

# VASCULAR ENDOTHELIN CONVERTING ENZYME-1 EXPRESSION AND ACTIVITY IS UPREGULATED IN CLINICAL DIABETES

Circulating and vascular endothelin-1 (ET-1) levels are elevated in diabetes, but the molecular components of the enzymatic activation of ET-1 in the vasculature remains unknown. Furthermore, the distribution of ET receptors favors a contractile phenotype in African Americans with diabetes. Whether there is any difference in local ET-1 activation in this population is unknown. This study examined the expression and activity of ET converting enzyme-1 subisoforms (ECE-1) in the internal mammary artery specimens obtained from patients undergoing coronary artery bypass grafting. The study groups included African-American (AA) and Caucasian (CA), nondiabetic (ND) and diabetic (D) patients: AAND  $N=10$ , CAND  $N=9$ , AAD  $N=9$ , and CAD  $N=11$ . The expression of ECE-1a, ECE-1b and ECE-1c subisoforms was studied by RT-PCR. ECE-1a was upregulated 2- and 4-fold in the CAD and AAD groups, respectively ( $P<.05$ ). In African-American patient groups, ECE-1 activity (fmol/mg protein.h) was augmented from  $2,804 \pm 185$  in nondiabetic tissue samples to  $6,857 \pm 393$  in the diabetic tissue ( $P<.05$ ). There was a similar increase in the CAD group, which did not significantly differ from AA diabetics. ECE-1 inhibitors, phosphoramidon and FR-901533, inhibited vascular ECE-1 activity by more than 80%. While neutral endopeptidase (NEP) and matrix metalloproteinase-2 (MMP-2) are able to process big ET-1, inhibitors of NEP (thiorphan) and MMP (batimistat) did not affect ECE-1 activity. In conclusion, the enzymatic pathway essential for generating vascular ET-1 is activated in the vasculature of both AA and CA diabetic patients and this activation is highly specific for ECE-1. (*Ethn Dis.* 2002;12[suppl3]:S3-5-S3-9)

**Key Words:** Diabetes, Endothelin Converting Enzyme, African-American

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## INTRODUCTION

The incidence of diabetes is higher in African Americans (AA) compared to Caucasians (CA) and AAs suffer higher complication rates, including retinopathy, nephropathy, severe limb ischemia, and stroke.<sup>1,2</sup> We have shown that circulating levels of endothelin-1 (ET-1) is significantly higher in African-American hypertensives compared to Caucasians.<sup>3</sup> ET-1 is generated from post-translational processing of a precursor protein, preproET-1 (PPET-1) to big ET-1 which is further processed to biologically active ET-1 by endothelin converting enzyme (ECE-1).<sup>4</sup> Recent investigations identified upregulated arterial PPET-1 expression in both African-American and Caucasian diabetic patients.<sup>5</sup> Also, the vasorelaxation-promoting ET<sub>B</sub> receptor subtype expression was decreased in African-American patients, providing evidence that the ET system is altered in favor of a contractile phenotype in this patient population. Whether and to what extent ECE-1 activity, a rate limiting step in ET-1 biosynthesis, is altered in the vasculature of diabetic patients and whether there are racial differences in the regulation of enzymatic activity remain unclear. Moreover, there are four splice variants of ECE-1<sup>6</sup> and the activity and expression of vascular ECE-1 subisoforms in diabetes remain to be defined. Therefore, this study was designed to determine ECE-1 activity and subisoform expression profiles in the peripheral vasculature of diabetic African-American and Caucasian patients.

## METHODS

### Materials

Synthetic MMP inhibitor (Batimistat) was provided by British Biotech Inc. (Oxford, UK). ECE inhibitor (FR901533) was a gift from Fujisawa Pharmaceuticals (Osaka, Japan). Thiorphan and phosphoramidon were purchased from Sigma Chemical Co. (St. Louis, Mo).

### Patient Enrollment and Tissue Collection

Internal mammary artery specimens were obtained from patients undergoing coronary artery bypass grafting surgery. Twenty type 2 diabetic (AAD=9 and CAD=11) and 19 nondiabetic (AAND=10 and CAND=9) patients were enrolled in the study. All patients were on combination therapy, which included calcium channel blockers, angiotensin converting enzyme inhibitors, diuretics, lipid-lowering agents and anti-thrombotic therapy, as well as insulin and oral agents for diabetic patients. The use of medications, mean age and female/male ratio were similar in all groups. Specimens were snap frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Informed patient consent was obtained from all patients who participated in the study and the protocol was approved by the Medical College of Georgia Institutional Review Board for Human Research.

### Expression Studies by RT-PCR

RNA was extracted from internal mammary artery specimens (100 mg) using RNeasy kit from Qiagen (Valencia, Calif). First strand cDNA synthesis

was performed with 1  $\mu\text{g}$  of total RNA and oligo(dT)<sub>12-18</sub> primers by using AMV reverse transcriptase (Promega, Madison, Wis). The PCR was carried out in a reaction mixture containing 20 mM Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 500 nM of each sense and antisense primer, 3  $\mu\text{l}$  first strand cDNA, and 2.5 units of Taq DNA polymerase. In order to determine the linear range for the generation of desired PCR products, initially various PCR cycles (20–40) were employed for amplification. Based on these results, subsequent PCR amplification steps were set for 30 cycles. In order to ensure that an equivalent cDNA template was used in each reaction, amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. PCR products were electrophoresed on 2% agarose gels and, images were captured using Image-Pro software (Media Cybernetics, Silver Spring, Md). The density of PCR products were analyzed by Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, Md) and normalized over GAPDH expression.

### Vascular Membrane Preparation

Internal mammary artery specimens (200 mg) were homogenized in 5 ml Buffer A (20 mM Tris-HCl, pH 7.4, 20  $\mu\text{M}$  pepstatin A, 1 mM PMSE, and 250 mM sucrose). After an initial centrifugation at  $1000 \times g$  for 10 min, supernatant was centrifuged at  $100,000 \times g$  for 60 min. Pellet was resuspended in 500  $\mu\text{l}$  Buffer A and membrane aliquotes were stored at  $-80^\circ\text{C}$ . The protein content in the membrane preparation was measured using the Bradford Protein Assay from Bio-Rad Laboratories (Hercules, Calif).

### Measurement of ECE-1 Activity

Enzyme activity was determined by incubating 30  $\mu\text{g}$  membrane preparations with big ET-1 (0.1  $\mu\text{M}$ ) in 50  $\mu\text{l}$  reaction buffer (0.1 M sodium phosphate buffer, pH 6.8 and 0.5 M NaCl)

for 1 h at  $37^\circ\text{C}$ . To determine whether cleavage of big ET-1 by the membrane fractions was specific for ECE-1, enzyme reactions were repeated in the presence of a set of inhibitors. These included: phosphoramidon (100  $\mu\text{M}$ ), a nonspecific ECE inhibitor; thiorphan (100  $\mu\text{M}$ ), a nonspecific neutral endopeptidase inhibitor that does not inhibit ECE activity; a specific ECE inhibitor (100  $\mu\text{M}$ ), FR901533; and a matrix metalloprotease inhibitor (100  $\mu\text{M}$ ), batimistat. The reaction was terminated with 50  $\mu\text{l}$  of 5 mM EDTA and the mixture assayed for ET-1 by an ELISA kit described previously. All assays were performed in duplicate.

### Data Analysis

Values obtained for ECE-1 activity (fmol ET-1/mg protein.hour) in the 4 study groups were compared using analysis of variance. All statistical tests were performed using *Prism* software (GraphPad Software, San Diego, Calif). Results were expressed as mean  $\pm$  standard error of the mean (SEM). Values of  $P < .05$  were considered to be statistically significant.

## RESULTS

### Expression of ECE-1 Subisoforms

Standard RT-PCR analysis demonstrated expression of ECE-1a and ECE-1c subisoforms in arterial specimens (Figure 1A). The densitometric analysis of PCR products revealed mRNA for ECE-1a was upregulated by 2- and 4-fold in Caucasian and African-American diabetics, respectively, compared to their nondiabetic counterparts. There was no detectable change in ECE-1c expression in diabetics vs nondiabetics (Figure 1B). Furthermore, there was no difference in ECE-1a expression between African-American and Caucasian diabetic patients.

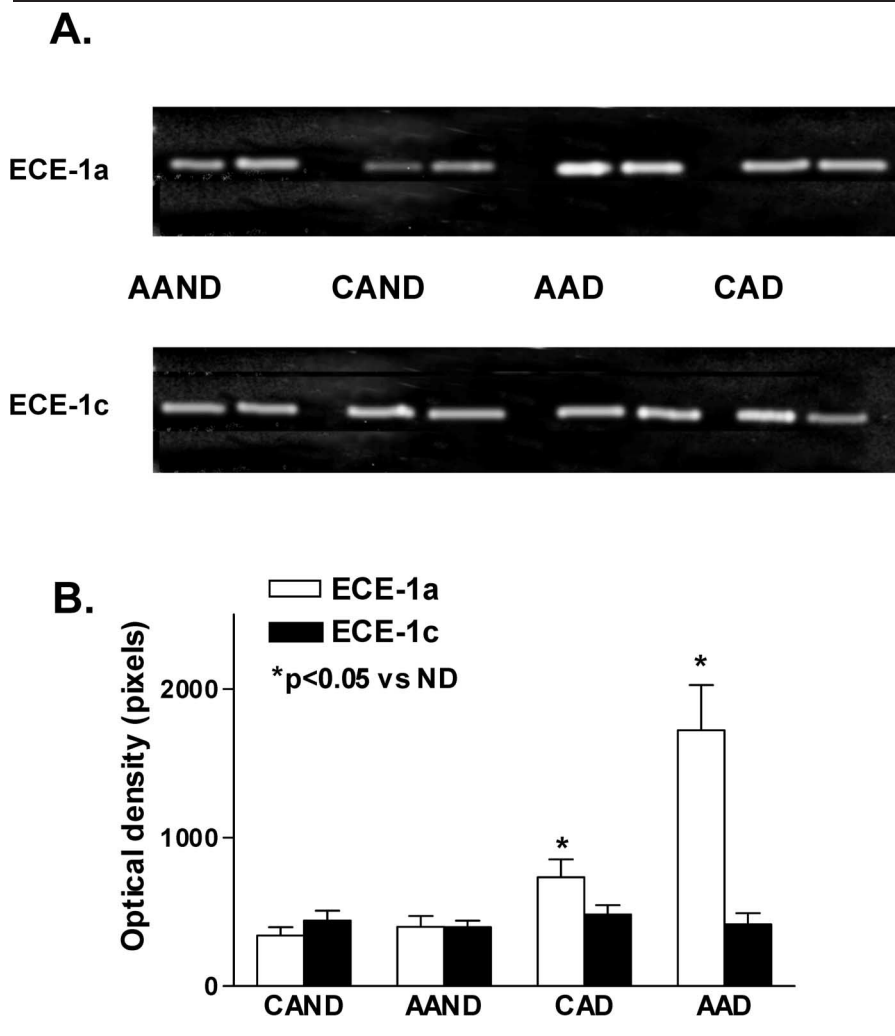
### ECE-1 Activity

ECE activity was increased nearly 2-fold in the diabetic tissue (Figure 2A) and there was no statistically significant difference between African-American and Caucasian patients. ECE activity in nondiabetic and diabetic specimens remained unchanged in the presence of thiorphan, a metalloprotease inhibitor that does not affect ECE activity (Figure 2B) and batimistat, a matrix metalloprotease inhibitor (Figure 2C). The non-specific ECE inhibitor phosphoramidon decreased ECE activity approximately by 90% in both normal and diabetic membranes (Figure 2D). A selective ECE-1 inhibitor also inhibited enzyme activity by more than 90% (Figure 2E). These results strongly suggest that the enzyme activity measured is specific for ECE-1.

## DISCUSSION

While plasma ET-1 levels are increased in diabetic patients and related animal models, the enzymatic regulation responsible for big ET-1 activation in diabetes remained unexplored. Previous studies provide evidence that the ET system, including ET-1 peptide and ET receptors, is differentially regulated in African-American hypertensive and diabetic patients.<sup>3,5,7</sup> Therefore, the current study investigated the subisoform specific expression of ECE-1 in normal and diabetic arterial tissue obtained from patients with different racial backgrounds. Moreover, inhibitors of other enzymes, such as NEP and MMP-2 that have been shown to cleave big ET-1,<sup>8,9</sup> were utilized to provide evidence that the processing of big ET-1 to ET-1 is specifically mediated by ECE-1. These results indicate that selective upregulation of ECE-1 to a subisoform accompanies increased vascular ET-1 synthesis in diabetes.

Several lines of evidence suggest that elevated plasma ET-1 levels may be involved in the pathogenesis of diabetic



**Fig 1. (A)** A representative standard RT-PCR analysis for ECE-1 isoforms in arterial tissue from diabetic and nondiabetic African-American and Caucasian patients. **(B)** Densitometric analyses of the PCR products normalized to GAPDH demonstrated increased ECE-1a mRNA levels in diabetic tissue (AAD and CAD). \* $P < .05$  vs AAND or CAND

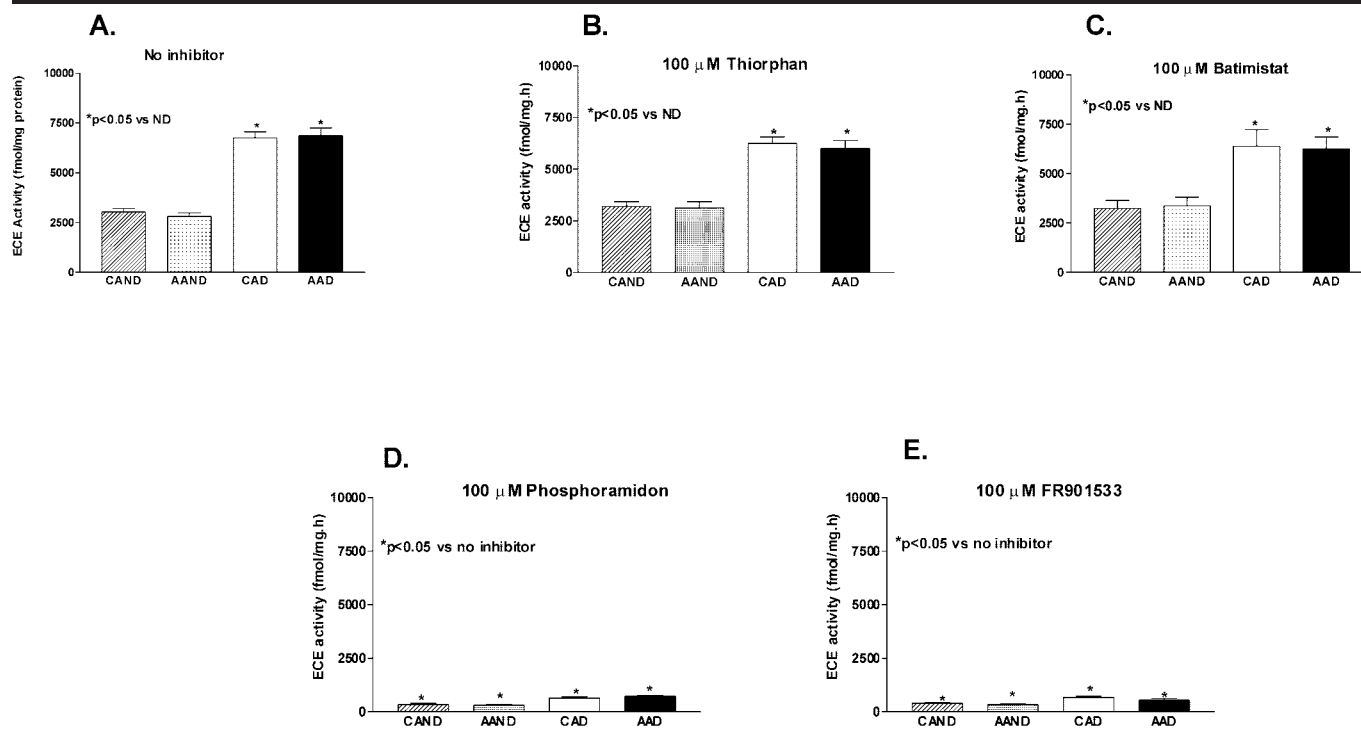
vasculopathy. First, plasma ET-1 levels are elevated in both type 1 and type 2 diabetes, as well as in streptozotocin (STZ)-induced diabetes in rats.<sup>10-12</sup> Basal ET-1 production in mesenteric arteries isolated from STZ-diabetic rats is significantly higher than in arteries from normal rats.<sup>13</sup> Furthermore, a significant correlation has been observed between plasma ET-1 levels and diabetic complications. For example, ET-1 levels are higher in patients with microalbuminuria and elevated glycosylated hemoglobin (Hb<sub>A1c</sub>) concentrations.<sup>10</sup> The risk of developing diabetes as well as vascular

complications of diabetes is 2- to 3-fold higher in African Americans.<sup>1</sup> Furthermore, hypertension, insulin resistance, and obesity cluster in this population. Although the reasons for increased incidence of complications in African Americans remains unclear, recent findings provide evidence that altered ET activity may contribute to increased cardiovascular complications in this patient population. For example, plasma levels of ET-1 are elevated<sup>3</sup> and the distribution of ET receptors in the saphenous veins of African Americans with coronary artery disease is altered. The den-

sity of the vasorelaxation-promoting endothelial ET<sub>B</sub> receptor is decreased and the vasoconstriction-promoting smooth muscle ET<sub>B</sub> receptor density is increased compared to age-matched Caucasian patients.<sup>7</sup> Following ET-1 contraction, endothelium-independent arterial relaxation is significantly impaired in African-American diabetics.<sup>5</sup> Recent findings provide insight that arterial ET-1 gene expression is upregulated in both Caucasian and African-American diabetics.<sup>5</sup> Since the enzymatic processing of big ET-1 is an important regulatory step in the biosynthesis of ET-1, the current study investigated whether an enhanced ECE-1 expression may contribute to the conversion of inactive big ET-1 to active ET-1, leading to elevated local peptide levels in diabetic African Americans.

Four subisoforms of ECE-1 are generated by alternative splicing of the same gene product and cannot be distinguished by enzyme activity assays.<sup>14,15</sup> Thus, RT-PCR was used to study the expression of ECE-1 subisoforms and demonstrated that ECE-1a mRNA is selectively increased in the diabetic arterial tissue. Future studies investigating the presence of ECE-1 subisoforms by immunohistochemical studies should provide information regarding ECE-1 subisoform localization on endothelial and vascular smooth muscle, as well as surrounding connective tissue.

In a recent study, Fernandez-Patron and colleagues reported that vascular matrix metalloproteinase-2 (MMP-2) can cleave big ET-1 between Gly32-Leu33 residues. The resulting peptide ET-1[1-31] is also a potent vasoconstrictor.<sup>8</sup> MMP-2 belongs to an endogenous family of enzymes responsible for extracellular collagen degradation and remodeling.<sup>16</sup> There are a number of MMP species that are abundant in various tissues and display differences in substrate specificity.<sup>16</sup> Clinical and experimental models of diabetes have demonstrated altered MMP activity associated with vascular remodeling. Based on these observations, the current



**Fig 2.** The conversion of big ET-1 to ET-1 by ECE-1 in membranes prepared from CAND (N=9), AAND (N=10), CAD (N=11) and AAD (N=9) patients. ECE-1 activity was increased in diabetic samples (\*P<.05 vs normal). In the presence of 100 μM thiorphan (B), and batimistat (C), enzyme activity remained unchanged. Phosphoramidon (D) and FR901533 (E) significantly inhibited the enzyme activity as compared to enzyme activity measured in the absence of any inhibitors (\*P<.05)

study investigated whether MMPs can contribute to the activation of big ET-1. Using a synthetic metalloproteinase inhibitor, batimistat, we demonstrated that inhibition of MMPs did not affect the conversion of big ET-1 to ET-1. In addition to ECE-1 and MMP-2, NEP, which is widely expressed in the vascular wall, has been reported to process big ET-1 to ET-1 and smaller fragments.<sup>9</sup> Therefore, we tested whether the presence of a NEP inhibitor, thiorphan, would influence the conversion rate of big ET-1 in the arterial vasculature and demonstrated that NEP does not contribute to the activation of big ET-1. Our findings that only ECE-1 inhibitors, phosphoramidon and FR901533, decrease the enzymatic activity provide further support that arterial enzyme activity measured in normal and diabetic samples is specific for ECE-1.

In summary, the present study demonstrated for the first time that upre-

gulated ECE-1a expression and activity accompanies elevated tissue ET-1 in diabetes. Furthermore, vascular activation of ET-1 is highly specific for ECE-1a in both African-American and Caucasian diabetic patients.

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