Protease-Activated Receptor-2 Involvement in Hypotension in Normal and Endotoxemic Rats In Vivo

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Background—The protease-activated receptor-2 (PAR-2) is expressed by vascular endothelial cells and upregulated by lipopolysaccharide (LPS) in vitro. PAR-2 is activated by a tethered ligand created after proteolytic cleavage by trypsin or experimentally by a synthetic agonist peptide (PAR-2AP) corresponding to the new amino terminus of the tethered ligand.

Methods and Results—Intravenous administration of PAR-2AP (0.1, 0.3, and 1 mg/kg) to rats caused a dose-dependent hypotension. A scrambled peptide was without effect. A specific trypsin inhibitor, biotin–SGKR-chloromethylketone, inhibited trypsin-induced hypotension but not that stimulated by PAR-2AP. In animals treated with LPS 20 hours earlier, we found an increased sensitivity to trypsin and PAR-2AP in the hypotensive response. In particular, PAR-2AP caused hypotension at a low concentration of 30 ng/kg. Moreover, PAR-2 was immunolocalized to endothelial and smooth muscle cells in aorta and jugular vein in LPS-treated rats, and increased levels of PAR-2 mRNA were shown by reverse transcription–polymerase chain reaction analysis.

Conclusions—Our findings suggest that PAR-2 is important in the regulation of blood pressure in vivo. A functional upregulation of PAR-2 by LPS was demonstrated by the activity of concentrations of PAR-2AP that were inactive in normal animals. We conclude that PAR-2 may play an important role in the hypotension associated with endotoxic shock and may represent a new therapeutic target. (Circulation. 1999;99:2590-2597.)

Key Words: receptors | trypsin | endotoxemia | hypotension | shock
myxin B, mepramine, cyproheptadine, and nordihydroguaiaretic acid (NDGA) were from Sigma Chemical Co. Activated CH-Sepharose 4B was from Pharmacia-LKB Biotechnology. Trypsin (porcine pancreas, EC 3.4.21.4, 13,000 to 20,000 BAEE U/mg), indomethacin, atropine, and methohexital sodium were purchased from Sigma. Glyceryl trinitrate (GTN) was a generous gift of Dr Vincenzo Greco (Cardiology Unit, Naples, Italy). The ET_4/ET_6 endothelin inhibitor (SB290670) was obtained from SmithKline Beecham, and HOE-140 was obtained from Hoechst. Hirstolog was a generous gift of Dr Maraganore (Biogen, USA). The biotinylated inhibitor biotin-Ser-Gly-Arg-CH₂CP, which is based on the P1-P4 residues of the PAR-2 cleavage site, was synthesized according to the method of Kay et al.14

Blood Pressure Measurement

Rats (Charles River, Wilmington, Mass., 250 to 300 g) were anesthetized with urethane (solution 15% wt/vol; 1.5 g/kg IP), and the left carotid artery and right jugular vein were cannulated for blood pressure measurement and drug administration, respectively. All the drugs tested were administered intravenously as a slow bolus injection except L-NAME, which was infused through the tail vein during the equilibration period, and a tension of 0.5 g (basal tone) was applied. For each ring mounted, the molar concentration that produced 80% of the maximum contraction (EC₈₀) was established. The rings were washed at 30-minute intervals and tension were recorded continuously with a polygraph linear recorder to an isometric transducer (model 7004, Basile), and changes in tension were measured as mean arterial blood pressure (MABP).

Aortas were cleaned of adherent connective tissue and cut into rings 1.46 mm in length. For each ring mounted, the molar concentration that was a generous gift of Dr Vincenzo Greco (Cardiology Unit, Naples, Italy). The ET_4/ET_6 endothelin inhibitor (SB290670) was obtained from SmithKline Beecham, and HOE-140 was obtained from Hoechst. Hirstolog was a generous gift of Dr Maraganore (Biogen, USA). The biotinylated inhibitor biotin-Ser-Gly-Arg-CH₂CP, which is based on the P1-P4 residues of the PAR-2 cleavage site, was synthesized according to the method of Kay et al.14

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Studies in LPS-Treated Rats

In Vitro Studies

Rats that had received saline or LPS were killed, and the thoracic aorta was removed and immersed in cold gassed (95% O₂/5% CO₂) Krebs solution composed of (in mmol/L) NaCl 115.3, KCl 4.9, CaCl₂ 1.46, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.0, and glucose 11.1. Aortas were cleaned of adherent connective tissue and cut into rings (5 mm long) and placed in 2 mL isolated organ bath filled with gassed Krebs solution maintained at 37°C. The rings were connected to an isometric transducer (model 7004, Basile), and changes in tension were recorded continuously with a polygraph linear recorder (WR 3310 Graphitec). The rings were washed at 30-minute intervals during the equilibration period, and a tension of 0.5 g (basal tone) was applied. For each ring mounted, the molar concentration that produced 80% of the maximum contraction (EC₈₀) was established. Briefly, arteries were contracted with cumulative concentrations of phenylephrine (PE, 0.01 to 3 μmol/L), and the EC₈₀ was determined from the curve obtained before the rings were washed with Krebs solution. Aortas were then contracted with PE EC₈₀, and when a stable tone was reached, a single concentration of acetylcholine (1 μmol/L) was applied to assess the endothelial response. Rings that showed relaxation of <70% were discarded. On the subsequent stable PE contraction, the effect of PAR-2AP or control peptide (10, 30, and 100 μmol/L) was assessed. Peptides were also tested in the presence of L-NAME (100 μmol/L). Data are expressed as percent of relaxation calculated as percent of the maximum relaxation induced by acetylcholine in the same tissue.

Preparation of PAR-2 Antibodies

A peptide corresponding to the activation site of human PAR-2 (SKGRSLIGKVDTSHTVTKG-NH₂ residues 33 to 51) was synthesized as a multiple antigenic construct and was used as an immunogen.14 Rabbit anti–PAR-2 IgGs were purified with an affinity column of the linear peptide coupled to activated CH-Sepharose 4B, as previously described.15 Preimmune sera were purified with a protein-A Sepharose column to yield control IgG.

Immunohistochemistry

Aorta and jugular veins removed from rats were stored at −70°C before use. Serial cryostat (Bright) sections (6 μm) were cut and mounted on APES-coated slides (Sigma) and dried overnight at room temperature. Sections were fixed in cold acetone for 1 minute before blocking with 10% FCS in 0.2 mol/L Tris-HCl buffer for 15 minutes to reduce nonspecific staining. Anti–factor VIII rabbit IgG (Dako), affinity-purified rabbit anti–PAR-2 IgG, or preimmune control IgG was diluted in 2% FCS Tris-HCl (1, 5, 10, and 20 μg/mL), applied to sections, and incubated overnight in a humid chamber at 4°C. After a washing step with PBS, sections were dried and incubated with biotinylated anti-rabbit IgG (Dako) for 30 minutes, followed by avidin–biotinylated alkaline phosphatase complex (Dako) incubated for a further 30 minutes at room temperature. Color was developed with Vector Red (Vector Laboratories) containing 1 mmol/L levamisole to block endogenous alkaline phosphatase activity. The sections were counterstained with hematoxylin, mounted, and examined with a light microscope (Photomath FX4, Nikon).

RT-PCR Analysis

Aortas and jugular veins were removed from control and LPS-treated rats, snap-frozen in liquid nitrogen, and stored at −70°C. Total RNA was isolated with RNAzol solution (Biogenesis) according to the manufacturer’s instructions. RNA purity was estimated by measurement of optical density at 260/280 nm. Total RNA (5 μg) was subjected to first-strand cDNA synthesis in a 10-μL reaction containing 250 mmol/L Tris-HCl (pH 8.3 at 20°C), 375 mmol/L KCl, 15 mmol/L MgCl₂, 1 mmol/L DTT, 1 mmol/L of each dNTP, and 20 U RNase inhibitor, in the presence of 1.5 μg oligo dT₁₂–₁₈ primer and 200 U Superscriptase (all from Life Technologies). After completion of first-strand cDNA synthesis, the reaction was stopped by heat inactivation (5 minutes, 95°C) and diluted to 50 ng/μL RNA equivalents with water. cDNA amounts equivalent to 100 ng of total RNA were subjected to PCR in a 50-μL reaction volume containing 10 mmol/L Tris-HCl (pH 9 at 25°C), 50 mol/L KCl, 1.5 mmol/L MgCl₂, 0.01% (wt/vol) gelatin, 0.1% (vol/vol) Triton X-100, 2 mmol/L DTT, 200 μmol/L of each dNTP, 1 μmol/L of each primer, and 0.2 U of Taq DNA polymerase (AB Biotechnology) under the following conditions: denaturation for 30 seconds at 94°C, primer annealing for 1 minute at 58°C, and primer extension for 1 minute at 72°C. The PCR products (10 μL) were electrophoresed through 1% agarose gels and visualized by UV illumination. Primers used were 5'-ATGCGAAGTCTCAGCCTG-3' (sense) and 5'- TCAGT AGGAGTTTCG-3' (antisense) to amplify a 1900-bp PCR product for rat PAR-2, and 5'- ACCACAGTCCAT-GCCAATC-3' (sense) and 5'- TCACCACCTGGTTTGTG-3' (antisense) to amplify a 400-bp product for GAPDH.

Statistics

Data were analyzed by ANOVA, 1- or 2-way as appropriate, followed by Dunnett’s test.

Results

Effect of PAR-2AP and Trypsin on MABP

As shown in Figure 1A, intravenous administration of human PAR-2AP (0.1, 0.3, and 1 mg/kg IV) caused a dose-related hypotension. The change in MABP was characterized by a rapid fall lasting ~30 seconds for 0.1 mg/kg, 1 minute for 0.3 mg/kg, and 2 minutes for 1 mg/kg (Figure 2A). Trypsin-
induced hypotension (Figure 1B) was very similar to that caused by PAR-2AP. The kinetics of the hypotension and the return to basal blood pressure levels for the doses of 0.2 and 0.6 mg/kg occurred at almost the same time intervals as seen with PAR-2AP (Figure 2B). A dose of 2 mg/kg took ~3 minutes to return to the basal value. There was no tolerance to the hypotensive effect of both PAR-2AP and trypsin (data not shown).

Effect of Inhibitors on Hypotension

Atropine (0.5 mg/kg), HOE-140 (0.1 mg/kg), NDGA (15 mg/kg), and mepyramine (5 mg/kg IV) had no effect on the hypotension caused by trypsin (data not shown). The doses chosen are able to cause at least 50% inhibition of the response to the specific agonist. However, indomethacin (5 mg/kg IV) administered 1 hour before trypsin (2 mg/kg) caused a reduced hypotensive response (Figure 3A), from 47±2.4 to 16±2.2 mm Hg (n=4; P<0.01). The mixed endothelin (ET<sub>A</sub>/ET<sub>β</sub>) antagonist SB209670, at a dose of 1 mg/kg IV given 30 minutes before trypsin (2 mg/kg) administration, caused a reduction of hypotensive response (Figure 3B) from 38±7 to 18±3.4 mm Hg (n=4; P<0.01). L-NAME was given as an infusion at a rate 3 μg · kg⁻¹ · min⁻¹. This dose was determined, in a separate set of experiments, not to cause hypertension (data not shown). Trypsin (2 mg/kg IV)-induced hypotension before the L-NAME infusion was started was 46±8.2 mm Hg. After L-NAME, the hypotension was 29.3±10.4 mm Hg (n=4; P<0.02), 28.7±5 mm Hg (n=4; P<0.02), and 25.7±5.6 mm Hg (n=4; P<0.02) after 20, 30, and 40 minutes after administration, respectively (Figure 3C). Biotin-SKGR-chloromethylketone (0.1 mg/kg IV), a potent irreversible inhibitor of trypsin proteolysis, given 30 minutes before trypsin (2.4 mg/kg) reduced the hypotensive response significantly, from 41.5±6.4 to 21±6.9 mm Hg (n=5; P<0.01). Strikingly, none of the drugs tested, including the trypsin inhibitor, caused a significant inhibition of the PAR-2AP-induced hypotension. Indeed, at a dose of 0.3 mg/kg IV, PAR-2AP gave a hypotension of 27.3±6.3 mm Hg (n=6) that was not significantly inhibited.
obtained from normal and LPS-treated rats. Control and PAR-2 antibodies at a range of dilutions (1, 5, 10, and 20 μg/mL) were used for immunohistochemistry, and the staining intensities obtained with PAR-2 and control antibodies were compared at the same concentration. PAR-2 staining was apparent in endothelial cells and vascular smooth muscle cells within the blood vessels obtained from normal rats and rats treated with LPS 20 hours before death. Endothelial cells were identified in sequential sections by staining for factor VIII antigen (Figure 4A).

The expression of PAR-2 after LPS administration in vivo was investigated by semiquantitative RT-PCR analysis (Figure 4B). Primers based on the rat PAR-2 sequence were used with cDNA of total RNA obtained from the aortas and jugular veins of rats treated with LPS or saline 20 hours before death. The increased intensity of the PAR-2 PCR product bands (1900 bp) obtained (Figure 4) indicated that expression of PAR-2 mRNA was increased in both the aorta and jugular veins of LPS-treated rats compared with saline-treated controls.

**Effect of PAR-2AP and Trypsin on LPS-Treated Rats**

PAR-2AP- and trypsin-induced hypotensive responses were significantly increased in those rats given LPS 20 hours earlier. Initial MABP in LPS-treated rats was 127.4±4.12 mm Hg. However, if experiments were performed 6 or 12 hours after LPS administration, there was no change in hypotension induced by either PAR-2AP or trypsin. As shown in Figure 5A, in LPS-treated rats (13.5×10^6 U/kg IV 20 hours earlier), the hypotension caused by PAR-2AP (0.1 mg/kg IV) was 21.7±1.87 mm Hg, versus 6.2±1.59 mm Hg in saline-treated rats (n=4; P<0.001). PAR-2AP given at 0.03 mg/kg did not cause hypotension in normal rats, but strikingly, the hypotension in LPS-treated rats was 20.7±3.2 mm Hg (n=4; P<0.001; Figure 5A). The hypotensive response to higher doses (0.3 and 1 mg/kg) was not affected significantly by LPS (data not shown). As shown in Figure 5B, the potency of trypsin in the induced hypotensive response was also significantly increased in LPS-treated rats compared with normal rats. At the doses of 0.2 and 0.6 mg/kg, trypsin hypotension was 26.3±3.64 and 36.2±1.99 mm Hg in LPS-treated rats versus 9.87±1.03 and 23.9±1.55 mm Hg in controls (n=4; P<0.001); a lower dose (0.02 mg/kg) was inactive. All the inhibitors tested had no effect on the hypotension induced by trypsin (0.2 to 0.6 mg/kg) or PAR-2AP (0.3 and 0.1 mg/kg). In separate experiments, the hypotensive response in LPS-treated rats to GTN (0.5 mg/kg IV) and bradykinin (10 μg/kg IV) was also evaluated. Hypotension produced by GTN in normal rats (Figure 5C) was not significantly different from that produced in LPS-treated rats (26±2.93 versus 27.1±2.43 mm Hg). Conversely, bradykinin-induced hypotension was enhanced significantly, from 36.3±1.29 mm Hg in normal rats to 50±2.29 mm Hg (P<0.001) in LPS-treated rats (Figure 5D).

The 10-fold reduction in the minimum effective dose of agonist peptide to induce hypotension in LPS-treated rats was striking. As shown in Figure 6, both the kinetics and
magnitude of the hypotension induced by PAR-2AP in LPS-treated rats were identical to those seen in control animals at a 10-fold higher concentration of peptide. These data suggest a functional upregulation of PAR-2 in vivo.

In Vitro Studies

Aortic rings from normal rats precontracted with PE relaxed in a concentration-dependent manner to PAR-2AP (10, 30, and 100 μmol/L) and trypsin. Both responses were significantly potentiated in aortic rings from LPS-treated rats and abolished by L-NAME 100 μmol/L (Figure 7). Removal of endothelium caused a complete loss of the relaxing effect of both trypsin and PAR-2AP.

Discussion

Intravenous administration of trypsin was followed almost immediately by a dose-related hypotension. When PAR-2AP was tested under the same experimental conditions, it caused a hypotensive response that was very similar. Our results are consistent with those obtained previously by others, showing that in vivo activation of PAR-2 causes hypotension.16–18 On the basis of the earliest studies of Rocha e Silva,1–3 we evaluated the involvement of histamine in the trypsin effect on blood pressure. The H1 antagonist mepyramine had no effect on trypsin or PAR-2AP–induced hypotension. These data are in agreement with an earlier study that demonstrated that trypsin-induced hypotension in vivo is resistant to anti-
thistaminic drugs. We investigated the possible mediators involved in the hypotensive response induced by trypsin or PAR-2AP, using a set of inhibitors as pharmacological tools. For this purpose, we used atropine; a cyclooxygenase inhibitor, indomethacin; a bradykinin B2 antagonist, HOE140; an ETα/ETβ endothelin antagonist, SB-209670X; and a nitric oxide inhibitor, L-NAME. Trypsin hypotension was inhibited by indomethacin, SB-209670X ETA/ETB, and L-NAME. We also used a trypsin inhibitor, biotin–SKGR-chloromethylketone, a peptide that specifically blocks the proteolytic activity of trypsin. The finding that trypsin-induced hypotension was inhibited by biotin–SKGR-chloromethylketone indicates that proteolysis is required for the induction of hypotension. Unexpectedly, and strikingly, none of these inhibitors affected the hypotension caused by PAR-2AP. Similarly, in vitro experiments have shown that PAR-2AP–induced relaxation of rat vascular tissues is not affected by a broad range of inhibitors, including atropine, indomethacin, chlorpheniramine, genistein, propranolol, or ritanserin, but only by nitric oxide synthase inhibition. When we tested the effect of PAR-2AP in vitro on precontracted rat aortic rings, the relaxant effect was clearly endothelium-dependent and blocked by L-NAME, confirming the in vitro data obtained previously. Furthermore, we observed a potentiation of relaxation of aortic rings from LPS-treated rats similar to that observed in vivo. The discrepancy between the effects of L-NAME on PAR-2AP actions in vitro and in vivo most likely reflects other interactions that contribute to the hypotension in vivo, whereas in vitro, the effect is completely dependent on nitric oxide. It also seems likely that trypsin activates multiple signaling pathways in vivo, whereas the agonist peptide–induced PAR-2 activation involves specific pathways, unaffected by the inhibitors, which remain to be identified.

To exclude the possibility that thrombin generated after the administration of trypsin may be mediating the hypotensive response, we treated rats with Hirulog, a specific inhibitor of thrombin, before administration of trypsin and PAR-2AP. Neither the PAR-2AP nor trypsin responses were inhibited in rats pretreated with Hirulog. However, we cannot exclude a role for the thrombin receptor, because high concentrations of trypsin can cleave and activate PAR-1 as well as PAR-2. Importantly, by contrast, the PAR-2–activating peptide PAR-2AP is relatively specific and does not activate PAR-1. Therefore, the differences between the responses to trypsin compared with those induced by PAR-2AP in vivo may be due, in part, to the simultaneous activation of PAR-1 and PAR-2 by trypsin but of PAR-2 alone by PAR-2AP. This raises interesting questions about what effects PAR-1 and PAR-2 activation together may have on blood pressure. Some authors have suggested that PAR-1 activation produces a biphasic change in blood pressure, whereas PAR-2 activation alone may cause a single-phase hypotensive response, and our data would support the latter.

After stimulation of endothelial cells with LPS, PAR-2 mRNA is elevated 5- to 10-fold, whereas the thrombin PAR-1 receptor expression is unaffected. Therefore, we extended this study to test the possible role of PAR-2 in the hypotension of endotoxemic rats and to evaluate whether endotoxin has a role in the regulation of PAR-2 receptor in vivo. Because hypotension is one of the main features of septic shock, we evaluated in vivo whether the hypotensive response to PAR-2AP and trypsin was modified by LPS and whether expression of PAR-2 was concomitantly increased in artery and vein ex vivo. PAR-2 was immunolocalized to

Figure 6. Comparison between a representative tracing of effect of PAR-2AP in (A) rats treated with LPS 20 hours before PAR-2AP administration with a 10-fold lower dose of PAR-2AP (0.03 mg/kg IV; n=4) vs B, control rats (0.3 mg/kg IV; n=4). Dose of 0.03 mg/kg in normal rats was without effect.
Interestingly, a drug called ulinastatin, a trypsin inhibitor, is currently used in Japan to treat septic shock. However, a key question that remains to be resolved is the identity of relevant pathophysiological protease ligand for PAR-2, because trypsin is not found in the circulation, except perhaps during gastrointestinal tract surgery.

In conclusion, we have shown that trypsin and a PAR-2AP cause a dose-dependent hypotension in normal rats and that the hypotensive effect was exacerbated by pretreating rats with LPS. PAR-2AP caused a sustained hypotension at a concentration as low as 30 ng/kg, well within the concentration range used in vitro to assess the effects of LPS on PAR-2AP responses in endothelial cells. Increased vascular expression of PAR-2, immunolocalized to endothelial and smooth muscle cells, was also demonstrated in LPS-treated rats by RT-PCR, and a marked functional upregulation of the receptor was shown by a 10-fold lower dose of PAR-2AP inducing hypotension in LPS-treated rats. These data strongly indicate a potentially important role for PAR-2 hypotension and endotoxemia. Unraveling the roles of PAR-2 in endotoxemia, along with those of PAR-1 and the newly cloned PAR-3, may lead to the development of new therapeutic strategies and targets.

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References


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