Inhibition of Neutral Endopeptidase Causes Vasoconstriction of Human Resistance Vessels In Vivo

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**Background**—Neutral endopeptidase (NEP) degrades vasoactive peptides, including the natriuretic peptides, angiotensin II, and endothelin-1. Systemic inhibition of NEP does not consistently lower blood pressure, even though it increases natriuretic peptide concentrations and causes natriuresis and diuresis. We therefore investigated the direct effects of local inhibition of NEP on forearm resistance vessel tone.

**Methods and Results**—Four separate studies were performed, each with 90-minute drug infusions. In the first study, 10 healthy subjects received a brachial artery infusion of the NEP inhibitor candoxatrilat (125 nmol/min), which caused a slowly progressive forearm vasoconstriction (12±2%; P=0.001). In a second two-phase study, 6 healthy subjects received, 4 hours after enalapril (20 mg) or placebo, an intra-arterial infusion of the NEP inhibitor thiorphan (30 nmol/min). Thiorphan caused similar degrees of local forearm vasoconstriction (P=0.6) after pretreatment with both placebo (13±1%, P=0.006) and enalapril (17±6%, P=0.05). In a third three-phase study, 8 healthy subjects received intra-arterial thiorphan (30 nmol/min), the endothelin ET<sub>α</sub> antagonist BQ-123 (100 nmol/min), and both combined. Thiorphan caused local forearm vasoconstriction (13±1%, P=0.0001); BQ-123 caused local vasodilatation (33±3%, P=0.0001). Combined thiorphan and BQ-123 caused vasodilatation (32±1%, P=0.0001) similar to BQ-123 alone (P=0.98). In a fourth study, 6 hypertensive patients (blood pressure >160/100 mm Hg) received intra-arterial thiorphan (30 nmol/min). Thiorphan caused a slowly progressive forearm vasoconstriction (10±2%, P=0.0001).

**Conclusions**—Inhibition of local NEP causes vasoconstriction in forearm resistance vessels of both healthy volunteers and patients with hypertension. The lack of effect of ACE inhibition on the vasoconstriction produced by thiorphan and its absence during concomitant ET<sub>α</sub> receptor blockade suggest that it is mediated by endothelin-1 and not angiotensin II. These findings may help to explain the failure of systemic NEP inhibition to lower blood pressure. (Circulation. 1998;97:2323-2330.)

Key Words: natriuretic peptides ■ vasoconstriction ■ endothelin ■ angiotensin II ■ human

Neutral endopeptidase (EC 3.4.24.11, enkephalinase) is a plasma membrane-bound zinc metalloprotease that was initially isolated from renal epithelial brush border cells and cleaves peptide substrates at the amino side of hydrophobic amino acids. It catalyzes the degradation of a number of endogenous vasodilator peptides, including ANP, brain natriuretic peptide, C-type natriuretic peptide, substance P, and bradykinin, as well as vasoconstrictor peptides, including ET-1 and Ang II. In addition to degrading vasoactive peptides to inactive breakdown products, NEP can also convert big ET-1 to the active peptide ET-1. Therefore, the physiological actions of NEP in vivo will be the balance of its effects on the breakdown of vasodilators and vasoconstrictors and on the synthesis of ET-1 from big ET-1 (Figure 1).

NEP is inhibited by several agents, including candoxatrilat; thiorphan and its prodrug, sinorphan; and phosphoramidon. ANP has potent natriuretic and vasodilator properties and inhibits activity of the renin-angiotensin-aldosterone system by reducing both renin and aldosterone release. Therefore, increasing the circulating concentrations of ANP through inhibition of NEP is an attractive therapeutic approach to a number of cardiovascular diseases such as hypertension and heart failure. However, although NEP inhibitors increase circulating ANP concentrations in humans and cause the expected natriuresis, they do not generally lower blood pressure in normotensive subjects. Indeed, both candoxatrilat and candoxatrilat have been reported as raising blood pressure in normotensive subjects. Although NEP inhibitors have been reported to lower blood pressure in patients with essential hypertension, this finding has not been universal. Thus, the therapeutic value of NEP inhibitors in hypertension remains uncertain. In patients with heart failure, these agents do not reduce afterload although they do reduce pulmonary capillary wedge pressure, presumably because of natriuresis.
We hypothesized that if the predominant substrates for vascular NEP were vasodilator peptides, then local inhibition of this enzyme should cause peripheral vasodilatation. However, in previous studies using brachial artery administration of the NEP inhibitor thiorphan, we observed a modest vasoconstriction, suggesting accumulation of vasoconstrictor peptides such as Ang II or ET-1. Therefore, in the present study, we examined the effects of brachial artery administration of a structurally different NEP inhibitor, candoxatrilat, on forearm blood flow to determine whether the vasoconstriction produced by thiorphan is a class effect of NEP inhibitors. We also investigated whether an accumulation of Ang II was the cause of the forearm vasoconstriction produced by thiorphan by infusing thiorphan into the brachial artery in the presence or absence of concurrent systemic ACE inhibition. Furthermore, we investigated whether accumulation of ET-1 was the cause of the forearm vasoconstriction in response to thiorphan by confusing an ET₄ antagonist, BQ-123, together with thiorphan. We also examined the effects of brachial arterial administration of thiorphan in a group of hypertensive patients to confirm the clinical relevance of our findings in healthy subjects.

Methods

Subjects

Twenty-four healthy male subjects and 6 hypertensive male patients (mean age, 45±3 years; 24-hour ambulatory mean arterial pressure, 116±3 mm Hg) who had not yet received any treatment participated in these studies, which were conducted with the approval of the local ethics committee and with the written informed consent of each subject. None of the subjects received vasoactive or nonsteroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 hours and from food, caffeine-containing drinks, and cigarettes for at least 3 hours before any measurements were made. All studies were performed in a quiet room maintained at a constant temperature of between 22°C and 25°C.

Drugs

Candoxatrilat (Pfizer) and thiorphan (Sigma) were administered intra-arterially dissolved in physiological saline (0.9%; Baxter Healthcare Ltd). We used (+)-candoxatrilat (UK-73,967) in this study; this eutomer has twice the potency as an NEP inhibitor than (-)candoxatrilat (UK-69,578), and is the active metabolite of the orally available prodrug candoxatrilat. The dose of candoxatrilat (125 nmol/min) was chosen to achieve forearm blood concentrations >50-fold higher than the IC₅₀ (40 nmol/L) of (+)candoxatrilat in vitro. The dose of thiorphan (30 nmol/min) used in this study has been shown to produce ~20% reduction in forearm blood flow when infused via the brachial artery. This dose is known to achieve local concentrations in forearm blood after brachial artery administration, >10-fold higher than the IC₅₀ of thiorphan (35 nmol/L) for NEP in vitro.

Figure 1. NEP catalyzes the metabolism of the vasoconstrictor peptides ET-1 and Ang II, as well as the metabolism of several vasodilator peptides, including bradykinin (BK), ANP, brain and C-type natriuretic peptides (BNP and CNP, respectively), and substance P (SP). NEP is also involved in the enzymatic conversion of big ET-1 to its active form, the vasoconstrictor peptide ET-1. The balance of effects of NEP inhibition on vascular tone, therefore, will depend on whether the predominant substrate(s) degraded by NEP are vasodilators or vasoconstrictors and on the extent of NEP involvement in the processing of big ET-1.

The peptide ET₄ antagonist BQ-123 (Cyclo[−D-Asp−L-Pro−D-Val−L-Leu−D-Trp−]; American Peptide Co) was administered intra-arterially (100 nmol/min) dissolved in physiological saline. This dose achieves local concentrations in the forearm >10-fold higher than the Pₐ₈ of the ET₄ receptor and is known to produce ~40% increase in blood flow when infused via the brachial artery.

The ACE inhibitor enalapril (Merck, Sharp & Dohme Ltd) was administered orally in ascending, single, daily doses of 2.5, 5, 10, and 15 mg over a period of 4 days. This ascending dose design was used to minimize the already low risk of hypotension. On the fifth day, subjects were admitted to the clinical research center and, after lying supine for 30 minutes, received 20 mg enalapril orally at 8:30 AM. The final dose of 20 mg was chosen because it reduces plasma concentrations of Ang II to a level close to the detection limit of radioimmunoassay 4 hours after administration.

Intra-arterial Administration

The left brachial artery was cannulated under local anesthesia (1% lidocaine) with a 27-standard wire gauge needle attached to a 16-gauge epidural catheter. Patency was maintained by infusion of physiological saline via a Welmed P1000 syringe pump. The total rate of intra-arterial infusion was maintained constant throughout at 1 mL/min. In all studies, physiological saline was infused for 30 minutes before infusion of vasoactive agents.

Measurements

Forearm Blood Flow

Blood flow was measured in both forearms by venous occlusion plethysmography by use of indium/gallium-in-Silastic gauges as previously described. Recordings of forearm blood flow were made repeatedly over 3-minute periods. Voltage output from a dual-channel Vasculab SPG 16 strain-gauge plethysmograph (Medasonics, Inc) was transferred to a Macintosh personal computer by use of a MacLab analogue digital converter and Chart software (AD Instruments). Calibration was achieved by use of the internal standard of the Vasculab plethysmography units. In all studies, forearm blood flow was recorded in both arms every 5 minutes during infusion of the study agents.

Blood Pressure

A well-validated semiautomated noninvasive oscillometric sphygmanometer (Takeda UA 751) was used to make duplicate measurements of blood pressure in the noninfused arm, which were then averaged. In all studies, blood pressure was measured at 10-minute intervals during infusion of the study agents.

Plasma Assays

Venous blood samples (40 mL) were obtained at intervals for assay of concentrations of plasma active renin, Ang II, aldosterone, ANP,
and ET from both arms. This technique of bilateral venous sampling, from deep veins in the antecubital fossae and with intra-brachial artery infusion of locally active agents, has been reported previously. Samples were collected into chilled tubes, centrifuged at 1500g for 20 minutes at 4°C, and stored at −80°C until assay. Samples for renin were collected into tubes coated with potassium EDTA, and plasma active renin concentration was measured by an antibody trapping technique. The intra-assay CV for this assay is 3.4%. Samples for Ang II were collected into plain tubes containing potassium EDTA/phenanthrolime, and plasma concentration of Ang II was measured by radioimmunoassay. The intra-assay CV is 10%. Venous blood samples for plasma aldosterone concentrations were collected into lithium heparin tubes, and plasma aldosterone was measured by use of a solid-phase (coated tube) radioimmunoassay with a commercially available kit (Diagnostic Products UK Ltd). The intra-assay CV is <8.3%. Samples for ANP were collected into EDTA/trasyol tubes, and plasma ANP concentration was measured by radioimmunoassay. The intra-assay CV is 3.9%. Samples for ET assay were collected into tubes coated with EDTA, and plasma ET was assayed with a commercially available kit (Endothelin-1 Radioimmunoassay, Peninsula Laboratories) as previously described, except samples were extracted with acetic acid. This method gives an extraction recovery of ET-1 of 89%. The intra-assay CV is <6%, and the cross-reactivities of this assay with ET-1, ET-2, ET-3, and big ET-1 are 100%, 7%, 7%, and 10%, respectively. All assays were done in single batches.

**Study Design**

Four single-blind studies were performed.

**Protocol 1: Intra-arterial Candoxatrilat**

Ten subjects participated in this single-phase, single-blind study. Candoxatrilat (125 nmol/min) was infused for 90 minutes.

**Protocol 2: Intra-arterial Thiorphan and Systemic ACE Inhibition**

Six subjects participated in this two-phase, single-blind, crossover study. In each phase, subjects were administered orally either increasing single daily doses of enalapril (as detailed above) or matching placebo. On the fifth day, subjects were admitted to the clinical research center at 8:00 AM, and deep veins in both antecubital fossae were cannulated with 18-gauge intravenous cannulas for blood sampling. After the subject lay recumbent for 30 minutes (8:30 AM), a venous blood sample was taken from the right (noninfused) arm for assays of renin, Ang II, aldosterone, ANP, and ET concentrations. Blood pressure was measured, and enalapril 20 mg or placebo was administered at 8:30 AM. Blood pressure was then measured at 30-minute intervals for 3.5 hours with the subjects remaining recumbent. At midday, physiological saline was infused via the left brachial artery for 30 minutes. At 12:30 PM, thiorphan (30 nmol/min) was infused for 90 minutes, 4 hours after administration of the final dose of enalapril or placebo. Before the start of the thiorphan infusion, a venous blood sample was taken for aldosterone, renin, and clinical biochemistry from the right (noninfused) arm. Blood samples were also taken from both arms at the beginning and end of the period of thiorphan infusion for measurement of plasma Ang II, ANP, and ET concentrations.

**Protocol 3: Intra-arterial Thiorphan and Intrabrachial BQ-123**

Eight subjects participated in this randomized three-phase, single-blind study. In random order and on separate occasions at least 1 week apart, either thiorphan (30 nmol/min) or BQ-123 (100 nmol/min) alone or both in combination was infused for 90 minutes.

**Protocol 4: Intra-arterial Thiorphan in Hypertensive Patients**

Six hypertensive patients participated in this single-phase, single-blind study. Thiorphan (30 nmol/min) was infused for 90 minutes.
did not change during the intra-arterial infusion of thiorphan in either phase (Table 1).

Plasma active renin concentrations were higher after 4 days of treatment with enalapril than with placebo. Plasma active renin concentration increased further 4 hours after administration of 20 mg enalapril, with no change during the placebo phase (Table 1). Plasma Ang II concentration tended to be lower after 4 days of enalapril, although this difference between phases did not reach statistical significance \( (P=0.09; \text{Table 2}) \). Plasma Ang II concentrations did not change significantly during the placebo phase in either the infused or noninfused arm. Four hours after administration of 20 mg enalapril, there was a substantial reduction in plasma Ang II concentration (Table 2). Plasma Ang II concentration did not change further during the 90-minute thiorphan infusion in the enalapril phase in either the infused or noninfused arm (Table 2). Venous aldosterone concentration was lower after 4 days of enalapril than after 4 days of placebo (Table 2). During both phases, aldosterone concentration tended to decrease after 4 hours of supine posture. However, this decrease was significant only after 20 mg enalapril compared with basal (Table 2).

Neither oral enalapril nor intra-arterial thiorphan had any effect on plasma ANP or plasma ET concentrations in either the infused or noninfused arm (Table 2).

Basal forearm blood flow in the infused arm tended to be lower during the enalapril phase than the placebo phase, although this was not statistically significant (2.9±0.4 and...
Blood flow in the noninfused arm did not change significantly after infusion of thiorphan, confirming that drug effects were confined to the infused arm. Brachial artery administration of thiorphan caused a slowly progressive forearm vasoconstriction, with blood flow decreasing during both the enalapril phase (mean, 17 ± 6%; maximum, 33 ± 7%; P = 0.05) and placebo phase (mean, 13 ± 3%; maximum, 24 ± 2%; P = 0.006). The reductions in blood flow were similar during either phase (P = 0.6; Figure 3).

Protocol 3: Intra-arterial Thiorphan and Intra-arterial BQ-123
Brachial artery administration of BQ-123 alone caused a progressive forearm vasodilatation (mean, 33 ± 3%; maximum, 47 ± 9%; P = 0.0001), whereas thiorphan caused a slowly progressive vasoconstriction (mean, −14 ± 1%; maximum, −22 ± 4%; P = 0.0001). Coinfusion of BQ-123 and thiorphan caused a vasodilatation (mean, 32 ± 2%; maximum, 48 ± 6%; P = 0.0001) that was not different from that observed with BQ-123 alone (P = 0.98; Figure 4).

Protocol 4: Intra-arterial Thiorphan in Hypertensive Patients
In hypertensive patients, brachial artery administration of thiorphan caused a slowly progressive forearm vasoconstriction (mean, −10 ± 2%; maximum, −20 ± 3%; P = 0.0001). This was not significantly different from that observed in the healthy volunteers in the third study (P = 0.39; Figure 5).

Discussion
We have shown that the specific NEP inhibitors candoxatrilat and thiorphan cause slowly progressive vasoconstriction when given by direct brachial artery infusion to healthy subjects and patients with essential hypertension. The vasoconstriction caused by thiorphan was not reversed by systemic ACE inhibition but was abolished by ET receptor antagonism. Our findings are unlikely to be due to other actions of these agents because both candoxatrilat and thiorphan are highly specific for NEP. Furthermore, the finding that two structurally independent inhibitors of NEP produce vasoconstriction strongly suggests that this is a class effect of NEP inhibition on human resistance vessels. It is possible that different effects may be obtained in other blood vessels, although responses in forearm resistance vessels are generally thought to be broadly representative of those in other vascular beds. Our findings have potential implications both for the physiological role of NEP and for the therapeutic use of NEP inhibitors.

Although it was initially thought that the most important site of natriuretic peptide metabolism by NEP was the kidney, candoxatrilat is just as effective in reducing clear-
ance of ANP in nephrectomized animals,\textsuperscript{55} implying other, nonrenal sites of action. NEP is now known to be expressed in blood vessels by both endothelial\textsuperscript{56} and vascular smooth muscle cells.\textsuperscript{57} Despite the clear evidence for vascular generation and metabolism of natriuretic peptides, we found that local NEP inhibitors caused vasoconstriction rather than vasodilatation. This finding implies that under physiological conditions, vasconstrictor peptides, such as Ang II and ET-1, are more important substrates for vascular NEP than dilator substances, such as the natriuretic peptides and bradykinin (Figure 1). However, our finding that brachial artery administration of thiorphan produces forearm vasoconstriction in the presence of substantial systemic ACE inhibition implies that Ang II accumulation is not responsible for the observed vasoconstriction. In addition, ANP blocks activity of the renin-angiotensin system by reducing renin release\textsuperscript{14} and blocking aldosterone secretion,\textsuperscript{35} so Ang II generation is likely to be decreased by NEP inhibition.

The vasoconstriction to candoxatrilat and thiorphan was slowly progressive, which is more in keeping with an effect of ET-1 than Ang II, on the basis of the known rate of onset of forearm vasoconstriction after brachial artery infusion of these peptides.\textsuperscript{58} This is supported by a recent study in which systemic oral doses of candoxatril in healthy men produced an increase in both systolic blood pressure and venous plasma ET concentration.\textsuperscript{22} In another recent study, systemic administration of candoxatrilat in healthy subjects produced a significant increase in systemic blood pressure.\textsuperscript{23} However, because this rise was prevented by pretreatment with enalapril, it was suggested that the increase in blood pressure was caused by potentiation of Ang II. Our findings do not support this conclusion. Indeed, in our study, thiorphan produced arterial vasoconstriction in the presence of systemic ACE inhibition despite the very low Ang II concentrations. Furthermore, we did not detect any increase in Ang II concentrations in venous blood draining the infused arm during the placebo phase of our study, suggesting that NEP inhibition does not cause an accumulation of Ang II.

We did not demonstrate a significant decrease in blood pressure after 20 mg enalapril orally despite the very low concentrations of Ang II produced. However, our study was not designed to specifically measure changes in systemic hemodynamics. The hypotensive effect of enalapril would be expected to have been greatest when subjects were being prepared for the intra-arterial stage of the study. This involved subjects standing to pass urine and having the blood pressure cuff repositioned over the rapid inflation cuffs required for forearm plethysmography, as well as insertion of an intra-arterial needle.

In this study, enalapril had no effects on plasma ANP concentrations. This is in agreement with other published reports.\textsuperscript{23,50,60} Intra-arterial thiorphan did not produce a detectable increase in ANP concentrations in venous blood draining the infused arm. However, any changes in local ANP concentrations are likely to be small and may have been below the sensitivity of the assay. In addition, not all studies of acute NEP inhibition have demonstrated an increase in ANP concentrations.\textsuperscript{21,22,35} ANP may also be metabolized by an aminopeptidase, which is insensitive to thiorphan.\textsuperscript{61} Although incomplete local NEP inhibition is possible, it is highly unlikely because the doses of both candoxatrilat and thiorphan used were chosen to achieve local blood concentrations in the forearm >50-fold and >10-fold higher than the IC\textsubscript{50} of (+)candoxatrilat and thiorphan, respectively, for ANP in vitro.\textsuperscript{39} Consistent with earlier work,\textsuperscript{62} systemic ACE inhibition with enalapril had no effect on plasma ET concentrations. In addition, intra-arterial thiorphan did not increase plasma ET concentrations in samples collected from the infused arm. However, ET produced by endothelial cells is preferentially secreted abluminally,\textsuperscript{63} and inhibition of local ET degradation may not have resulted in increased plasma ET concentrations. Furthermore, any measurable increase in plasma ET concentrations is likely to be rapidly reduced through tissue receptor binding.\textsuperscript{39} Therefore, the absence of any detectable increase in plasma ET does not exclude local accumulation of the peptide, and it is still possible that decreased ET-1 breakdown is the cause of the vasoconstriction produced by NEP inhibitors.

ET-1 mediates vasoconstriction primarily by effects on the vascular smooth muscle ET\textsubscript{A} receptor.\textsuperscript{64} We find that the selective ET\textsubscript{A} receptor antagonist BQ-123 abolishes the vasoconstriction produced by thiorphan. This provides strong evidence that accumulation of ET-1, resulting from an inhibition of its degradation, mediates the vasoconstriction caused by local NEP inhibition. Nevertheless, it is also possible that accumulation of an as-yet undiscovered vasocostrictr may contribute to the observed vasoconstriction, although its abolition by BQ-123 makes this unlikely.

In clinical trials, NEP inhibitors have been shown to cause a natriuresis and diuresis.\textsuperscript{10,17} However, a reduction in blood pressure has not been clearly demonstrated in normotensive subjects,\textsuperscript{10,18,20,21} and two studies have even reported an increase in blood pressure,\textsuperscript{22,23} despite the potent vasodilator actions of the natriuretic peptides.\textsuperscript{12,13,16} Also, several studies on hypertensive patients\textsuperscript{10,32-35} have not demonstrated a reduction in blood pressure. Interestingly, and perhaps relevant to our own findings, a recent study in patients with chronic heart failure showed that candoxatril increases systemic vasoconstriction and decreases cardiac index.\textsuperscript{55} Our results help to explain these apparent contradictions. The hemodynamic effects of systemic NEP inhibition will depend on the balance between its cardiac, renal, and vascular actions. We have shown that local NEP inhibition causes forearm vasoconstriction in healthy subjects and, of greater clinical relevance, that this effect occurs in untreated essential hypertensive patients. Thus, peripheral vasoconstriction may play an important role in counteracting the antihypertensive actions of NEP inhibition.

Our study shows that the vasoconstriction produced by NEP inhibitors may be mediated by ET-1 or other vasocostrictr peptides. Given that systemic NEP inhibition has been shown to increase venous ET concentrations,\textsuperscript{22} it is possible that the combination of NEP inhibition and ET antagonism may be useful therapeutically. Indeed, phosphoramidon, a combined ET-converting enzyme and NEP inhibitor, is known to produce substantial vasodilatation when infused intra-arterially in humans\textsuperscript{37,38} and can lower blood pressure in

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normotensive and hypertensive rats.\textsuperscript{32} Nevertheless, even without reducing blood pressure, NEP inhibition may offer therapeutic benefits in hypertension and heart failure. For example, infusion of ANP causes sympathoinhibition in humans.\textsuperscript{22} In addition, NEP inhibitors appear to possess favorable antimitogenic effects in models of left ventricular hypertrophy\textsuperscript{66} and atherosclerosis.\textsuperscript{67} Such effects would need to be counterbalanced against potential mitogenic actions of ET-1.\textsuperscript{68}

In conclusion, local inhibition of NEP causes slowly progressive vasoconstriction in healthy subjects and essential hypertensive patients, suggesting that the predominant physiologcal substrates for vascular NEP are vasoconstrictor peptides. The slowly progressive nature of the vasoconstriction, together with the finding that it is not blocked by systemic ACE inhibition but is abolished by ET antagonism, supports accumulation of ET-1 as the cause. Vasoconstriction produced by NEP inhibitors may help to explain some of the apparently contradictory hemodynamic results obtained after systemic dosing with NEP inhibitors.

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