Phosphoramidon-Sensitive Conversion of Big Endothelin-1 and Degradation of Endothelin-1 in Rat Kidney

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Abstract We investigated the intrarenal conversion of big endothelin-1 (ET-1) to ET-1 in the isolated perfused rat kidney. Big ET-1 caused a concentration-dependent increase in perfusion pressure, and the pressor molar potency of the peptide was 50-fold less than that of ET-1. The big ET-1 (2×10⁻⁵ mol/L)-induced pressor action was accompanied by increases in immunoreactive endothelin levels in both the perfusate and renal tissues. Phosphoramidon (10⁻⁴ mol/L), a metalloproteinase inhibitor, significantly suppressed the big ET-1-induced pressor action and the accumulation of immunoreactive endothelin in renal tissues. On the other hand, phosphoramidon slightly but significantly sustained the ET-1-induced pressor effect. The effect of kelatorphan (10⁻⁴ mol/L), a specific inhibitor of neutral endopeptidase 24.11, on the ET-1-induced pressor effect was the same as that seen with phosphoramidon. When ET-1 was exogenously added to the perfusate, phosphoramidon or kelatorphan significantly increased the immunoreactive endothelin levels in renal tissues after perfusion, without affecting the disappearance rate of immunoreactive endothelin from the perfusate. Therefore, the phosphoramidon-sensitive ET-1→ET-1 converting enzyme in the kidney seems to contribute to the functional local conversion of big ET-1 to ET-1, and neutral endopeptidase 24.11 may be responsible for the proteolytic degradation of ET-1 in the kidney. In addition, immunoreactive endothelin levels in renal tissues but not in the perfusate can account for the functional conversion of big ET-1 to ET-1 and for the local proteolytic degradation of ET-1 in the kidney. (Hypertension. 1994;24:227-233.)

Key Words • endothelins • kidney • vasoconstriction • metalloproteinases • enzyme inhibitors

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide originally isolated from the supernatant of cultured porcine aortic endothelial cells. Sequence analysis of cDNA encoding ET-1 suggested that ET-1 is produced from an intermediate form termed big ET-1 by a specific endothelin-converting enzyme (ECE). Several candidates for ECE have been identified in cultured endothelial cells and tissue homogenates, and attention has focused on a neutral metalloproteinase. We and other investigators consider that phosphoramidon-sensitive metalloproteinase is the most plausible candidate for ECE in endothelial cells and other tissues.

Big ET-1 is a weaker vasoconstrictor than ET-1 in vitro by two orders of magnitude but has a pressor effect comparable to that of big ET-1 in vivo. Phosphoramidon was seen to markedly suppress big ET-1-induced hypertensive effects without affecting the response to ET-1 in anesthetized rats. These findings suggest that phosphoramidon-sensitive conversion of big ET-1 to mature ET-1 is essential for the expression of full biological activities in vivo.

ET-1 has diverse biological activities in the kidney. Administration of ET-1 into the renal artery in intact animals induces intense renal vasoconstriction, and ET-1 has a direct effect on renal tubules. In addition, ET-1 is produced in the kidney by both endothelial cells and nonendothelial cells, including mesangial and tubular epithelial cells. ET-1 is most likely an autocrine/paracrine hormone regulating renal function. Several reports suggest that an overproduction of ET-1 plays a pathophysiological role in renal dysfunction, including acute renal failure and cyclosporine nephrotoxicity. Thus, the kidney could serve as a therapeutic target organ for the action of putative ECE inhibitors.

We used an in vitro model, isolated perfused rat kidney, to examine the pressor response to big ET-1 and the production of ET-1 and evaluated the functional role of phosphoramidon-sensitive ECE.

Methods

Isolated Perfused Rat Kidney

Experiments were performed on male Sprague-Dawley rats weighing 250 to 350 g. Animals were anesthetized with sodium pentobarbital (50 mg/kg IP), and the abdomen was opened by a midline incision. The ureter was cannulated with a polyethylene catheter. The right renal artery was cannulated via the mesenteric artery, and the kidney was removed from the animal and perfused at a constant flow rate of 4 ml/min with Dulbecco's modified Eagle medium containing penicillin (100 U/ml), streptomycin (0.1 mg/ml), and fraction V bovine serum albumin (1 g/dl). The perfusate was constantly bubbled with 97% O₂/3% CO₂, to adjust the pH to 7.4 to 7.6 and for oxygenation. Just before use, the perfusion medium was passed through 0.45-μm filters. A kidney was placed in a siliconized 30-ml organ bath maintained at 37° to 38°C and perfused in an open system for 20 minutes to avoid contamination by plasma components; thereafter, the perfusion system.
was changed to a closed system (effluent from the renal vein was reperfused via the perfusion reservoir). All experiments were done in the closed system. We ended the experimental period within 120 to 150 minutes to avoid any functional damage of the kidney. Changes in mean perfusion pressure were measured at a point close to the kidney by a pressure transducer (AP 601G, Nihon Kohden), and a polygraph (RM 6000G, Nihon Kohden) was used for recording.

**Experimental Protocol**

After an equilibration period of 25 minutes in the closed system, perfusion pressure was measured. In dose-response experiments, six ET-1 concentrations were cumulatively perfused, the next dose being perfused when the effects of the preceding one had subsided. In the cases of addition of big ET-1, only two selected doses of six doses of big ET-1 were cumulatively added per one isolated kidney over 120 to 150 minutes because of limitations of the experimental period. Perfusion of the vehicle had no effect on perfusion pressure. In time course experiments, big ET-1 (2×10^{-8} mol/L) or ET-1 (5×10^{-8} mol/L) was perfused after a 25-minute stabilization period. In this case, sampling of perfusate (0.25 mL) and measurements of perfusion pressure were done just after the perfusion of big ET-1 or ET-1 and thereafter at 5, 15, 30, and 60 minutes, respectively. In the case of pretreatment with phosphoramidon (10^{-4} mol/L) or kelatorphan (10^{-4} mol/L), each inhibitor was added 15 minutes before perfusion of big ET-1 or ET-1.

**Tissue Extraction**

ET-1 was extracted from the kidney according to our method described elsewhere, with minor modifications. Briefly, after a 60-minute perfusion with big ET-1 (2×10^{-8} mol/L) or ET-1 (5×10^{-8} mol/L) in the presence or absence of inhibitor, rat kidneys were weighed and homogenized for 60 seconds in 8 vol ice-cold organic solution (chloroform/methanol, 2:1, including 1 mmol/L N-ethylmaleimide). The homogenates were left overnight at 4°C, then 0.4 vol distilled water was added to the homogenate. The homogenate was centrifuged at 3000 rpm for 30 minutes, and the supernatant was stored. Aliquots of the supernatant were diluted 1:10 with a 0.09% phosphoramidon (Peptide Institute Inc); kelatorphan (synthesized in our laboratory); and 125I-ET-1 (Amersham). Antibodies for EIA (30821 and 30846) were obtained from International Reagents Corp. Big ET-1 and ET-1 were added as solutions in 0.9% saline, including 0.1% bovine serum albumin. Phosphoramidon was dissolved in physiological saline and added to the perfusate. Kelatorphan was added as solution in ethanol. The final ethanol concentration was 0.3%; we verified that this concentration had no effect on the pressor response to ET-1.

**Measurement of ET-1**

For measurement of ET-1, perfusate samples were subjected to a sandwich-type enzyme immunoassay (EIA). Briefly, microtest plates coated with rabbit anti-ET-1 C-terminal heptapeptide (15-21) antibody (30821) were incubated at 37°C for 1 hour with 100 μL of standard solutions or perfusate samples. After washing with phosphate-buffered saline (PBS), the plates were allowed to react at 37°C for 30 minutes with 100 μL horseradish peroxidase-labeled rabbit anti-ET-1 N-terminal loop domain antibody (30846). After the plates were washed with PBS, the bound enzyme activity was measured using o-phenylenediamine as a chromogen. The EIA did not cross-react with big ET-1.

**Drugs**

Drugs used were ET-1, big ET-1, and phosphoramidon (Peptide Institute Inc); kelatorphan (synthesized in our laboratory); and 125I-ET-1 (Amersham). Antibodies for EIA (30821 and 30846) were obtained from International Reagents Corp. Big ET-1 and ET-1 were added as solutions in 0.9% saline, including 0.1% bovine serum albumin. Phosphoramidon was dissolved in physiological saline and added to the perfusate. Kelatorphan was added as solution in ethanol. The final ethanol concentration was 0.3%; we verified that this concentration had no effect on the pressor response to ET-1.

**Statistics**

Results are expressed as mean±SEM and were examined by ANOVA. The difference of means was further assessed with Duncan's multiple comparison test (when three groups were compared: Figs 3, 5, 6, and 7) or Student's unpaired t test (when two groups were compared: Fig 2). A value of P<.05 was considered statistically significant.

**Results**

**Pressor Effects of ET-1 and Big ET-1**

The basal perfusion pressure of the rat kidney preparation used in this study was 60.5±2.2 mm Hg (n=53). Time control studies indicated that the renal perfusion pressure remained stable throughout the experiments. Kidney weight after perfusion did not differ among the groups. As shown in Fig 1, ET-1 (5×10^{-11} to 1×10^{-8} mol/L) caused a concentration-dependent increase in perfusion pressure in the isolated perfused rat kidney. The increase in perfusion pressure observed after administration of the peptide was slow in onset and
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A)

B)

reached a maximum after approximately 15 minutes. At 1×10⁻⁸ mol/L, the observed change in perfusion pressure was 175.0±10 mm Hg. Big ET-1 (10⁻⁸ to 1×10⁻⁷ mol/L) also caused a concentration-dependent increase in perfusion pressure, although the increases were slower than those seen with ET-1. The maximal dose of big ET-1 (1×10⁻⁷ mol/L) produced an increase of 165.0±15 mm Hg. The pressor molar potency was 50-fold lower than that of ET-1 in the perfused kidney.

Effect of Phosphoramidon on Pressor Action and ET-1 Accumulation in Perfusate During Kidney Perfusion With Big ET-1

As shown in Fig 2A, big ET-1 (2×10⁻⁸ mol/L) caused a slow onset of and time-dependent increases in perfusion pressure. After 60 minutes of perfusion of big ET-1, the observed change was 84±9.4 mm Hg. The pressor response to big ET-1 was markedly suppressed by pretreatment with phosphoramidon (10⁻⁴ mol/L). In the presence of phosphoramidon, the observed increase was 18.8±10.8 mm Hg. As shown in Fig 2B, the pressor responses to big ET-1 were accompanied by increases of IR-ET in the perfusate. The IR-ET level after 60 minutes increased to approximately 30-fold above that of baseline. Phosphoramidon did not significantly attenuate the increase of IR-ET in the perfusate. In our preparation, neither the vehicle nor phosphoramidon modified basal perfusion pressure or IR-ET level in the perfusate.

HPLC Analysis

Fig 4 shows a typical HPLC profile of IR-ET that accumulated in renal tissue after perfusion of big ET-1 (2×10⁻⁸ mol/L). As shown in Fig 4A, the IR-ET component in the tissue eluted as two major peaks. The eluting position of one peak was identical to that of the synthetic ET-1, whereas another peak (probably the Met'sulfoxide form of ET-1) eluted approximately 1 minute earlier than seen with the synthetic ET-1. After pretreatment with phosphoramidon, both IR-ET components were markedly suppressed (Fig 4B).

Effects of Enzyme Inhibitors on Pressor Action of ET-1

As shown in Fig 5, ET-1 (5×10⁻¹⁰ mol/L) caused a significant increase in perfusion pressure. Maximal response was seen at 15 minutes after the start of perfusion, and the observed change in perfusion pressure was 63.4±9.3 mm Hg. Thereafter, the increase in perfusion pressure gradually reverted to the basal level. Phosphoramidon (10⁻⁴ mol/L) did not affect the maximal pressor response to ET-1 but did significantly augment the duration of the ET-1-induced increases in perfusion pressure. At 60 minutes after the perfusion of ET-1, with or without phosphoramidon, the observed changes were 29.0±4.0 and 11.2±1.5 mm Hg, respectively. Ketorolaphan (10⁻⁴ mol/L), the inhibitor of the neutral endopeptidase 24.11 (NEP), showed a similar potentiation of the ET-1-induced pressor effect.

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Effects of Enzyme Inhibitors on Disappearance of ET-1 From the Perfusate

When ET-1 (5 × 10^(-10) mol/L) was exogenously applied to the perfusion system, the peptide rapidly disappeared from the perfusate, and only 3.2% of the applied ET-1 remained after 60 minutes. The apparent half-life of synthetic ET-1 in the perfusate was 4 to 5 minutes. No differences in the disappearance rate of ET-1 from the perfusate were observed with or without enzyme inhibitors (Fig 6).

Effects of Enzyme Inhibitors on ET-1 Accumulation in Tissue Perfused With ET-1

Fig 7 shows the effects of phosphoramidon or kelatorphan on IR-ET levels in renal tissues after 60 minutes of perfusion of ET-1. Only 10% of the applied ET-1 was recovered in the kidney. Pretreatment of the kidney with phosphoramidon or kelatorphan significantly increased the IR-ET levels that accumulated in the tissues.

Discussion

Considerable evidence now exists for the specific conversion of big ET-1 to ET-1 by a phosphoramidon-sensitive ECE. We have recently demonstrated that the big ET-1-induced vasoconstriction is caused by the phosphoramidon-sensitive conversion of big ET-1 to ET-1 in isolated vascular tissues. In the present study we investigated the functional role of phosphoramidon-sensitive ECE in the isolated rat perfused kidney. Our results clearly show that big ET-1 is enzymatically converted in the renal vasculature and that this converting activity is fully sensitive to phosphoramidon, thereby suggesting the presence of a functional phosphoramidon-sensitive ECE in the rat kidney.

In the isolated perfused kidney, the pressor molar potency of big ET-1 is 30- to 50-fold less than that seen
with ET-1. We reported that in the isolated perfused rat lung, big ET-1 has only a fivefold less potent pressor molar potency than ET-1. These results may indicate that ECE activity in the kidney is less potent than that in the lung and that the converting activity is likely to be variable throughout several vascular tissues. In line with these observations, Pollock and Opgenorth noted that the conversion of big ET-1 may occur at a relatively low rate in the kidney compared with other vascular beds.

In the present study big ET-1 caused a time-dependent increase in perfusion pressure in the isolated rat kidney. Big ET-1-induced pressor action was accompanied by an increase of IR-ET levels in both the perfusate and renal tissue. Thus, in this preparation the conversion of big ET-1 to ET-1 apparently occurred. Phosphoramidon significantly suppressed the big ET-1-induced pressor effect. On the other hand, the inhibitor decreased the accumulation of IR-ET during perfusion of big ET-1 in renal tissues but not in the perfusate. It is reasonable to consider that in the kidney, exogenously applied big ET-1 is converted to ET-1 by the action of phosphoramidon-sensitive ECE and that the generated ET-1 causes vasoconstriction. Our results further suggest that IR-ET levels in the tissue rather than in the perfusate account for the functional conversion of big ET-1 to ET-1 in the isolated rat kidney. The ineffectiveness of phosphoramidon on IR-ET accumulation in the perfusate may be related to the existence of a phosphoramidon-insensitive enzyme in the perfusates. In recent work we noted that the presence of a chymotrypsin-like enzyme released from the lung can generate a small amount of ET-1 in the perfusate, although generation of this ET-1 did not influence perfusion pressure. In the isolated perfused kidney, although the precise location of ET-1 generation within the kidney is unknown, ET-1 converted from big ET-1 by a phosphoramidon-sensitive ECE could accumulate in tissue rather than in the perfusate. One study suggested that big ET-1 has direct vasoconstrictor effects without conversion to ET-1 in the rat kidney. Although our results do not exclude this possibility, most if not all of the pressor effect of big ET-1 in the kidney is attributed to ET-1 converted from big ET-1. Further studies should address the point of whether big ET-1 has an intrinsic direct vasoconstrictor effect in vivo.

With our preparation it is important to keep in mind the amount of ET-1 converted from exogenous big ET-1. Kidney perfusion with big ET-1 for 60 minutes resulted in approximately a 0.27% conversion of big ET-1 to ET-1 in the tissues. However, this value probably underestimates the extent of conversion because of simultaneous ET-1 degradation. In the same preparation, only 10% of exogenously applied ET-1 was recovered in the tissue after 60 minutes of perfusion of the peptide (15 pmol). If one assumes that newly formed ET-1 (converted from big ET-1) disappears to the same extent as the exogenously applied ET-1, a conversion rate of big ET-1 to ET-1 after 60 minutes of perfusion can be calculated at 2.7%. This estimate is supported by comparing equipressor doses of ET-1 and big ET-1.

In the present study phosphoramidon slightly but significantly sustained the ET-1-induced pressor effect. It is well known that phosphoramidon potently inhibits the activity of NEP, the enzyme reported to be involved in the metabolism of a number of vasoactive peptides. It has been shown that NEP degrades ET-1 in vitro and that it may play a role in the inactivation of ET-1 in vivo. Therefore, we hypothesize that the effect of phosphoramidon on the ET-1-induced pressor effect may be caused by inhibition of NEP responsible for degrading ET-1. To gain support for this hypothesis, we examined the effect of another inhibitor of NEP, kelatorphan, which has no inhibitory activity on ECE, on the ET-1-induced pressor effect. The effect of kelatorphan on the ET-1-induced pressor effect was the same as that of phosphoramidon, so NEP probably participates in the enzymatic degradation of ET-1 in the rat kidney. Our results obtained using isolated kidney are in contrast to the finding that phosphoramidon does not affect the renin effect of ET-1 in intact animals. The reason for this discrepancy is unclear but may be due to differences in experimental protocol or conditions. In addition, the absence (isolated kidney) or presence (in vivo) of extrarenal humoral or nervous influences may be involved in the above discrepancy. On the other hand, Salvati et al reported that phosphoramidon potentiates the renal vasoconstrictive effect of ET-1 in vivo. This result supports the hypothesis that NEP is responsible for the degradation of ET-1 in the kidney. Our previous studies have shown that phosphoramidon does not affect the ET-1-induced pressor effect in the isolated perfused rat mesenteric artery and lung. The contribution of NEP to the degradation of ET-1 might not be uniform in various tissues.

Asaad et al have reported that SQ 28,603, a selective NEP inhibitor, does not affect the circulatory clearance of ET-1 administered intravenously in vivo. They suggested that NEP does not contribute to the in vivo clearance of ET-1. In the present study, when ET-1 was exogenously added to the perfusate, phosphoramidon or kelatorphan did not affect the disappearance of IR-ET in the perfusate. On the other hand, both phosphoramidon and kelatorphan significantly increased IR-ET accumulation in the tissues after perfusion of ET-1. Therefore, the ET-1 level in circulating blood or perfusate may not accurately reflect the local proteolytic degradation of ET-1. It has been reported that ET-1 administered to anesthetized rats rapidly disappears from the circulating blood and is trapped by various organs. Therefore, we consider that exogenously administered ET-1 is mainly trapped by vascular tissues, and thereafter ET-1 is degraded by enzymes.

Results from the present study suggest that the pressor effect of big ET-1 in the kidney is caused by its conversion to ET-1 by phosphoramidon-sensitive ECE. This enzyme is thought to be distinct from NEP on the basis of inhibitory profiles of phosphoramidon and thiorphan, another NEP inhibitor, on the pressor response to big ET-1 and on ECE activities in vascular endothelial cells. In addition, Abassi et al demonstrated that recombinant NEP has no ECE activity. On the other hand, phosphoramidon significantly sustained the pressor response to ET-1. Kelatorphan, another NEP inhibitor, also showed a similar effect on the pressor response to ET-1. Therefore, it is reasonable to consider that different enzymes contribute to the conversion of big ET-1 and to the degradation of ET-1 in the kidney.
It has been proposed that a specific inhibitor of ECE may have beneficial effects in the prevention and/or treatment of certain diseases, based on a possible relation between the accelerated formation of ET-1 and various vascular diseases. Several investigators reported that phosphoramidon attenuates impairment of renal function in postischemic renal failure. They suggested that the protective action of phosphoramidon is most likely mediated by the inhibition of endothelin formation. These results suggest the pathophysiological significance of phosphoramidon-sensitive ECE in the kidney and that inhibition of the phosphoramidon-sensitive ECE may have clinical benefits. However, further study is required to accurately define the pathophysiological role of ET-1 in vivo, and the availability of more specific and potent ECE inhibitors will facilitate a better understanding of the pathophysiological role of ECE.

In conclusion, our results indicate that phosphoramidon-sensitive ECE in the kidney contributes to the functional local conversion of big ET-1 to ET-1 and that NEP may be responsible for the proteolytic degradation of ET-1 in the kidney. In addition, IR-ET levels in tissues but not in the perfusate can account for the functional conversion of big ET-1 to ET-1 and for the local proteolytic degradation of ET-1 in the kidney.

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