indomethacin and acetylsalicylic acid fully blocked thromboxane synthesis, coronary vasoconstrictor response, and depression of myocardial contractility; and (3) the same effects on heart physiology were caused by the specific inhibitors daltroban and phentolamine.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control Mean (SEM)</th>
<th>α-Toxin Mean (SEM)</th>
<th>α-Toxin + Indomethacin Mean (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK, U/L</td>
<td>61.7 (18.1)</td>
<td>47.8 (9.0)</td>
<td>54.0 (5.7)</td>
</tr>
<tr>
<td>LDH, U/L</td>
<td>34.3 (8.8)</td>
<td>52.0 (9.8)</td>
<td>48.2 (8.0)</td>
</tr>
<tr>
<td>K⁺, mmol/L</td>
<td>5.14 (0.06)</td>
<td>5.00 (0.07)</td>
<td>5.07 (0.03)</td>
</tr>
<tr>
<td>Lactate, mg/dL</td>
<td>5.4 (1.0)</td>
<td>6.8 (1.2)</td>
<td>6.5 (1.2)</td>
</tr>
</tbody>
</table>

Figure 6. Influence of indomethacin 100 μmol/L, daltroban 10 μmol/L, phentolamine 5 μmol/L, and L-NMMA 25 μmol/L on α-toxin 0.5 μg/mL–induced increase of CPP after 45 minutes. Control experiments using identical inhibitor concentrations were performed in absence of α-toxin. In these control studies (not shown in detail), no influence of indomethacin, daltroban, phentolamine, or L-NMMA on baseline CPP was noted. Mean ± SEM of at least 4 experiments each is given. *Significant difference from control. **Significant difference from control and α-toxin.
thromboxane receptor antagonist daltroban, whereas thromboxane generation was unaffected by this agent. These findings are in line with previous studies of rabbit lungs, in which thromboxane-mediated vasoconstriction was noted to be the main contributor to α-toxin–elicited acute pulmonary hypertension.30,31 The strong vasoconstrictive potency of thromboxane evidently surpasses the vasodilatory capacity of prostacyclin, which is generated with comparable kinetics but in higher quantities in response to the α-toxin challenge; low numbers of prostacyclin receptors in the coronary circulation might contribute to this finding. In contrast, cysteinyl-leukotrienes, another eicosanoid species with vasoconstrictive potency, were not discovered in α-toxin–challenged hearts, and the lipoxygenase inhibitor NDGA did not suppress the cardiac abnormalities. Although thromboxane generation in isolated rat hearts has been described in several studies, the source of thromboxane in the rat hearts still remains unclear. However, thromboxane generation by endothelial cells of rat aorta, in addition to rat vascular smooth muscle cells, was described.32,33 Moreover, endothelial cells were identified as the source of α-toxin–induced prostanoid liberation.34 The coronary endothelium may well be a candidate for the α-toxin–induced thromboxane generation in the isolated rat heart. The mode of action by which α-toxin induces synthesis of this prostanoid remains to be established. In vitro studies in different cell types, not originating from the coronary vascular bed, suggested that transmembrane calcium flux via the toxin-elicited discrete pores may be a decisive step in the induction of eicosanoid synthesis.34,35

The coronary vasoconstriction in α-toxin–perfused hearts was accompanied by edema formation, plateauing after 40 minutes. At first glance, this could be attributed to enhanced pressure-induced fluid filtration; however, the edema was largely unaffected by the pharmacological interventions blocking the exotoxin-elicited pressor response, thus characterizing this finding as an independent event. In endothelial monolayers, α-toxin was shown to increase permeability for water and albumin by forming large intercellular gaps.36 This event was clearly independent of hydrostatic pressure and was explained by direct activation of endothelial cells with subsequent cytoskeleton rearrangement. Further studies are necessary to address the question of whether the α-toxin–evoked increase in endothelial permeability, as noted, for example, in rabbit lungs in response to this agent,30,37 is the responsible underlying event.

Our most impressive finding was that staphylococcal α-toxin causes a rapid, dose-dependent decrease in myocardial contractility. Moreover, there is strong evidence that this negative inotropism is strictly related to the α-toxin–induced formation of thromboxane. Time- and dose-dependence of
the decrease in LVDP and dP/dt\(_{\text{max}}\) matched that of the toxin-induced coronary vasoconstriction very well, and all contractile abnormalities were fully blocked on cyclooxygenase inhibition and by a specific thromboxane receptor antagonist. This observation largely rules out a direct effect of α-toxin on cardiomyocyte function as underlying event. Because of the constant perfusion flow, supervening the overall increase in coronary vascular resistance, global ischemia of the myocardium may also not account for the cardiodepression in the present study. In toxin-exposed rabbit lungs, the thromboxane-mediated pulmonary vasoconstriction is accompanied by a dramatic maldistribution of perfusion,\(^3\) and such a phenomenon, resulting in regions of lungs, the thromboxane-mediated pulmonary vasoconstriction and depression of contractility were even further enhanced in the presence of L-NMMA, suggesting that endogenous vascular NO partly antagonizes the strong and hypothetically regionally uneven vasoconstriction by α-toxin–elicited thromboxane. Such beneficial vascular effects of NO might therefore overcome putative disadvantageous effects of this agent on cardiomyocyte function, as discussed above. Although perfusion maldistribution offers an attractive explanation for the present findings, further studies are clearly necessary to verify this hypothesis.

In conclusion, purified staphylococcal α-toxin exerts profound effects on rat cardiac function in the absence of circulating blood cells, plasmatic mediator systems, and endotoxin-elicited cytokine generation. Coronary vasoconstriction and depression of myocardial contractility represent the key changes, and both mediator analysis and pharmacological interventions strongly suggest that α-toxin–elicited thromboxane formation is largely responsible for both events. Perfusion maldistribution in the toxin-exposed hearts offers an attractive explanation for the present findings, but this requires further elucidation. The large family of pore-forming exotoxins from Gram-positive but also from Gram-negative bacteria may thus be implicated in the loss of cardiac performance encountered in septic shock.

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**References**


**Figure 9.** Photomicrographs of myocardial sections of Langendorff rat hearts treated with either vehicle (left) or 1 µg/mL α-toxin for 2 hours. Left, Myocardial tissue from a Langendorff rat heart subjected to 2 hours of perfusion and treated with vehicle. Normal cardiac myocytes with cross-striations. Right, Myocardial tissue from a Langendorff rat heart subjected to 2 hours of perfusion and treated with 1 µg/mL α-toxin. Cardiac myocytes demonstrated slight injury, with loss of cross-striation and contraction bands.


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