Role of Neutral Endopeptidase in the Metabolism of Endothelin

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Endothelin is a potent vasoconstrictor produced by endothelial cells. Although endothelin has been studied extensively, little is known about its metabolism in vivo. Neutral endopeptidase EC.3.4.24.11 is reported to degrade endothelin in vitro. Therefore, we studied the effect of neutral endopeptidase inhibition by SQ29,072 on plasma levels and urinary excretion of endogenous and exogenous endothelin.

Injection of 30 or 60 mg/kg SQ29,072 into anesthetized rats increased the urinary excretion of endothelin nearly 14-fold. The response was maximal during the first 30 minutes of collection and lasted for 90 minutes. The larger dose of inhibitor caused a 37–43% increase (p≤0.05) in the plasma concentration of endothelin. Only 0.20±0.04% of the total radioactivity injected as 125I-endothelin (1 μCi; 1,308 pg) into normal rats was recovered in the urine within 30 minutes. Urinary radioactivity increased to 0.54–0.63% (p≤0.05) of the total infused in rats pretreated with SQ29,072. Chromatographic analysis of radioactivity in the urine revealed that intact endothelin accounted for only 6–9% of the total counts in control rats but 50–56% in rats pretreated with the inhibitor. We also studied the effects of another inhibitor of neutral endopeptidase, SQ28,063, on the distribution of radioactivity in the urine, kidney, and lung of rats injected with 125I-endothelin. SQ28,063 increased urinary excretion of labeled endothelin and increased total radioactivity accumulated in the lung and kidney from 157 and 105 pg to 234 and 157 pg, respectively. Intact endothelin accounted for 90% or more of the accumulated counts in both tissues. These results indicate that 1) little circulating endothelin is cleared into the urine, 2) endothelin in the urine is likely of renal origin, and 3) neutral endopeptidase EC.3.4.24.11 plays a major role in the inactivation of endothelin. (Hypertension 1992;89:93–98)

KEY WORDS • endothelin • metabolism • peptide peptidohydrolases • protease inhibitors

Methods

Male Munich Wistar rats (Harlan Sprague Dawley, Inc., Indianapolis, Ind.) weighing 250–300 g were kept in cages in a room with controlled temperature and light cycle, fed a standard rat diet, and given tap water ad libitum.

Experimental Protocol

Effect of SQ29,072 on plasma levels and urinary excretion of endothelin. On the day of the experiment the rats were anesthetized with 100 mg/kg i.p. thiobutabarbitol (Inactin, Abbott Laboratories, North Chicago, Ill.) and placed on a temperature-regulated table, and a tracheostomy was performed. Polyethylene catheters (PE-50, Clay Adams, Parsippany, N.J.) were inserted into a carotid artery for blood pressure monitoring (model 79D, Grass Instrument Co., Quincy, Mass.) and blood sampling, into a jugular vein for drug infusion, and into the bladder for urine collection. Normal saline was infused at a rate equal to 1% of body weight per hour. After a 60-minute equilibration period, one baseline 60-minute clearance period was obtained, followed by four successive 30-minute periods. After the baseline collection, a single dose of either 30 or 60 mg/kg of the NEP inhibitor (NEP-I) SQ29,072 (Bristol-Myers Squibb Co., Princeton, N.J.) dissolved in 1% NaHCO3 was administered as an intravenous bolus. An identical volume of vehicle was administered to control rats. To avoid volume depletion, urinary losses were replaced by infusion of an equal volume of 0.9% saline. Urine was
collected into preweighed vials kept on ice. Urine samples (0.2–2 ml) were applied to a C-2 minicolumn (500 mg) (Amersham Corporation, Arlington Heights, Ill.) previously activated with 2 ml of 100% methanol followed by 2 ml cold distilled water. The minicolumn was subsequently washed with 5 ml of 0.1% trifluoroacetic acid (TFA). The adsorbed ET was eluted with 4 ml of 80% acetonitrile in 0.1% TFA. The eluates were collected and evaporated in a vacuum. The residue was reconstituted in 100 μl radioimmunoassay (RIA) buffer, and ET levels were determined by a highly sensitive RIA using a commercial kit (Amersham). Blood samples for assay of plasma levels of ET and atrial natriuretic factor (ANF) were collected in tubes containing EDTA (3 mg/ml) and aprotonin (500,000 IU/ml) 30 and 120 minutes after infusion of SQ29,072.

Effect of SQ29,072 on urinary excretion of exogenous endothelin. To evaluate the effect of NEP-I on the metabolism of exogenous ET, 1 μCi (1,308 pg) of 125I-ET-1 (porcine ET-1, 2,000 Ci/mmol, Amersham) was administered intravenously to another group of rats pretreated with either 30 or 60 mg/kg SQ29,072 or an equal volume of vehicle as an intravenous bolus after an equilibration period of 60 minutes. Thirty minutes later 1 μCi (1,308 pg) of 125I-ET-1 was infused, and urine was collected for 30 minutes. Thirty minutes later 1 μCi (1,308 pg) of the labeled compound. Periodic blood samples (200 μl) were obtained and centrifuged, and intact 125I-ET-1 in 100 μl plasma was separated from its hydrolytic products by using a C-2 minicolumn as described above. Both SQ29,072 and SQ28,063 are NEP-I supplied by Bristol-Myers Squibb. They have similar structures and potencies. We used SQ29,072 until the supply ran out, and then we switched to SQ28,063.

Analytical Methods

The concentration of ET in plasma (PET) and urine was determined by RIA using a kit (Amersham). The plasma level of ANF (PANF) was measured using a sensitive RIA kit (Peninsula Laboratories, Belmont, Calif.).

Statistics

All values are expressed as mean±SEM. For statistical evaluation of repeated measurements, analysis of variance was used followed by Fisher’s post hoc test; a value of p<0.05 was considered significant.

Results

Effect of SQ29,072 on Plasma Levels and Urinary Excretion of Endothelin

Both doses of NEP-I significantly increased urinary excretion of ET (UETV) at 30, 60, and 90 minutes but...
not at 120 minutes after administration (Figure 1). The maximal effect occurred 30 minutes after NEP-I administration, when UETV was nearly 14-fold higher than in animals given vehicle. Mean arterial blood pressure did not differ between control and NEP-I-treated rats throughout the experiment (data not shown).

PET increased very little after the injection of SQ29,072 (Figure 2A). PET increased nonsignificantly after the smaller dose and significantly (37% at 30 minutes and 43% at 120 minutes) after the larger dose of NEP-I; PET values for the two times were not significantly different. PANF values were not significantly different from those in control animals, values for the two doses or the two sample times were not significantly different.

**Effect of SQ29,072 on Urinary Excretion of Exogenous Endothelin**

Only a small amount of the total radioactivity injected as $^{125}$I-ET was recovered in the urine, e.g., $0.20\pm0.04\%$ in the first 30 minutes and $0.40\pm0.04\%$ in the second 30 minutes. In rats pretreated with the smaller dose of NEP-I, urine radioactivity increased to $0.42\pm0.05\%$ and $0.56\pm0.03\%$ of the total amount injected in the first and second periods, respectively ($p<0.05$ versus control). After the larger dose of NEP-I, urinary radioactivity increased further to $0.54\pm0.1\%$ and $0.63\pm0.05\%$ of the total radioactivity injected in the first and second periods, respectively ($p<0.05$ versus control). Fractionation of the radioactivity in the urine revealed that intact $^{125}$I-ET accounted for only $6-9\%$ of the total counts ($0.16-0.47$ pg) in control rats, while the remainder of the radioactivity was in free iodine (Figure 3). The amount of intact $^{125}$I-ET increased nearly fivefold ($p<0.05$) to $50\pm4\%$ and $35\pm4\%$ of the total radioactivity in the urine ($2.8\pm0.2$ and $2.6\pm0.3$ pg) at 30 and 60 minutes, respectively, in rats pretreated with the smaller dose of NEP-I and to $56\pm4\%$ and $51\pm5\%$ ($4.0\pm0.3$ and $4.2\pm0.4$ pg), respectively, in rats pretreated with the larger dose of NEP-I.

**Effect of SQ28,063 on Radioactivity in Urine, Plasma, Kidney, and Lung of Rats Injected With $^{125}$I-Endothelin**

In a typical reverse-phase HPLC radiochromatogram a free iodine standard has a retention time of 4 minutes,
Figure 4. High-performance liquid chromatograms of radioactivity recovered in urine from rats after injection with 125I-endothelin-1. Panel A: Radiochromatogram of standards. Rats were given either vehicle (panel B) or 60 mg/kg SQ28,063 (panel C) 30 minutes before injection of 1.0 μCi i.v. 125I-endothelin-1, and radioactivity was recovered in urine 30 minutes later. Data are from one typical experiment, which was replicated five times. cpm, Counts per minute; NEP-I, neutral endopeptidase inhibitor (SQ28,063).

Figure 5. Line graph shows disappearance of intact 125I-endothelin-1 (125I ET) from plasma after bolus injection of 1.0 μCi i.v. labeled peptide into control rats and rats pretreated with neutral endopeptidase inhibitor SQ28,063 (NEP-I). Intact ET was separated from free iodine by using a C-2 minicolumn and further analyzed by high-performance liquid chromatography. Data are expressed as mean±SEM. Blood samples were collected 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 20.0, and 30.0 minutes after injection of labeled ET. cpm, Counts per minute.

whereas a 123I-ET standard has a retention time of 30 minutes (Figure 4A). HPLC of the radioactivity excreted in the urine from control rats yielded a radiochromatogram with only one major peak having a retention time of 4 minutes, typical for free iodine (Figure 4B). HPLC of the radioactivity recovered in the urine from rats pretreated with NEP-I yielded two peaks. Approximately 67% of the radioactivity in the larger peak corresponded to 125I-ET; however, there was also a significant, incompletely separated peak that eluted at 28–29 minutes. The smaller peak, accounting for 10% of the total radioactivity, had a retention time of 4 minutes and corresponded to free iodine (Figure 4C).

Plasma decay of intact 123I-ET after a single bolus intravenous injection of 1.0 μCi labeled peptide into control and NEP-I-pretreated rats is presented in Figure 5. No significant differences in this parameter were observed between the groups.

Extraction of radiolabeled compounds from the lungs and kidneys of control rats revealed that 157 pg of the total infused radioactivity accumulated in the lungs, and 105 pg was found in the kidneys (Figures 6A and 6C). This pattern changed markedly in rats pretreated with NEP-I; i.e., accumulated radioactivity increased to 234 pg (49% increase) in the lungs and 157 pg (50% increase) in the kidneys (Figures 6B and 6D). HPLC of the radiolabeled compounds extracted from the kidneys or lungs revealed a major peak having a retention time
corresponding to intact ET (Figure 6). This peak was accompanied by a negligible peak corresponding to free iodine. No significant differences were found between the radiochromatograms of lung extracts obtained from rats pretreated with NEP-I or vehicle; however, in kidney extracts from NEP-I-pretreated rats there was an additional, incompletely separated peak in the same position as that found in urine.

Discussion

The present study reports that administration of NEP-I in rats 1) induces large increases in UETV associated with only small increases in PET, 2) increases the amount of intact ET recovered in the urine, and 3) increases the amount of intact ET recovered in the lungs and kidneys.

Although previous studies have documented that NEP plays a significant role in the degradation of ET in vitro, the present study is, to the best of our knowledge, the first to show that a similar pathway occurs in vivo. Vijayaraghavan et al showed that purified NEP from rat kidney membranes rapidly degrades ET-1, ET-2, and ET-3 and that the affinities of NEP for ETs in vitro are among the highest reported for this enzyme. We show that PET was modestly increased, by approximately 40%, while UETV was greatly increased, by about 14-fold, in rats treated with NEP-I compared with rats that received vehicle alone. This suggests that NEP plays a significant role in ET elimination in vivo. The fact that PANF increased along with PET after NEP-I indicates that NEP is the same enzyme that is important in ANF metabolism.

UETV is of particular interest because it provides a way to study the extrarenal and renal synthesis and the clearance of ET. Our data from radiolabeled ET indicate that very little circulating ET is excreted in the urine; of 1,308 pg labeled ET injected into control rats 0.16–0.47 pg was excreted in the urine within 30 minutes. Thus, it is likely that UETV under basal conditions reflects renal synthesis. After NEP-I, UETV increased and accounted for 0.2–0.3% of the amount of 125I-ET injected per hour (2.8–4.2 pg of 1,308 pg infused). This increase may indicate that after NEP-I a small but significant portion of excreted ET is of nonrenal origin.
P<sub>ET</sub> increases after NEP-I, and this increases the filtered load of the peptide, which in the presence of NEP-I escapes renal degradation and appears in the urine as intact ET. These findings confirm and extend a previous report by Benigni et al.\textsuperscript{10} who found that less than 0.3% of total infused I<sup>125</sup>-ET was recovered in the urine of normal rats. However, the authors did not determine the nature of the recovered radioactivity, which we found to be mainly (more than 90%) free iodine.

While NEP-I had a profound effect on U<sub>ET</sub>V, P<sub>ET</sub> from either endogenous or exogenous origins was not dramatically altered. The lack of a marked effect of NEP-I on P<sub>ET</sub> indicates that mechanisms other than NEP play the major role in the elimination of circulating ET. It should be borne in mind that ET is mainly a paracrine substance and is not abundant in the blood.

It was also shown previously that bilateral nephrectomy of rats leads to delayed disappearance of exogenous ET\textsuperscript{11,12} and potentiation of its vasoconstrictive action.\textsuperscript{12} The authors concluded that degradation in the kidney is in part responsible for the removal of ET from the circulation, without further identifying the mechanism. The present findings are consistent with these studies and indicate the importance of NEP in the inactivation of ET. Furthermore, our data are compatible with recent reports showing that patients with chronic renal failure have increased P<sub>ET</sub>\textsuperscript{13,14} as well as increased U<sub>ET</sub>V.\textsuperscript{15,16} Chronic renal failure is a pathophysiological condition characterized by reduction of functional renal mass and probably activities of renal parenchymal enzymes such as NEP. Therefore, it is likely that the observed increases in P<sub>ET</sub> and U<sub>ET</sub>V in chronic renal failure are secondary to renal damage, which in this regard could be mimicked by NEP-I.

Pretreatment of rats with NEP-I increased the amounts of ET radioactivity accumulated in the lungs and kidneys. This indicates that more labeled material was reaching these organs. HPLC showed that more than 90% of the accumulated radioactivity was intact ET and that, although there was no difference in the radiochromatograms from the lung between the control group and rats treated with NEP-I, an additional metabolite of ET appeared in the kidney after NEP-I administration. These findings are similar to what we saw in the urine of rats treated with NEP-I. Since the metabolite peaks have the same retention times, they are probably the same substance. Although the nature of that metabolite has not been established, it may represent the methionine-sulfoxide form of ET, which reportedly elutes slightly before native ET.\textsuperscript{16,17}

Our findings in the control group are in contrast to the findings of Benigni et al.,\textsuperscript{10} who reported that HPLC of the radioactivity accumulated in the kidney of normal rats yielded one major peak, which corresponded to the methionine-sulfoxide form of ET, and three minor peaks. The reasons for the differences in experimental protocols or to interlaboratory or interaninal variability. These authors infused rats with 5 μCi of I<sup>125</sup>-ET-1 over 50 minutes and killed them immediately after the infusion. Radioactivity accumulated in the tissues was measured up to 3 days later, whereas we injected rats with 1 μCi of I<sup>125</sup>-ET-1 as a bolus, killed them after 30 minutes, and measured the radioactivity accumulated in the tissues on the same day. Nevertheless, these differences between findings are negligible in light of the lack in both studies of major peaks corresponding to hydrolysis products of ET after 30–50 minutes. Taken together, these findings suggest that ET is degraded on the brush border of the renal proximal tubules and that hydrolysis products appear in the urine rather than in renal tissue. The amounts of I<sup>125</sup>-ET in the kidney and lung probably relate to ET receptors, which are concentrated in these organs.\textsuperscript{18–20}

NEP EC.3.4.24.11 is a ubiquitous membrane-bound enzyme present in kidney, lung, brain, endothelial cells, thyroid, intestine, neutrophils, male genital tract, and fibroblasts.\textsuperscript{21} However, in rodents the enzyme is found predominantly in the kidney, principally within the brush border vesicles of the proximal tubules. In a recent study Scicli et al.\textsuperscript{22} found that kidney, and to a lesser extent lung, were the tissues that exhibited significant "endothelinase" activity in vitro as measured by the destruction of ET biological activity. Our finding that NEP-I increases the urinary excretion of intact ET without affecting the nature of the ET radioactivity accumulated in the lung supports this conclusion and indicates that the kidneys are the main site for ET inactivation, at least in rodents.

In summary, these studies demonstrate that the NEP system plays a significant role in the metabolism of ET in vivo. Further investigation is required to identify additional routes of metabolism of the peptide and determine the relative contribution of NEP.

References

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