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Plasma Kallikrein and Angiotensin I-converting enzyme N- and C-terminal domain activities are modulated by the insertion/deletion polymorphism

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ABSTRACT

Angiotensin I-converting enzyme (ACE) is recognized as one of the main effector molecules involved in blood pressure regulation. In the last few years some polymorphisms of ACE such as the insertion/deletion (I/D) polymorphism have been described, but their physiologic relevance is poorly understood. In addition, few studies investigated if the specific activity of ACE domain is related to the I/D polymorphism and if it can affect other systems. The aim of this study was to establish a biochemical and functional characterization of the I/D polymorphism and correlate this with the corresponding ACE activity. For this purpose, 119 male brazilian army recruits were genotyped and their ACE plasma activities evaluated from the C- and N-terminal catalytic domains using fluorescence resonance energy transfer (FRET) peptides, specific for the C-domain (Abz-LFK(Dnp)OH), N-domain (Abz-SDK(Dnp)P-OH) and both C- and N-domains (Abz-FRK(Dnp)P-OH). Plasma kallikrein activity was measured using Z-Phe-Arg-AMC as substrate and inhibited by selective plasma kallikrein inhibitor (PKSI). Some physiological parameters previously described related to the I/D polymorphism such as handgrip strength, blood pressure, heart rate and BMI were also evaluated. The genotype distribution was II $n = 27$, ID $n = 64$ and DD $n = 28$. Total plasma ACE activity of both domains in II individuals was significantly lower in comparison to ID and DD. This pattern was also observed for C- and N-domain activities. Difference between ID and DD subjects was observed only with the N-domain specific substrate. Blood pressure, heart rate, handgrip strength and BMI were similar among the genotypes. This polymorphism also affected the plasma kallikrein activity and DD group presents high activity level. Thus, our data demonstrate that the I/D ACE polymorphism affects differently both ACE domains without effects on handgrip strength. Moreover, this polymorphism influences the kallikrein–kinin system of normotensive individuals.

1. Introduction

ACE (EC 3.4.15.1) is a zinc metallopeptidase that converts the inactive decapeptide angiotensin I (Ang I) to the potent vasopressor angiotensin II (Ang II) and inactivates the vasodilator bradykinin (Yang et al., 1970; Dorer et al., 1974). Although, plasma ACE activity is normally constant when measured in the same individual, there are large inter-individual differences (Alhenc-Gelas et al., 1991; Tiret et al., 1992), suggesting that plasma ACE is under genetic control. In fact, a insertion (I)/deletion (D) polymorphism of a 287-bp *Alu*-repeat sequence in intron 16 of the ACE gene was identified and three different genotypes (II, ID, DD) have been characterized. Since then, several studies have been carried out to investigate if the different levels of ACE are involved in physiological and pathophysiological actions of the renin–angiotensin (RAS) and kallikrein–kinin systems (KKS) (Yang et al., 1970; Dorer et al., 1974). Nevertheless, these three genotypes display distinct ACE plasma activities (Rigat et al., 1990; Danser et al., 1995), the greater enzymatic activity of DD subjects (as compared to II) however not resulting in higher levels of circulating Ang II in DD.

In this context, van Esch et al. (2008) investigated an alternative explanation for the absence of a D allele-related change in Ang II in humans. Somatic ACE has two domains, the C- and the N-domains, which have 60% sequence identity, except for 36 amino acid residues at its N-terminus (Ehlers et al., 1989). Each domain contains the typical zinc-binding motif (His-Glu-X-X-His) found in many zinc peptidases (Fig. 1). This led these authors to investigate the possibility that both contribute differently to Ang II production in D vs. I allele carriers. In spite of the high degree of homology shared by the two homologous domains, each possess a functional active site and differ in relation to substrate and inhibitor specificities (Wei et al., 1991, 1992). For example, Ang I, substance P, luteinizing

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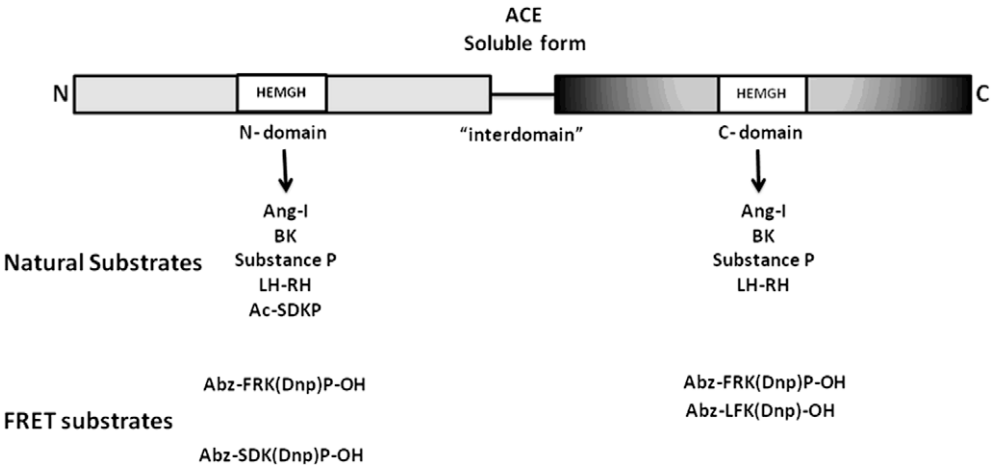

Fig. 1. Schematic representation of somatic ACE. sACE comprises an N-domain (gray bar) and a C- domain (black bar); The zinc-binding motif (HEMGH) is repeated twice. The natural substrates: Ang I; angiotensin I, BK; bradykinin, LH–RH; luteinizing hormone-releasing hormone, Ac-SDKP; N-acetyl-seryl-aspartyl-lysyl-proline, FRET; fluorescence resonance energy transfer. Modified from Woodman (2005).

hormone–releasing hormone (LH–RH) and bradykinin are cleaved by both domains (Jaspard et al., 1993), but only the N-domain cleaves N-acetyl-seryl-aspartyl-lysyl-proline (Rousseau et al., 1995). However, the activity of the C-domain is highly dependent on chloride ion concentration, whereas the N-domain is still active in the absence of chloride and is fully activated at relatively low concentrations of this anion (Wei et al., 1991). Thus, the differential activity of both domains could be important not only to explain the conversion of Ang II, but also the effects on health and disease attributed to the RAS (Jones and Woods, 2003; Yang et al., 2003) and KKS or the putative influence of these systems on physical exercise. An example for the latter could be the recent description of an association between the ACE I/D polymorphism and physical performance (Alvarez et al., 2000; Williams et al., 2005).

It is important to note that most of the previous studies that investigated the involvement of ACE levels in the actions attributed to the RAS and the KKS used techniques that were not able to distinguish between the catalytic activities of the two domains. Thus, as few studies investigated if the specific activity of ACE domains is related to the I/D polymorphism. The aim of present work was to investigate if the ACE I/D polymorphism can modulate C- and N-domain activities or activities of plasma kallikrein. In addition, we evaluated if the specific ACE domains activities are involved in physiological parameters.

2. Material and methods

2.1. Volunteers

This study was approved by the Research Ethics Committee of the University of Mogi das Cruzes. The procedures followed were in accordance with institutional guidelines. After exclusion of smokers and subjects who had received antihypertensive drugs, data were collected and analyzed from the remaining 119 healthy male subjects (Brazilian Army recruits with a mean age of 19 ± 0 years). Each subject gave informed consent prior to collection of DNA and the beginning of the experimental procedures.

2.2. Measurement of blood pressure and heart rate

Blood pressure (BP) and heart rate (HR) were measured after participants had rested for 20 min in sitting position by the a calibrated automated oscillometric non-invasive blood pressure device (model Microlife 3AC1–1, Microlife AG, Widnau, Switzerland) (Topouchian et al., 2005). BP and HR were measured three times in 2 min intervals on the left arm, and the mean of the readings was used as representative value. Systolic (SBP), diastolic blood pressure (DBP) and HR were measured before, and immediately after each trial of HS test. From these data mean arterial pressure (MAP) was calculated.

2.3. Handgrip strength

Handgrip strength (HGS) was assessed using a Jamar Hydraulic Hand Dynamometer (Model PC5030J1, Lafayette Instrument, Inc., Lafayette, IN), adjusted for each subject so that the angle of flexion of the middle finger at the second joint from the tip was as close to 90° as possible. Each individual performed the test with the dominant hand during three trials; the mean value was considered the grip strength. BP and HR were measured before, and immediately after trial of the HGS.

2.4. Genotyping

Genomic DNA was extracted from circulating leukocytes using a blood DNA extraction kit (ChargeSwitch® gDNA Blood kits - Invitrogen). The ACE I/D polymorphism was determined by polymerase chain reaction (PCR). Briefly, primers (ECAS 5′-CTGGAGACC ACTCCCATCCTTTCT-3′), (ECAR 5′-GATGTGGCCATCACATTCGTCAG AT-3′) flanking the polymorphic region outside of the *alu* insert in intron 16, were used to PCR-amplify a portion of the ACE gene, and the amplified product was analyzed by gel electrophoresis and ethidium bromide staining to determine the I/D pattern. Since the D allele in heterozygotes is preferentially amplified, each DD genotype was confirmed by a second independent PCR with another primer pair (ECAint 5′-GTCTCGATCTCCTGACCTCGTG-3′) and (ECAS 5′-CTGGAGACCACTCCCATCCTTTCT-3′) that amplifies the insertion-specific sequence (Shanmugam et al., 1993).

2.5. Determination of plasma ACE activity

Fasting 10 mL blood samples were obtained from a superficial forearm vein. All subjects abstained from alcohol and caffeine intake, and from exercise, for at least 24 h before blood tests. Plasma was separated immediately from 10 mL of the whole blood by centrifugation at 1500 rpm for 10 min, and stored at −20 °C until analysis. ACE activity was assayed using FRET substrates containing *ortho*-aminobenzoic (Abz) and dinitrophenyl (Dnp) as fluorescence

donor/acceptor pair, namely Abz-FRK(Dnp)P-OH for somatic ACE, Abz-SDK(Dnp)P-OH for the N-domain and Abz-LFK(Dnp)-OH for the C-domain (Alves et al., 2005; Carmona et al., 2006). The methodology was adapted for a 96-well fluorescence plate reader format. Briefly, 5 μL plasma was incubated separately with 10 μM of the specific substrates at 37 °C in 0.1 M Tris–HCl, pH 7.0, containing 50 mM NaCl and 10 μM $ZnCl_2$, in a final volume of 200 μL. Enzymatic activity was continuously monitored in an automated microtiter plate spectrofluorimeter (λ_{ex} = 320 nm; λ_{em} = 420 nm) equipped with a temperature control device and a plate shaker. Black polystyrene 96-well microtiter plates with flat bottom (NUNC) were used and analyzed using the GraFit version 5.0 software (Erithacus Software Ltd.). The slope measured was converted into a reaction rate (nmol substrate hydrolyzed per minute) based on a calibration curve obtained by complete hydrolysis of the peptide as reported previously (Araujo et al., 2000). Measurements were made in triplicate with differences accounting for less than 5%. ACE activity was reported as mU/ml plasma (1 mU = nmol of Abz-FRK(Dnp)P-OH, Abz-SDK(Dnp)P-OH and Abz-LFK(Dnp)-OH hydrolyzed per minute).

2.6. Determination of plasma kallikrein activity

Plasma kallikrein activity (PKA) was quantified spectrofluorimetrically by cleavage of Z-Phe-Arg-7-amido-4-methylcoumarin (Z-Phe-Arg-AMC, Sigma, St. Louis, USA) (Lottenberg et al., 1981), and the activity attributed to plasma kallikrein was that inhibited by the highly specific plasma kallikrein serine protease inhibitor PKSI (Okada et al., 1999). The assays were performed by adding 5 μl plasma samples to 50 mM Tris–HCl buffer (pH 7.4) containing 100 mM NaCl (final volume of 2 ml) in the absence or presence of 10 μM PKSI. A pre-incubation period of 3 min was followed by addition of the fluorogenic substrate Z-Phe-Arg-AMC. The reaction was monitored continuously in a Hitachi F-2500 spectrofluorimeter (Hitachi, Tokyo, Japan) for 5–10 min at λ_{ex} = 380 nm and λ_{ex} = 460 nm (slit width 5 nm for both) in a thermostatically controlled cell compartment set at 37 °C. PKA activity, expressed in arbitrary units of fluorescence per minute (AFU/min), was calculated as the rate of substrate hydrolysis measured without PKSI minus the rate of substrate hydrolysis measured with PKSI (Wanaka et al., 1990). Plasma kallikrein is quickly activated in vitro by contact with negatively charged surfaces (Henriques and Allan, 1972) and in the cold (Omran et al., 1996). The concentration of PKSI used in the experiments (10 μM) inhibited more than 95% of the activity of purified plasma kallikrein under similar conditions (Okada et al., 1999). Furthermore, the inhibition constants (K_i) reported for PKSI for a series of other plasma peptidases that can also cleave the fluorogenic substrate used are: glandular kallikrein, 500 μM; plasmin, 390 μM; urokinase 200 μM; tissue plasminogen activator, 500 μM (Wanaka et al., 1990). Thus, the PKSI concentration used in our experiments is 20- to 50-fold lower than these K_i values, resulting in highly specific inhibition of PKA. In addition, PKSI has been shown to inhibit other effects related to plasma kallikrein, such as BK release, coagulation and fibrinolysis in human plasma (Wanaka et al., 1990). Intra- and inter-assay coefficients of variation were <5%.

2.7. Statistics analysis

ANOVA with repeated measurements was used for statistical analyses, and the Tukey *post hoc* test was used to identify significant data points ($p < 0.05$). Association between relative values for plasma kallikrein and BP and grip strength was determined by the Pearson coefficient correlation. Statistical analyses were performed using SPSS 10.0 for Windows package (SPSS, Inc., Chicago, IL, USA). Differences were considered significant for $p < 0.05$. All data are expressed as mean ± SEM.

Table 1
Subject characteristics.

	ACE II (n = 27)	ACE ID (n = 64)	ACE DD (n = 28)
Age (yr)	19 ± 0	19 ± 0	19 ± 0
Weight (kg)	75 ± 2	72 ± 1	75 ± 2
Height (cm)	176 ± 0	176 ± 0	178 ± 0
BMI (kg/m^2)	24 ± 0	23 ± 0	23 ± 1

BMI, body mass index.

Table 2
Relationship between the ACE gene I/D polymorphism and handgrip strength, mean blood pressure and heart rate.

	II	ID	DD
MAP-Rest (mm Hg)	91 ± 2	90 ± 1	91 ± 2
MAP-Post (mm Hg)	91 ± 2	91 ± 2	93 ± 2
HR-Rest (beats/min)	69 ± 2	71 ± 2	67 ± 2
HR-Post (beats/min)	76 ± 2	77 ± 2	71 ± 2
HGS (kgf)	49 ± 2	49 ± 1	48 ± 1

MAP, mean arterial pressure; HR, heart rate; HGS, hand grip strength; kgf, kilogram-force; Rest, measure before HGS; Post, measured immediately after HGS.

3. Results

3.1. Subjects characteristics, handgrip strength and ACE genotype

A total of 119 male subjects were studied and the distribution of ACE genotypes in our sample was II 23%, ID 54% and DD 23%. There were no observable differences of age, weight, height, and BMI among the groups with subjects with different ACE genotypes (Table 1).

Our results demonstrated that there were no differences related to handgrip strength in subjects with different ACE genotypes (Table 2).

There were no observed differences for MAP, SBP and DBP from individuals from the three ACE genotypes in rest and after the handgrip strength test (Table 2).

3.2. Plasma ACE activity

Fig. 2 presents plasma ACE activities measured using the three different substrates, Abz-FRK(Dnp)P-OH for somatic ACE (A), Abz-LFK(Dnp)-OH for the C-domain (B) and Abz-SDK(Dnp)P-OH for the N-domain (C). Our results demonstrate that the enzymatic activity increases in the dependence of D alleles. In this sense, a significant difference was observed between II and ID/DD subjects for the substrate of both domains and the C-domain specific substrate. The use of N-domain specific substrate demonstrated a significant difference among II, ID and DD subjects.

3.3. Plasma kallikrein activity

As demonstrated in Fig. 3, DD subjects presented a 30% and 60% increase of plasma kallikrein activity in comparison to ID and II subjects, respectively.

4. Discussion

The main finding of the present study was that the activities of the ACE C- and N-domains are different depending on the ACE genotypes when specific domain substrates were used. In accor-

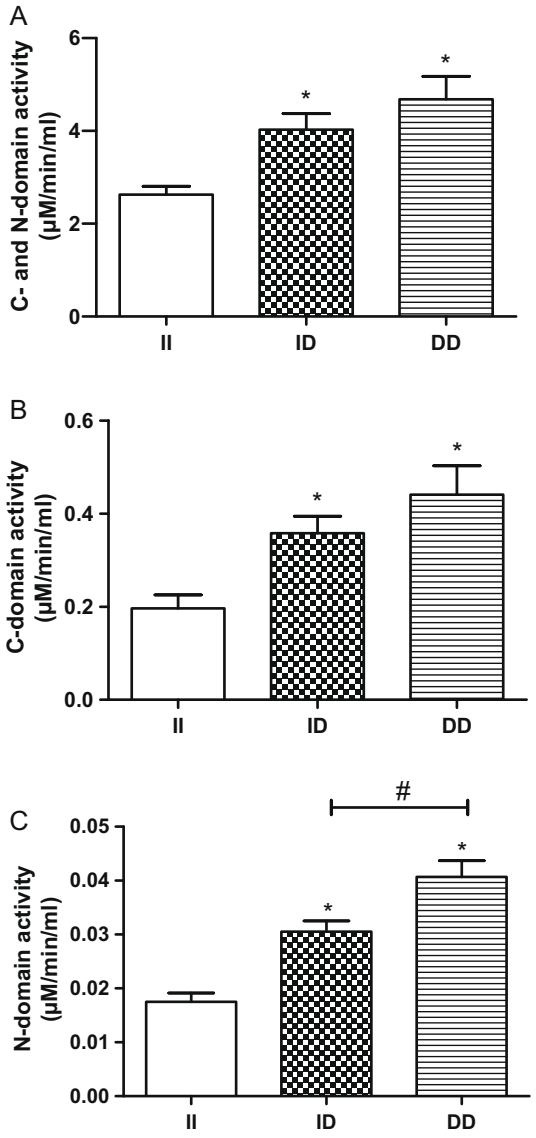

Fig. 2. Determination of ACE activities. Activity for the C- and N-domain using Abz-FRK(Dnp)P-OH as substrate (A) Activity of the C-domain using Abz-LFK(Dnp)-OH as substrate (B) and activity for the N-domain using Abz-SDK(Dnp)P-OH as a substrate (C). The values are the means ± SEM $^{*}p < 0.05$ compared to II, $\#p < 0.01$.

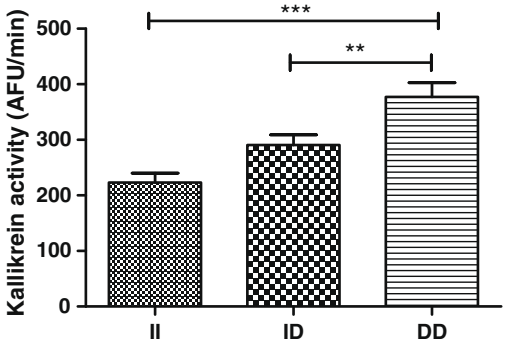

Fig. 3. Plasma kallikrein activity. Determination of plasma kallikrein of II, ID and DD groups. The values are the means ± SEM $^{**}p < 0.01$, $^{***}p < 0.001$.

dance with our data, Van Esch et al. (2008) using specific inhibitors reported that the activity of ACE domains is affected by the I/D polymorphism. Thus, the issue of ACE domains deserves further investigation since most of the previous studies on this topic used colorimetric, fluorimetric or radioactive assays using synthetic or natural substrates; all those methods cannot be used to make distinctions between the ACE domains.

We observed that the N-domain activity was more affected by the ACE polymorphism and the DD subjects presented 133% of ID and 228% of II N-activity. Ac-SDKP is a strong inhibitor of stem cells and is metabolized by the N-domain (Fig. 1). Based in our results we believe this peptide to be one of the molecules most affected by the ACE polymorphism. Azizi et al. (2002) showed that the plasmatic level of Ac-SDKP is not influenced by ACE activity but DD metabolized more rapidly exogenously infused Ac-SDKP than II. We can speculate that II subjects could present different responses after hematological challengers than DD. Corroborating this point of view, Van der Meer et al. (2005) showed that decrease ACE activity and increased Ac-SDKP levels correlated with anemia in heart failure patients. The author also showed that II patients presented much less erythroid progenitor cell proliferation than ID or DD patients.

Different from Rigat et al. (1990), we are not able to demonstrate differences between ID and DD genotypes with the substrate for both domains. The difference among the data could be related to ethnic differences because the majority of studies investigated Caucasians or African Americans differently from us. Interesting, the unique study with Latin Americans presented results similar to ours (Jalil et al., 1999).

We did not observe differences on blood pressure regarding the ACE polymorphism at the basal stage or after the handgrip exercise. The reason for these results could be related to the inability of this polymorphism to induce changes in levels of plasma RAS components other than ACE itself (Alhenc-Gelas et al., 1991; Lachurie et al., 1995; Rankinen et al., 2000). The fact that studies with hypertensive individuals demonstrated an association of blood pressure with the ACE polymorphism (O'Donnell et al., 1998; Jalil et al., 2002), could be a consequence of the different mechanisms in pathophysiological conditions. In fact, plasma ACE activity is involved in the development of many neurological and metabolic diseases associated with this polymorphism (Kakoki and Smithies, 2009). Disturbance of tissue RAS or of other peptides metabolized by ACE could be the reason for the relation between the ACE polymorphism and these diseases.

Kinins are much better ACE substrates than angiotensin I (Jaspard et al., 1993) and the presence of the D allele increases bradykinin degradation (Murphey et al., 2000). In this work we are showing for the first time that this polymorphism affects plasma kallikrein activity and it was 30% and 60% higher in DD subjects in comparison to ID and II subjects, respectively. Taken this into account, we agree with the theory of Kakoki and Smithies (2009) that kinins probably are related with the effects of the ACE D allele. Curiously, bradykinin (BK) metabolism appears to be increased in DD subjects, even though the basal levels of this vasodilator is similar in all genotypes (Murphey et al., 2000). Because our volunteers were all normotensive, it is tempting to speculate that the higher kallikrein activity observed in DD individuals could be a mechanism to compensate their increased BK degradation.

In the present study, we did not find associations between the ACE polymorphism and parameters such as handgrip strength, heart rate and BMI in young untrained individuals. It is interesting to note that the ACE I/D polymorphism has been associated with physical performance or physiological responses to exercise in the last few years. Examples include baseline muscular strength (Williams et al., 2005), endurance performance (Myerson et al., 1999) and response to physical training (Montgomery et al., 1999; Foschini et al., 2009). However, other studies have not observed such associations (Rankinen et al., 2000; Thomis et al.,

2004). On the other hand, plasma kallikrein activity and kinins have been involved with blood pressure control using different exercise models (Moraes et al., 2007; Pontes et al., 2008).

In conclusion, our data suggest that while the I/D ACE polymorphism affects differently the C- and N-domains as well as the kallikrein–kinin system of young normotensive individuals, this polymorphism can not be associated with effects on handgrip strength.

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