Activated Protein C Prevents Endotoxin-Induced Hypotension in Rats by Inhibiting Excessive Production of Nitric Oxide

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Background—Excessive production of nitric oxide (NO) by the inducible isoform of NO synthase (iNOS) is critically involved in endotoxin (ET)-induced hypotension. Tumor necrosis factor-α (TNF-α) plays an important role in induction of iNOS. Because activated protein C (APC), a physiological anticoagulant, inhibits TNF-α production, it might prevent hypotension by inhibiting excessive production of NO. In this study, we examined this possibility using a rat model of septic shock.

Methods and Results—Intravenous administration of APC prevented both ET-induced hypotension and the increases in plasma levels of NO₂⁻/NO₃⁻. The hypotension was also inhibited when APC was administered 30 minutes after ET administration. APC inhibited the increases in lung levels of iNOS activity by inhibiting expression of iNOS mRNA in animals given ET. APC significantly inhibited the increases in lung tissue levels of TNF-α and expression of TNF-α mRNA in animals given ET. Neither DEGR-F.Xa, a selective inhibitor of thrombin generation, nor DIP-APC, an active site–blocked APC, showed any effect on these ET-induced changes. Both inhibition of TNF-α production by leukocytopenia and treatment with anti–rat TNF-α antibody produced effects similar to those induced by APC. Aminoguanidine, a selective inhibitor of iNOS, inhibited both the hypotension and the increases in plasma levels of NO₂⁻/NO₃⁻ in this animal model.

Conclusions—These observations strongly suggest that APC inhibits iNOS induction by decreasing TNF-α production, leading to the prevention of ET-induced hypotension. Furthermore, such effects of APC were not dependent on its anticoagulant effects but rather on its serine protease activity. (Circulation. 2001;104:1171-1175.)

Key Words: anticoagulants • infection • shock • nitric oxide

Septic shock associated with Gram-negative, Gram-positive, and fungal infections is characterized by hypotension, organ dysfunction, and disseminated intravascular coagulation leading to multiple organ failure and consequently to a high mortality rate. The mechanism of septic shock is now considered to be the marked reduction of vascular reactivity to vasoconstrictors. 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. NO activates soluble guanylyl cyclase, thereby increasing the cytoplasmic concentration of cGMP, followed by reduction of intracellular calcium concentration. Furthermore, myocardial depression induced by NO might also contribute to hypotension induced by endotoxin (ET).

iNOS can be induced by tumor necrosis factor-α (TNF-α), a proinflammatory cytokine elaborated by monocytes stimulated with ET. Thus, hypotension is one of the deleterious effects induced by TNF-α under the pathological conditions of sepsis.

Activated protein C (APC) is an important physiological anticoagulant that inactivates factor Va and factor VIIIa. APC is generated from protein C by the action of thrombin-thrombomodulin complex on endothelial cells. We have previously demonstrated that APC prevents pulmonary vascular injury by inhibiting neutrophil activation through inhibiting production of TNF-α in rats. These observations raise a possibility that APC prevents ET-induced hypotension by inhibiting the induction of iNOS.

In this study, we examined this possibility using a rat model of septic shock. Because the lung is one of the main organs expressing large amounts of iNOS in response to ET, we investigated the effects of APC on the changes in iNOS activity, expression of iNOS mRNA, and tissue levels of TNF-α and its mRNA in the lung tissue of rats given ET.

Methods

Reagents
APC was a generous gift from the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). Aminoguanidine (AG) was...
purchased from Sigma Chemical Co; anti–rat TNF-α antibody (Ab) was from Genzyme-Technie; ET (lipopolysaccharide, Escherichia coli, serotype 055:B5) was from Difco; and nitrogen mustard N oxide was from Yoshitomi Pharmaceutical Co Ltd. All reagents used were of analytical grade.

Preparation of Active Site– Blocked Factor Xa
5-Dimethylaminonaphthalene-1-sulfonyl-glutamylglycylarginyl chloromethyl ketone (DEGR)–treated factor Xa (DEGR-F.Xa) was prepared according to a previously described method. 13

Preparation of Diisopropyl Fluorophosphate– Treated APC
APC was inactivated with diisopropyl fluorophosphate (DIP; Sigma Chemical) according to a previously described method. 14

Reduction in the Number of Circulating Leukocytes Induced by Nitrogen Mustard N Oxide
Rats were made leukocytopenic by administration of nitrogen mustard according to a method described previously. 15

Induction of Hypotension by ET in Rats
The study protocol was approved by the Kumamoto University School of Medicine Animal Care and Use Committee, and the care and handling of the animals were in accordance with the guidelines of the National Institutes of Health. Specific pathogen– free male Wistar rats weighing 220 to 280 g were obtained from Kyudo (Kumamoto, Japan). Animals were anesthetized with pentobarbital sodium (50 mg/kg IP). The right femoral artery was cannulated and connected to a pressure transducer for the measurement of mean arterial blood pressure (MAP). MAP equals the diastolic pressure plus one third of the pulse pressure, the difference between the systolic and diastolic pressure.

Measurement of Plasma Levels of NO 2 /NO 3
NO 2 and NO 3 are the primary oxidized products of NO reacting with water, and therefore total concentration of NO 2 /NO 3 in plasma was used as an indicator of NO production in vivo. 16

Measurement of Lung Levels of iNOS Activity
The lungs were removed after perfusion via the right cardiac ventricle and frozen in liquid nitrogen. 3 These lung samples were homogenized on ice in HEPES buffer (pH 7.5, 30 mmol/L). The homogenate was sonicated and centrifuged at 12 500 g for 15 minutes at 4°C. Conversion of [3H]-i-arginine to [3H]-i-citrulline was measured in the supernatant as described by Szabó et al. 3

Isolation of RNA and Northern Blotting Analysis
Total RNA from rat lungs was prepared by the acid guanidinium– phenol–chloroform extraction procedure. 17 Hybridization was performed with digoxigenin–labeled rat iNOS antisense RNA 18 and rat TNF-α antisense RNA as probes.

Data Analysis
Data are presented as mean±SD. The results were compared by either ANOVA followed by Scheffé’s post hoc test or paired t test. A level of P<0.05 was accepted as statistically significant.

Results
Effects of APC, DEGR-F.Xa, and DIP-APC on MAP and Plasma Levels of NO 2 /NO 3
MAP decreased markedly from the pre-ET level (110±4 mm Hg) to 79±7 mm Hg 90 minutes after ET administration. This hypotension was sustained until 180 minutes after ET administration (Figure 1A).

Although intravenous administration of 25 or 50 μg/kg APC 30 minutes before ET administration did not inhibit the ET– induced decrease in MAP (data not shown), the intravenous administration of 100 μg/kg APC showed an inhibitory effect (Figure 1A). Although APC (100 μg/kg) also inhibited the ET– induced decrease in MAP when given 30 minutes after ET administration, it did not show any inhibitory effect when administered 60 minutes after ET administration (data not shown). Neither DEGR-F.Xa (3 mg/kg), a selective inhibitor of thrombin generation, nor DIP-APC (100 μg/kg), inactivated APC, showed any effect (Figure 1A).

Plasma levels of NO 2 /NO 3 were increased significantly 90 minutes after ET administration, reaching a maximum at 180 minutes (data not shown). Although APC (100 μg/kg) significantly inhibited the increase in plasma levels of NO 2 /NO 3 90 minutes (data not shown) and 180 minutes (Figure 2) after ET administration compared with those of animals given ET plus saline, neither DEGR-F.Xa (3 mg/kg) nor DIP-APC (100 μg/kg) showed any effect.

Effects of APC, DEGR-F.Xa, and DIP-APC on Lung Tissue Levels of iNOS Activity and iNOS mRNA After ET Administration
Lung tissue levels of iNOS activity were increased significantly with time after ET administration compared with those of control animals (data not shown). These increases were inhibited significantly in animals given APC (100 μg/kg) compared with those of animals given ET plus saline 90
minutes (data not shown) and 180 minutes (Figure 3) after ET administration. Neither DEGR-F.Xa (3 mg/kg) nor DIP-APC (100 μg/kg), however, inhibited these increases.

Chemiluminograms for typical expression of iNOS mRNA are shown in Figure 4. The chemiluminograms were quantified by comparison with the values seen in the ET plus saline group, arbitrarily set at 100. Expression of iNOS mRNA in the lung increased significantly 180 minutes after ET administration. This increase was inhibited in animals given APC (100 μg/kg). Neither DEGR-F.Xa (3 mg/kg) nor DIP-APC (100 μg/kg) inhibited the increase in expression of iNOS mRNA in the lung (Figure 4).

Effects of APC, DEGR-F.Xa, and DIP-APC on Lung Tissue Levels of TNF-α and TNF-α mRNA After ET Administration

Lung tissue levels of TNF-α began to increase 60 minutes after ET administration, peaking at 90 minutes, and gradually decreased to pre-ET levels 180 minutes after ET administration (data not shown). Intravenous administration of APC (100 μg/kg) significantly inhibited the increase observed 90 minutes after ET administration, but neither DEGR-F.Xa (3 mg/kg) nor DIP-APC (100 μg/kg) showed any effect (Figure 5).

Expression of TNF-α mRNA in the lung increased significantly 30 minutes after ET administration, peaking at 60 minutes after ET administration (data not shown). This increase was inhibited significantly in animals given APC (100 μg/kg) compared with that of animals given ET plus saline (Figure 5).

Effects of Leukocytopenia, Anti–Rat TNF-α Ab, and AG on MAP, Plasma Levels of NO₂⁻/NO₃⁻, Lung Tissue Levels of iNOS Activity, and Lung Tissue Levels of TNF-α in Animals Given ET

The ET-induced decrease in MAP was prevented in animals with leukocytopenia and in those given anti–rat TNF-α Ab
(0.25 mg/kg) or AG (5 mg/kg plus 10 mg \cdot kg^{-1} \cdot h^{-1}) (Figure 1B). The increases in both plasma levels of NO$_2$\-/NO$_3$ and lung tissue levels of iNOS activity were inhibited in animals with leukocytopenia and in those given anti–rat TNF-\(\alpha\) Ab or AG 90 minutes (data not shown) and 180 minutes (Figures 2 and 3) after ET administration. Although the increases in lung tissue levels of TNF-\(\alpha\) were not inhibited in animals given AG, the increases were inhibited in animals with leukocytopenia (Figure 5).

**Discussion**

In the present study, APC, a physiological anticoagulant, inhibited ET-induced hypotension by inhibiting excessive production of NO. Excessive production of NO by iNOS has been shown to play an important role in ET-induced hypotension.\(^{19,20}\) Because AG, a selective inhibitor of iNOS,\(^{21}\) significantly inhibited the increases in plasma levels of NO$_2$\-/NO$_3$ as well as hypotension in rats given ET in this study, iNOS could play a role in ET-induced hypotension in this animal model. The lung is one of the main organs expressing large amounts of iNOS in response to ET.\(^{3}\) Consistent with this observation, lung iNOS activity increased significantly 180 minutes after ET administration when plasma levels of NO$_2$ /NO$_3$ were increased significantly compared with those of control animals in the present study. Expression of iNOS mRNA in the lungs was enhanced in animals 180 minutes after ET administration.

APC partially, but significantly, inhibited the ET-induced increases in plasma levels of NO$_2$ /NO$_3$ as well as hypotension. APC also inhibited the increases in iNOS activity and expression of iNOS mRNA in the lungs of animals given ET. These observations suggest that APC prevents ET-induced hypotension mainly by inhibiting the induction of iNOS. Neither DEGR-F.Xa, a selective inhibitor of thrombin generation, nor DIP-APC, an inactive derivative of APC, affected these changes induced by ET. DEGR-F.Xa (3 mg/kg) inhibited the ET-induced coagulation abnormalities to the same extent as APC (100 \(\mu\)g/kg).\(^{11}\) Thus, APC might inhibit these ET-induced changes not by inhibiting thrombin generation.
but rather by some other actions in which the serine protease activity of APC is critically involved.

ET increases production of TNF-α in circulating monocytes and resident macrophages,24 TNF-α, a proinflammatory cytokine, plays an important role in induction of iNOS.6 Recombinant human TNF-α has been shown to cause hypotension and death in the rat sepsis model.23 Thiemermann et al24 reported that a monoclonal antibody against TNF-α ameliorated the hypotension induced by ET in rats. Both reduction of TNF-α production by leukocytes and treatment with anti-rat TNF-α Ab significantly inhibited increases in both plasma levels of NO₂⁻/NO₃⁻ and lung tissue levels of iNOS activity and further ameliorated the subsequent hypotension as shown in the present study. These observations strongly suggest that TNF-α could play a causal role in ET-induced hypotension by inducing iNOS in this animal model of septic shock.

APC has been shown to inhibit TNF-α production in a manner dependent on its serine protease activity in vitro and in vivo.11,12 Consistent with these observations, APC inhibited the increases in lung tissue levels of both TNF-α and TNF-α mRNA in rats given ET in this study. These observations strongly suggest that APC could prevent ET-induced hypotension by inhibiting TNF-α production in this study.

In the present study, the degrees of inhibition of the increases in both plasma levels of NO₂⁻/NO₃⁻ and lung tissue activity of iNOS by APC were small but statistically significant. Although the level of inhibition of iNOS activity sufficient to prevent ET-induced hypotension in this animal model is not known, the degrees of inhibition of the increases in both plasma levels of NO₂⁻/NO₃⁻ and lung tissue iNOS activity in animals with leukocytopenia and in those pre-treated with anti-rat TNF-α Ab were comparable to those seen in animals given APC, suggesting that the inhibition of iNOS activity by APC might be sufficient to prevent the hypotension.

The precise mechanism by which APC inhibits TNF-α production is not well understood at present. Using a THP-1 cell line, White et al25 showed that APC inhibited lipopolysaccharide-induced TNF-α production by inhibiting the nuclear translocation of nuclear factor-κB. We are currently investigating the precise mechanism by which APC inhibits TNF-α production by monocytes.

Acknowledgment
This study was supported, in part, by departmental funds from Kumamoto University School of Medicine.

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Circulation. 2001;104:1171-1175
doi: 10.1161/hc3501.093799
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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