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ACE I/D genotype-related increase in ACE plasma activity is a better predictor for schizophrenia diagnosis than the genotype alone

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ABSTRACT

Background: Angiotensin-I converting enzyme (ACE) is a key component of the renin–angiotensin system (RAS). Although the several contradictory data, ACE has been associated with schizophrenia (SCZ) pathophysiology. Here the ACE activity of SCZ patients and healthy controls (HCs), and its possible correlations with the *ACE* polymorphism genotype and symptomatic dimensions, was investigated.
Methodology: ACE activity of 86 SCZ patients and 100 HCs paired by age, gender and educational level was measured, using the FRET peptide substrate and the specific inhibitor lisinopril. The *ACE* insertion/deletion (I/D) genotypes were assessed by the restriction fragment length polymorphism (RFLP) technique.
Results: Significantly higher ACE activity was observed in SCZ patients compared to HCs ($t = -5.09$; $p < 0.001$). The area under the receiver operating characteristic (ROC) curve was 0.701. Mean ACE activity levels were higher for the D-allele carriers ($F = 5.570$; $p = 0.005$), but no significant difference was found among SCZ patients and HCs for genotypes frequencies (Chi-squared = 2.08; df = 2; $p = 0.35$). Interestingly, we found that the difference between the measured ACE activity for each SCZ patient and the expected average mean value for each respective genotype group (for control subjects) was a better predictor of SCZ than the ACE dichotomized values (high/low) or *ACE* I/D.
Conclusion: Our results suggest that higher levels of ACE activity are associated with SCZ with stronger impact when the genetic background of each individual is considered. This may explain the heterogeneity of the results on ACE previously reported.

1. Introduction

The renin–angiotensin system (RAS) was classically characterized as the principal regulator of the systemic blood pressure (Basso and Terragno, 2001). Angiotensin I-converting enzyme (ACE) has a central role in this system by producing the angiotensin II, one of the main effector of RAS. The interest in RAS, beyond its classical functions, was renewed by the identification of several RAS effectors in different human tissues, especially in the brain (Dupont and Brouwers, 2010; Bali and Jaggi, 2013; Wright et al., 2013).

Brain RAS has been implicated in several functions, including regulation of cerebral blood flow and cerebroprotection, stress, depression, seizure, alcohol consumption, memory consolidation, and possible roles in the etiology of Alzheimer's and Parkinson's diseases were also suggested (as reviewed by Wright and Harding, 2011). Both ACE and angiotensin receptors were identified in dopaminergic neurons of basal ganglia (Allen et al., 1992). Significant decreased dopamine release determined by ACE inhibitors was also demonstrated (Obata et al., 2008). Therefore, the interaction with the dopaminergic system suggests that RAS and ACE are both promising targets for schizophrenia (SCZ) investigations.

Abbreviations: SCZ, schizophrenia; ACE, angiotensin I-converting enzyme; RAS, renin angiotensin system; RFLP, restriction fragment length polymorphism; HCs, healthy controls; ROC, receiver operating characteristic; FRET, fluorescence resonance energy transfer; BDs, bipolar disorders; ELISA, enzyme-linked immuno assay; PANSS, positive and negative syndrome scale; SCID, structured diagnostic interview; CGI, Clinical Global Impression; GAF, Global Assessment of Functioning; IPAP, International Psychopharmacological Algorithm Project; LSD, Fisher's least significant difference; TR, treatment-resistant; non-TR, non-treatment-resistant; IPAP, International Psychopharmacological Criteria; PCR, polymerase chain reaction; AUC, area under the curve; BMI, Body Mass Index; SPSS, Statistical Package for the Social Sciences; CSF, cerebrospinal fluid.

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The insertion/deletion (I/D) polymorphism in the 16th intron of *ACE* gene was shown to influence ACE activity levels (Rigat et al., 1990). In SCZ, genetic association studies with *ACE* investigated mainly the I/D polymorphism, and only two out of ten studies showed significant major effects of I/D *ACE* genotypes in SCZ diagnosis (Crescenti et al., 2009; Kucukali et al., 2010). One study suggested the D allele of *ACE* gene as a protective factor for SCZ in a Spanish sample (Crescenti et al., 2009), while the other proposed the I allele as protective against the development of SCZ and bipolar disorders (BDs) in a Turkish sample (Kucukali et al., 2010), suggesting the potential influence of population stratification on the reported results. Baskan et al. (2010) described significantly higher levels of serum ACE protein concentrations, determined by enzyme-linked immunoassay (ELISA), in SCZ patients, but with no association to SCZ diagnosis or I/D polymorphism genotypes. In other words, no significant difference in serum ACE concentration could be observed according to the genotypes (Baskan et al., 2010).

The few studies addressing the potential role of ACE protein levels or activity in SCZ have yielded inconclusive results, with no robust replication (Beckmann et al., 1984; Wahlbeck et al., 1993, 1998, 2000). None have used the current available and more sensitive FRET substrates to detect the ACE activity (Carmona et al., 2006).

Therefore, our main objective here is to investigate whether ACE enzymatic levels are different between SCZ patients and HCs in the Brazilian admixture population using FRET substrates. Secondarily, we also wanted to evaluate if this measure correlates with the symptomatic dimensions and *ACE* I/D polymorphism genotypes.

2. Materials and methods

2.1. Study participants

Patients were recruited from an outpatient clinic, The Schizophrenia Program (PROESQ) of Universidade Federal de São Paulo (UNIFESP/EPM). Inclusion criteria were: 1) age between 18 and 65 years old; 2) fulfill Schizophrenia (SCZ) or Schizoaffective Diagnoses; and 3) at least one year of follow-up. Healthy control (HC) volunteers were selected paired by age, gender, and educational level from a governmental unemployment agency. First they were submitted to a phone screening for psychiatric diagnosis and then they were invited for full psychiatric interview. Inclusion criteria were: 1) age between 18 and 65 years old; 2) no current or lifetime psychiatric diagnosis; and 3) no family history of psychosis of any degree.

For both groups exclusion criteria were: 1) diagnosis of hypertension, 2) use of any anti-hypertensive medication, and 3) doubt or lack of consensus on diagnosis after full psychiatric interview.

Trained psychiatrists applied the Structured Diagnostic Interview (SCID), to assess the psychiatric diagnosis; clinical assessment also included Positive and Negative Syndrome Scale (PANSS), Calgary Depression Scale, Clinical Global Impression (CGI), and Global Assessment of Functioning (GAF). A questionnaire adapted from SCID screening questions was used to investigate family history of mental disease.

The criteria proposed by Andreasen et al. (2005) were used to assess remission. Treatment resistance (TR) was defined following the International Psychopharmacological Criteria (IPAP) [www.ipap.org].

At first, 92 SCZ patients agreed to participate and were initially enrolled, 2 were out of age range and were excluded. 102 HCs were initially enrolled and all met the inclusion criteria. 4 SCZ patients and 2 HCs were excluded due to hypertension diagnosis and/or use of anti-hypertensive. None was excluded by doubt or lack of consensus on diagnosis. Thus, 86 patients (80 with SCZ and 6 with schizoaffective diagnoses) and 100 HCs were included in the analyses. All the patients were using at least one antipsychotic medication. 24 SCZ patients and 18 healthy controlHCs reported previous cannabis use, 1 patient reported previous use of cocaine and 2 healthy controlHCs reported the use of other drugs (cocaine and amphetamine). No subject in the healthy controlHC group fulfilled the criteria for alcohol or drug dependence. No one subject in either group reported drug use of in the two weeks before the blood collection.

The interviewer inferred ethnic background and four groups were considered: Caucasian, African, Native American (in this group we included Chinese and Japanese ancestry), and a miscellaneous group (for the combinations of Caucasian + African, Native American + African, Native American + Caucasian). We opted to group the Native-Americans and Asian descendants because the South American Amerindian population shares more SNPs with Eastern Asian populations than with European or African populations (Ribeiro-dos-Santos et al., 2013).

This study was approved by the Research Ethics Committee of UNIFESP [CEP No. 1883/10]. We obtained written informed consent from all participants recruited, and clinical and laboratory investigations were strictly conducted according to the principles expressed in the Declaration of Helsinki.

2.2. Blood samples

Blood samples from all subjects were collected into heparin vacuum tubes (BD Vacutainer®, BD, NJ, USA) for ACE activity measurements, and into EDTA vacuum tubes (BD Vacutainer®) for *ACE* genotype analysis. All blood samples collected into vacuum tubes were kept at 4 °C for up to 12 h, or they were immediately centrifuged at 1500-2000 ×*g*, for 10–15 min, at room temperature, to recover the plasma that were stored at −80 °C until use. The stored samples were defrosted in wet ice, soon before use.

2.3. Enzyme activity measurements

Angiotensin-converting enzyme (ACE) activity in human plasma samples of HC volunteers and SCZ patients were measured by a fluorimetric assay, using the Fluorescence Resonance Energy Transfer (FRET) peptide substrate Abz-FRK(Dnp)P-OH essentially as previously described (Carmona et al., 2006). The plasma sample was handled and prepared as described in Gadelha et al. (2013).

2.4. DNA extraction and genotyping

DNA was isolated from whole blood white cells using Gentra Puregene (Qiagen, Germantown, MD) according to the manufacturer's instructions. I/D *ACE* genotyping was performed using the restriction fragment length polymorphism (RFLP) technique. Primers and polymerase chain reaction (PCR) conditions are available upon request.

2.5. Statistical analysis

Variable distribution was first checked for Gaussian distribution using Kolmogorov–Smirnoff tests for the whole sample and in each comparison group. For measuring the mean differences on ACE activity between SCZ patients and HC groups, we employed the Student's *t*-test. The possible associations of ACE activity and variables that present non-parametric distributions were investigated using non-parametric correlations (Spearman's).

In order to verify the clinical usefulness of ACE activity measurements to discriminate SCZ patients and HCs, we built a receiver operating characteristic (ROC) curve for SCZ patients/HCs condition as the outcome. Aiming to further evaluate the applicability of ACE activity to diagnosis, we dichotomized it using the median value for the whole sample. Then logistic regression models were fitted considering the clinical condition as the dependent variable, ACE activity dichotomized as predictor, and age and sex as covariates. Nagelkerke R-square was used to report overall model variance explained in the logistic regression model. Significance was defined at $p < 0.05$.

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