Tissue Endothelin-Converting Enzyme Activity Correlates With Cardiovascular Risk Factors in Coronary Artery Disease

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Methods and Results—Vascular reactivity to big endothelin-1 (bigET-1; 10−8 to 10−7 mol/L) and ET-1 (10−9 to 10−7 mol/L) were examined in the internal mammary artery (IMA, n=33) and saphenous vein (SV, n=27) of patients with coronary artery disease with identified cardiovascular risk factors. Vascular ECE activity was determined by conversion of exogenously added bigET-1 to ET-1. Tissue contents of bigET-1 and ET-1 were measured by radioimmunoassay. In addition, the effects of LDL and oxidized LDL on ECE-1 protein levels were determined by Western blot analysis in human IMA endothelial cells. In the IMA, vascular ECE activity showed an inverse correlation with serum LDL levels (r=−0.76; P<0.01) and systolic and diastolic blood pressure and a positive correlation with fibrinogen (r=0.58; P<0.05). In the SV, fibrinogen was the only parameter to be correlated with vascular ECE activity. Vascular tissue content of bigET-1 was attenuated in the IMA of patients with hyperfibrinogenemia but increased in patients with elevated systolic blood pressure and increased serum LDL levels (P<0.05). Most interestingly, LDL and oxidized LDL downregulated ECE-1 protein levels in human IMA endothelial cells (P<0.05).

Conclusions—These data demonstrate, for the first time, that vascular ECE activity is (1) inversely correlated with serum LDL levels and blood pressure and (2) positively associated with fibrinogen in human vascular tissue. Hence, ECE-1 activity may modulate cardiovascular risk in patients with coronary artery disease. (Circulation. 2000;102:1086-1092.)

Key Words: endothelin enzymes arteries lipoproteins fibrinogen blood pressure

The endotelin congeners are a family of biologically active peptides implicated in the pathogenesis of cardiovascular disorders as a result of their vasoconstrictor and growth-promoting properties.1,2 Endothelin (ET)-1 is produced by endothelial cells,2 smooth muscle cells,3,4 and macrophages3 and acts through activation of G protein–coupled ETa and ETb receptors.6 ET-1 is cleaved from its precursor polypeptide bigET-1 by 3 different endothelin-converting enzymes (ECEs).7 ECE-1 has recently been cloned and is the major form of ECE in most tissues.7,8 ECE-1 exists in 2 isoforms, ECE-1α and ECE-1β, with differing N-terminal cytoplasmic domains but similar enzymatic properties and tissue distribution.9 The more recently cloned ECE-2 is a homologous protein that differs from ECE-1 in its sensitivity for ECE inhibitors, such as phosphoramidon.10 ECE-1 and ECE-2 are homologous with neutral endopeptidase-24.11 and have been localized to endothelial cells in a variety of tissues. A novel ECE-3 that has specificity for bigET-3 was purified from bovine iris microsomes; however, ECE-3 has not as yet been detected in human endothelial cells.11 Because of differences in pH optimum and cellular distribution, ECE-2 is unlikely to play a pathogenic role in diseases in which cellular pH is reduced, eg, ischemic heart disease.12 Hence, ECE-1 appears to be the predominant ECE in human vascular tissue. ECE-1 is clustered along the plasma membrane and upregulated and redistributed to an intracellular compartment, probably the Golgi apparatus, after treatment with a metalloprotease inhibitor, phosphoramidon or thiorphan.10 In addition, ECE-1 immunostaining is associated with membranes of vesicles that were immunopositive for bigET-1, suggesting that at least a proportion of the intermediate peptide is processed intracellularly and delivered to the cell surface through the secretory pathway.8

Although bigET-1 is widely expressed in the human vascular endothelium,13 it exhibits little vasoconstrictor activity compared with ET-1 in human isolated blood vessels.14

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TABLE 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Risk Factors</th>
<th>Men (n=28)</th>
<th>Women (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>62±9</td>
<td>69±5</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>23 (82)</td>
<td>2 (40)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>11 (40)</td>
<td>4 (80)</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>5 (18)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>25 (89)</td>
<td>5 (100)</td>
</tr>
<tr>
<td>Hypertriglyceridemia, n (%)</td>
<td>19 (68)</td>
<td>2 (40)</td>
</tr>
<tr>
<td>Serum cholesterol, mmol/L</td>
<td>5.3±1.2</td>
<td>5.8±0.5</td>
</tr>
<tr>
<td>Serum HDL, mmol/L</td>
<td>1.1±0.3</td>
<td>1.6±0.4</td>
</tr>
<tr>
<td>Serum LDL, mmol/L</td>
<td>3.4±1.0</td>
<td>3.5±0.4</td>
</tr>
<tr>
<td>Serum triglycerides, mmol/L</td>
<td>2.4±1.4</td>
<td>1.8±0.7</td>
</tr>
<tr>
<td>Plasma fibrinogen, mmol/L</td>
<td>3.4±1.2</td>
<td>4.4±0.6</td>
</tr>
<tr>
<td>Drugs, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrate</td>
<td>20 (71)</td>
<td>4 (80)</td>
</tr>
<tr>
<td>β-Blockers</td>
<td>9 (32)</td>
<td>2 (40)</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>20 (71)</td>
<td>4 (80)</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>11 (39)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Angiotensin II antagons</td>
<td>1 (4)</td>
<td>1 (20)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>17 (61)</td>
<td>3 (60)</td>
</tr>
<tr>
<td>Coumarin</td>
<td>4 (14)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Statins</td>
<td>18 (64)</td>
<td>2 (40)</td>
</tr>
</tbody>
</table>

Indeed, in the human forearm, bigET-1 causes a slow-onset, long-lasting vasoconstriction with a potency of only ~10% of that of ET-1, suggesting that its conversion to ET-1 by ECE appears to be essential to elicit full hemodynamic effects.14

The expression of ET-1 in cultured vascular endothelial cells is stimulated by several factors associated with the formation of atherosclerotic lesions, such as transforming growth factor-β, interleukin-1, and oxidized LDL (oxLDL).15

In addition, ET-1 and ECE-1 are expressed in vascular smooth muscle cells and macrophages in human atherosclerotic plaques and rat atherosclerosis models of allografts.3,5,16 Although an increasing body of evidence suggests a pivotal role of the endothelins in coronary atherosclerosis, their implication in hypertension remains controversial.13

Hence, the goal of the present study was to further delineate whether ET-1, its precursor bigET-1, and ECE, the key enzyme in ET generation, are related to cardiovascular risk factors in patients with coronary atherosclerosis, in particular high blood pressure and serum lipoproteins. For this purpose, tissue contents of bigET-1 and ET-1, vascular reactivity to ET-1 and bigET-1, and vascular ECE activity were determined in arteries and veins obtained from patients undergoing coronary artery bypass surgery.

Methods

Patient Characteristics and Risk Factors

Thirty-three patients underwent elective bypass surgery and gave written consent for inclusion into the study. The study protocol was approved by the Human Ethics Committee of the University Hospital Zurich. Serum lipids, including total cholesterol, LDL, HDL, lipoprotein (α), and triglycerides, and fibrinogen were determined in all patients (Table 1). Cigarette smoking was excluded as a risk factor if patients had stopped smoking >6 months before surgery. In addition, a history of either hypertension or diabetes was considered a risk factor. Almost all patients were on multiple drug therapy.

Because all patients underwent elective bypass surgery, drugs that impact endothelial function, ie, statins, ACE inhibitors, angiotensin II antagonists, and calcium antagonists, were suspended for the last 3 and aspirin for the last 7 days before the operation. β-Blockers and occasional use of nitrates were maintained until the day of admission to our institution, ie, on average, 1 day before operation. No woman was receiving estrogen replacement therapy. Hypertensive subjects were moderate essential hypertensives on medication who had had the condition for a mean duration of 8.5 years. None of the patients had renovascular hypertension or renal failure. Seven patients presented with a history of myocardial infarction; 2 in the hypertensive group had suffered a minor stroke.

Vascular Function

Internal mammary arteries (IMAs) and saphenous veins (SVs) were obtained during surgery, and studies of isometric tension development were performed on IMA and SV ring segments by use of methods previously reported.17 To study contractions to different agonists, ie, bigET-1, ET-1, and norepinephrine, quiescent arterial and venous vessel segments were exposed to either cumulative doses of bigET-1 (10⁻⁷ to 10⁻⁵ mol/L), ET-1 (10⁻⁷ to 10⁻⁵ mol/L), or norepinephrine (10⁻⁶ to 10⁻⁴ mol/L). Each contraction to bigET-1 and ET-1 was allowed 30 minutes to develop, and a plateau was obtained before the next dose was given.

Vascular ECE activity was assessed as the ratio of the maximal vascular responses of exogenously added bigET-1 to ET-1 in IMA and SV. Because ECE activity cannot be assessed solely by contractions to bigET, this ratio gives an approximation of the functional enzyme activity by dividing the response to the substrate by that of the enzyme product. Assuming that equimolar concentrations of bigET are completely converted to ET-1, the ratio theoretically should be 1. This ratio includes both conversion of bigET (enzyme kinetics, enzyme activity) and receptor activation by ET-1.18 Because ECEs are expressed predominantly in endothelial cells, only ring segments with preserved endothelial function were included. Preserved endothelial function was determined as relaxation to acetylcholine (10⁻⁷ mol/L) in IMA or bradykinin (10⁻⁷ mol/L) in SV >50% of norepinephrine-induced preconstriction. Similarly, a patient was included only if ≥4 segments of IMA and SV were valid simultaneously. Hence, vascular ECE activity could be determined in 120 vessel rings of 30 IMAs and SVs.

In addition, to further investigate a possible correlation of endothelial function with cardiovascular risk factors, dose-response curves to acetylcholine (10⁻¹⁰ to 10⁻⁴ mol/L) in IMA and bradykinin (10⁻⁵ to 10⁻⁴ mol/L) in SV were obtained in rings precontracted with norepinephrine (10⁻⁷ mol/L). Because IMA and SV obtained during bypass graft operation varied in length (<2 cm), vessel rings of a patient were randomly assigned to the determination of vascular reactivity to bigET-1 and ET-1 and vascular ECE activity, acetylcholine-induced vasorelaxation, or determination of bigET-1 and ET-1 tissue levels.

ET-1 and BigET-1 Tissue Levels

ET-1 and bigET-1 were determined as previously described.19

Lipoprotein Isolation and Modification

LDL (d=1.019 to 1.063 g/mL) was prepared from plasma of normolipidemic blood donors by ultracentrifugation and was oxidatively modified as described previously.20

Western Blot Analysis of ECE-1 Expression in Human IMA Endothelial Cells

Human IMA endothelial cells were cultivated and used for experiments after the first passage when confluent. Forty-eight hours before the experiments, cells were kept in lipoprotein-free medium with 5% FCS. Cells were preincubated with either cerivastatin (10⁻⁶ mol/L) or placebo for 1 hour, followed by an incubation period of 24 hours with 100 mg/mL LDL or oxLDL, respectively. Equal amounts of protein were used for electrophoresis, and comparable loading
was confirmed by silver staining. Optical density of ECE protein bands was detected by NIH imaging software, and optical density in control cells was regarded as 100%.

Materials
Acetylcholine, bradykinin, L-norepinephrine bitartrate, and indomethacin were obtained from Sigma Chemicals, cerivastatin from Bayer, and ET-1 and bigET-1 from Novabiochem. The concentrations of the drugs are expressed as final concentrations in the bath solution. All drugs were dissolved in distilled water and stored in ice.

Statistics
Data are given as mean±SEM; n equals the number of patients from whom blood vessels were obtained. Contractions were expressed as percentage of the increase in tension to 100 mmol/L KCl. Relaxations to acetylcholine are expressed as percentage of norepinephrine-induced preconstriction. Responses to each agent were obtained in 4 vascular segments and averaged for each patient. Statistical analysis was performed by a t test for unpaired observations or by a Mann-Whitney U test when the distribution of variances was heterogeneous, as determined by a z test for proportions. Simple linear regression analysis was used for determining the relation between 2 variables. A value of P<0.05 was considered significant.

Results

Patient Characteristics
For initial studies examining vascular contractions to bigET-1, ET-1, and norepinephrine, 60 vessels (33 IMAs and 27 SV) of patients undergoing coronary artery bypass surgery were studied. Male and female populations differed by the frequency of evident cardiovascular risk factors (Table 1).

Vascular Reactivity

Vascular ECE Activity
In IMA, vascular ECE activity showed an inverse correlation with serum LDL (r = 0.76; P<0.01; n = 15). In contrast, vascular ECE activity (b) was positively correlated with plasma fibrinogen (r = 0.58; P<0.05; n = 15). Systolic (c) (r = 0.59; P<0.05) and diastolic (d) (r = 0.51; P<0.05) blood pressures were inversely correlated with vascular ECE activity.

Figure 1. Vascular ECE activity and cardiovascular risk factors. a, Vascular ECE activity (as assessed by ratio of vascular response to bigET-1/ET-1) showed inverse correlation with serum LDL (r = 0.76; P<0.01; n = 15). In contrast, vascular ECE activity (b) was positively correlated with plasma fibrinogen (r = 0.58; P<0.05; n = 15). Systolic (c) (r = 0.59; P<0.05) and diastolic (d) (r = 0.51; P<0.05) blood pressures were inversely correlated with vascular ECE activity.
and diastolic (Figure 1d) blood pressures were correlated with vascular ECE activity. Conversely, vascular ECE activity was positively correlated with plasma fibrinogen (Figure 1b), whereas serum levels of Lp(a), total cholesterol, and triglycerides were not associated with ECE activity in IMA. In SV, fibrinogen was the only parameter to be correlated with vascular ECE activity ($r=0.59; P<0.05$; not shown). However, vascular reactivity of SV and IMA to ET-1 did not differ between patients with and without any of the cardiovascular risk factors studied (Figure 2).

**Vascular Response to BigET-1**

Vascular reactivity to bigET-1 was blunted in IMA of patients with elevated serum LDL levels (Figure 2a) and increased blood pressure (Figure 2b), but was enhanced in IMA of patients with hyperfibrinogenemia (Figure 2c). In saphenous vein, contractions to bigET-1 were attenuated in hypercholesterolemia and enhanced in hyperfibrinogenemia, but they did not differ between patients with normal and increased blood pressure levels (not shown). No such correlations were detectable for total cholesterol, HDL, Lp(a), triglycerides, or fibrinogen in IMA.

**Vascular Response to ET-1**

Contractions to ET-1 were significantly greater than those induced by bigET-1 in all concentrations studied (Figure 2). However, vascular reactivity of SV and IMA to ET-1 did not differ between patients with and without any of the cardiovascular risk factors (Figure 2).

**Contractions to Norepinephrine and KCl**

In SV and IMA, contractions to norepinephrine did not show any correlation with any cardiovascular risk factor studied. The response to KCl was not altered in the different groups studied (not shown).

**BigET-1 and ET-1 Tissue Levels**

BigET-1 but not ET-1 tissue levels were increased in IMA of patients with hypercholesterolemia (Figure 3a) and elevated blood pressure (Figure 3b). In contrast, bigET-1 content was reduced and that of ET-1 was augmented in IMA of patients with hyperfibrinogenemia (Figure 3c). Tissue content of bigET-1 in SV did not differ between patients with different cardiovascular risk factors (Figure 3). Vascular ET-1 levels were increased in patients with hyperfibrinogenemia only (Figure 3c).

**Endothelium-Dependent Relaxation**

Endothelium-dependent relaxation to acetylcholine was blunted in IMA of patients with increased LDL levels (Figure 4a) and elevated blood pressure (Figure 4b). Most interestingly, we demonstrate, for the first time, decreased endothelial function in patients with hyperfibrinogenemia (Figure 4c). In addition, endothelial function was inversely correlated with systolic blood pressure and cholesterol (Table 2).

**Effects of LDL and oxLDL on ECE Expression in Human IMA Endothelial Cells**

In human IMA endothelial cells, Western blot analysis revealed that both LDL and oxLDL downregulate ECE protein levels (Figure 5). Effects of LDL and oxLDL were prevented by cerivastatin.

**Discussion**

These data demonstrate, for the first time, that in patients with coronary artery disease, vascular ECE activity is inversely correlated with serum LDL levels and blood pressure in human arteries and positively associated with plasma fibrinogen in both arteries and veins of patients with coronary artery disease.

There is experimental and clinical evidence that endothelins are activated in atherosclerosis, myocardial infarction, coronary spasm, congestive heart failure, and pulmonary hypertension. The formation of ET-1 is regulated by endothelin-derived vasoactive substances, components of the coagulation cascade, and growth factors, all of which are implicated in...
atherosclerosis. Moreover, circulating ET-1 and semiquantitative immunoreactive staining for ET-1 of atherosclerotic plaque are increased in patients with coronary atherosclerosis. However, the role of vascular ECE in patients with atherosclerosis remains to be determined. Intriguingly, we here provide the first evidence that vascular ECE activity is inversely correlated with serum LDL in patients with cardiovascular disease.

Table 2. Correlation of Endothelium-Dependent Relaxation to Acetylcholine (Maximal Response) With Cardiovascular Risk Factors in Arteries and Veins of Patients With Coronary Artery Disease

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>IMA</th>
<th>SV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP</td>
<td>−0.743, &lt;0.05</td>
<td>−0.115, NS</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>−0.064, NS</td>
<td>0.060, NS</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>−0.605, &lt;0.05</td>
<td>−0.298, NS</td>
</tr>
<tr>
<td>HDL</td>
<td>0.461, NS</td>
<td>0.460, NS</td>
</tr>
<tr>
<td>LDL</td>
<td>−0.494, NS</td>
<td>0.150, NS</td>
</tr>
<tr>
<td>Lipoprotein (a)</td>
<td>0.257, NS</td>
<td>−0.100, NS</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>−0.102, NS</td>
<td>−0.439, NS</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.482, NS</td>
<td>0.400, NS</td>
</tr>
</tbody>
</table>
endothelial cells.\textsuperscript{25} Thus, in certain pathophysiological conditions, regulation of ECE-1 expression by ET-1 in vivo may serve as a negative feedback system to prevent an increase of tissue ET-1 in the vessel wall, particularly in patients with cardiovascular risk factors.

Because all patients underwent elective bypass surgery, drugs that impact endothelial function were suspended for the last 3 days before operation. However, because some drugs may affect endothelial function after long-term treatment, e.g., with ACE inhibitors and statins, we cannot exclude a contribution of chronic drug-induced effects.\textsuperscript{26,27} In the present study, cerivastatin prevented the effects of LDL and oxLDL on vascular ECE in cultured human IMA endothelial cells. Hence, even if the effects of statins were persistent for more than 3 days after the medication was suspended, the inverse correlation observed between serum LDL and vascular ECE would actually be even stronger in patients never treated with these drugs.

Because of its potent vasoconstrictor action and effects on vascular hypertrophy, ET-1 is thought to be involved in blood pressure regulation as well as functional and structural changes in hypertension.\textsuperscript{13} Although some studies found increased plasma levels of ET-1, particularly in patients with hemangioendothelioma and in some forms of salt-sensitive hypertension,\textsuperscript{28,29} many others found no differences from controls.\textsuperscript{13} Because ET-1 is released primarily abluminally,\textsuperscript{30} tissue ET-1 as opposed to circulating ET-1 may reflect its possible activation in disease states. In the present study, however, vascular tissue levels of ET-1 and vascular responsiveness to ET-1 did not show any association with blood pressure. This is in line with the findings of others, except for 1 study by Schiffrin and coworkers,\textsuperscript{31} who showed enhanced ET-1 gene expression in small arteries of patients with moderate to severe hypertension. However, ET-1 gene expression was similar in control subjects and untreated mild hypertensives whose blood pressure levels were comparable to those of the present study. Thus, tissue ET-1 levels may be increased only in the presence of marked hypertension. Intriguingly, in the present study, vascular bigET-1 but not ET-1 levels were increased in the IMA of patients with elevated blood pressure. Because conversion of the precursor polypeptide bigET-1 to mature ET-1 by ECE-1 is the final key step in the activation of this pressor system, a modulation of ECE activity may explain a dissociation between ET-1 and bigET-1 levels. Accordingly, rats with hypertension induced by hepatic overexpression of human preproET-1 have a high ratio of bigET-1 to mature ET-1.\textsuperscript{32} Moreover, in patients with chronic hemodialysis, strikingly elevated bigET-1 levels with only slightly elevated ET-1 levels in plasma are observed, suggesting decreased ECE activity.\textsuperscript{33} Correspondingly, in the present study, vascular ECE activity showed an inverse correlation with blood pressure in IMA. In SVs, which are not exposed to systemic blood pressure values, no such correlation was detectable. This suggests that blood pressure exerted on the vessel wall is involved in the regulation of vascular ECE activity.

Another new finding of the present study is that vascular ECE was positively correlated with plasma fibrinogen levels. Accordingly, vascular ET-1 but not bigET-1 content was augmented in patients with hyperfibrinogenemia, which further suggested enhanced vascular ECE activity. Although ET-1 increases immunoreactive von Willebrand factor in endothelial cells,\textsuperscript{34} these data are the first to show that ECE activity in the vessel wall is associated with clotting factors in patients with coronary artery disease. Because fibrinogen is an acute-phase protein, these findings may also suggest that vascular ECE is implicated in the inflammatory response of the vessel wall, which further extends findings that ET-1 formation is stimulated by interleukins and tumor necrosis factor, which are all implicated in atherogenesis.\textsuperscript{1} However, prolonged effects of aspirin on vascular ECE activity cannot be excluded but seem unlikely, because aspirin was suspended for the last 7 days before the operation, and correlation between vascular ECE and fibrinogen did not differ between patients with and without aspirin treatment.

In addition, we demonstrate, for the first time, decreased endothelium-dependent relaxation to acetylcholine in patients with hyperfibrinogenemia. Furthermore, endothelial function was blunted in IMA of patients with elevated blood pressure as well as increased LDL levels, which further extends the findings of Huraux and coworkers,\textsuperscript{35} who showed an inverse correlation between endothelium-dependent relaxation and the number of cardiovascular risk factors present in patients with coronary artery disease. Because elevated LDL cholesterol levels are directly and causally related to coronary artery disease,\textsuperscript{36} reduced endothelium-dependent relaxation in the presence of elevated serum LDL levels indicates that NO availability may be an important component of endothelial injury.\textsuperscript{37}

In summary, the present studies are the first to demonstrate that vascular ECE activity shows an inverse correlation with serum LDL levels and blood pressure in human arteries and a positive association with plasma fibrinogen levels in both arteries and veins of patients with coronary artery disease. Vascular ECE activity may represent a future pharmacological target in the treatment of patients with coronary artery disease, particularly when ECE activity is positively correlated with cardiovascular risk. In hypertension and hypercholesterolemia, however, ET-receptor antagonism rather than ECE inhibition may be the superior strategy to block the effects of the vascular endothelin system in humans.
Acknowledgment
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References
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