Recombinant human activated protein C improves endotoxemia-induced endothelial dysfunction: a blood-free model in isolated mouse arteries

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Sennoun N, Baron-Menguy C, Burban M, Lecompte T, Andriantsitohaina R, Henrion D, Mercat A, Asfar P, Levy B, Meziani F. Recombinant human activated protein C improves endotoxemia-induced endothelial dysfunction: a blood-free model in isolated mouse arteries. Am J Physiol Heart Circ Physiol 297: H277–H282, 2009. First published April 24, 2009; doi:10.1152/ajpheart.01133.2008.—Recombinant human activated protein C (rhAPC) is one of the treatment panels for improving vascular dysfunction in septic patients. In a previous study, we reported that rhAPC treatment in rat endotoxemia improved vascular reactivity, although the mechanisms involved are still under debate. In the present study, we hypothesized that rhAPC may improve arterial dysfunction through its nonanticoagulant properties. Ten hours after injection of LPS in mice (50 mg/kg ip), aortic rings and mesenteric arteries were isolated and incubated with or without rhAPC for 12 h. Aortic rings were mounted in a myograph, after which arterial contractility and endothelium-dependent relaxation were measured in the presence or absence of nitric oxide synthase or cyclooxygenase inhibitors. Flow (shear stress)-mediated dilation with or without the above inhibitors was also measured in mesenteric resistance arteries. Protein expression was assessed by Western blotting. Lipopolysaccharide (LPS) reduced aortic contractility to KCl and phenylephrine as well as dilation to acetycholine. LPS also reduced flow-mediated dilation in mesenteric arteries. In rhAPC-treated aorta and mesenteric arteries, contractility and endothelial responsiveness to vasodilator drug and shear stress were improved. rhAPC treatment also improved LPS-induced endothelial dysfunction; this effect was associated with an increase in the phosphorylated form of endothelial nitric oxide synthase and protein kinase B as well as cyclooxygenase vasodilatory pathways, thus suggesting that these pathways, together with the decrease in nuclear factor-κB activation and inducible nitric oxide synthase expression in the vascular wall, are implicated in the endothelial effect of rhAPC. In conclusion, ex vivo application of rhAPC improves arterial contractility and endothelial dysfunction resulting from endotoxemia in mice. This finding provides important insights into the mechanism underlying rhAPC-induced improvements on arterial dysfunction during septic shock.

endotoxiaemia; endothelial dysfunction; endothelial nitric oxide synthase

SEPTIC SHOCK IS ASSOCIATED with hypotension and frequently with disseminated intravascular coagulation contributing to multiple organ failure and a high mortality rate (20, 36). Endothelial dysfunction with impaired release of endothelial nitric oxide (NO) and prostacyclin, reduction of vascular reactivity to vasoconstrictors associated with leukocytes, and platelet aggregation are all hallmarks of the pathophysiology of this syndrome (14). The protein C pathway has been implicated as having a pivotal role in these disturbances (29).

Furthermore, the release of reactive oxygen and nitrogen species by various pathways has been shown to contribute to a number of organ failures such as lung, heart, brain, and liver (7). Data collected from clinical studies and various endotoxemia models suggest that this phenomenon is related to the activation of the nuclear factor (NF)-κB pathway, enabling the expression of several specific genes involved in the pathogenesis of septic shock leading to the production of cytokines, adhesion molecules, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) (33, 34). The induction of iNOS along with an overproduction of NO has also been shown to play a major role in endotoxemia-induced vascular hyporeactivity in several experimental models (31, 38) as well as in small vessels in patients with septic shock (37).

Activated protein C (APC), an endogenous vitamin K-dependent serine protease with multiple biological activities, is an important modulator of the host systemic response to severe infection (32). In a previous experimental study (30), we reported that recombinant human APC (rhAPC) improved cardiovascular function 1 by modulating the endotoxin-induced proinflammatory/proxioxidant state, 2) by decreasing cell adhesion, and 3) by favoring stabilization of the extracellular matrix. These results may well be related to APC anticoagulant and/or nonanticoagulant activities observed in experimental endotoxemia (16, 19). Indeed, rhAPC could act directly on endothelial dysfunction via endothelial protein C receptor (EPCR)/protease activated receptor-1 (PAR-1), by direct modulation of leukocyte migration and cell adhesion expression, thereby blocking cytokine signaling and decreasing vascular permeability and cell apoptosis (18). Moreover, rhAPC has been shown to exhibit multiple biological activities as evidenced both in vivo in several animal studies as well as in vitro in endothelial, smooth muscle, and immune cells (29). Thus the ability of APC to suppress proinflammatory processes and to enhance cellular survival suggests that APC could play a role in adaptive vascular response (32).

The present study was aimed at investigating, ex vivo, the potential protective, blood free, and nonanticoagulant proper-

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ties of rhAPC on vascular dysfunction induced by bacterial lipopolysaccharide (LPS) in isolated mouse arteries. Because differences in their specific roles in blood pressure and tissue specificity induce a specialization of vascular cells (2), the study was performed on conductance (the aorta) as well as on small mesenteric resistance arteries (SMA).

METHODS

Animals and Protocols

All animal experimentations were performed in accordance with institutional guidelines and protocols approved by the French Animal Care Committee in keeping with European regulations. Experiments were conducted in compliance with statutory requirements by accredited research scientists. The procedure for the care and death of study animals was in accordance with the European Community Standards on the Care and Use of Laboratory Animals (authorization no. 6422; Ministère de l’Agriculture).

As described previously (25), experimental male Swiss mice (35–40 g) were injected with LPS (50 mg/kg ip, LPS from Escherichia coli 055:B5; Sigma Aldrich, Saint Quentin Fallavier, France). Mice exhibited lethargy, piloerection, and glassy eyes from the 5th h after LPS injection. Control mice received an equivalent volume of vehicle (0.9% NaCl solution) (control group).

After 10 h, mice were anesthetized with isoflurane gas and killed by decapitation to harvest aortic rings and SMA. Aortic rings (2–3 mm) and second-order SMA were emptied of blood and randomly incubated for 12 h in RPMI/medium 199:1:1 (blood free) supplemented with 10% bovine fetal serum with (LPS group) rhAPC (drotrecogin α activated, Xigris; Lilly, Indianapolis, IN) (50 ng/ml). This dosage corresponds to human plasma levels and within therapeutic concentration ranges, as well as to the concentration used in our previous study (30).

Vascular Reactivity

Aortic rings. Vascular reactivity of aortic rings was studied on a wire myograph (Danish Myo Technology, Arhus, Denmark) as previously described (25). The experiments were performed at 37°C in a physiological salt solution (PSS) with the following composition (in mM): 119 NaCl, 4.7 KCl, 1.8 MgSO4, 2.5 CaCl2, 1.18 KH2PO4, and 5.5 glucose, continuously bubbled with 95% O2 and 5% CO2. After an equilibration period (at least 20 min) vessels. After 20-min washout period, concentration-response curves to the combination of KCl depolarization (100 mM) and phenylephrine (PE) (10 M) were generated by a peristaltic pump. The presence of a functional endothelium was assessed by applying ACh (10 µM) in arteries precontracted with PE (10 µM). To determine FMD at 75 mmHg of pressure, arteries were contracted with PE (50% reduction in diameter) and submitted to stepwise increases in intraluminal flow (0–100 µl/min). Diameter measurements were continuously collected for analysis (Biopac MP 100).

To investigate the mechanisms involved in the endothelial effect of rhAPC, particularly with regard to the role of NO- and COX-induced prostanoids, all of the above vascular experiments were consecutively conducted in the presence or absence of the NO synthase inhibitor Nω-nitro-l-arginine methyl ester (l-NAME) 100 µM; Sigma-Aldrich) or COX inhibitor (indomethacin 10 µM; Sigma-Aldrich). The inhibitor was added in the bath 30 min before the addition of ACh. The first curve from ACh was thus taken as control, and the effect of the inhibitor was tested on the second curve.

Western Blot Analysis

Proteins from aortic lysates were subjected to SDS-PAGE using 9% gels. After electrophoresis, proteins were transferred to nitrocellulose membranes and probed with the following antibodies: monoclonal murine anti-endothelial nitric oxide synthase (eNOS), monoclonal anti-eNOS phosphorylated (eNOS Ser1177), monoclonal anti-protein kinase B (Akt) and anti-Akt phosphorylated (Akt Ser473) (BD Biosciences, San Jose, CA), polyclonal anti-NF-κB p65 (Abcam, Cambridge, UK), and monoclonal anti-inhibitory factor (I-κB) α-phosphorylated (US Biological, Swampscott, MA). The membranes were then washed at least three times in Tris-buffered solution containing 0.05% TWEEN and incubated for 1 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham, Piscataway, NJ). The protein-antibody complexes were detected by the enhanced chemiluminescence system (Amersham, Buckingham, UK) and quantified by densitometry according to the protocol recommended by the manufacturer.

Data Analysis

Data obtained during measurements of vascular reactivity were compared using two-way ANOVA for repeated measurements (data were tested for homogeneity of variance by Levene’s statistics). The nonparametric Kruskal Wallis test was used for comparison of Western blotting measurements between groups. When a significant difference was found between groups, subsequent post hoc tests were performed. All values are presented as means ± SE, with n representing the number of experiments or the number of animals, accordingly.

All of the above analyses were performed with the Statview version 5.0 software (SAS Institute, Cary, NC). P < 0.05 was considered statistically significant.

RESULTS

rhAPC Improves Contractile Capacity and Endothelial Dysfunction Induced by LPS

LPS blunted KCl- and PE-stimulated contraction in aortic rings, whereas rhAPC significantly restored the maximal contractile capacity of aortic rings from LPS-treated mice (3.1 ± 0.5 vs. 1.5 ± 0.3 mN/mm) (P < 0.05) to control levels (3.8 ± 0.6 mN/mm) (Fig. 1A). ACh produced a concentration-dependent relaxation of isolated aortic rings (Fig. 1C). Compared with the control group, vascular responses to ACh were significantly decreased in aorta of LPS-treated mice (60 ± 7 vs. 27 ± 3%) (P < 0.05). Conversely, the addition of rhAPC significantly countered the endothelial dysfunction induced by LPS (58 ± 7 vs. 27 ± 3%; P < 0.05) (Fig. 1C and Table 1). In SMA, stepwise increases in flow induced a progressive vasodilatation in these vessels. Compared with the control group,
FMD was lower in SMA from LPS-treated mice (29 ± 2 vs. 13 ± 4.1 μm, respectively, for a flow rate of 100 μl/min, P < 0.05). Incubation of SMA with rhAPC significantly increased FMD to control levels (28 ± 2.1 μm for a flow rate 100 μl/min (P < 0.05) (Fig. 1D and Table 1). rhAPC did not alter vascular reactivity in either aorta or SMA harvested from the control group (Table 1).

After a pretreatment with rhAPC, l-NAME abolished ACh-induced vasodilatation in the aorta (P < 0.05) and the FMD in SMA (P < 0.01). Addition of indomethacin only affected this response by one-half (Fig. 2, A and B) while the combination of l-NAME plus indomethacin did not alter FMD in SMA (Fig. 2B and Table 1). Conversely, in non-rhAPC-incubated vessels harvested from endotoxemic mice, neither l-NAME nor indomethacin or their combination modified this reactivity. Finally, maximal relaxation of aorta to acetylcholine and FMD in SMA harvested from control mice was similarly altered in the presence or absence of rhAPC (Table 1).

rhAPC restores endothelial NO synthase activation and reduces LPS-induced upregulation of NF-κB and iNOS expression. To investigate the mechanisms involved in the beneficial effect of rhAPC on endothelial-dependent dilatation, the role of the eNOS NO-Akt/phosphatidylinositol 3-kinase pathway was assessed, since the decrease in activity of this pathway may be attributable to altered eNOS phosphorylation. In the aorta, endotoxemia lowered eNOS and phosphorylated eNOS (eNOS Ser1177 phosphorylation) expression levels as well as the phosphorylated eNOS-to-eNOS ratio, consistent with a lower enzyme activity (P < 0.05) (Fig. 3, A and D).

Akt, phosphorylated Akt (Akt Ser473 phosphorylation) expression levels, and the phosphorylated Akt-to-Akt ratio were also decreased in the aorta of endotoxemic mice (Fig. 3, B and E). rhAPC-treated aorta exhibited an enhanced activation of Akt (Fig. 3, B and E) that can phosphorylate eNOS on Ser1177. The difference in the ratio was 2-fold higher in rhAPC-treated

Table 1. Vascular reactivity to ACh (maximal relaxation and effective concentration 50:EC50) of aorta and to FMD of SMA

<table>
<thead>
<tr>
<th>Arteries</th>
<th>Maximal relaxation, %</th>
<th>EC50, μM</th>
<th>SMA (FMD)</th>
<th>Diameter of rhAPC+, μm</th>
<th>Diameter of rhAPC−, μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>61 ± 5</td>
<td>0.1</td>
<td>30 ± 3</td>
<td>29 ± 2</td>
<td></td>
</tr>
<tr>
<td>Control + l-NAME</td>
<td>35 ± 7</td>
<td>1.2</td>
<td>17 ± 3</td>
<td>19 ± 2</td>
<td></td>
</tr>
<tr>
<td>Control + indomethacin</td>
<td>52 ± 4</td>
<td>0.7</td>
<td>20 ± 7</td>
<td>21 ± 5</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>56 ± 3*</td>
<td>0.8*</td>
<td>28 ± 2*</td>
<td>13 ± 4</td>
<td></td>
</tr>
<tr>
<td>LPS + l-NAME</td>
<td>3 ± 1†</td>
<td>23†</td>
<td>6 ± 2†</td>
<td>10 ± 3</td>
<td></td>
</tr>
<tr>
<td>LPS + indomethacin</td>
<td>39 ± 3†</td>
<td>1.2†</td>
<td>12 ± 3†</td>
<td>12 ± 3</td>
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Values are means ± SE; n = 6–8 mice/group. SMA, small mesenteric resistance arteries; FMD, flow-mediated dilatation; rhAPC, recombinant human activated protein C (APC) improves arterial contractility and dilation to ACh and flow-mediated dilatation (FMD). A: KCl- and phenylephrine (PE)-induced contraction in aorta (A, n = 8 mice/group). LPS, lipopolysaccharide. B: Western blot analysis of inducible nitric oxide synthase (iNOS) in aortic rings isolated from control or endotoxemic mice incubated in the presence or absence of rhAPC. Values are expressed as means ± SE. Concentration-response curves to ACh in aortic rings (C, n = 8/group) and FMD in mesenteric resistance arteries (D, n = 8/group). Blood vessels were harvested from control mice with (■) or without (○) rhAPC or endotoxemic mice incubated with (●) or without rhAPC (○). *P < 0.05, LPS + rhAPC vs. LPS group.

Fig. 1. Recombinant human (rh) activated protein C (APC) improves arterial contractility and dilation to ACh and flow-mediated dilatation (FMD). A: KCl- and phenylephrine (PE)-induced contraction in aorta (A, n = 8 mice/group). LPS, lipopolysaccharide. B: Western blot analysis of inducible nitric oxide synthase (iNOS) in aortic rings isolated from control or endotoxemic mice incubated in the presence or absence of rhAPC. Values are expressed as means ± SE. Concentration-response curves to ACh in aortic rings (C, n = 8/group) and FMD in mesenteric resistance arteries (D, n = 8/group). Blood vessels were harvested from control mice with (■) or without (○) rhAPC or endotoxemic mice incubated with (●) or without rhAPC (○). *P < 0.05, LPS + rhAPC vs. LPS group.
aortas in phosphorylated eNOS/eNOS and 2.5-fold higher in the phosphorylated Akt-to-Akt ratio (data not shown).

The NF-κB heterodimer is colocalized in the cytoplasm with the inhibitory protein IκB. Upon cell stimulation, IκB is phosphorylated, removed, and degraded, allowing NF-κB to induce transcription.

Western blotting of the p65 subunit of NF-κB and phosphorylated IκB was therefore used as an index of NF-κB activation (Fig. 3, C and F). No significant expression could be found in aortas from the control group. In endotoxemic mice, however, LPS induced a marked blotting of the p65/RelA subunit of NF-κB and of phosphorylated IκB in the aorta. This blotting pattern was almost completely reversed in aortic rhAPC-treated rings (Fig. 3, C and F). In addition, rhAPC decreased iNOS expression in the aorta (Fig. 1B).

**DISCUSSION**

The present study suggests a nonanticoagulant protective role of rhAPC on endothelial dysfunction during LPS-induced shock, including a potential effect on arterial contractility.

Prompt restoration of organ perfusion is an early goal in the treatment of patients with septic shock. rhAPC has been associated with a more rapid reduction in cardiovascular failure (29) and was initially thought to be related to its anticoagulant and anti-inflammatory effects in severe sepsis. It has since been...
associated with multiple cytoprotective effects including: 1) downregulation of proinflammatory gene expression (17, 41); 2) ant apoptotic activity (3); 3) antioxidant properties (43), and 4) protection of the endothelial barrier function (21, 35), mediated by EPCR and the effectors’ receptor PAR-1 (12).

Recent animal (13, 30, 40) and human (28) studies have suggested that rhAPC may improve hemodynamic and vascular reactivity to catecholamines during septic states. However, much of our current knowledge concerning rhAPC vascular activity is still much debated, especially with respect to its nonanticoagulant properties. For this reason, we designed an ex vivo, blood-free model application of rhAPC on isolated vessels from endotoxemic mice.

A major finding of this study was that rhAPC improved vascular dysfunction with a beneficial effect on both endothelial dysfunction and arterial contractility. To our knowledge, this is the first study reporting new vascular insight on the benefits of the ex vivo nonanticoagulant effect of rhAPC on endothelial dysfunction induced by LPS, which involves several related cellular mechanisms. As previously reported, rhAPC likely contributes in limiting the process of refractory vasoplegia in patients with septic shock (28) as well as in endotoxemic rats (30). Hence, rhAPC is able to blunt iNOS expression and probably subsequent NO production, resulting in an improvement in PE curve-dose contractility. The present results are also supported by Wiel et al. (42), who reported that the contractile response to PE was improved by in vivo administration of rhAPC in a rabbit model of endotoxin-induced shock and that this effect was maximal in the presence of a functional endothelium. Indeed, both EPCR and PAR-1 are expressed in arterial endothelial cells. However, Bretschneider et al. (4) recently reported that vascular smooth muscle cells in vitro also express EPCR and suggested that this receptor-bound APC may modulate PAR-1-mediated responses of SMC via a rise in intracellular calcium. Furthermore, a previous study reported that sepsis causes a progressive and profound reduction in phosphorylation of eNOS in rabbit mesenteric arteries (24). Alterations in endothelial cell surface receptors and modified signal transduction pathways such as receptor-eNOS uncoupling have been shown to result in a loss of the protective role of NO. Although protein expressions were not performed herein in small mesenteric arteries, our results nevertheless support such a hypothesis, since LPS-induced endothelial dysfunction is due in part to decreased eNOS activity, which is most likely associated with a decrease in Akt phosphorylation in the arterial wall. Interestingly, rhAPC overcomes the deleterious endothelial effect of LPS. Indeed, the beneficial effect of rhAPC on both ACh-induced relaxation in the aorta and FMD in SMA could be explained via eNOS activation and COX vasorelaxant metabolites. NO is one of the major endothelial relaxant factors produced by eNOS, allowing the endothelium to regulate smooth muscle tone and proliferation, leukocyte recruitment, and platelet aggregation (27). The expression of eNOS is increased severalfold by multiple factors, including shear stress, vascular endothelial growth factor, ACh, and estrogens. Phosphorylated eNOS, via the Akt/phosphatidylinositol 3-kinase pathway, is known to regulate enzyme activity (11). Endotoxin challenge involves eNOS activity in its early phase and induces a later increase in iNOS expression (8, 10) that can exert a negative feedback on eNOS expression during endotoxemia (6, 23). It is well documented that LPS impairs endothelium-dependent relaxation to ACh as well as flow, leading to endothelial dysfunction in both resistance and conductance arteries (6, 22). Endothelial dysfunction has been associated with a number of pathophysiological processes (34), among which inflammation and oxidative stress appear to be common pathways underlying such cell disturbances. However, the mechanisms underlying this endothelial dysfunction can be markedly different depending on the pathology and the studied vascular bed.

A reduced bioavailability of endothelial NO, an alteration in the production of prostanooids, and impairment in endothelium-dependent hyperpolarization, either individually or in association, can lead to endothelial dysfunction (6, 7). Furthermore, during inflammatory states, COX-derived prostanooids, like thromboxane and prostaglandins, are released and could interact with iNOS-derived NO, resulting in cell disturbances (26). In the present model, nonspecific inhibition of the COX pathway was found to be implicated in the rhAPC effect, confirming the data of Brueckmann et al. (5), who demonstrated that rhAPC upregulates endothelial COX-2 mRNA expression and synthesis of COX-2 enzyme in human umbilical vein endothelial cells with subsequent release of vasodilator prostacyclin. Moreover, in keeping with reported data in the field of LPS-induced endothelial dysfunction (6, 15), we can hypothesize herein, as others (39), that rhAPC reduces NF-kB activation and iNOS expression in the aorta, leading to improved endothelial dysfunction and vascular contractility. Furthermore, the mechanism whereby LPS induces endothelial dysfunction is linked to its ability to activate the NF-kB/Rel transcription family. NF-kB-dependent gene expression may be upregulated in pathological situations and hence impair vascular cell function (9). Conversely, inhibition of NF-kB activation improves cardiovascular functional abnormalities reported in septic shock (1). In vitro studies have shown that incubation of cultured endothelial cells with APC leads to a number of potentially anti-inflammatory cellular changes, including downregulation of the expression of adhesion molecules, improvement of barrier function, and decreased susceptibility to apoptosis (12).

In conclusion, the use of rhAPC has been shown to play a key role in endotoxemia-induced vascular dysfunction. We report herein that rhAPC-induced improvement of endothelial dysfunction is associated with an increase in eNOS activation, through anti-inflammatory but not anti-coagulant properties.

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