Recombinant Tissue Factor Pathway Inhibitor Prevents Lipopolysaccharide-induced Systemic Hypotension in Rats by Inhibiting Excessive Production of Nitric Oxide

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Keywords

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Summary

Excessive production of nitric oxide (NO) by the inducible form of NO synthase (iNOS) plays a key role in the development of endotoxin shock. Tumor necrosis factor-α (TNF-α) induces iNOS, thereby contributing to the development of shock. We recently reported that recombinant tissue factor pathway inhibitor (r-TFPI), an important inhibitor of the extrinsic pathway of the coagulation system, inhibits TNF-α production by monocytes. In this study, we investigated whether r-TFPI could ameliorate hypotension by inhibiting excessive production of NO in rats given lipopolysaccharide (LPS). Pretreatment of animals with r-TFPI prevented LPS-induced hypotension. Recombinant TFPI significantly inhibited the increases in both the plasma levels of NO$_2$/$NO_3^-$ and lung iNOS activity 3 h after LPS administration. Expression of iNOS mRNA in the lung was also inhibited by intravenous administration of r-TFPI. However, neither DX-9065a, a selective inhibitor of factor Xa, nor an inactive derivative of factor VIIa (DEGR-F.VIIa) that selectively inhibits factor VIIa activity, had any effect on LPS-induced hypotension despite their potent anticoagulant effects. Moreover, neither the plasma levels of NO$_2$/$NO_3^-$ nor lung iNOS activity were affected by administration of DX-9065a and DEGR-F.VIIa. These results suggested that r-TFPI ameliorates LPS-induced hypotension by reducing excessive production of NO in rats given LPS and this effect was not attributable to its anticoagulant effects, but to the inhibition of TNF-α production.

Introduction

Septic shock associated with infection with Gram-negative bacteria is characterized by hypotension, organ dysfunction and disseminated intravascular coagulation (DIC) leading to multiple organ failure, and consequently to a high mortality rate (1). The mechanism of septic shock is now considered to be the marked reduction of vascular reactivity to vasoconstrictors (2). The hyporeactivity has been shown to be attributable to the action of nitric oxide (NO) excessively produced by the inducible isoform of NO synthase (iNOS) expressed within the vasculature (3). NO activates soluble guanylyl cyclase, thereby increasing the cytoplasmic concentration of cyclic GMP followed by reduction of intracellular calcium concentration (4). Cyclic GMP-independent mechanisms of vasodilation have also been postulated. For example, peroxynitrite, an oxidant produced by the reaction of NO and superoxide, has been suggested to activate membrane potassium channels leading to vasodilation (5). Furthermore, myocardial depression induced by NO might also contribute to hypotension induced by lipopolysaccharide (LPS) (6).

The inducible isoform of NO synthase can be induced by tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, which is elaborated by monocytes stimulated with LPS (7). Thus, hypotension is one of the deleterious effects induced by TNF-α under the pathological conditions of sepsis.

Tissue factor pathway inhibitor (TFPI) is a multivalent, Kunitz-type plasma protease inhibitor known to inhibit factor Xa (F.Xa) and factor VIIa bound to tissue factor (TF/F.VIIa) (8). Since depletion of endogenous TFPI sensitized rabbits to DIC induced by tissue factor or endotoxin (9, 10), TFPI might play an important role in alleviating DIC seen in sepsis. Carr et al. (11) reported that infusion of recombinant TFPI (r-TFPI) reduced mortality rate as well as the coagulation abnormalities in baboons injected with lethal doses of *Escherichia coli*. We have recently shown that r-TFPI inhibits TNF-α production in vivo and in vitro (12). Since r-TFPI has been shown to attenuate hypotension in a baboon model of septic shock (11), it is possible that r-TFPI prevents LPS-induced hypotension by inhibiting the induction of iNOS through inhibition of TNF-α production.

In this study, we examined this possibility using a rat model of septic shock. Since the lung is one of the main organs expressing large amounts of iNOS in response to LPS (13), we investigated the effect of r-TFPI on the changes in iNOS activity and expression of iNOS mRNA in the lung tissue of rats given LPS.

Materials and Methods

**Materials**

Human r-TFPI was obtained from the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan. Recombinant TFPI was expressed in CHO cells and purified according to the method described previously (14). Recombinant TFPI used in the present study was carboxyl-terminus truncated TFPI showing a single band at 42.5 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol. The amino acid sequences of r-TFPI were confirmed by sequence analyses of the peptides produced by digestion with lysyl endopeptidase. The results indicated that truncated r-TFPI used in this study ends at Lys$^{216}$ (14). Inhibition of F.Xa and TF/F.VIIa by r-TFPI was measured using Z-Pyr-Gly-Arg-MCA and Boc-Leu-Thr-Arg-MCA, respectively (15). The Ki values of r-TFPI for the inhibition of F.Xa and TF/F.VIIa were 5.6 and 17.4 nM, respectively (15). DX-9065a, a synthetic, potent anticoagulant and selective inhibitor of F.Xa, was a generous gift from...
Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan) (16). Recombinant factor VIIa treated with dansyl-glutamyglycylarginyl-chloromethyl ketone (DEGR-F.VIIa) was kindly provided by Dr. Bregengaard (Novo Nordisk, Gentofte, Denmark). Lipopolysaccharide (Escherichia coli, serotype 055:B5) was from Difco (Detroit, MI). All other reagents used were of analytical grade.

Animal Model of LPS-induced Hypotension

The study protocol was approved by the Kumamoto University Animal Care and Use Committee, and the care and handling of the animals were performed in accordance with the National Institutes of Health guidelines. Adult, pathogen-free male Wistar rats (body mass, 200-220 g) (Kyudo, Kumamoto, Japan) were given an intravenous injection of LPS (5 mg/kg) via the tail vein. Recombinant-TFPI (1 mg/kg, intravenously), DX-9065a (3 mg/kg, subcutaneously), DEGR-F.VIIa (3 mg/kg, intravenously) or saline was injected 30 min prior to the injection of LPS. Animals were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg), and exsanguinated via the abdominal aorta. Blood and lung tissue samples were obtained at various time points after LPS injection. Blood samples were collected in tubes containing 1/10 (vol/vol) of 3.8% (wt/vol) sodium citrate and were centrifuged at 3,000 × g for 15 min. The right carotid artery was cannulated for measurement of mean arterial pressure (MAP). Mean arterial pressure equals the diastolic pressure plus one-third of the pulse pressure, the difference between the systolic and diastolic pressure. Control animals received saline instead of the study drugs.

Measurement of MAP

For measurement of MAP, the right carotid artery was cannulated and connected to a pressure transducer. Upon completion of the surgical procedure, MAP was allowed to stabilize for 15 min. After recording the baseline MAP, animals were treated with vehicle (saline) or reagents such as r-TFPI (1 mg/kg), DX-9065a (3 mg/kg) and DEGR-F.VIIa (3 mg/kg). Thirty minutes after administration of vehicle or reagents, animals received LPS (5 mg/kg, intravenously). Mean arterial pressure was continuously monitored for 180 min after LPS administration.

Measurement of Plasma Levels of NO\textsubscript{2}/NO\textsubscript{3}

For measurement of plasma levels of NO\textsubscript{2}/NO\textsubscript{3}, blood samples were taken at various times after LPS administration and centrifuged at 1,000 × g for 20 min. Total concentration of NO\textsubscript{2}/NO\textsubscript{3} was measured using a Griess reaction kit (Boehringer Mannheim, Mannheim, Germany) (17). Briefly, plasma protein was removed by adding 5% ZnSO\textsubscript{4} and NaOH (0.3 M) solution. After centrifugation (2,300 × g for 10 min), the supernatant was incubated with nitrate reductase (0.24 U/ml), nicotinamide adenine dinucleotide (3.2 g) and flavin adenine dinucleotide (3.2 µM) for 30 min at room temperature to reduce NO\textsubscript{2}/NO\textsubscript{3}. After incubation, the samples were placed in 96-well plates and incubated with 1% sulfanilic acid and 0.2% naphthylethylenediamine dihydrochloride/20% H\textsubscript{3}PO\textsubscript{4} for 5 min at room temperature. The absorbance of the mixture at 550 nm was determined using a microplate reader. NO\textsubscript{2} concentration was calculated by comparison with the absorbance of a standard solution of KNO\textsubscript{2}.

NOS Assay

Recombinant TFPI, DX-9065a, DEGR-F.VIIa or saline was injected 30 min prior to LPS administration. The lung samples were taken at various times after injection of LPS. Lung vasculature was perfused via the right cardiac ventricle with 10 ml of cold 0.9% NaCl. The lungs were frozen in liquid nitrogen and homogenized on ice in HEPES buffer (pH 7.5, 30 mM). The homogenate was sonicated and centrifuged at 12,500 × g for 15 min at 4° C. Conversion of [\textsuperscript{3}H]-L-arginine to [\textsuperscript{3}H]-L-citrulline was measured in the supernatant as described by Szabo et al. (6). Briefly, tissue homogenate was incubated in the presence of [\textsuperscript{3}H]-L-arginine (0.5 mM) with calmodulin (30 mM), tetrahydrobiopterin (50 µM), flavin mononucleotide (20 µM), flavin adenine dinucleotide (20 µM), L-valine (60 mM) and CaCl\textsubscript{2} (2 mM) for 20 min at 37° C in HEPES buffer (pH 7.5, 30 mM). Reactions were stopped by adding Hepes buffer (pH 5.5, 100 mM) containing ethylene glycol tetraacetic acid (2 mM) and ethylene diamine tetraacetic acid (2 mM). Reaction mixtures were applied to Dowex 50 W (sodium form) columns (Bio-Rad Laboratories, Hercules, CA) and the radioactivity of eluted [\textsuperscript{3}H]-L-citrulline was measured using a scintillation counter (TRI-CARB 2300TR, Packard, Meriden, CT). Experiments, without calcium and with ethylene glycol tetraacetic acid (5 mM), were performed to determine calcium-independent iNOS activity. Protein concentration was determined spectrophotometrically by the method of Lowry with bovine serum albumin as a standard.

RNA Isolation

For measurement of iNOS mRNA, samples of lung tissue were taken from rats treated with saline or r-TFPI (1 mg/kg, i.v.) 30 min prior to 5 mg/kg LPS administration and were frozen at −80° C. Total RNA was isolated by the technique described by Chomczynski and Sacchi (18). Briefly, frozen rat lung tissue was homogenized in 4 ml of RNA-zol B (Biotex Laboratories, Houston, TX), and 400 µl of chloroform was added to the homogenate before centrifugation at 12,000 × g for 15 min at 4° C. RNA was precipitated from the aqueous layer by adding an equal volume of isopropanol, incubating the mixture at 4° C for 15 min, and centrifugation at 12,000 × g for 15 min at 4° C. The RNA pellet was washed with 500 µl of 70% ethanol, and RNA concentration was determined by measuring the absorbance at 260 nm.

Northern Blotting and RNA Hybridization

Amounts of 20 µg of total RNA per sample were loaded onto 1% agarose/formaldehyde denaturing gels, electrophoresed at a constant voltage, and transferred onto nylon membranes. Hybridization was performed using digoxigenin-labeled rat iNOS antisense RNA (19) as a probe. The antisense RNA was synthesized using complementary DNA cloned by reverse transcription-polymerase chain reaction and subcloned into pcDNAII as templates. Probes were labeled using a digoxigenin-RNA labeling kit (Boehringer Mannheim, Mannheim, Germany). Chemiluminescence signals derived from hybridized probes were detected on x-ray film using a digoxigenin luminescence detection kit (Boehringer Mannheim, Mannheim, Germany) and quantified by densitometry.

Statistical Analysis

Data are presented as means ± SD. Results were compared using analysis of variance and Scheffe’s post hoc test or Student’s unpaired t-test. A level of p < 0.05 was accepted as statistically significant.

Results

Effects of r-TFPI, DX-9065a and DEGR-F.VIIa on LPS-induced Systemic Hypotension

Intravenous injection of 5 mg/kg LPS induced marked and sustained decreases in the MAP from 90 min up to 3 h following injection (Fig. 1). This decrease in blood pressure was significantly inhibited by intravenous administration of r-TFPI (1 mg/kg). However, neither DX-9065a, a selective inhibitor of F.Xa, nor DEGR-F.VIIa, a selective inhibitor of F.VIIa, affected the fall in blood pressure in animals given LPS (Fig. 1).

Effects of r-TFPI, DX-9065a and DEGR-F.VIIa on the LPS-induced Increases in Plasma Levels of NO\textsubscript{2}/NO\textsubscript{3} and Lung iNOS Activity

Both plasma levels of NO\textsubscript{2}/NO\textsubscript{3} (Fig. 2A) and lung iNOS activity (Fig. 2B) increased with time 90 min after administration of LPS (Figs. 2A and 2B). Both the increases in plasma levels of NO\textsubscript{2}/NO\textsubscript{3}...
Fig. 1 Effects of r-TFPI, DX-9065a and DEGR-F.VIIa on the LPS-induced increase in MAP. Recombinant TFPI (1 mg/kg, intravenously), DX-9065a (3 mg/kg, subcutaneously) or DEGR-F.VIIa (3 mg/kg, intravenously) was injected 30 min before LPS administration (5 mg/kg, intravenously). Solid circles, LPS + Saline; open circles, LPS + r-TFPI; open squares, LPS + DX-9065a; open triangles, LPS + DEGR-F.VIIa. Data are expressed as means ± SD of six animals. * p < 0.05 vs. LPS.

Fig. 2 Changes in plasma levels of NO$_2$-/-NO$_3$- and lung iNOS activity after administration of LPS in rats. Animals were administered saline (open circles) or LPS (5 mg/kg) (solid circles). Changes in the plasma levels of NO$_2$-/-NO$_3$-, stable metabolites of NO (A) and lung iNOS activity (B), were determined at the indicated time points. Data are expressed as means ± SD of five animals. * p <0.01 vs. control.

Fig. 3 Effects of r-TFPI, DX-9065a and DEGR-F.VIIa on LPS-induced increases in plasma levels of NO$_2$-/-NO$_3$- and lung iNOS activity. Saline, r-TFPI 1 mg/kg, intravenously), DX-9065a (3 mg/kg, subcutaneously) or DEGR-F.VIIa (3 mg/kg, intravenously) was administered 30 min prior to intravenous injection of LPS (5 mg/kg). Control animals received saline instead of LPS. Plasma levels of NO$_2$-/-NO$_3$- (A) and lung iNOS activity (B) were determined 3 h after LPS administration. Data are expressed as means ± SD. Numbers in parentheses indicate the number of animals in each experiment. * p <0.01 vs. control. † p <0.01 vs. LPS.

(Fig. 3A) and lung iNOS activities (Fig. 3B) observed 180 min after LPS administration were significantly inhibited in animals given r-TFPI (1 mg/kg). However, neither DX-9065a nor DEGR-F.VIIa had any effect on the changes in these parameters (Fig. 3).

Effect of r-TFPI on the LPS-induced Increase in Lung iNOS mRNA Level

The level of expression of iNOS mRNA in the lung was significantly increased 180 min after LPS administration (Fig. 4). This increase was inhibited in animals given r-TFPI (Fig. 4). Neither DX-9065a nor DEGR-F.VIIa inhibited the increase in expression of iNOS mRNA in the lung (data not shown).

Discussion

In the present study, r-TFPI inhibited LPS-induced hypotension by inhibiting excessive production of NO in rats. Consistent with this observation, Carr et al. (11) reported that r-TFPI reduces the drop in MAP as well as coagulopathic responses, thereby decreasing the mortality rate in baboons treated with Escherichia coli. Since r-TFPI inhibited the LPS-induced increases in iNOS activity and its mRNA level, inhibition of excessive production of NO by r-TFPI could be accounted for by its inhibition of induction of iNOS in the lungs of animals given...
LPS. Since coagulation abnormalities were also induced by LPS administration in this rat model of sepsis (12), the inhibitory effect of r-TFPI on the LPS-induced hypotension could be dependent on its anticoagulant effects. However, this is unlikely since neither DX-9065a, a selective inhibitor of F.Xa, nor DEGR-F.VIIa, a selective inhibitor of F.VIIa bound to tissue factor (TF), showed any effect on LPS-induced hypotension. These two anticoagulants inhibited LPS-induced coagulation abnormalities more markedly than r-TFPI in this animal model (12). Thus, it is likely that r-TFPI prevents the LPS-induced hypotension by as yet unknown effects other than its anticoagulant effect.

It has been shown that excessive production of NO by iNOS plays an important role in LPS-induced systemic hypotension (6). Wang et al. (20) have shown that various NOS inhibitors including 1,4-PBIT, a relatively specific iNOS inhibitor, significantly inhibited the increase in the plasma level of NO2/NO3 as well as the decrease in MAP in animals given LPS. In this rat model of septic shock, aminoguanidine, a selective inhibitor of iNOS, inhibited the LPS-induced hypotension as well as the increase in plasma level of NO (data not shown). These observations strongly suggest that excessive NO production by iNOS plays a central role in LPS-induced hypotension.

LPS increases production of TNF-α in circulating monocytes and resident macrophages (21). TNF-α, a proinflammatory cytokine, plays an important role in induction of iNOS (4). Recombinant human TNF-α has been shown to cause hypotension and death in a rat model of sepsis (21). Thiemermann et al. (22) reported that a monoclonal antibody against TNF-α ameliorated the hypotension induced by LPS in rats. These observations strongly suggest that TNF-α is involved in LPS-induced hypotension in rats by inducing iNOS. Consistent with this hypothesis, we previously reported that the expression of TNF-α mRNA in the lungs of rats given LPS peaked prior to the occurrence of hypotension (12). These observations strongly suggest that TNF-α plays a causal role in LPS-induced hypotension by inducing iNOS in this rat model of septic shock.

We have recently shown that r-TFPI inhibits TNF-α production in rats given LPS, and this effect appears to be independent of its anticoagulant activity (12). Taylor et al. (23) demonstrated that DEGR-F.VIIa had no effect on TNF-α response to a lethal dose of Escherichia coli in baboons despite its potent anticoagulant effect. Although the mechanism(s) by which TFPI inhibits TNF-α production is not well understood, Park et al. (24) have reported that TFPI binds to endotoxin, thereby preventing interaction of endotoxin with both LPS-binding protein and CD14, leading to blocking cellular responses such as monocyctic elaboration of TNF-α.

Plasma levels of rTFPI, as determined by ELISA method, were 2.54 ± 0.56 μg/ml (mean ± SD) in rats pretreated with r-TFPI (1 mg/kg, intravenously) at 90 min after LPS administration (5 mg/kg, intravenously) (12). These levels were higher than concentrations of r-TFPI required to inhibit the production of TNF-α by LPS-stimulated monocytes in vitro (0.1 to 1.0 μg/ml) (12), suggesting that r-TFPI could inhibit TNF-α production in vivo.

Taken together, these observations strongly suggest that r-TFPI inhibits LPS-induced hypotension by inhibiting iNOS induction through inhibition of TNF-α production and this effect could be independent of its anticoagulant effect.

Taylor et al. (25) demonstrated that anti-TF monoclonal antibody attenuated the hypotension as well as the coagulopathic response induced by the lethal dose of Escherichia coli in baboons. Since DEGR-F.Xa failed to attenuate the hypotension in the baboon model of septic shock (26), TF might be involved in the development of hypotension induced by LPS independent of the activation of the coagulation system. Thus, it is possible that r-TFPI prevents the LPS-induced hypotension by inhibiting the TF-mediated signaling process leading to hypotension. Tissue factor activates the extrinsic pathway of the coagulation system to generate factor Xa (27). Since F.Xa has been shown to induce hypotension by increasing NO production via binding to effector cell protease receptor-1 on the endothelial cell (28), TF might induce hypotension by generating F.Xa in this rat model of septic shock. However, this possibility is less likely since neither DX-9065a, a selective inhibitor of F.Xa, nor DEGR-F.VIIa, which inhibits the generation of F.Xa, prevented the hypotension in the present study.

Recombinant-TFPI also reduces the LPS-induced pulmonary vascular injury by inhibiting TNF-α production (12). These observations strongly suggest that r-TFPI may be a useful therapeutic agent for sepsis in which acute respiratory distress syndrome, hypotension and DIC frequently occur. Opal et al. (29) reported that TFPI improved the mortality rate in models of superantigen-induced shock and polymicrobial intra-abdominal infection by reducing interleukin-6 response, suggesting that TFPI might prevent the Gram-positive bacteria-induced septic shock probably by attenuating responses of proinflammatory cytokines. de Jonge et al. (30) reported that bolus intravenous injection of 0.2 mg/kg of r-TFPI inhibited the coagulation abnormalities without influencing the cytokine response in healthy human volunteers given an important role in induction of iNOS (4). Recombinant human TNF-α has been shown to cause hypotension and death in a rat model of sepsis (21). Thiemermann et al. (22) reported that a monoclonal antibody against TNF-α ameliorated the hypotension induced by LPS in rats. These observations strongly suggest that TNF-α is involved in LPS-induced hypotension in rats by inducing iNOS. Consistent with this hypothesis, we previously reported that the expression of TNF-α mRNA in the lungs of rats given LPS peaked prior to the occurrence of hypotension (12). These observations strongly suggest that TNF-α plays a causal role in LPS-induced hypotension by inducing iNOS in this rat model of septic shock.

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endotoxin. This observation appears to be inconsistent with those in the present animal model of sepsis. Although the reason for this discrepancy is not known, r-TFPI may be a useful agent for the treatment of sepsis.

References