Endotoxin-Induced Myocardial Tumor Necrosis Factor-\(\alpha\) Synthesis Depresses Contractility of Isolated Rat Hearts
Evidence for a Role of Sphingosine and Cyclooxygenase-2–Derived Thromboxane Production

Ulrich Grandel, MD; Ludger Fink, MD; Andreas Blum, MD; Martina Heep; Michael Buerke, MD; Hans-Joachim Kraemer, MD; Konstantin Mayer, MD; Rainer M. Bohle, MD; Werner Seeger, MD; Friedrich Grimminger, MD, PhD; Ulf Sibelius, MD

Background—Although endotoxin (lipopolysaccharides, LPS) is recognized as a mediator of septic cardiodepression, its cardiac effects are still not fully elucidated.

Methods and Results—Perfusion of isolated rat hearts with LPS for 180 minutes resulted in a decline of left ventricular contractility after 90 minutes, whereas coronary perfusion pressure remained unaffected. This cardiodepression was paralleled by a release of tumor necrosis factor (TNF)-\(\alpha\) into the perfusate and preceded by myocardial TNF-\(\alpha\) mRNA upregulation as quantified by real-time polymerase chain reaction. The cardiodepression was abrogated when LPS was perfused with a TNF-\(\alpha\) antiserum or the ceramidase inhibitor \(N\)-oleoylethanolamine. In contrast, the cardiac release of nitric oxide (NO) was not augmented by LPS. Immunohistochemical studies of LPS-perfused hearts revealed a positive staining for the constitutive (NOSIII) but not for the inducible NO synthase (NOSII). Accordingly, NOSII mRNA levels commenced to increase only at the very end of the LPS perfusion period. Progressive liberation of thromboxane (Tx) \(A_2\) and prostacyclin was induced by LPS together with myocardial cyclooxygenase (Cox)-2 mRNA expression. Both nonselective inhibition of Cox by indomethacin and selective inhibition of the inducible Cox-2 by NS-398 abolished prostanoid release. Interestingly, the generation of TNF-\(\alpha\) and the associated cardiodepression caused by LPS were reduced by indomethacin, NS-398 and the Tx-receptor antagonist daltroban.

Conclusions—LPS depresses contractility of isolated rat hearts by inducing TNF-\(\alpha\) synthesis and subsequently activating the sphingomyelinase pathway, whereas no evidence for a role of NOSII- or NOSIII-generated NO was found. Moreover, Cox-2–derived TxA\(_2\) appears to facilitate TNF-\(\alpha\) synthesis in response to LPS. (Circulation. 2000;102:2758-2764.)

Key Words: contractility ■ nitric oxide ■ nitric oxide synthase ■ perfusion ■ heart failure ■ shock

Progressive myocardial depression contributes to the profound cardiocirculatory abnormalities of septic shock.\(^{1,2}\) Bacterial endotoxins, lipopolysaccharides (LPS) of Gram-negative bacteria, are important pathogenicity factors in this context and mimic the cardiac depression of septic shock in human volunteers\(^3\) or experimental animals.\(^4\) However, the pathophysiological sequelae underlying this cardiodepression are not fully elucidated. Two different mechanisms, focusing on either the appearance of myocardial depressant substances or the impairment of myocardial perfusion in septic shock, have been proposed, and LPS has been implicated as a causative agent in both settings.

Cardiodepressant properties of serum from septic patients have largely been attributed to cytokines, in particular tumor necrosis factor (TNF)-\(\alpha\) and interleukin-1\(\beta\).\(^5,6\) TNF-\(\alpha\) impairs contractile performance in intact animals, isolated hearts, and cardiomyocytes.\(^7-11\) Within minutes, TNF-\(\alpha\) reduces myocardial contractility in vitro by disturbing intracellular Ca\(^{2+}\) homeostasis,\(^10\) and activation of the sphingomyelinase pathway has been suggested as underlying mechanism.\(^8,11\) Sphingomyelinases yield ceramide from membrane-bound sphingomyelin, which may be converted by ceramidase into sphingosine. This agent may be involved in the regulation of intracellular calcium.\(^12\) As a different concept, negative inotropic effects of TNF-\(\alpha\) were attributed to enhanced synthesis of nitric oxide (NO) in the myocardium via the constitutively expressed NOSIII\(^16,14\) or the inducible isoenzyme (NOSII).\(^9,15\) Recent studies demon-
strated that the myocardial tissue itself synthesizes TNF-α in response to various challenges, including LPS exposure.16–18

Apart from circulating cytokines, sepsis-associated impairment of regional perfusion may extend to the coronary vasculature, and maldistribution of myocardial perfusion with disturbances in regional O2 supply may depress myocardial performance.19–23 Vasoactive prostanoids synthesized by the constitutively expressed cyclooxygenase (Cox)-1 and under septic conditions increasingly by the inducible Cox-2 may contribute to the perfusion maldistribution of sepsis. Recently, significant expression of Cox-2 has been demonstrated in septic human and endotoxic rat heart,24,25 and in a very recent study from our laboratory we showed that the myocardial depression of isolated rat hearts in response to staphylococcal α-toxin results from thromboxane (Tx)A₂ liberation with coronary vasoconstriction and perfusion mismatch.23

The purpose of this study was to investigate whether endotoxin depresses contractility of isolated buffer-perfused rat hearts and, if so, to study the mechanisms involved. We noted an LPS-induced cardiodepression associated with myocardial upregulation of TNF-α and subsequent activation of the sphingomyelinase pathway. In contrast, cardiac NO generation did not contribute to the negative inotropism of LPS. Moreover, Cox-2–derived TxA₂ is suggested to act as a paracrine facilitator of TNF-α synthesis, thereby contributing to LPS-evoked cardiac dysfunction.

Methods

Materials

Salmonella abortus equii lipopolysaccharide was provided by C. Gallanos (Max-Planck Institute for Immunology, Freiburg, Germany). The neutralizing rabbit anti-human TNF-α antisera, cross-reacting with rat TNF-α,26 was purchased from Genzyme Virotech, the ceramidase inhibitor N-oleoylthanolamine (NOE) from Sigma, indomethacin from ICN Biomedicals, the selective Cox-2 inhibitor NS-3987 from Calbiochem, and the TxA₂ receptor antagonist daltroban (BM 13,505) from Boehringer. Other materials used are stated below.

Isolated Heart Perfusion and Experimental Protocols

Materials and techniques of preparation, perfusion, and monitoring of physiological parameters have been described by us in detail.23 All hearts were perfused in a recirculating mode (total volume, 50 mL). LPS, 0.1 or 1.0 µg/mL, were perfused for 180 minutes. Perfusion samples for determination of TNF-α, TxB₂, 6-keto prostaglandin (PG)F₁₂, lactate dehydrogenase (LDH), and creatine kinase (CK) were taken twice before and at 30, 60, 90, 120, 150, and 180 minutes after LPS application. For pharmacological intervention, either TNF-α antisera (0.4% vol/vol), NOE (5 µmol/L), indomethacin (100 µmol/L), NS-398 (25 µmol/L), or daltroban (10 µmol/L) was admixed to the perfusate before LPS application (1 µg/mL). Control experiments included perfusion solely with perfusate and with perfusate enriched with the respective pharmacological inhibitors.

Measurement of TNF-α, TxA₂, Prostacyclin, CK, and LDH

TNF-α was measured with a rat TNF-α ELISA (Biosource). TxA₂ and prostacyclin were quantified by measuring their stable hydrolys products TxB₂ and 6-keto PGF₁₂, by ELISA (Cayman Chemical Co). LDH and CK were measured by routine techniques.

Measurement of NO

NO was detected as described by us previously.28 NO is rapidly converted to nitrite and nitrate, summarized as NOₓ, in the oxygen-containing perfusate. To monitor NO, perfusate, samples were transferred to a reaction vessel containing 80 mL of 0.1 mol/L vanadium (III) chloride in 2.0 mol/L HCL at 98°C. This solution quantitatively reduced NOₓ to NO. NO was removed from the reaction vessel by oxygen-free nitrogen continuously flushing through the liquid (160 mL/min), which entered a chemiluminescence detector (UPK300; UPK). Calibration was performed with known amounts of nitrite and nitrate.

Immunohistochemical Analysis of NOSII/NOSIII Expression in Myocardium

Specimens of the left ventricle were prepared for immunohistochemical analysis as plastic sections, as described by us previously.23 Immunohistochemical procedures were performed as described by Beckstead et al.29 with the avidin-biotin immunoperoxidase technique (Vectastain ABC Reagent, Vector Laboratories). Immunohistochemical analysis was performed with antibodies against NOSIII and NOSII (Stressgene). Incubation of the primary antibodies was carried out overnight at different dilutions, of which 1:50 gave the highest degree of immunolocalization and the least nonspecific background staining. The sections were lightly counterstained with Gill’s hematoxylin 3 (Sigma) and examined with a Zeiss light microscope at ×400.

Quantitative Analysis of mRNA

To determine mRNA expression, hearts were perfused for various time periods with or without LPS.

mRNA Extraction

Cryosections of the left ventricle were lysed in 300 µL lysis buffer of the Dynabeads mRNA direct kit (Dynal). mRNA was captured by attachment to oligo-dT fragments coupled to supermagnetic glass particles. Per sample, 100-µg beads were applied. mRNA was finally dissolved in 20 µL DEPC-treated H₂O.

mRNA Quantification

This was performed by the Sequence Detection System 7700 (PE Applied Biosystems) and real-time polymerase chain reaction (PCR), with comparative quantification (ΔCT) and normalizing the target gene to an internal standard gene according to the formula

\[ \frac{T_{R}}{R_{0}} = K(1 + E)^{CT_{R} - CT_{T}} \]

\( T_{R} \) is the initial number of target gene mRNA copies; \( R_{0} \), initial number of standard gene mRNA copies; \( E \), efficiency of amplification; \( CT_{T} \), threshold cycle of target gene; \( CT_{R} \), threshold cycle of standard gene; and \( K \), constant.

For internal calibration, porphobilinogen deaminase (PBGD) mRNA was used, a ubiquitously and consistently expressed standard gene without pseudogenes.30 In preliminary experiments, amplification efficiency of PBGD, TNF-α, NOSII, NOSIII, and Cox-2 primer/probe sets was approximately equal and amounted to 0.9±0.02 (90±2%).

cDNA Synthesis and Real-Time PCR

cDNA synthesis and real-time PCR were performed as described previously by us.31 Two µL cDNA was applied to each sample. Oligonucleotide primers (Table) were added to a final concentration of 300 nmol/L each and hybridization probes (Table) to a final concentration of 200 nmol/L in a volume of 50 µL. Cycling conditions were adapted to 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 61°C for 60 seconds.
Statistical Analysis
All data are given as mean±SEM and were analyzed by 1-way ANOVA followed by Tukey’s honestly significant difference test. A value of \( P < 0.05 \) was considered to be significant.

Results
LPS Depresses the Contractile Function of Isolated Rat Hearts
Left ventricular developed pressure (LVDP) and the maximum rate of left ventricular pressure rise (dP/dt max) of control hearts remained stable throughout the perfusion period (Figure 1, A and B). Administration of LPS to the recirculating perfusate (0.1 and 1.0 \( \mu \)g/mL) caused a delayed dose-dependent depression of LVDP and dP/dt max after \( \approx 90 \) minutes, which further progressed until terminating the experiments. In contrast, no significant change in coronary perfusion pressure (CPP) was observed (Figure 1C).

Perfusion of LPS Results in Liberation of TNF-\( \alpha \), Which Is Preceded by Myocardial Expression of TNF-\( \alpha \) mRNA
In the recirculating perfusate of control hearts, only trace amounts of TNF-\( \alpha \) accumulated over 180 minutes. In contrast, a dose-dependent rise in TNF-\( \alpha \) levels occurred in the presence of LPS, commencing after 90 minutes and progressing until the end of the experiments (Figure 2A).

Quantification of TNF-\( \alpha \) mRNA showed a significant expression after 60 minutes’ exposure to endotoxin, thus preceding the liberation of TNF-\( \alpha \) in the perfusate (Figure 2B). This expression increased considerably for the remainder of perfusion time. In contrast, no TNF-\( \alpha \) mRNA expression was observed in time-matched controls except for a minimal expression after 180 minutes.

In the Presence of LPS, Release of NO Is Not Augmented and NOSII mRNA Is Elevated Only After 180 Minutes
NO\( \no{X} \) accumulation in the perfusate of control hearts, signaling ongoing NO liberation, was not enhanced by LPS (Figure 3A). Moreover, no NOSII mRNA expression was found in control and LPS-challenged hearts, except for LPS-perfused hearts after 180 minutes (Figure 3B). Immunohistochemical analysis of LPS-treated hearts showed a positive staining for NOSIII in the coronary endothelium (Figure 4) but no significant staining for NOSII (not shown) after 180 minutes. Likewise, only positive staining for NOSIII but not NOSII was noted in time-matched control hearts (not shown), and NOSIII mRNA expression in LPS-challenged (1.0 \( \mu \)g/mL) hearts did not differ significantly from controls after 0, 60, 120, and 180 minutes (not shown).

LPS Provokes Release of TxA\( \no{2} \) and Prostacyclin and Augmentation of Cox-2 mRNA
Only minor quantities of TxB\( \no{2} \), the stable metabolite of TxA\( \no{2} \), accumulated in the perfusate of control hearts within 180 minutes. TxA\( \no{2} \) liberation was dose-dependently augmented by LPS after 60 minutes (Figure 5). Similarly, the basal release of 6-keto PGF\( \no{1} \) (343±50 pg/mL at 0 minutes, 3748±526 pg/mL at 180 minutes in control hearts) was markedly increased in the presence of 0.1 \( \mu \)g/mL LPS.
At 0 minutes, LPS was admixed to recirculating perfusate. Controls were perfused without endotoxin. A, LVDP; B, dP/dt max; and C, CPP. Values represent mean±SEM of at least 5 experiments. *P<0.05 vs control.

(485±73 pg/mL at 0 minutes, 8077±2131 pg/mL at 180 minutes) and 1.0 µg/mL LPS (205±64 pg/mL at 0 minutes, 12 258±1973 pg/mL at 180 minutes). At the end of the perfusion period, Cox-2 mRNA was augmented ≈9-fold (14.70±6.83 copies Cox-2 mRNA/1 copy PBGD mRNA) compared with control hearts (1.65±0.60 copies Cox-2 mRNA/1 copy PBGD mRNA).

Inhibitory Capacities of Anti-TNF-α, NOE, Indomethacin, NS-398, and Daltroban

TNF-α antiserum completely abolished the cardiodepression by LPS (Figure 6, A and B). Similarly, the LPS-induced cardiodepression was completely abrogated in the presence of the ceramidase inhibitor NOE. Indomethacin, the selective Cox-2 inhibitor NS-398, and the specific thromboxane-receptor antagonist daltroban partially prevented the LPS-induced cardiodepression. Indomethacin blocked cardiac TxB₂ (Figure 5) and 6-keto PGF₁α release (374±132 pg/mL at 0 minutes, 550±154 pg/mL at 180 minutes) to very low levels, and when LPS was perfused in the presence of NS-398, TxB₂ and 6-keto PGF₁α levels were also dramatically reduced (6-keto PGF₁α, 1018±518 pg/mL after 180 minutes; Figure 5). As anticipated, the cardiac prostanoïd generation was not affected by daltroban (not shown). CPP was not significantly affected by any compound (Figure 6C). In separate control experiments, none of the drugs/antiserum used were found to alter CPP, LVDP, and dP/dt max in the absence of LPS (data not shown).

Indomethacin, NS-398, and Daltroban Partly Inhibit LPS-Induced TNF-α Liberation

In the presence of indomethacin, the LPS-evoked TNF-α release was reduced by nearly 50% (Figure 7). Nearly identical inhibition was noted for the selective Cox-2 inhibitor NS-398 and the thromboxane receptor antagonist daltroban.

LPS Does Not Affect Endothelial Permeability and Release of CK and LDH

The weight gain of hearts undergoing 180 minutes of LPS (1.0 µg/mL) perfusion was 338±22 mg (baseline weight, 1018±66 mg), which did not significantly differ from 294±27 mg in buffer-perfused control hearts (baseline heart weight, 914±57 mg). CK and LDH perfusate levels did not differ significantly between control and LPS-perfused (1.0 µg/mL) hearts.

Discussion

Myocardial dysfunction encountered in septic shock has been either attributed to the cardiodepressive properties of circulating cytokines or the impairment of cardiac...
regional perfusion, and endotoxin may act as a causative agent in both settings. The current study, which used blood-free perfused hearts, suggests that LPS-induced upregulation of myocardial TNF-α synthesis with subsequent activation of the sphingomyelinase pathway is a major effector of myocardial depression. In contrast, no evidence for a contribution of NO to the early negative inotropic effect of LPS was obtained in this model. Endotoxin-elicited Cox-2-derived thromboxane generation is proposed as an autocrine facilitator of LPS-induced TNF-α generation.

The cardiodepression became obvious within ~90 minutes of LPS perfusion and progressed steadily until the end of the experiments, whereas CPP remained unaffected. This may be surprising, as endotoxin provoked a marked liberation of the vasoconstrictor thromboxane into the coronary bed. The most reasonable explanation for this observation is the finding that thromboxane release was accompanied by prominent prostacyclin liberation, and this vasodilatory agent might antagonize the vasomotor effects of TxA2. Thus, perfusion abnormalities are very unlikely to contribute to the negative inotropic effect of LPS in the buffer-perfused hearts.

In contrast, cardiac synthesis of TNF-α in response to LPS, preceded by myocardial expression of TNF-α mRNA, offers the most plausible explanation for the loss of contractility. TNF-α release into the coronary vasculature and negative inotropism progressed in a nearly superimposable fashion. Comparable dose dependence was noted for both events, and the myocardial depression was completely abrogated in the presence of a TNF-α antiserum. From our studies, we cannot deduce the origin of cardiac TNF-α synthesis. However, recent investigations have convincingly demonstrated that endothelial and smooth muscle cells of the coronary vasculature and particularly cardiomyocytes themselves are capable of synthesizing this cytokine.

Cardiodepressive effects of TNF-α have been ascribed to at least 2 different mechanisms. The immediate cardiopression by TNF-α might either be mediated by activation of the sphingomyelinase pathway or by activation of the constitutive NOSIII, whereas a more delayed cardiodepression of TNF-α might be attributed to “extra” NOSIII release. Monitoring of NO-release demonstrated ongoing accumulation of NO, however, kinetics were indistinguishable in control and LPS hearts, thus excluding any major impact of LPS per se or LPS-evoked TNF-α on baseline NO formation. Moreover, immunohistochemical studies showed a strong positive staining for NOSIII, distributed in the vascular endothelium of myocardial vessels, but no significant staining for NOSII in control or LPS-perfused hearts.
Accordingly, NOSII mRNA expression increased only at the very end of the perfusion period. Because the expression of NOSII is regulated at transcriptional levels and demands a few hours’ lag phase,9,15 the NOSII mRNA induction at the end of our experimental protocol might indicate the onset of “excessive” NO synthesis, which might then contribute to a more delayed LPS-mediated cardiodepression occurring beyond the time range studied here. However, under the current experimental conditions, a major role of NOSII- or NOSIII-mediated NO formation in the LPS-induced cardiodepression is largely excluded.

In contrast, the cardiodepression in LPS-perfused hearts was completely abolished in the presence of the specific ceramidase inhibitor NOE. This finding suggests a sequence of TNF-α–elicited activation of the sphingomyelinase pathway with formation of sphingosine as the underlying mechanism. It is well in line with this suggestion that sphingosine is known to be produced in TNF-α–exposed cardiomyocytes within minutes, together with a loss of contractility,11 and that superfusion of cardiomyocytes with the effluent of LPS-challenged hearts containing TNF-α provokes corresponding effects.15 Additional evidence for a role of sphingosine in TNF-α–induced myocardial depression was recently obtained from isolated rat hearts directly perfused with this cytokine.8 Another remarkable finding of the present study is the fact that inhibition of cyclooxygenase activity by indomethacin or the selective Cox-2 inhibitor NS-39827 inhibited the release of TxA2. Together with the fact that Cox-2 mRNA was upregulated in the myocardium of LPS-perfused hearts, this finding supports the notion that LPS-induced TxA2 liberation proceeds largely through upregulation of Cox-2. Interestingly, inhibition of Cox activity by a nonselective Cox inhibitor and the selective Cox-2 inhibitor as well as administration of a specific thromboxane receptor antagonist reduced TNF-α formation and the cardiodepression in response to LPS. This observation is reminiscent of the recent finding in human monocytes that blocking TxA2 decreased zymosan-induced TNF-α production, which was partly reproduced by a stable Tx receptor agonist.33 Supporting this notion, suppression of TNF-α synthesis by a TxA2 inhibitor was also observed in liver ischemia34 and alcoholic liver disease35 in the rat. Taken together, these data suggest that TxA2, largely originating from upregulated Cox-2, may act as a paracrine facilitator of TNF-α synthesis.

In summary, the present study provides evidence that TNF-α is both generated and biologically active in the myocardium of endotoxin-exposed rat hearts. The LPS-induced early loss in contractile force is suggested to proceed largely through TNF-α–elicited sphingomyelinase formation and not through impact on cardiac NO synthesis. Interestingly, Cox-2–derived TxA2 may act as a paracrine facilitator of LPS-induced TNF-α liberation.
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