Inhibition of the renin–angiotensin system prevents seizures in a rat model of epilepsy

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ABSTRACT

The RAS (renin–angiotensin system) is classically involved in BP (blood pressure) regulation and water–electrolyte balance, and in the central nervous system it has been mostly associated with homoeostatic processes, such as thirst, hormone secretion and thermoregulation. Epilepsies are chronic neurological disorders characterized by recurrent epileptic seizures that affect 1–3% of the world’s population, and the most commonly used anticonvulsants are described to be effective in approx. 70% of the population with this neurological alteration. Using a rat model of epilepsy, we found that components of the RAS, namely ACE (angiotensin-converting enzyme) and the AT1 receptor (angiotensin II type 1 receptor) are up-regulated in the brain (2.6- and 8.2-fold respectively) following repetitive seizures. Subsequently, epileptic animals were treated with clinically used doses of enalapril, an ACE inhibitor, and losartan, an AT1 receptor blocker, leading to a significant decrease in seizure severities. These results suggest that centrally acting drugs that target the RAS deserve further investigation as possible anticonvulsant agents and may represent an additional strategy in the management of epileptic patients.

INTRODUCTION

The RAS (renin–angiotensin system) is classically involved in BP (blood pressure) regulation and water–electrolyte balance, acting mainly by activation of the AT1 receptor [AngII (angiotensin II) type 1 receptor] after binding of the agonist peptide AngII [1]. Inhibition of ACE (angiotensin-converting enzyme), the enzyme responsible for AngII production, and blocking the AT1 receptor are effective strategies to control BP and associated target-organ damage. In the CNS (central nervous system), the RAS has been mostly associated with the regulation of homoeostatic processes, such as thirst, hormone secretion and thermoregulation [2,3]. The existence of a functional RAS in different tissues and organs raises new possibilities of interfering with different pathophysiological events using specific drugs routinely used in anti-hypertensive therapies that target the RAS. For example, it has been reported that losartan, a specific AT1 receptor antagonist, has beneficial effects in patients with Marfan syndrome [4].

Key words: angiotensin-converting enzyme (ACE), angiotensin II type 1 receptor (AT1 receptor), central nervous system, epilepsy, neurological disorder, renin–angiotensin system.

Abbreviations: ACE, angiotensin-converting enzyme; AngII, angiotensin II; AngIV, angiotensin IV; AT1 receptor, AngII type 1 receptor; BP, blood pressure; CNS, central nervous system; RAS, renin-angiotensin system; SBP, systolic BP; WAR, Wistar audiogenic rat.

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Epilepsies affect 1–3% of the world’s population and are characterized by recurrent epileptic seizures that are triggered by abnormal, excessive and/or hypersynchronous neuronal activity [5]. The first choice of treatment for epilepsy is drug-based, and approx. 70% of the anticonvulsants are described to be effective. Hence approx. 30% of the epileptic population is non-responsive to classical anticonvulsants, which are also known to yield a panel of severe side effects, including neurotoxicity (for a review, see [6]).

Different experimental models are used to study epilepsy, including the WAR (Wistar audiogenic rat) strain [7], a genetic-based model with a good correlation with behavioural and electroencephalographic features of human epilepsies. In WAR and other audiogenic strains, chronic acoustic stimulation [8,9] transforms brainstem-dependent tonic clonic seizures into limbic ones. These newly developed limbic seizures are associated with behavioural, structural and electroencephalographic alterations that are coupled to recruited forebrain regions, such as the hippocampus, and seizures mimicking temporal lobe epilepsy [10,11], the most common type of epilepsy in humans [12]. A recent study reported that RAS components were up-regulated in the brain of patients with temporal lobe epilepsy [13], which prompted us to investigate a possible role for the angiotensinergic pathway in epilepsy.

MATERIALS AND METHODS

Animals
Experiments were conducted with 200–250 g female Wistar rats and WARs, as well as 2-day-old neonates. Animals were maintained in a controlled environment with a constant 12 h/12 h light/dark cycle and were provided food and water ad libitum. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were reviewed and approved by the local Animal Care and Use Committee (protocol FMRP-USP 200/2005).

Drugs and treatments
Enalapril and losartan were purchased from Galena. In order to avoid biased results, litters of WARs and Wistar rats were randomly divided into distinct experimental groups, which were subjected to the different treatments. Likewise, experimental groups were subjected to seizure induction with collection of data in a blinded manner. Wistar rats and WARs were treated orally once a day (between 12:00 and 14:00 hours) by gavage with the ACE inhibitor enalapril (10 mg · kg⁻¹ of body weight · day⁻¹), the AT₁ receptor antagonist losartan (50 mg · kg⁻¹ of body weight · day⁻¹) or vehicle (water) for 21 days. The efficacy of the doses used was validated in vitro in SHRs (spontaneous hypertensive rats), where both enalapril and losartan induced a significant anti-hypertensive effect (reduction of approx. 30% in mean BP).

SBP (systolic BP) measurements
SBP was measured using the tail-cuff plethysmography method (MK-II; E & M Instrument) in conscious rats pre-warmed for 10 min at 38 °C in a thermostatically controlled heating cabinet. Measurements were performed before the beginning of treatment and on day 7 of treatment with enalapril, losartan or the vehicle. Final SBP values were obtained by averaging three successful readings. In groups that received vehicle, no significant differences in SBP values collected before and after treatment were observed.

ACE activity
Hippocampi were homogenized by hand using a Teflon glass homogenizer in ice-cold 0.4 M sodium borate buffer (pH 8.3) containing 0.9 % NaCl and 0.32 M sucrose. Samples were stored at −20 °C until assay processing. ACE activity was determined as described previously [14,15]. Briefly, plasma (15 μl) and hippocampus extracts (50 μl) from Wistar rats and WARs that received vehicle or treatment for 7 days were incubated with 5 mM Hip-His-Leu (hippuryl-L-histidyl-L-leucine) in 250 μl of 0.4 M sodium borate buffer (pH 8.3) containing 0.9 % NaCl for 15 min. The enzyme reaction was stopped by the addition of 1.0 ml of 0.5 M NaOH. The blank samples were prepared by reversing the order of addition of the enzyme and NaOH. After the addition of 100 μl of o-phthalaldehyde in methanol (10 mg/ml), followed 10 min later by the addition of 200 μl of 6 M HCl, the reaction mixture was measured fluorimetrically (364 nm for excitation and 486 nm for emission; SPF-500C; SLM-Aminco). Standard curves for His-Leu (0–20 nmol) were prepared under the same conditions. All measurements were made in duplicate. Results were expressed in nmol of His-Leu · min⁻¹ · ml⁻¹ of plasma or nmol of His-Leu · min⁻¹ · g⁻¹ of tissue.

Acoustic stimulation and seizure evaluation
Acoustic stimulation was performed singly (single stimulus group) or twice a day at fixed times (between 08:00–09:00 hours and 16:00–17:00 hours) over the course of 2 weeks (14-day stimuli). In experimental groups, the acoustic stimulation was initiated at day 7 of treatment with enalapril, losartan or vehicle in Wistar rats and WARs. An acoustically isolated chamber was used to individually expose animals to a high-intensity sound (120 dB SPL) until tonic seizure appearance or for the maximum time of 1 min. Animal behaviour was recorded during the stimulus and for an additional 1 min. The severity of tonic clonic seizures was evaluated using the mesencephalic severity index [16]. Racine’s scale (limbic...
Analyses of the expression levels of transcripts for ACE (forward, 5′-CTAAGGCAACCGTGAAAA-GA-3′; and reverse, 5′-ATTGCGCGATGTGACCTG-3′), the AT₁ receptor (forward, 5′-AACACTGCCTGAACCTCT-3′; and reverse, 5′-ACTGGCTCTTGGGTCTGAG-3′) and β-actin (forward, 5′-GCTCGACTCTACTCTG-3′; and reverse, 5′-CCTAGCGGTGAGCAAGATTG-3′) using Platinum Taq polymerase. After separation on agarose gels, PCR products were quantified densitometrically using ImageJ software (http://rsb.info.nih.gov/ij).

Real-time PCR
Reactions were performed using Platinum SYBR Green qPCR SuperMix-UDG with ROX, according to the manufacturer’s recommendations (Invitrogen). Briefly, reaction conditions were as follows: initial (50°C, 2 min; 95°C, 10 min), followed by 40 cycles of denaturation (95°C, 15 s) and annealing/extension (60°C, 1 min) using an Applied Biosystems 7500 Fast Real-time PCR System. Specific primers (see above) were designed based on GenBank® sequences and were used to amplify fragments from AT₁ receptor, ACE and β-actin. Amplification of β-actin was used as the internal control, and samples with total RNA were used as a control for genomic contamination.

Statistical analyses
Results are given as means ± S.E.M. of the indicated number of independent experiments. Statistical analyses were performed using one- or two-way ANOVA, followed by the Newman–Keuls multiple comparison test, or an unpaired Student’s t test, as appropriate. Statistical analysis was performed using GraphPad software, and statistical significance was accepted where P < 0.05.

RESULTS
Expression of ACE and AT₁ receptor transcripts in epileptic and Wistar rats
Our first step to address the possible functional participation of RAS in epilepsy was to evaluate the expression levels of transcripts of ACE and the AT₁ receptor in the hippocampus of newborn WARs (2 days post-birth). For these analyses, the use of newborn rats is essential, since the triggering of seizures in this strain occurs through activation of the auditory pathways and it is known that the auditory meatus of rats opens around day 14 [18]. Our results revealed that the hippocampus of WARs did not have an innate modulation of ACE and the AT₁ receptor compared with newborn Wistar rats (Figure 1A).

The same analyses were conducted in adult WARs subjected to a single stimulus or 14-day stimuli compared with naïve WARs (no stimulus). As shown in Figure 1(B), the results suggested a relevant involvement of RAS in the establishment and/or maintenance of temporal lobe epilepsy, which is established after multiple seizures, since a significant up-regulation in the expression level of transcripts for ACE (2.6-fold) and for the AT₁ receptor (8.2-fold) was observed exclusively in this group. Analyses of the hearts showed no modulation in the expression levels of these molecules in any of the groups studied (Figure 1C).
Effect of orally administrated enalapril on epileptic seizures, ACE activity and SBP

On the basis of the results found in the temporal lobe epilepsy group (14-day stimuli group), we investigated further whether an ACE inhibitor, which is widely used as an anti-hypertensive agent (enalapril), would affect the triggering and/or maintenance of seizures in this group. As shown in Figure 2(A), treatment with enalapril significantly suppressed temporal lobe epilepsy seizures, with a reduced severity of limbic seizure index, as compared with the control group that received vehicle (water). With regard to the tonic clonic seizures (mesencephalic index), although not altered in the first few days, from day 6 they were significantly attenuated in the enalapril-treated group (Figure 2B). Wistar rats treated with enalapril or vehicle had no evidence of seizure activity (Figures 2A and 2B).

To evaluate whether the dose of enalapril used was effective in inhibiting ACE, we performed enzymatic assays with an ACE-specific substrate in plasma and hippocampus homogenates. As shown in Figures 2(C) and 2(D), the dose of enalapril used was able to significantly impair activity of ACE from plasma and hippocampus respectively. Nevertheless, no significant reduction in SBP was observed in either strain (Figure 2E).

Effect of orally administrated losartan on epileptic seizures and SBP

We examined further the role of the AT1 receptor in this phenomenon by assessing the effects of losartan, a specific AT1 receptor antagonist. Figure 3(A) shows that, similar to enalapril, losartan was able to significantly suppress temporal lobe epilepsy seizures (limbic seizure index). Notably, a drastic reduction in tonic clonic seizures was achieved from the first day of stimulus in the losartan-treated group (Figure 3B), as compared with the group treated with the ACE inhibitor enalapril ($P = 0.0004$). Wistar rats treated with losartan or vehicle had no evidence of seizure activity (Figures 3A and 3B). Interestingly, treatment with losartan caused a moderate reduction in SBP in WARs (Figure 3C).

DISCUSSION

The results of the present study demonstrate that the functional inhibition of the RAS, by an enzymic inhibitor...
or a receptor antagonist, was able to significantly impair the triggering and maintenance of epileptic seizures. Although the findings described in the present study are experimental, and therefore future evaluations in humans are mandatory, these novel findings suggest a possible new application for RAS inhibitors, drugs that are commonly used as anti-hypertensive agents and are known to yield relatively low and non-severe side-effects. On the other hand, classical anti-epileptic drugs are known to yield severe side effects, including neurotoxicity [6]. An early study reported a stimulatory effect of AngII in the hippocampus [19], and it has been reported that AT1 receptors located pre- and post-synaptically are able to modulate GABAergic (where GABA is γ-aminobutyric acid) and glutamatergic transmission [20,21]. Hence, knowing that losartan and other AT1 receptor antagonists are able to cross the blood-brain barrier [22] and that the blockade of AngII action may indirectly interfere in the modulation of excitatory and inhibitory components of CNS, this can be proposed as a possible mechanism of action by which the inhibition of the RAS impaired the epileptic seizures described in this study. In our present study, no innate differences in ACE or the AT1 receptor were detected in WARs compared with Wistar rats (see Figure 1A). The up-regulation profile found for ACE and AT1 receptor expression in the hippocampus of WARs (see Figure 1B) occurred after repetitive seizures induced in our experimental model of temporal lobe epilepsy. However, neither ACE nor AT1 receptor expression was modulated in the heart, a tissue where RAS plays relevant functional roles (see Figure 1C). It is interesting to note that new classes of drugs that are able to act centrally on the RAS, such as the aminopeptidase A inhibitors [23,24], may also become useful tools to control seizures. The observed moderate differences in the impairments of seizure indices by enalapril or losartan could be related to the functional blockade of different sites in the RAS (enzyme or receptor respectively) or differences in pharmacokinetics within the organism, such as absorption, half-life and elimination of the studied drugs and/or active metabolites. Peak enalapril concentrations in plasma occurs approx. 1 h after oral administration, whereas its active metabolite peaks approx. 4 h after administration [25,26], with a half-life of more than 12 h. A similar profile was described for losartan, with a peak in plasma occurring 1–2 h after oral administration, with a half-life of the corresponding active metabolite (EXP3174) to be approx. 9 h [27]. Besides that, differences in physicochemical properties of the drugs studied, reflected in their abilities to cross the blood–brain barrier, could account for the differences in pharmacodynamics and therefore their effective activities. Those issues certainly deserve to be thoroughly addressed in future studies.

A recent study indicates that the peptide AngIV (angiotensin IV) (a metabolite of AngII) possesses a wide range of central functions, including protection against cerebral ischaemia, and anticonvulsant and anti-epileptogenic actions [28]. In addition, intracerebroventricular administration of AngIV in rats leads to an increase in the hippocampal extracellular dopamine and serotonin levels that protect against pilocarpine-induced seizures [29]. Combining this observation with our present results suggests that the anticonvulsant actions of AngIV might be at least partially derived from its binding to the AT1 receptor and thus competing with endogenous AngII. Nevertheless, it is also possible that binding of AngIV to its own receptor (insulin-regulated aminopeptidase) may lead to anticonvulsant actions.

It is important to note that the results of the present study were obtained with an audiogenic model of epilepsy induction. Therefore future studies with other experimental models of epilepsy, such as chemical (e.g. pilocarpine) or electrical induction, should be evaluated in order to reinforce the relevance of the present findings. However, it is also important to discuss that one of the strongest criticisms concerning the use of maximal electroshock and chemical-induced models to screen new antiepileptic drugs is that these models use normal animals (for a review, see [30]). Meldrum [31] also highlights that levetiracetam, an anticonvulsant drug introduced into clinical use, was initially shown to be inactive in electroshock and PTZ (pentylenetetrazol) models, but highly active in genetic models such as audiogenic seizures in DBA/2 mice. Therefore the use of genetically selected animals, such as the WAR strain, can bring important contributions to the development of new anti-epileptic therapies.

We believe that it would be interesting to investigate the clinical relevance of the RAS by evaluating clinical data, although it may include some bias and generate misinterpretations since epileptic patients regularly undergo concurrent treatments along with the anticonvulsant drugs. Nevertheless, the recent finding that epileptic patients with temporal lobe epilepsy have an up-regulation of RAS components in the brain [13] corroborates and gives clinical relevance to our results. Our present study uncovers new functions for the RAS in the pathophysiology of the CNS, and we believe that future investigations on the subject may shed light on new therapies for epilepsies and associated pathologies.

**AUTHOR CONTRIBUTION**

Marilia Pereira and Christiane Becari performed and interpreted the experiments; José Oliveira performed and interpreted the seizure induction experiments; and Maria Salgado, Norberto Garcia-Cairasco and Claudio Costa-Neto conceived the study, interpreted the results and wrote the paper.
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