Modulation of Functionally Active Endothelin-Converting Enzyme by Chronic Neutral Endopeptidase Inhibition in Experimental Atherosclerosis

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Background—Endothelin-converting enzyme-1 (ECE-1) processes big endothelin-1 (ET-1) to ET-1, a peptide implicated in atherogenesis. ECE-1 exists in 2 isoforms, ECE-1α and ECE-1β, the result of alternative splicing of a common gene. Neutral endopeptidase (NEP) is a genetically distinct metallopeptidase that degrades the natriuretic peptides. These peptides mediate antiproliferative and vasodilating actions. We sought to demonstrate the distribution of the 2 ECE-1 isoforms in experimental atherosclerosis, to determine the effects of chronic NEP-I on plasma cGMP concentrations, vascular wall ECE-1 activity, and ET-1 concentration, and to correlate these actions with atheroma formation. Our hypothesis was that chronic NEP-I, in association with augmented cGMP, would inhibit ECE-1 conversion of big ET-1 to active ET-1, thus reducing tissue ET-1 concentrations and associated atheroma formation.

Methods and Results—Cholesterol-fed New Zealand White rabbits (n=8, 1% cholesterol diet) and NEP-I–treated cholesterol-fed New Zealand White rabbits (n=8; candoxatril, 30 mg/kg per day, Pfizer) were euthanized after 8 weeks of feeding. ECE-1α and ECE-1β immunoreactivity was present in the aortas of both groups. Compared with control values, plasma cGMP concentrations were increased (2.8±0.6 versus 8.4±1.2 pmol/mL, P<0.05), ECE-1 activity was attenuated (68±3% versus 32±8%, P<0.05), aortic tissue ET-1 concentrations were reduced (4.6±0.5 versus 3.2±0.3 pg/mg protein, P<0.05), and atheroma formation was attenuated (62±6% versus 34±5%, P<0.01) by NEP-I.

Conclusions—These studies suggest that ECE-1 is present and functionally active in the vascular wall in atherosclerosis. Inhibition of ECE-1 by NEP-I represents a novel approach to interruption of the endothelin system in this cardiovascular disease state. (Circulation. 2000;101:1976-1981.)

Key Words: atherosclerosis ■ endothelin ■ natriuretic peptides ■ vasoconstriction

Endothelin-1 (ET-1) is a potent mitogenic and vasoconstricting factor that is activated in atherosclerosis. ET-1 is cleaved from the prohormone (big ET-1) by endothelin-converting enzyme-1 (ECE-1) into its biologically active form. ECE-1 exists in 2 isoforms, ECE-1α and ECE-1β, which are the products of alternative splicing of a common gene. Recent studies have demonstrated that the expression of the ECE-1β isoform can be induced by cytokines implicated in atherogenesis. ECE-1 is upregulated after vascular wall injury produced by balloon inflation, and ECE-1 mRNA is present in human vascular tissues from patients with atherosclerosis and hypertension.

Atrial, brain, and C-type natriuretic peptides constitute a family of vasodilating and antiproliferative peptides of cardiovascular cell origin, which generate cGMP in response to receptor binding on endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and myocardial and renal epithelial cells. The natriuretic peptides have been shown to possess endothelin-inhibiting actions, underscoring their counterregulatory actions to ET-1. The natriuretic peptides are degraded by neutral endopeptidase (NEP), an enzyme that shares structural similarity and colocalization with ECE-1.

We have previously characterized the presence and distribution of ECE-1 in early experimental atherosclerosis, demonstrating the localization of ECE-1 in cells of fatty streaks. The present study was designed to determine the presence and distribution of ECE-1α and ECE-1β by using the same model. Furthermore, we sought to define interactions between the natriuretic peptide system and the endothelin system by chronically inhibiting NEP with the oral NEP-I Candoxatril (Pfizer) to augment cGMP. We also determined the effects of chronic NEP-I on vascular wall ECE-1, ET-1, and atheroma formation testing the hypothesis that chronic
NEP-I will inhibit the ability of ECE-1 to convert big ET-1 to biologically active ET-1 in association with reductions in vascular wall ET-1 concentrations and atheroma formation.

Methods
All animal studies were performed in accordance with the guidelines for animal use in research set forth by the American Association for Accreditation of Laboratory Animal Care and approved by the Mayo Clinic Animal Care and Use Committee.

Animal Model of Hypercholesterolemic Atherosclerosis
Studies were conducted in male New Zealand White rabbits weighing 2 to 4 kg. Rabbits received a 1% cholesterol diet (PMI Feeds Inc) for 8 weeks (Athero group, n=8). NEP-I-treated rabbits also received Candoxatril (30 mg/kg per day, Pfizer) for 8 weeks (NEP-I group, n=6). Candoxatril was administered via drinking water, and the dose was confirmed each day by assessment of water intake. Adjustments to the daily dose were made on the basis of these determinations. Candoxatril is a specific inhibitor of NEP, as demonstrated in previous in vivo and vitro studies.16 At the end of the 8-week feeding period, each rabbit was euthanized with an overdose of pentobarbital (30 mg/kg). The aorta was excised from the arch to the level of the diaphragm. A section of the descending thoracic aorta was removed and placed in chilled Krebs-Ringer solution of the following composition (mmol/L): NaCl 118.3, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25.0, calcium disodium edetate 0.26, and glucose 11.1 for use in organ chamber studies. The remaining descending thoracic aorta was cut and immediately placed in liquid nitrogen for freezing before storage at −70°C until processing for tissue peptide analysis. A single 4- to 6-mm ring was preserved in 10% buffered formalin for immunohistochemical analysis. The aortic arch was fixed in 10% buffered formalin, rinsed with distilled water, and then stained with oil red O (2 g/12 mL) for 20 minutes. The aortic arch from the aortic valve to the second intercostal artery was mounted en face, and the percent plaque area was quantified by threshold analysis using true color image analyzer software.

Immunohistochemical Staining
After fixation, the tissue was dehydrated and embedded in paraffin. Serial sections were cut at a thickness of 6 µm. The presence of ECE-1α and ECE-1β isoforms in aortic tissue was documented by a specific immunohistochemical staining technique for each peptide.17 We used polyclonal antibodies to human ECE-1α and ECE-1β (BASF Pharma) with cross-reactivity to other species raised in rabbits to assess by immunohistochemistry their presence and distribution in vascular tissue.

Functional Studies of ECE-1 Activity in Isolated Rabbit Aorta as Assessed by Contraction to Big-ET and ET-1
Adventitial tissue was trimmed, and the aorta was cut into 4- to 5-mm rings; care was taken to avoid touching the luminal surface. Some rings were mechanically denuded of endothelium with a pair of blunt forceps; care was taken not to damage the smooth muscle. Aortic rings with and without endothelium from a single rabbit were studied in parallel. Each ring was mounted between a fixed point and a force transducer (UC-2, Gould Inc; Hewlett-Packard Co) in an organ bath filled with 3.5 mL modified Krebs-Ringer solution at 37°C, which was aerated with 95% O2 and 5% CO2. Each ring was progressively stretched to the optimum point on the length-tension curve, as determined by the active tension developed to potassium chloride (20 mmol/L). Maximal contraction to 60 mmol/L KCl was then determined to confirm the intact function of VSMCs. ET-1 (10−12 mol/L) or big ET-1 (10−15 mol/L) was added to the bath in the presence and absence of phosphoramidon (10−4 mol/L). The presence or absence of endothelium was confirmed by addition of the calcium ionophore A23187 (10−6 mol/L). Peak response to agonists was recorded as the percentage of the maximal KCl (60 mmol/L) response. To determine ECE-1 activity, the maximal big ET-1 response was divided by the maximal ET-1 response at equimolar concentrations multiplied by 100 and expressed as arbitrary units.

Plasma Analysis
Arterial blood for hormone analysis was obtained after the 8-week protocol. Plasma samples were collected from the dorsal ear artery in heparin and EDTA tubes and immediately placed on ice. After centrifugation at 2500 rpm at 4°C, the plasma was decanted and stored at −20°C until analysis. Specific plasma radioimmunoassays for ET-1 were performed as described below. Total plasma cholesterol was assessed by the Mayo Lipid Core Laboratory Facility.

Radioimmunoassay
Plasma and tissue ET-1 concentration was determined by the [125I]ET-1 or [125I]big ET-1 assay system from Phoenix Pharmaceuticals, as previously described after supernatants were acidified with 0.5% trifluoroacetic acid.18 The recovery of the extraction procedure was 81% and 80%, as determined by the addition of synthetic ET-1 and big ET-1, respectively, to plasma. Interassay and intra-assay variations for ET-1 were 9% and 5%, respectively. The minimal level of detection was 0.5 pg per tube. The cross-reactivity of ET-2, ET-3, and big ET-1 was <5%, <3%, and <27%, respectively.

Plasma cGMP was determined by cGMP assay, as previously described,19 after the supernatants were acidified with 0.5% trifluoroacetic acid. The recovery of the extraction procedure was 99%, as determined by the addition of synthetic cGMP to plasma; interassay and intra-assay variations were 8% and 5%, respectively. The minimal level of detection was 0.5 pmol per tube.

Statistical Analysis
Results were expressed as mean±SEM. Multiple ANOVA between conditions in organ chamber studies was analyzed by the Bonferroni test to determine significance between groups while allowing for multiple comparisons in a single study. Statistical comparison of assays within groups was made by the Student paired t test, and comparisons between groups were made by unpaired t test. Where appropriate, data were analyzed by 1-factor ANOVA for repeated measures followed by the Fisher least significant difference test.

Results
Model of Atherosclerosis and Effect of Chronic NEP-I
The Table shows the model of atherosclerosis and the effects of NEP-I. Plasma cholesterol concentrations and mean arterial pressure were not influenced by NEP-I. Chronic NEP-I augmented cGMP concentrations, without change in plasma ET-1 concentrations but with a 30% reduction in aortic tissue ET-1 concentration. Atheroma formation was attenuated by chronic NEP-I (62±6% versus 34±5%, P<0.05).

Presence and Distribution of ECE-1 Isoforms
Immunohistochemical staining for total ECE-1, ECE-1α, and ECE-1β is shown in Figure 1. Compared with nonimmune controls (panels A and E), the nonselective antibody (panels B and F) demonstrates the presence of ECE-1 in the atherosclerotic plaques. Both ECE-1 isoforms (ECE-1α, panels C and G; ECE-1β, panels D and H) are also present in the atheromatous plaques. In the cholesterol-fed rabbits (Athero group), ECE-1β immunoreactivity more closely parallels the nonselective antibody immunoreactivity.
General Responsiveness of Isolated Aorta
There were no differences between the groups in resting tension at the optimum point of the length-tension curve. Additionally, the maximal tension developed to 60 mmol/L KCl was not different (8.1 ± 1.2 versus 8.4 ± 0.6 g for Athero group versus NEP-I–treated cholesterol-fed rabbits [NEP-I group], respectively; P < NS). The calcium ionophore A23187 resulted in complete relaxation of vessels with endothelium and no relaxation of vessels denuded of endothelium.

Time Course of Big ET-1 and ET-1 Responses
Figure 2A is a representative tracing of the vasoconstrictor response to big ET-1 and ET-1 in isolated aortic rings from the Athero group in the presence and absence of endothelium. The response to big ET-1 was delayed compared with the response to ET-1 (time to maximal response 64.0 ± 5.6 versus 19.3 ± 1.4 minutes, P < 0.001). Time to maximal contraction was not affected by the endothelium. Phosphoramidon completely blocked the big ET-1 response, independent of the presence or absence of endothelium in all but 1 isolated aortic ring in the Athero group (data not shown). In that experiment, the maximal big ET-1 response was 18% of the maximal ET-1 response. Phosphoramidon did not alter the ET-1 response.

Figure 2B is a representative tracing of the vasoconstrictor response to big ET-1 and ET-1 in isolated aortic rings from the NEP-I group in the presence and absence of endothelium. The response to big ET-1 was again delayed compared with the response to ET-1 (time to maximal response 56.0 ± 3.6 versus 23.0 ± 3.1 minutes, P < 0.001). Time to maximal contraction was not affected by the endothelium and was not different from that in the Athero group. Phosphoramidon completely blocked the big ET-1 response, independent of the endothelium, in all experiments in the NEP-I group and did not alter the ET-1 response.

Maximal Big ET-1 and ET-1 Response
Big ET-1 mediated a maximal vasoconstriction response of 61 ± 3% of the KCl maximum in the presence of endothelium and 74 ± 6% of the KCl maximum in the absence of endothelium. ET-1 resulted in a maximal response of 91 ± 3% of the KCl contraction in the presence of endothelium and 105 ± 6% of the KCl contraction in the absence of endothelium. In the NEP-I group, big ET-1 resulted in a maximal vasoconstriction response of 58 ± 4% (P = NS versus Athero group with endothelium), which was not different from the actions of big ET-1 in the untreated Athero group with endothelium. However, with the endothelium removed in the NEP-I–treated group, big ET-1–mediated contraction was only 34 ± 10% (P < 0.05 versus Athero group without endothelium) of the maximal KCl response, consistent with a reduction in functional ECE-1 activity. The maximal response to equimolar ET-1 was 84 ± 2% and 104 ± 7% of the KCl maximum in the presence and absence of endothelium, respectively.

ECE-1 Activity as Assessed by Big ET-1–to–ET-1 Response Ratio and Modulation by NEP-I
Figure 3 shows ECE-1 activity of the 2 groups as determined by the ratio of the big ET-1 to ET-1 response in the presence (solid bars) and absence (open bars) of endothelium. In the presence of endothelium, ECE-1 activity was preserved in the NEP-I group compared with the Athero group (65 ± 5% [Athero group] versus 64 ± 7% [NEP-I group], P = NS). In the

<table>
<thead>
<tr>
<th>Group</th>
<th>Chol, mg/dL</th>
<th>MAP, mm Hg</th>
<th>P cGMP, pmol/mL</th>
<th>P ET-1, pg/mL</th>
<th>Ao ET-1, pg/mg Protein</th>
<th>Atheroma, % Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Athero</td>
<td>1490 ± 136</td>
<td>81.4 ± 2.0</td>
<td>2.8 ± 0.6</td>
<td>5.8 ± 0.9</td>
<td>4.6 ± 0.5</td>
<td>62 ± 6</td>
</tr>
<tr>
<td>NEP-I</td>
<td>1368 ± 104</td>
<td>83.3 ± 2.1</td>
<td>8.4 ± 1.2*</td>
<td>6.1 ± 0.7</td>
<td>3.2 ± 0.3*</td>
<td>38 ± 5*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Chol indicates total cholesterol; MAP, mean arterial pressure; P cGMP, plasma cGMP concentration; P ET-1, plasma ET-1 concentration; Ao ET-1, aortic ET-1 concentration; and atheroma, percent surface area of the aortic arch with positive staining by oil red O.

*P < 0.05 vs Athero group.

Figure 1. Immunoreactivity for ECE-1 by use of nonimmune serum (A and E), nonselective anti–ECE-1 antibody (B and F), anti–ECE-1α antibody (C and G), and anti–ECE-1β antibody (D and H) in Athero (top) and NEP-I (bottom) groups (magnification ×400).
Atherosclerotic vascular tissues and by determining the presence and distribution of the α and β isoforms of ECE-1. Shimada and colleagues reported the expression of ECE-1 in rat ECs and human umbilical vein ECs and, later, isolated two isoforms of ECE-1 in humans. This discovery led to the development of monoclonal ECE-1 antibodies. Newer antibodies to the α and β isoforms of human ECE-1 that cross-react with other species were used in the present study to demonstrate the presence of these isoforms in the hypercholesterolemic rabbit. The selective antibodies demonstrate that both the α and β isoforms of ECE-1 are distributed in the atherosclerotic rabbit aorta. ECs, VSMCs, and foam cells contained within atherosclerotic lesions demonstrated ECE-1 immunoreactivity; however, it is apparent after inspection of the immunohistochemical staining for ECE that ECE-1β immunoreactivity more closely parallels that of the nonselective antibody. Although no conclusion can be drawn regarding the relative importance of these isoforms on ET-1 production in atherosclerosis, this observation may suggest that ECE-1β contributes to ET-1 production in atherosclerosis. The presence of ECE-1 immunoreactivity in atheromatous lesions is consistent with previous reports of ECE-1 immunoreactivity and mRNA expression in atherectomy specimens from human coronary arteries and, more recently, a report by Rossi et al confirming the presence of ECE-1 mRNA in diseased, but not nondiseased, human arteries.

The endothelin system interacts with other vascular peptide and cytokine systems relevant to the progression of atherosclerosis. Previous reports have demonstrated antimitogenic actions of the natriuretic peptide system on endothelin-induced smooth muscle cell proliferation. Suenobo et al have recently defined the antiapoptotic actions of the natriuretic peptides and the proapoptotic actions of ET-1 in ECs. They proposed a counterregulatory role for these systems in the maintenance of EC turnover through apoptosis. The observation that stimulated macrophages release ET-1 and ET-1 in Athero (A) and NEP-I (B) groups in presence and absence of endothelium.

The model of atherosclerosis used in the present study is well established and reproducible. Rabbits fed a 1% cholesterol diet for 8 weeks developed fatty streaks involving 60% of the surface area of the aortic arch, which were characterized by infiltration by lipid-laden foam cells and VSMCs. The absence of increased circulating ET-1 is further evidence of the early nature of this model because circulating ET-1 concentration correlates with atherosclerosis disease burden. The present study confirms and extends previous investigations by demonstrating ECE-1 immunoreactivity in atherosclerotic vascular tissues and by determining the presence and distribution of the α and β isoforms of ECE-1.
able to assume that the reductions in the aortic arch atheroma area were also present in the descending thoracic aorta in the present study.

The mechanism of attenuated ECE-1 activity remains undefined but was likely in part due to cGMP. In cultured bovine ECs, atrial natriuretic peptide inhibits ET-1 production and release.\textsuperscript{33} It is important to note that the time course of the big ET-1 contractions was not altered by chronic NEP-I or the presence of endothelium. This observation suggests that the concentration of ECE-1 available for substrate processing remained constant and that the affinity for the substrate or access to the substrate may have been impaired. Furthermore, the effect was not likely a nonspecific action of the NEP-I Candoxatril, inasmuch as this compound has been demonstrated to be specific for NEP\textsuperscript{34} and does not cross-react with ECE-1 in human tissue. The role of oxidized LDL in the regulation of ET-1 release and ECE-1 activity remains controversial.\textsuperscript{35,36} There were no differences in total serum cholesterol concentrations between the Atero and the NEP-I groups in the present study, suggesting that ECE-1 inhibition and attenuated atheroma formation by NEP-I occurred independent of alterations in lipids.

The present study used an assay of ECE-1 activity that may have been dependent on mechanical factors that participate in ET-1 release.\textsuperscript{37} However, there were no apparent differences in the mean arterial pressure or the degree of resting and maximal tension of the aortic rings between the groups that could explain the attenuated ECE-1 activity in the NEP-I group. Taken together, the data support a cGMP-related hypothesis that chronic NEP-I results in a net antiproliferative effect independent of alterations in lipids.

Whereas NEP-I has been shown to inhibit the degradation of the natriuretic peptides, it has also been shown to inhibit ET-1 degradation, at least during acute administration.\textsuperscript{38} We observed no increase in circulating or tissue ET-1 concentrations but did show increased cGMP concentrations in the group treated with chronic oral NEP-I compared with the untreated Atero group. Together with the evidence suggesting attenuated ECE-1 activity by chronic NEP-I, the hypothesis that chronic NEP-I results in a net antiproliferative effect via the augmentation of natriuretic peptide concentrations or the enhanced cGMP-generating capacity of these proteins is plausible. These actions of chronic oral NEP-I further support the notion proposed by others that important interactions exist\textsuperscript{28} between the natriuretic peptide system and the endothelin system that have pathophysiological and potential therapeutic relevance in atherosclerosis. Indeed, the ability to inhibit the tissue ET-1 system in atherosclerosis, independent of effects on cholesterol lowering, also suggests the need to explore possible synergistic relations between NEP-I and lipid-lowering agents in the treatment of atherosclerosis.

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**References**


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