Saphenous Vein Endothelin System Expression and Activity in African American Patients

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Objective—Plasma endothelin (ET)-1 levels are significantly higher in African American hypertensive patients than in white hypertensive patients. However, whether the molecular components of vascular ET-1 biosynthesis and function are altered in this population remains to be established. Accordingly, the overall goal of this study was to investigate the effects of race on vascular mRNA and protein levels of ET-converting enzyme (ECE)-1 subisoforms, ET-1, and ET receptor profiles in hypertension.

Methods and Results—Saphenous vein samples were obtained from African American (n = 13) and white (n = 15) patients undergoing coronary artery grafting surgery. The expression of preproET-1 and of ECE-1α was upregulated 2- and 3-fold, respectively, in African Americans. In endothelium-intact vessels, the ET<sub>A</sub> expression was higher in whites. In endothelium-denuded vessels, the ET<sub>B</sub> mRNA was 3-fold higher in African Americans, suggesting that vasoconstriction-promoting ET<sub>B</sub> receptors are upregulated in this population. Vascular tissue ET-1 levels and ECE-1 activity were also augmented in African American patients.

Conclusions—This study demonstrated that the biosynthetic pathway of ET-1 is activated to a higher degree and that the ET<sub>B</sub> receptor subtype expression is altered in the peripheral vasculature of African American hypertensive patients. The augmented synthesis and altered expression of ET<sub>B</sub> receptors may both contribute to the increased incidence of hypertension and related complications in this patient population. (Arterioscler Thromb Vasc Biol. 2002;22:1122-1127.)

Key Words: endothelin-converting enzyme • receptor subtype • race • hypertension • gene expression

The prevalence of essential hypertension is significantly higher and the severity of cardiovascular complications is greater in the African American population than in whites. These differences in the development and progression of hypertension in blacks have been proposed to be related to abnormal hemodynamic reactivity characterized by increased peripheral vascular resistance and diminished vasodilatation in response to environmental stress. In a previous study, we found that plasma levels of the potent vasoconstrictor endothelin (ET)-1 were significantly higher in a black hypertensive group than in white hypertensive and black normotensive groups. ET-1 acts locally in a paracrine/autocrine fashion and is predominantly secreted from the endothelial cells toward the underlying smooth muscle cells. Local vascular ET-1 levels in this patient population remain unknown.

The ET system consists of 3 major components: (1) ET-1, (2) ET-converting enzyme (ECE)-1, which is responsible for the biosynthesis of the active ET peptide, and (3) ET receptors, which mediate the biological effects of this peptide. Therefore, in addition to peptide levels, regulation of ECE-1 and regulation of ET receptor subtype expression in the vasculature in the setting of hypertension are equally critical. To date, there are 4 splice variants of ECE-1, with ECE-1α and ECE-1c being the most commonly expressed subisoforms. However, the expression and activity of the ECE-1 subisoforms in the setting of hypertension are not known. Moreover, the potent contractile effect of ET-1 is regulated by the relative ratio of vasoconstriction-promoting ET<sub>A</sub> and ET<sub>B</sub> receptors on vascular smooth muscle cells to vasodilatation-promoting ET<sub>B</sub> receptors on endothelial cells. Accordingly, the overall purpose of the present study was to investigate vascular ET-1, ET receptor, and ECE-1 subisoform profiles at mRNA and protein levels in hypertensive patients.

Methods

Tissue Source

Saphenous vein specimens were obtained from African American (n = 13) and white (n = 15) patients with essential hypertension undergoing coronary artery bypass grafting surgery at the Medical University of South Carolina and Medical College of Georgia. Patients with diabetes, chronic heart failure, and kidney disease were excluded. Patient characteristics are given in the Table. All patients were on antihypertensive therapy, which included calcium channel blockers, ACE inhibitors, angiotensin II receptor blockers, β-blockers, and diuretics. Most of the patients were on combination
therapy, and the use of antihypertensive medications in African American and white patient groups was similar (Table). In addition, tissue samples from African American (*n*=3) and white (*n*=3) normotensive patients were included. After fat tissue was removed, vessels were cut into smaller sections, and in some rings, endothelium was denuded by scraping the inner surface of the rings. Tissue samples were then snap-frozen in liquid nitrogen and stored at −80°C until use. Patient consent was obtained in all cases.

### Expression Studies by Reverse Transcription–PCR

RNA was extracted from 50 mg vascular tissue by using the RNAsolo kit from Qiagen. First-strand cDNA synthesis was performed with 1 µg total RNA and oligo(dT)primers by using AMV reverse transcriptase (Promega). Polymerase chain reaction (PCR) was carried out in a reaction mixture containing 20 mmol/L Tris-HCl (pH 8.5), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L of each dNTP, 500 nmol/L of each sense and antisense primer, 3 µL first-strand cDNA, and 2.5 U Taq DNA polymerase. The amplification primers and conditions used have recently been described.° To ensure that equivalent cDNA template was used in each reaction, amplification of GAPDH was used as an internal control. The PCR products were electrophoresed on 2% agarose gels, and image was captured with the use of Image-Pro software (Media Cybernetics). The density of PCR products was then analyzed by Gel-Pro Analyzer software (Media Cybernetics) and normalized over GAPDH expression.

### ET-1 Measurement

Vascular ET-1 content was determined by an extraction method. Briefly, 50 mg tissue was weighed and homogenized in 3 mL homogenization buffer that consisted of 1N NaOH, 0.15% trifluoroacetic acid, 1% formic acid, and 1% NaCl. After centrifugation at 1500g for 30 minutes, the protein content in the supernatant was measured by using the Bradford Protein Assay from Bio-Rad Laboratories. ET-1 levels were determined by using the Bradford Protein Assay from Bio-Rad Laboratories. ET-1 levels were also measured by using the Bradford Protein Assay from Bio-Rad Laboratories.

### Membrane Preparation

Vascular tissue (50 mg) was homogenized in buffer A (20 mmol/L Tris-HCl, pH 7.4, 20 µmol/L pepstatin A, 1 mmol/L phenylmethylsulfonyl fluoride, and 250 mmol/L sucrose). After an initial centrifugation at 1000g for 10 minutes, the resulting supernatant was centrifuged at 100 000g for 60 minutes. The pellet was resuspended in 50 µL buffer A, and membrane aliquots were stored at −70°C. The protein content in the membrane preparation was measured by using the Bradford Protein Assay from Bio-Rad Laboratories.

### Measurement of ECE-1 Activity

Enzyme activity was measured by incubating 50 µg total membrane protein with 0.1 µmol/L big ET-1 in 50 µL reaction mixture containing 0.1 mol/L sodium phosphate buffer, pH 6.8, and 0.5 mol/L NaCl at 1 hour at 37°C. The reaction was terminated with 50 µL of 5 mmol/L EDTA, and the assay was mixed and assayed for ET-1 by an ELISA kit described above. All assays were performed in duplicate.

### Receptor Binding Experiments

Membrane protein (30 µg) prepared from endothelium-denuded tissue was incubated with 100 pmol/L of [125I]ET-1 in the presence and absence of 1 µmol/L BQ-123 at 37°C for 2 hours. Nonspecific binding was determined in the presence of 2 µmol/L unlabeled ET-1.

### Data Analysis

Values obtained for ET-1 levels (femtomoles per milligram protein) and ECE-1 activity (femtomoles ET-1 per milligram protein per hour) in vascular tissue obtained from white and African American hypertensive patients were compared by use of the Student *t* test. Similarly, expression levels of the components of the ET system in white and African American hypertensive patients were analyzed by the Student *t* test. Because of the small number of normotensive patients, African American and white normotensive subjects were not included in the statistical comparisons. All statistical procedures were performed with the use of Prism software (GraphPad Software). Results are presented as mean±SEM. Values of *P*<0.05 were considered to be statistically significant.

### Results

**PreproET-1 (PPET-1) Expression and ET-1 Levels**

PPET-1 mRNA by densitometric analysis of the PCR products (Figure 1A) indicated a 3-fold increase in saphenous veins obtained from African American hypertensive patients (Figure 1B). Tissue ET-1 levels were also higher in the same patient group (Figure 1C). There was a trend for increased PPET-1 expression in white hypertensive compared with normotensive patients, but statistical significance was not achieved because of the small number of white normotensive patients included in the study.

**ECE-1 Expression and Activity**

Reverse transcription (RT)-PCR analysis of saphenous vein specimens demonstrated the expression of ECE-1a and ECE-1c subisofoms. The densitometric analysis of PCR
products revealed that the ECE-1a subisoform was increased 3-fold in African American patients compared with white hypertensive and normotensive patients, whereas there was no detectable change in ECE-1c expression (Figure 2A). ECE activity was also increased by nearly 3-fold in African American hypertensive patients (Figure 2B).

**ET Receptor Expression and Subtype Distribution**

Both ET<sub>A</sub> and ET<sub>B</sub> receptor mRNAs were amplified by semiquantitative RT-PCR in endothelium-intact and -denuded vascular tissue. In endothelium-intact tissue, the ET<sub>A</sub> receptor expression was 2-fold higher in white hypertensive than African American hypertensive patients (Figure 3A). There was no detectable difference in ET<sub>B</sub> receptor expression between the groups (Figure 3A). In endothelium-denuded tissue, however, the ET<sub>B</sub> mRNA was 3-fold higher in African American hypertensive patients compared with white hypertensive patients (Figure 3B). To compare our previous results and to relate them to the gene expression profile of ET receptors, total specific binding was determined by use of membrane fractions from endothelium-denuded tissue. Because of the limited amount of tissue samples, in each group 8 patients were included. Results shown in Figure 3C demonstrate that total binding counts were higher in white patients and represent only the ET<sub>A</sub> subtype. However, in specimens from African American patients, only 70% of the specific binding was sensitive to the ET<sub>A</sub> receptor antagonist BQ-123. These results suggest that 30% of ET receptors belong to the ET<sub>B</sub> receptor subtype and provide support for the RT-PCR experiments.

**Discussion**

Increased plasma ET-1 levels occur with essential hypertension in African American patients. However, the expression and synthesis of the components of the vascular ET system in this patient population have not been examined previously. The present study demonstrated that ECE-1a and ECE-1c subisoforms are both expressed in saphenous veins obtained from hypertensive patients undergoing coronary artery bypass grafting surgery and that ECE-1a mRNA was increased in African American hypertensive patients. ECE-1 activity, a determinant of ET-1 biosynthesis, was also increased in this patient population and was accompanied by elevated tissue PPET-1 mRNA and ET-1 levels. In addition, the presence of increased ET<sub>B</sub> receptor expression in endothelium-denuded vessels indicated that vasoconstriction-promoting ET<sub>B</sub> receptors on smooth muscle cells are upregulated in African American hypertensive patients. Thus, an augmented capacity for vascular ET-1 biosynthesis and an alteration in ET receptor subtypes may contribute to the increased incidence of hypertension and related complications in this high-risk patient population.

In experimental hypertension, the circulating levels of ET-1 are elevated only in salt-dependent models of hypertension, including deoxycorticosterone acetate (DOCA) salt hypertensive rats, DOCA salt-treated spontaneously hypertensive rats (see review<sup>10</sup>), and Dahl salt-sensitive rats. Consistent with these findings, ET receptor antagonists lower blood pressure in these models. Kassab et al<sup>11</sup> have reported that chronic administration of an ET<sub>A</sub>-selective antagonist (A-127722) significantly attenuates the increase in blood pressure and reduces renal injury. In DOCA salt-sensitive models, bosentan, a nonselective receptor antagonist, is effective in lowering blood pressure.<sup>10</sup>

In humans, early studies demonstrated an elevation in plasma ET-1 levels in hypertensive patients,<sup>12</sup> but a careful analysis of patient characteristics revealed that increased circulating levels were secondary to the impairment of renal clearance.<sup>13</sup> Schiffrin et al<sup>14</sup> reported that chronic administration of an ET<sub>A</sub>-selective antagonist (A-127722) significantly attenuates the increase in blood pressure and reduces renal injury. In DOCA salt–sensitive models, bosentan, a nonselective receptor antagonist, was effective in lowering blood pressure.<sup>10</sup>
uals. Patients with elevated creatinine levels were excluded from that study, indicating that increased plasma ET-1 levels were not due to renal disease. In addition to our studies, a recent report has provided further evidence indicating that the regulation of the ET system in African Americans may be different than the regulation of the ET system in the white population. Treiber et al. have demonstrated that plasma ET-1 levels are elevated not only in hypertensive patients but also in African American adolescents with family histories of essential hypertension in response to acute stress. Both video game challenge and forehead cold stimulation resulted in a higher increase in circulating ET-1 concentrations in black compared with white subjects. Furthermore, black individuals exhibited higher diastolic blood pressure and total peripheral resistance than did white individuals, and changes in ET-1 levels paralleled the changes in hemodynamic parameters. Although there is no definitive proof that ET-1 caused increased peripheral resistance in that study, it clearly demonstrates racial differences in ET-1 levels in response to stress and supports our findings.

To the best of our knowledge, this is the first study that has investigated ECE-1 expression and activity in vascular tissue in hypertension and that has related them to changes observed in ET-1 synthesis and ET receptor profiles. Past studies have clearly shown that ECE-1 is the major enzyme involved in the biosynthesis of ET-1. Therefore, the increased ECE-1 activity detected in the present study may contribute to the conversion of inactive big ET-1 to active ET-1, leading to elevated tissue and circulating peptide levels. There are at least 4 subisoforms of ECE-1 (ECE-1a, ECE-1b, ECE-1c, and ECE-1d) generated by alternative splicing. ECE-1a and ECE-1c are the most commonly expressed subisoforms, and on the basis of the sequence analysis of the promoter region, ECE-1a has been proposed to be the subisoform that can be induced under pathophysiological conditions. The present study has provided evidence that ECE-1a expression and activity are increased in African American hypertensive patients, who also present with higher plasma and tissue ET-1 levels.

The potent contractile effects of ET-1 are exerted by 2 distinct receptor subtypes in a complex fashion. ET<sub>A</sub> receptors are localized predominantly on vascular smooth muscle cells and mediate the vasoconstrictive and proliferative response to ET-1. ET<sub>B</sub> receptors, mainly located on endothelial
cells, mediate vasodilation via the release of NO and are also involved in the clearance of ET-1.18,19 Conversely, this receptor subtype can cause vasoconstriction when located on smooth muscle cells.5,20 Thus, the relative ratio of vasoconstriction-promoting receptors on smooth muscle cells to vasodilatation-promoting ET\(\alpha\) receptors on endothelial cells is critical for ET-1-mediated contractility. We have previously reported that the total vascular ET receptor density, including endothelial and smooth muscle cells, was higher in the saphenous veins of white patients and that the only receptor subtype on vascular smooth muscle cells was the ET\(\alpha\) subtype.21 Black patients, on the other hand, had both receptor subtypes on vascular smooth muscle cells, yet the total number of ET\(\alpha\) receptors was lower than in white patients, indicating a significantly lower ET\(\alpha\) receptor density on endothelial cells. This decrease in the ratio of endothelial to smooth muscle ET\(\alpha\) suggested a shift in favor of vasocconstriction-promoting receptors. The present study investigated the expression of ET receptors in saphenous vein specimens obtained from normotensive and hypertensive African American and white patients and provides evidence that the alterations in receptor profile that we reported previously are accompanied by changes at the mRNA level. We also measured specific binding of labeled ET-1 to endothelium-denuded tissue in the presence of the ETA receptor antagonist BQ-123. Our findings demonstrated that in white patients, total binding was completely blocked by BQ-123. In African American patients, on the other hand, only 70% of the ligand was displaced. These results confirm our previous findings and indicate that the density of ET\(\alpha\) receptor on smooth muscle cells is upregulated in this patient population. The functional significance of this finding remains unknown, but along with decreased endothelial ET\(\alpha\) receptors (as shown in our previous study21), this may result in enhanced vasoconstriction and/or decreased vasorelaxation. Alternatively, because this receptor subtype is involved in ET-1 clearance, it may be a compensation mechanism to remove ET-1. In addition to the effects of ET-1 on arteries, ET-1-mediated constriction in the venous system is enhanced in hypertensive patients.22 Haynes et al22 studied the responses to local infusion of ET-1 into hand veins and found that maximal contraction in response to ET-1 was significantly greater in the hypertensive group than in the normotensive group. Furthermore, ET-1 potentiated sympathetically mediated vasoconstriction in hypertensive patients, and a positive correlation was observed between ET-1 responses to local infusion of ET-1 in hand veins and found that maximal contraction in response to ET-1 was significantly greater in the hypertensive group than in the normotensive group. Whether similar findings also occur in arterial tissue remains to be determined. Third, because of the small amount of tissue available, we used a semiquantitative RT-PCR technique to study gene expression. However, RT-PCR conditions were optimized to prevent nonspecific amplification. Our findings that changes in gene expression of PPET-1, ECE-1 subisoforms, and ET receptor subtypes are accompanied by changes in protein levels provide support that RT-PCR is a sensitive approach for the study of mRNA levels. It also must be emphasized that only the most common ECE-1 subisoforms, ECE-1a and ECE-1c, were investigated because of the low levels of expression of the other subisoforms. Last, the functional consequences of an altered receptor ratio need to be investigated. Nevertheless, the findings of the present study demonstrate for the first time that heighted vascular ET-1 biosynthesis, which occurs in African American hypertensive patients, is accompanied by an increase in ECE-1 enzyme activity as well as changes in ET receptor profile, which would favor increased ET-mediated vasoconstriction.

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