Hirudin and Nitrates Inhibit the Thrombin-Induced Release of Endothelin From the Intact Porcine Aorta

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In intact porcine aorta, endothelium-derived nitric oxide released on thrombin stimulation inhibits the concomitant production of endothelin. Experiments were designed to examine the effect of hirudin (which inactivates thrombin) and the nitrovasodilators nitroglycerin and 3-morpholinosydnonimine on the spontaneous and thrombin-stimulated release of endothelin in intact blood vessels. Endothelin was detected by radioimmunoassay in the incubating medium of intact porcine aortas with endothelium. The spontaneous release of endothelin was not affected by hirudin (0.1 µg/ml) but that induced by thrombin (4 units/ml) was prevented. Nitroglycerin (10⁻⁴ M) and the active metabolite of molsidomine, 3-morpholinosydnonimine (10⁻⁵ M), did not modify the basal production of endothelin from the intima of intact porcine aortas. However, the nitrates fully inhibited the release of the peptide induced by thrombin (4 units/ml). The inhibitory effects of both 3-morpholinosydnonimine and nitroglycerin on the thrombin-stimulated release of endothelin were abolished in the presence of an inhibitor of soluble guanylate cyclase, methylene blue (10⁻⁴ M). Thus, the thrombin-stimulated release of endothelin is inhibited by inactivation of thrombin with hirudin or by agents that mimic the effect of endothelin-derived nitric oxide. In contrast, the spontaneous production of endothelin is not modulated by the drugs. (Circulation Research 1991;68:1768–1772)

Endothelin, a 21–amino acid peptide purified from the culture medium of endothelial cells, is a potent endothelium-derived contracting factor.¹⁻⁴ The peptide is released spontaneously and on stimulation with thrombin or the calcium ionophore A23187 from endothelial cells in culture and the intima of the intact porcine aorta.⁵⁻⁷ Both in cultured endothelial cells and in intact blood vessels, the nonselective inhibitor of protein synthesis cycloheximide abolishes the basal release and that induced by thrombin suggesting that endothelin is not stored in endothelial cells and that its release involves de novo protein synthesis.³⁻⁵⁻⁷

Thrombin not only stimulates the release of endothelin, but also that of endothelium-derived nitric oxide.⁶⁻⁸ Nitric oxide activates soluble guanylate cyclase and subsequently increases cGMP in vascular smooth muscle and in endothelial cells.⁹⁻¹¹ In the porcine aorta, endothelium-derived nitric oxide released after activation of the thrombin receptors inhibits the production of the peptide via a cGMP-dependent pathway.⁵

The purpose of the present experiments was to examine whether the production of endothelin from the intact porcine aorta induced by thrombin could be inhibited by hirudin, which specifically inhibits thrombin by the formation of a stable complex¹² or by nitrates such as nitroglycerin and 3-morpholinosydnonimine (SIN-1, the active metabolite of molsidomine), which mimic the action of endothelin-derived nitric oxide. Both classes of drugs are of great interest as they could be used as therapeutic tools aiming at inhibiting the formation of the peptide in the blood vessel wall.

Materials and Methods

Blood Vessels

Aortas were obtained from farm pigs killed at the nearby slaughterhouse and placed in modified Krebs-Ringer solution (control solution, millimolar composition: NaCl 118.3, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.0, Ca-EDTA 0.026, and glucose 11.1, pH 7.4). The blood vessels were cleaned...
of connective tissue and opened longitudinally. Great care was taken to preserve the intimal surface.

**Measurement of Endothelin**

The blood vessels (10 cm² intimal surface) were incubated at 37°C in 3 ml control solution containing 0.1% bovine serum albumin, aerated with a mixture of 95% O₂–5% CO₂, as previously described. As in previous studies, both spontaneous and stimulated release of endothelin could be observed only in preparations with endothelium because the release of the peptide was below the detection limit of the radioimmunoassay in denuded blood vessels. For each animal, part of the aorta was used for control experiments and the remaining parts for the various treatments. To study the effect of hirudin, nitroglycerin, SIN-1, and/or methylene blue on the thrombin-stimulated production of endothelin, the blood vessels were incubated for 15 minutes with the compounds before the addition of thrombin. Thrombin was used at a concentration (4 units/ml) that induced maximal stimulation of the production of endothelin in the porcine aorta. In intact porcine aortic strips, the release of endothelin requires de novo protein synthesis since no detectable amount of peptide was released from tissues incubated with cycloheximide. Accordingly, the incubation media were collected after 4 hours of incubation, and the amount of peptide was measured using a radioimmunoassay kit for detection of endothelin (Peninsula Laboratories, Merseyside, UK) as previously described. Under the experimental conditions described above, the amount of peptide released after 2 hours of incubation from intact preparations was below the detection limit of the assay. For the sake of clarity, the peptide detected by radioimmunoassay (immunoreactive endothelin) will be called endothelin.

**Drugs**

Bovine serum albumin and thrombin (human) were purchased from Sigma Chemical Co., St. Louis; recombinant hirudin was supplied by CIBA-GEIGY Ltd., Basel, Switzerland; methylene blue was purchased from Merck, Zurich; and nitroglycerin was obtained from the pharmacy of the University Hospital, Basel, Switzerland. Nitroglycerin stock solution (1%) was in ethanol, and dilutions were obtained in control solution with 0.1% bovine serum albumin. SIN-1 was obtained from Hoechst Laboratories, Paris. All other drugs were dissolved in control solution containing bovine serum albumin (0.1%). All concentrations are expressed as final molar concentrations in the incubation buffer.

**Statistical Analysis**

Results are expressed as the mean±SEM of picograms immunoreactive endothelin released per square centimeter of intimal surface after 4 hours of incubation. In all experiments, n refers to the number of pigs from which the aortas were obtained. Each sample was measured in duplicate. Statistical evaluation of the data was performed using the t test for paired observations and Scheffe’s test for multiple comparisons. Differences were considered significant if p<0.05.

**Results**

**Spontaneous and Stimulated Formation of Endothelin**

In porcine aortas with endothelium, the spontaneous release of endothelin over 4 hours of incubation was 37±3 pg/cm² (n=22). The basal production of the peptide was not affected by hirudin (0.1 μg/ml) (Figure 1), nitroglycerin (10⁻⁵ M) (Figure 2), or SIN-1 (10⁻⁵ M) (Figure 3, n=6 each). Thrombin (4 units/ml) significantly increased the production of the peptide from 37±3 to 67±5 pg/cm² (n=18; p<0.05).
Effect of Hirudin on the Production of Endothelin

Hirudin (0.1 μg/ml) did not modify the spontaneous release of the peptide but prevented that induced by thrombin (4 units/ml) (Figure 1, n=6, p<0.05). Hirudin (0.1 μg/ml) did not affect the release of endothelin induced by A23187 (10^{-6} M) (n=4). The amount of endothelin released by A23187 averaged 121±11 pg/cm^2 in the absence and 115±10 pg/cm^2 in the presence of hirudin (p=NS).

Effect of Nitrates on the Production of Endothelin

Nitroglycerin (10^{-5} M) and SIN-1 (10^{-5} M) prevented the thrombin-induced release of endothelin (Figures 2 and 3, p<0.05, n=6). Methylene blue (10^{-5} M) did not affect the spontaneous production of the peptide (43±5 pg/cm^2, n=12, NS) but significantly increased that induced by thrombin (4 units/ml) from 77±5 to 89±7 pg/cm^2 (n=12, p<0.05). Preincubation of porcine aortic strips with methylene blue (10^{-5} M) abolished the inhibitory effect induced by SIN-1 and nitroglycerin on the thrombin-induced release of endothelin (Figures 2 and 3). Under these experimental conditions, the amount of peptide released was not different from that induced by thrombin in the presence of methylene blue (89±5 pg/cm^2).

Discussion

The present experiments demonstrate that, in the intact porcine aorta, the release of endothelin induced by thrombin can be prevented by hirudin as well as by nitrates, such as nitroglycerin and SIN-1. Hirudin, a 65–amino acid polypeptide, is the most potent and specific inhibitor of thrombin. It rapidly forms a stable equimolar complex and in turn inactivates thrombin. In vitro, hirudin reduces endothelium-dependent responses induced by thrombin in canine arteries. Similarly, in cultured porcine aortic endothelial cells, hirudin prevents the increase in cGMP induced by thrombin. The present study demonstrates that hirudin abolishes the release of endothelin induced by thrombin in the intact porcine aorta. In contrast, hirudin by itself did not modify the spontaneous production of the peptide, nor that induced by A23187. This suggests that hirudin specifically inhibits the thrombin-stimulated release of endothelin—and concomitantly inhibits that of endothelium-derived relaxing factor by inactivating thrombin before it binds to its receptor on the endothelium, rather than by impairing the capacity of the endothelial cells to produce the vasoconstrictor peptide.

Nitric oxide and agents releasing endothelium-derived relaxing factor increase the level of cGMP in endothelial and vascular smooth muscle cells. This increase in cGMP results from the activation of the soluble guanylate cyclase since it is blocked by methylene blue, an inhibitor of the enzyme. In the intact porcine aorta, endothelium-derived nitric oxide, which is concomitantly released with endothelin on stimulation with thrombin, inhibits the production of the peptide through a mechanism involving cGMP. In line with this interpretation, methylene blue increased the thrombin-induced release of endothelin as previously reported. This may provide an explanation for the physiological role of the cGMP rise induced by nitric oxide in endothelial cells. However, it cannot be excluded that an activation of guanylate cyclase in vascular smooth muscle cells leads to the formation of a second messenger, which then exerts a negative feedback on the production of the peptide in endothelial cells.

This regulatory role of nitric oxide on the formation of endothelin could be used therapeutically with agents supplying exogenous nitric oxide such as nitrovasodilators. The active metabolite of molsidomine, SIN-1, releases the free radical nitric oxide via a nonenzymatic reaction, whereas nitroglycerin requires a complex enzymatic biotransformation involving thiol groups to achieve full biological activity. SIN-1 and nitroglycerin mimic the effects of nitric oxide in cultured endothelial and vascular smooth muscle cells by activating soluble guanylate cyclase leading to increased intracellular levels of cGMP. Indeed, in endothelial cells the rise in cGMP induced by SIN-1 is abolished in the presence of methylene blue. In this study, SIN-1 and nitroglycerin prevented the thrombin-stimulated release of endothelin as previously reported for endothelium-derived nitric oxide. The involvement of a cGMP-dependent mechanism is supported by the fact that methylene blue, either by inhibiting soluble guanylate cyclase or by inactivating nitric oxide, abolished both the inhibitory effect of SIN-1 and nitroglycerin on the thrombin-stimulated release of endothelin. However, both SIN-1 and nitroglycerin did not affect the basal release of the peptide. This is in good agreement with the previous observa-
tion that the inhibitor of endothelium-derived nitric oxide, N⁶-monomethyl-L-arginine, methylene blue, as well as 8-bromo-cGMP did not affect the spontaneous production of the peptide under comparable experimental conditions.

Thus, the spontaneous production of endothelin from the intact porcine aorta is insensitive to a pharmacological intervention involving activation of soluble guanylyl cyclase.

Activation of the coagulation cascade with concomitant formation of thrombin is a known event in unstable angina and myocardial infarction. Thrombin can exert effects both in platelets (to cause aggregation) and in endothelial cells of the blood vessel wall. Although the concentration of thrombin used in this study is rather high, increased levels of the enzyme may be reached locally in the vicinity of the endothelium at sites where platelets aggregate and the coagulation cascade is activated, particularly when cyclic flow variations occur and local blood flow decreases.

If so, the thrombin-induced endothelin production may contribute to vasospastic events occurring under these conditions. This may be particularly pronounced under conditions of an impaired formation of endothelium-derived relaxing factor, such as hypercholesterolemia and atherosclerosis. Indeed, even threshold and low levels of the peptide potentiate the contractions induced by norepinephrine and serotonin in human arteries. Hirudin and nitric oxide donors may represent new therapeutic tools to interfere with this vascular action of thrombin.

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