Brazilian Journal of Medical and Biological Research (2008) 41: 351-356
ISSN 0100-879X

Association of urinary 90 kDa angiotensin-converting enzyme with family history of hypertension and endothelial function in normotensive individuals

A.M.S. Teixeira[1], F.L. Plavnik[1], F.B. Fernandes[1], O. Marson[1], D.M.J. Christofalo[2], S.A. Ajzen[2], R. Sesso[1], M.C. Franco[1] and D.E. Casarini[1]

[1]Disciplina de Nefrologia, Departamento de Medicina, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brasil
[2]Departamento de Radiologia, Universidade Federal de São Paulo e Hospital do Rim e Hipertensão, Fundação Oswaldo Ramos, São Paulo, SP, Brasil

Correspondence to: D.E. Casarini, Disciplina de Nefrologia, Departamento de Medicina, Escola Paulista de Medicina, UNIFESP, Rua Botucatu, 740, 04023-900 São Paulo, SP, Brasil
Fax: +55-11-5573-9652. E-mail: dulce@nefro.epm.br

We described angiotensin-I-converting enzyme (ACE) isoforms with molecular masses of 190, 90, and 65 kDa in the urine of normotensive offspring of hypertensive subjects. Since they did not appear in equal amounts, we suggested that 90 kDa ACE might be a marker for hypertension. We evaluated the endothelial response in normotensive offspring with or without family history of hypertension and its association with the 90 kDa ACE in urine. Thirty-five normotensive subjects with a known family history of hypertension and 20 subjects without a family history of hypertension, matched for age, sex, body weight, and blood pressure, were included in the study. Endothelial function was assessed by ultrasound and a sample of urine was collected for determination of ACE isoforms. In the presence of a family history of hypertension and detection of 90 kDa ACE, we noted a maximal flow mediated dilation of 12.1 ± 5.0 *vs* 16.1 ± 6.0% in those without a previous history of hypertension and lacking urinary 90 kDa ACE ($P < 0.05$). In subjects with a family history of hypertension and presenting 90 kDa ACE, there were lower levels of HDL-cholesterol ($P < 0.05$) and higher levels of triglycerides ($P < 0.05$). Subjects with 90 kDa ACE irrespective of hypertensive history presented a trend for higher levels of triglycerides and HDL-cholesterol ($P = 0.06$) compared to subjects without 90 kDa ACE. Our data suggest that the 90 kDa ACE may be a marker for hypertension which may be related to the development of early atherosclerotic changes.

Key words: Endothelial dysfunction; Angiotensin-I-converting enzyme; Hypertension; Normotension; N-domain angiotensin-converting enzyme

A.M.S. Teixeira is the recipient of a scientific initiation scholarship from FAPESP (#01/13840-0). This project was supported by FAPESP (#02/13290-2) and CNPq.

Received April 11, 2007. Accepted April 7, 2008

Introduction

The term endothelial dysfunction is used to define early changes in endothelial function in different arterial sites in humans, such as coronary, renal and peripheral circulation (1). Endothelial cells have important properties, such as providing a protective barrier between tissues and circulating blood, functioning as a selective filter to facilitate the transport of macromolecules and blood gases between tissues and blood and also releasing vasoactive substances such as nitric oxide (NO), endothelium-derived relaxing factors, angiotensin II, endothelin, among

others (2-5). A disruption in the balance of these vasoactive substances leads to vasospasm, vasoconstriction, thrombogenesis, and abnormal vascular proliferation. Specifically, an impairment is associated with an increase in NO production or a degradation caused mainly by endothelial cell lesions and the increased production of free radicals (reactive superoxide species) (6,7).

Zizek et al. (8) reported that vasodilator response of the brachial artery was blunted during reactive hyperemia in normotensive offspring of subjects with essential hypertension (EH) compared to controls. They also found that offspring of subjects with EH showed increased blood pressure levels although within the normal range (8). Several studies developed in our laboratory (9-11) have shown that normotensive subjects with no family history of EH consistently present urinary angiotensin-I-converting enzyme (ACE) isoforms with molecular masses of 190 and 65 kDa, which differ from those presented by normotensive subjects with a positive history of EH (190, 90, and 65 kDa) and presented by hypertensive subjects (90 and 65 kDa). The 190 kDa isoform would correspond to somatic ACE and both 90 and 65 kDa would be N-terminal fragments. Animal studies performed in our laboratory have also shown that 90 kDa ACE could be a possible genetic marker of hypertension in different rat strains (11).

Several studies have shown that impaired endothelium-dependent vasodilation is probably genetically predetermined, which is a primary phenomenon not directly related to blood pressure levels (12-14). The events preceding the development of high blood pressure involve very subtle and progressive endothelial changes that may jeopardize baseline production as well as NO release under stimuli (15).

Based on available data from the literature that consistently show an impairment of endothelial response in normotensive offspring of subjects with EH and also based on our previous data that have suggested urinary 90 kDa ACE as a possible marker for hypertension, we evaluated the profile of ACE isoforms in urine of normotensive subjects with and without a family history of EH. Our objective was to assess whether these isoforms could be used as an indicator of different patterns of endothelial response assessed by high resolution ultrasound in young normotensive subjects.

Subjects and Methods

Subjects

Healthy male subjects, aged 18 to 40 years old, were initially divided into two groups according to the presence (FHH+, N = 35) or absence (FHH-, N = 20) of a family history of essential hypertension (FHH) from first-degree relatives. Hypertension was defined as blood pressure levels equal to or greater than 140 mmHg systolic and/or 90 mmHg diastolic and/or ongoing pharmacological treatment for hypertension. Women were not included in this study due to the protective effect of estrogen on vascular reactivity. The protocol of this cross-sectional study was approved by the Ethics Committee on Human Research of the Federal University of São Paulo (#095/02). All subjects received a full explanation of the study protocol and purposes and gave written informed consent.

None of the subjects had any significant past medical history nor were they receiving any prescribed medication or vitamins. There was also no previous history of tobacco use. Subjects underwent a physical examination consisting of weight and height measurements to determine body mass index, measurement of waist and hips and calculation of their ratio. Following a 5-min rest, heart rate and blood pressure were measured in the arm in a seated position recording the higher blood pressure level at intervals of 1 min for 3 min. The mean blood pressure was used for further analyses. Biochemical blood profile was determined from fasting serum samples. Subjects with a laboratory abnormality were excluded from this study. The subjects were subsequently scheduled for a brachial artery reactive test in order to evaluate flow-mediated dilation (FMD) in response to reactive hyperemia.

Endothelial function assessment

Brachial artery-mode ultrasound. The endothelial function test was performed by high-resolution B-mode ultrasound images using a non-invasive methodology described by Celermajer et al. (16). The equipment used in this study was an Ultramark HDI 3000 (Phillips-ATL Ultrasound Inc., USA), with a linear transducer of L7-4 MHz. This test assessed FMD after reactive hyperemia. Diameter and blood flow velocity were determined in triplicate. The maximum blood flow (mm/min) was determined in the first 15 s after cuff release. Ninety seconds after ischemia, 3 measurements of the diameter of the brachial artery were taken during the diastolic period. This FMD response was reported as the change in percent of end-diastolic diameter of the brachial artery during reactive hyperemia compared to the baseline measurement. It was used as a measure of endothelium-dependent vasodilation.

Purification of ACE

Urine preparation. Urine volume was measured and the pH was corrected to 8.0 with 1 M Tris buffer. Urine was centrifuged (3000 rpm). The supernatant was concentrated 10X in an Amicon concentrator (USA) and dialyzed in the same equipment against 50 mM Tris-HCl, pH 8.0,

containing 150 mM NaCl, with the use of a 30-kDa molecular mass exclusion membrane.

Gel filtration on an AcA-44 column. Individual samples of the concentrated urine (1.0 mL) were submitted to gel filtration on an AcA-44 column (1.6 x 84 cm) previously calibrated with standard proteins (Sigma-Aldrich, USA), equilibrated with 50 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl. Fractions (1.0 mL) were collected at a flow rate of 20 mL/h. Protein concentration was monitored by absorbance at 280 nm, and ACE activity was measured using Hippuryl-His-Leu and Z-Phe-His-Leu as substrates (17,18). The column was previously calibrated with standard protein markers.

Protein concentration. Protein concentration was determined by the Bradford method (19) (Bio-Rad Protein Assay, USA) using bovine albumin as standard.

Immunoblotting. Using 100 µg of total urinary protein, SDS-PAGE (7.5%) was performed under dissociating conditions with dithiotreitol as described by Laemmli (20). After the electrophoretic transfer of the proteins from the polyacrylamide gel to a nitrocellulose membrane (Immobilon P, Millipore, USA), the nitrocellulose membrane was incubated in 10 mM Tris, pH 8.0, containing 150 mM NaCl, 0.05% Tween 20 and 3 mg/mL bovine serum albumin, for 90 min followed by incubation for 2 h at room temperature with antiserum Y4 (Dr. François Alhenc-Gelas, Unité 367, INSERM, Paris, France) diluted 1:500. The subsequent steps were carried out using the biotin/streptavidin system (Amersham, USA) as recommended by the manufacturer.

Statistical analyses

Data were stored at SigmaStat for Windows, version 2.0 (Jandel Corporation, USA). All continuous variables were examined for normality with the Kolmogorov-Smirnov test. Data were analyzed by the Student *t*-test and χ^2 test when applicable, at a significance level of $P < 0.05$. Values are reported as means ± standard deviation (SD), unless otherwise specified. Data analysis was conducted initially in the subgroups of subjects considering the presence (+) or absence (-) of a family history of high blood pressure levels. The next step was based on the presence (+) or absence (-) of the 90 kDa ACE isoform, irrespective of FHH, and, finally, we combined the presence of FHH and the presence of 90 kDa ACE isoform and compared this to the group in which both conditions were absent.

Results

The presence of ACE found in most FHH+ subjects was characterized by gel-filtration chromatography in which three peaks with ACE activity were detected with an estimated molecular mass of 190, 90, and 65 kDa, confirmed by the expression of the three immunoreactive protein bands by Western blot analysis as shown in Figure 1. The same analysis was done for the FHH- subjects who presented 190 and 65 kDa ACE forms (Figure 1).

Table 1 shows demographic and clinical data from healthy volunteers based solely on family history of high blood pressure. Subjects with FHH+ showed a statistically significant difference in heart rate compared to FHH- (68.5 ± 9.2 *vs* 62.8 ± 8.0, $P < 0.05$) and a lower total cholesterol level compared to FHH- (164.4 ± 37.7 *vs* 186.0 ± 26.3, $P < 0.05$). We did not find any difference in flow-mediated dilation between FHH+ and FHH- (12.3 ± 5.6 *vs* 13.8 ± 6.0, respectively). Similarly, there was no difference in brachial artery diameter (4.0 ± 0.5 *vs* 3.8 ± 0.3 mm).

Subjects with the 90 kDa ACE isoform were compared to subjects without the 90 kDa ACE isoform (Table 1). There was a trend towards higher levels of triglycerides and lower levels in HDL-cholesterol in 90 kDa+ ($P = 0.06$ for both), and a statistically significant difference in flow-mediated dilation when the groups were compared (11.7 ± 4.8 *vs* 15.1 ± 6.7%, 90 kDa+ and 90 kDa-, respectively, $P < 0.05$). No differences in brachial artery diameter were detected (3.9 ± 0.4 *vs* 4.0 ± 0.5 mm).

The final analysis consisted of a comparison between subjects with 90 kDa ACE and FHH+ (FHH+/90+) or the absence of both (FHH-/90-; Table 1). In this analysis, we observed a statiscally significant difference with lower levels in HDL-cholesterol in FHH+/90+ (1.27 ± 0.32 *vs* 1.55 ± 0.36, $P < 0.05$) and higher triglyceride levels (1.24 ± 0.69 *vs* 0.92 ± 0.21, $P < 0.05$).

The FHH+/90+ group showed a significant difference in FMD compared to the FHH-/90- group (12.1 ± 5.0 *vs* 16.1 ± 6.0%, $P < 0.05$) indicating a reduction of 24.8% in FMD in the former group. There were no differences in brachial artery diameters between groups (3.9 ± 0.4 *vs* 3.9 ± 0.3 mm).

Since our data showed a consistent decrease in FMD in the presence of FHH and 90 kDa ACE isoform, we performed a χ^2 test to assess the association of these findings. There were 28 (50.9%) subjects with both conditions, 8 (14.5%) subjects with 90 kDa ACE isoform without FHH, 7 (12.7%) subjects with FHH and without 90 kDa ACE isoform, and 12 (21.8%) subjects who did not have either of these conditions. Our data showed that the proportion of observations in the different categories was significantly different confirming the association of FHH and the presence of urinary 90 kDa ACE ($P = 0.003$).

In order to test which variables could influence endothelial response to reactive hyperemia, we used a stepwise

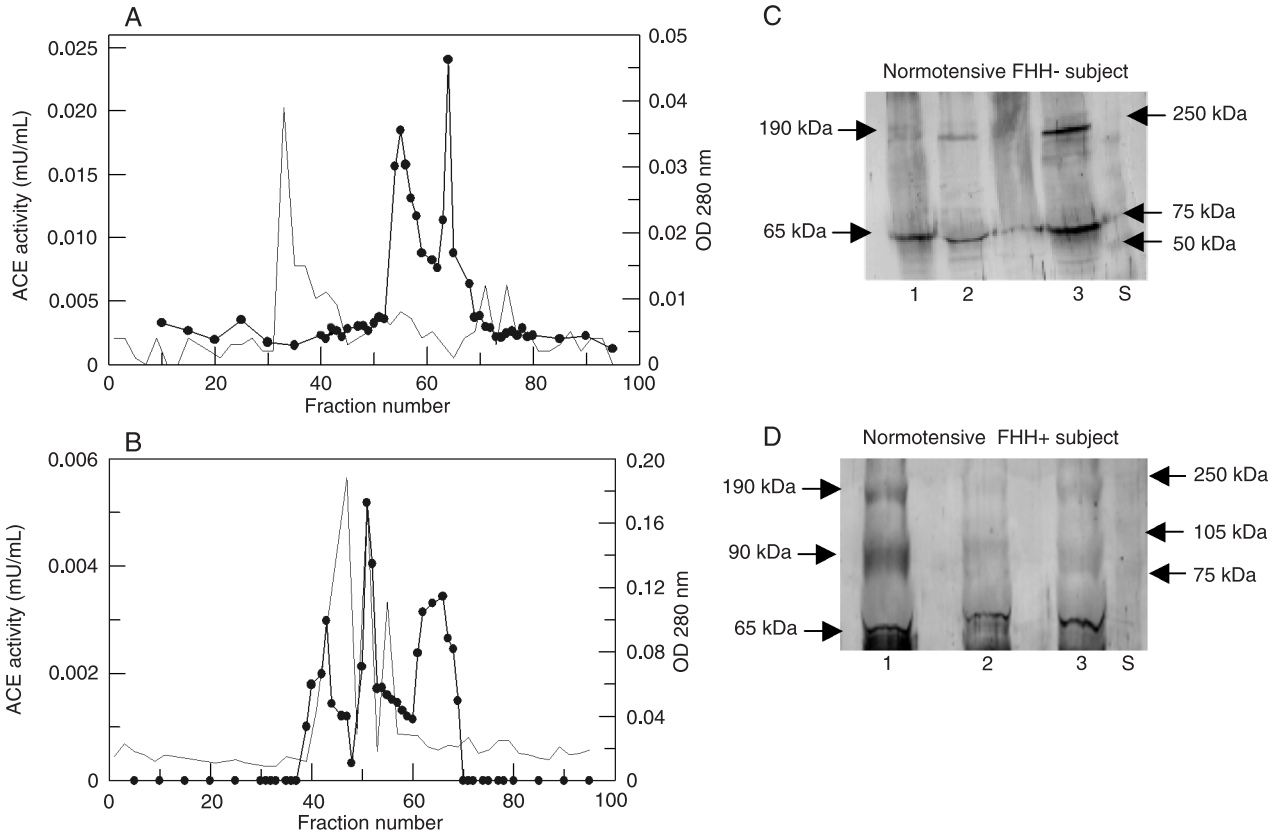

Figure 1. Gel filtration and SDS-PAGE of human urine from a normotensive subject without a family history of hypertension (FHH-; A) and a normotensive subject with a family history of hypertension (FHH+; B). Concentrated urine (1.0 mL) was submitted to gel filtration on an AcA-44 column, equilibrated, and eluted as described in the Methods section. Fractions of 1.0 mL were collected at a flow rate of 20 mL/h. Absorbance at 280 nm is indicated by the thin line and ACE activity on Z-Phe-His-Leu by the thick line with black circles. Western blot analysis of urine was carried out with the Y4 polyclonal anti-ACE antibody. C, Different normotensive FHH- subjects (*lanes 1, 2,* and *3*). *D*, Different normotensive FHH+ subjects (*lanes 1, 2,* and *3*). Arrows indicate bands recognized by the antibody and the positions of the standards (S, Rainbow molecular weight markers, from 250 to 10 kDa).

Table 1. Clinical features of subjects in groups with or without a family history of hypertension (FHH+ and FHH-), with or without urinary 90 kDa ACE isoforms (90 kDa+ and 90 kDa-), and with both FHH and urinary 90 kDa ACE isoforms (FHH+/90+) or without both (FHH-/90-).

Characteristics	FHH+ (N = 35)	FHH- (N = 20)	90 kDa+ (N = 36)	90 kDa- (N = 19)	FHH+/90+ (N = 28)	FHH-/90- (N = 12)
Age (years)	28.5 ± 5.5	27.2 ± 6.8	28.5 ± 5.9	27.2 ± 6.1	29.0 ± 5.8	27.5 ± 7.4
BMI (kg/m^2)	24.8 ± 2.9	24.8 ± 3.3	24.8 ± 3.0	24.9 ± 3.1	25.1 ± 3.0	25.8 ± 3.3
WHR	0.87 ± 0.47	0.87 ± 0.05	0.87 ± 0.04	0.87 ± 0.06	0.87 ± 0.05	0.88 ± 0.06
Systolic BP (mmHg)	111.5 ± 7.6	112.3 ± 6.6	111.1 ± 6.9	113.0 ± 7.8	111.6 ± 7.3	114.3 ± 6.7
Diastolic BP (mmHg)	74.5 ± 7.1	75.8 ± 5.3	75.4 ± 5.7	74.0 ± 7.8	74.9 ± 6.0	74.8 ± 6.0
Heart rate (bpm)	68.5 ± 9.2	62.8 ± 8.0*	67.1 ± 9.2	65.5 ± 9.2	68.4 ± 9.8	63.2 ± 10.3
Creatinine (µM)	97.24 ± 8.0	97.24 ± 7.1	97.24 ± 7.07	97.24 ± 7.07	97.2 ± 7.07	97.2 ± 7.07
Glucose (mM)	4.3 ± 0.42	4.24 ± 0.34	4.25 ± 0.4	4.28 ± 0.38	4.21 ± 0.4	4.14 ± 0.3
Total cholesterol (mM)	4.25 ± 0.97	4.8 ± 0.68*	4.41 ± 0.96	4.52 ± 0.83	4.31 ± 1.0	4.82 ± 0.66
HDL-C (mM)	12.67 ± 0.32	13.86 ± 0.37	1.24 ± 0.31	1.43 ± 0.38	1.27 ± 0.32	1.55 ± 0.36$^+$
LDL-C (mM)	2.49 ± 0.83	2.75 ± 1.02	2.63 ± 0.79	2.51 ± 1.12	2.55 ± 0.85	2.66 ± 1.28
Triglycerides (mM)	1.22 ± 0.7	1.2 ± 0.69	1.33 ± 0.75	1.0 ± 0.5	1.24 ± 0.69	0.92 ± 0.21$^+$

Data are reported as means ± SD for N subjects in each group. BMI = body mass index; WHR = waist-hip ratio; BP = blood pressure. *P < 0.05 *vs* FHH+; $^+$P < 0.05 *vs* FHH+ /90+ (Student *t*-test).

regression model entering FMD as a dependent variable and age, history of hypertension, 90 kDa isoform, systolic blood pressure and diastolic blood pressure as independent variables. This analysis showed that 90 kDa ACE isoform (ß = -3.452; SD = 1.596; P = 0.035) was the independent determinant factor affecting vascular function, indicating that the presence of this isoform is associated with a decrease in 3.5% of the vascular dilatation capacity in this population.

Discussion

The main objective of the present study was to determine if there is an association between endothelial response triggered by reactive hyperemia and the presence of urinary 90 kDa ACE. Because we were not expecting a change in smooth muscle cell response to exogenous nitrate donors in this population, we did not evaluate nitrate-mediated dilation as proposed by the Celermajer method and used by Li et al. (16,21). At this time, there are no data in the literature describing any other urinary protein that may represent a marker for hypertension or atherosclerotic processes.

There are several inflammatory markers such as C-reactive protein, von Willebrand factor, vascular cell adhesion molecule, intercellular adhesion molecule, among others that have been linked to a blunted FMD even in normotensive subjects with a family history of essential hypertension (22).

Our data have some limitations due to the small number of subjects studied, which may be responsible for the lack of statistical significance in some analyses, especially those related to FMD. However, we showed a decrease of 24.8% in FMD in the FHH+ group compared with FHH-. Millgard et al. (23) also failed to demonstrate a significant difference between normotensive subjects with and without a family history of hypertension regarding forearm blood flow induced by methacholine. They studied a similar number of subjects. Another possible explanation refers to the age of our subjects. As extensively described in the literature (22), aging is an important factor related to decreasing NO release. Since most of our subjects were less than 40 years old we did not expect to obtain a more significant impairment in FMD because we consider this change to be age-related. In fact, we did not find any correlation between age and maximal dilation in any subgroup analysis by Pearson correlation when we took the presence of EH, or 90 kDa ACE or both into account.

In the second subgroup analysis, when we considered the presence of 90 kDa ACE irrespective of family history, although there was a lack of a statistical significance, there was also a trend in lipid profile indicating a role for hypertension not as a marker but possibly more importantly as an indicator of an early atherosclerotic process. Indeed, we detected a significant difference in triglyceride levels in the presence of 90 kDa ACE, and the endothelial dysfunction was very similar to that seen in the initial analysis, i.e., a 21% reduction in FMD.

When we pooled conditions, family history and 90 kDa ACE these conclusions were reinforced. We not only detected a significant impairment in FMD (31%) but also lower levels of HDL-cholesterol, which has a protective lipoprotein, and higher levels of triglycerides. However, some of our findings are components of a metabolic syndrome, which is considered by some investigators to be relevant for endothelial dysfunction itself (24,25).

Thus, our data indicate a reduction in vasodilating effect when subjects presented a family history of high blood pressure levels, a deleterious lipid profile and urinary 90 kDa ACE. Whether this urinary isoform is a reliable genetic marker needs further evaluation with larger numbers of subjects.

We are starting two new studies in different populations. The first one aims to confirm the presence of this isoform in normal volunteers with a family history of primary hypercholesterolemia and the second study is planned to be a prospective study on a large population (1150 volunteers) to evaluate the prevalence and incidence of this 90 kDa isoform, a genetic marker for hypertension. This study will be based, at least in part, on the detection of the 90 kDa ACE in the urine of healthy subjects at risk of developing disease, particularly hypertension and coronary artery disease. Subjects enrolled in this second study are intended to be followed-up for at least 5 years.

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