INHIBITION OF CENTRAL ANGIOTENSIN CONVERTING ENZYME EXERTS ANXIOLYTIC EFFECTS BY DECREASING BRAIN OXIDATIVE STRESS

INHIBICIJA CENTRALNOG ENZIMA KOJI KONVERTUJE ANGIOTENZIN DELUJE KAO ANKSIOLITIK SMANJUJUĆI OKSIDATIVNI STRES U MOZGU

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Summary: This study investigated the effects of angiotensin II and captopril intracerebroventricular administration on anxiety status and brain oxidative stress. Elevated plus maze was used in order to asses the anxiety-like behavior, while the biochemical analysis included the determination of some antioxidant defense enzymes like superoxide dismutase and glutathione peroxidase and also a lipid peroxidation product (malondialdehyde). Our results provide additional evidence of angiotensin II induced anxiety-like effects and increased prooxidant status. Moreover, the blockade of angiotensin II, by the administration of an angiotensin converting enzyme inhibitor (captopril) resulted in anxiolytic effects and decreased oxidative stress status. In addition, we found a significant correlation between the time spent by rats in the open arms of the elevated plus maze and oxidative stress markers. This could raise important therapeutic issues regarding the anxiolytic effects of some angiotensin converting enzyme inhibitors used primarily for hypertension, such as captopril. Also, it seems that oxidative stress could play an important part in these actions.

Keywords: angiotensin II, captopril, anxiety, oxidative stress

Kratak sadržaj: Ispitivan je uticaj intracerebroventrikularne administracije angiotenzina II i kaptoprila na napetost i oksidativni stres u mozgu. Za procenjivanje ponašanja bliskog anksioznosti korišćen je test uzdignutog lavirinta u obliku znaka plus, dok je biohemijska analiza obuhvatila određivanje nekih enzima antioksidantne odbrane kao što su superoksid-dismutaza i glutation-peroksidaza i proizvoda lipidne peroksidacije (malondialdehid). Naši rezultati predstavljaju još jedan dokaz da angiotenzin II izaziva posledice nalik na anksioznost i povišen prooksidantni status. Štaviše, blokiranje angiotenzina II davanjem inhibitora enzima koji konvertuje angiotenzin (kaptoprila) delovalo je kao anksiolitik i dovelo do sniženja statusa oksidativnog stresa. Pored toga, otkrivena je značajna korelacija između vremena koje su pacovi proveli u otvorenim »rukama« lavirinta i markera oksidativnog stresa. Ovo možda ukazuje na važnost pokretanja nekih važnih terapijskih pitanja koja se tiču anksiolitičkog dejstva nekih inhibitora enzima koji konvertuju angiotenzin a koji se pre svega koriste za hipertenziju, kakav je kaptopril. Takođe, sva je prilika da oksidativni stres u tome igra važnu ulogu.

Ključne reči: angiotenzin II, kaptopril, anksioznost, oksidativni stres

Introduction

The renin-angiotensin system (RAS) is one of the best-studied enzyme neuropeptide systems in the brain and can serve as a model for the action of peptides on neuronal function in general. It is now well established that the brain has its own intrinsic RAS with all its components present in the central nervous system (1). Also, it is believed that the RAS of the brain is involved not only in the regulation of blood pressure, but also in the modulation of multiple additional functions in the brain, including processes of sensory information, learning and memory and regulation of emotional responses (2).

The RAS generated a family of bioactive angiotensin peptides with variable biological and neurobiological activities. These mainly include angiotensin

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II (Ang II), angiotensin IV and angiotensin 1-7 (3). Since Ang II does not readily cross the blood–brain barrier, brain Ang II is unlikely to originate from the peripheral. Indeed, a complete brain RAS exists that is distinctly separate from the peripheral system and comprises all necessary precursors and enzymes required for the formation and metabolism of the biologically active forms of angiotensin (1–3).

Enhanced cognitive performance mediated by angiotensin-converting enzyme (ACE) inhibitors like captopril, which is commonly used as an antihypertensive drug, has been previously reported (4). Additionally, it seems that drugs affecting RAS influence the anxiety-related behavior, but this varies with the way of administration, the time of testing after drug administration and the animal strain used (5). Regarding captopril, reports indicating that it has antidepressive effects/enhances well-being in human patients (6) and exerts mood elevating effects in rats (7), are contradicted by studies showing no effect of captopril on subjective feeling or mood in humans (8) or on behavior and cognition in rats (9).

There is also evidence that oxidative stress accompanies Ang II infusion, but the role of central RAS in the generation of reactive oxygen species is not clear. It was previously reported that administration of Ang II increases the formation of free radical like superoxide (O^{2-}) (10), while captopril inhibits oxidative stress, probably in association with the inhibition of Ang II (11).

The aim of the present work was to evaluate the effects of Ang II and captopril intracerebroventricular (icv) administration on anxiety state and oxidative stress status in the temporal cortical area of rat brain, the cortical area most vulnerable to oxidative stress effects (12). Also, we were interested in determining a possible correlation between the anxiety state response in elevated plus maze and central oxidative stress markers.

Material and Methods

Subjects

The subjects (n=36) were experimentally naive, male Wistar rats, weighing approximately 200–250 g at the beginning of the experiment. The animals were housed in a temperature- and light-controlled room (22 °C, a 12-h cycle starting at 08:00 h) and were fed and allowed to drink water *ad libitum*. Rats were treated in accordance with the guidelines of animal bioethics from the Act on Animal Experimentation and Animal Health and Welfare Act from Romania and all procedures were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). This study was approved by the local Ethics Committee and also, efforts were made to minimize animal suffering and to reduce the number of animals used.

Neurosurgery

All surgical procedures were conducted under aseptic conditions, under sodium pentobarbital (45 mg/kg b.w., i.p., SIGMA) anesthesia. Rats were mounted in the stereotaxic apparatus with the nose oriented 11° below horizontal zero plane. Captopril and angiotensin II (SIGMA) were icv administered (7 consecutive days, 0.1 µg/kg b.w.) by freehand through a plastic (silastic) cannula (Portex, 0.44 inside diameter, 0.9 mm outer diameter), stereotaxically implanted in the left cerebral ventricle at the following coordinates: 0.5 mm posterior to bregma; 1.3 mm lateral to the midline; 4.3 mm ventral to the surface of the cortex (13). The cannula was positioned with acrylic dental cement and secured by one stainless steel screw.

After surgery the rats were isolated in separate cages and protected with large spectrum antibiotic. The sham-operated rats were injected with saline. The location of the icv cannulas in lesioned rats was verified by injecting a dye (Trypan Blue, SIGMA) through each cannula at the end of the experiment. Brains were removed and cut with a scalpel, and after the temporal lobes were removed for oxidative stress assays, the spread of the dye within the ventricles was examined. All cannulas were found to be in the right position.

Behavioral testing was performed after seven consecutive days of treatment.

Elevated plus maze

The elevated plus maze (Coulbourn Instruments) consists of four arms, 49 cm long and 10 cm wide, elevated 50 cm off the ground. Two arms were enclosed by walls 30 cm high and the other two arms were exposed. Rats were placed at the juncture of the open and closed arms and the amount of time spent on the open arms was recorded during a 5-min test. Time spent on the open arms is considered to be an index of anxiety (14).

Tissue collection

After behavioral tests, all rats were anesthetized, rapidly decapitated and whole brains were removed. The temporal lobes were collected. Each of the temporal tissue samples was weighed and homogenized with a Potter Homogenizer coupled with Cole-Parmer Servodyne Mixer in bidistilled water (1 g tissue/10 mL bidistilled water). Samples were centrifuged 15 min at 3000 rpm. Following centrifugation, the supernatant was separated and pipetted into tubes.

Biochemical estimations

Determination of superoxide dismutase. Superoxide dismutase (SOD) activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 substrate (a water soluble tetrazolium dye) and

xanthine oxidase using a SOD Assay Kit (Fluka, 19160) according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37 °C. The percent inhibition was normalized by mg protein and presented as SOD activity units.

Determination of glutathione peroxidase. Glutathione peroxidase (GPX) activity was measured using the GPX cellular activity assay kit CGP-1 (Sigma Chemicals). This kit uses an indirect method, based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPX, which is then coupled with recycling GSSG back to GSH utilizing glutathione reductase (GR) and NADPH. The decrease in NADPH at 340 nm during oxidation of NADPH to NADP is indicative of GPX activity.

Determination of malondialdehyde. Malondialdehyde (MDA) levels were determined by the thiobarbituric acid reactive substances (TBARs) assay. 200 μL of temporal lobe homogenate (supernatant) was added and briefly mixed with 1 mL of trichloroacetic acid at 50%, 0.9 mL of TRIS-HCl (pH 7.4) and 1 mL of thiobarbituric acid 0.73%. After vortex mixing, samples were maintained at 100 °C for 20 minutes. Afterwards, samples were centrifuged at 3000 rpm for 10 min and supernatant read at 532 nm. The signal was read against an MDA standard curve, and the results were expressed as nmol/mg protein (15).

Data Analysis

The animal's behavior in the elevated plus maze was tracked and recorded using the ANY-maze behavioral software (Stoelting Co., USA, version 4.5) and then statistically analyzed using one-way analysis of variance (one-way ANOVA). The results for antioxidant enzymes activity and MDA level were analyzed also using one-way ANOVA. All results are expressed as mean ± SEM. *Post hoc* analyses were performed using Tukey's honestly significant difference test in order to compare captopril and angiotensin II groups. F values for which $P < 0.05$ were regarded as statistically significant. Pearson's correlation coefficient and regression analysis were used to evaluate the connection between time spent in the open arms of the elevated plus maze and the central oxidative stress markers.

Results

The effects of angiotensin II and captopril administration on anxiety state in the elevated plus maze

Behavior in the elevated plus maze is mainly used to assess exploration and anxiety status. In our experiment, the rats treated with angiotensin II spent significantly less time ($F_{(1,22)}=19$, $p=0.0002$) in the open arms of the elevated plus maze, compared to control group. Also, the administration of captopril resulted in a significant increase ($F_{(1,22)}= 33$, $p=0.0009$) in the time spent in the open arms, in comparison with the control rats, suggesting that blocking of Ang II significantly diminished anxiety-like behavior (*Figure 1*). Also, *post hoc* analysis revealed statistically significant differences between Ang II and captopril groups ($p=0.0001$).
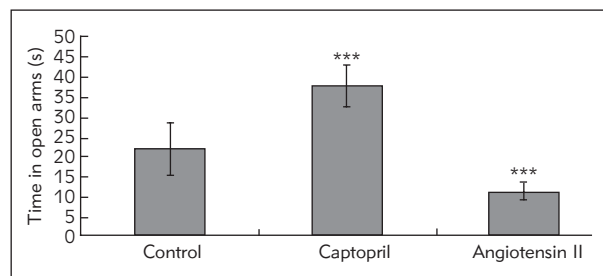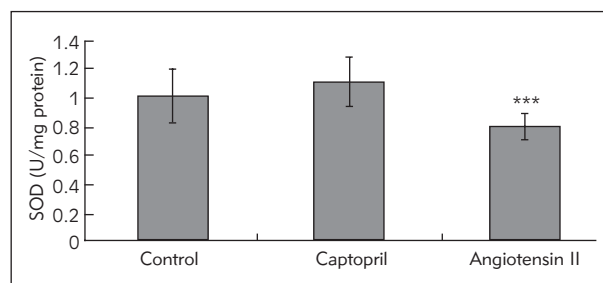
Figure 1 Effect of angiotensin II and captopril on the time spent in the open arms of the elevated plus maze. The values are mean ± S.E.M. (n=12 animals per group). ***p<0.001 vs. control group.
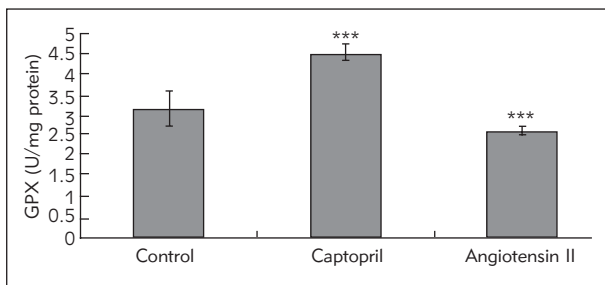
The effects of angiotensin II and captopril administration on oxidative stress status

Regarding the oxidative stress status, we observed a significant decrease ($F_{(1,22)}=15$, $p=0.0007$) of SOD specific activity in the angiotensin II group, compared to the control rats. Statistically insignificant increase ($F_{(1,22)}=2$, $p=0.14$) of SOD activity was observed in the case of captopril group, in comparison with control (*Figure 2*). However, *post hoc* analysis revealed significant differences between angiotensin II and captopril groups ($p< 0.0001$).

In addition, a significant decrease ($F_{(1,22)}=21$, $p=0.0001$) of the other important antioxidant enzyme, GPX, was observed in the case of angiotensin II group, compared to control rats. Also, we noticed a significant increase ($F_{(1,22)}=119$,

Figure 2 Effects of angiotensin II and captopril on SOD specific activity from the rat temporal lobe homogenates. The values are mean ± S.E.M. (n=12 animals per group). ***p<0.001 vs. control group.

Figure 3 Effects of angiotensin II and captopril on GPX specific activity from the rat temporal lobe homogenates. The values are mean ± S.E.M. (n=12 animals per group). ***p<0.001 vs. control group.
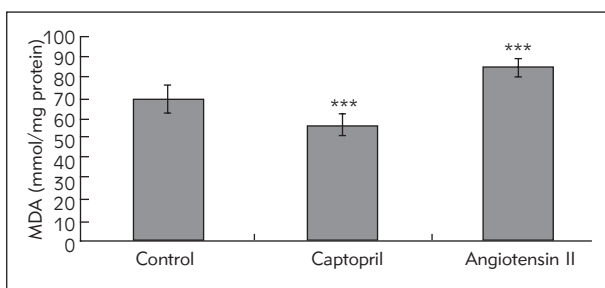
Figure 4 Effects of angiotensin II and captopril on MDA levels from the rat temporal lobe homogenates. The values are mean ± S.E.M. (n=12 animals per group). ***p< 0.0001 vs. control group.

$p=2.4^{-10}$) of GPX specific activity in the captopril group, compared to controls (*Figure 3*). *Post hoc* analysis showed significant differences between angiotensin II and captopril groups (p< 0.0001).

Concerning the levels of lipid peroxidation products, we observed a significant increase ($F_{(1,22)}= 44$, $p=1.06^{-6}$) of MDA concentration from the temporal lobe in the angiotensin II group, compared to control rats. Also, a significant decrease ($F_{(1,22)}= 24$, $p=7.02^{-5}$) of MDA levels was observed in the case of captopril group, in comparison with the controls, suggesting antioxidant effects (*Figure 4*). *Post hoc* analysis also revealed significant differences between the angiotensin II and captopril groups (p< 0.0001).

Interestingly, when we determined the linear regression between the time spent in the open arms of the elevated plus maze vs. oxidative stress markers (SOD, GPX and MDA), we found significant positive correlations between time in the open arms vs. SOD (n=36, r = 0.536, p=0.001) and also between time in the open arms vs. GPX (n=36, r = 0.818, p=0.0001). Moreover, we found a significant negative correlation between time in the open arms and MDA concentration (n=36, r = –0.777, p=0.0001).

Discussion

This study investigated the effects of angiotensin II and captopril icv administration on anxiety status and brain oxidative stress. Our results provide additional evidence of angiotensin II induced anxiety-like effects and increased prooxidant status. Furthermore, the blockade of angiotensin II, by the administration of an ACE inhibitor (captopril) resulted in anxiolytic effects and decreased oxidative stress status. In addition, we found a significant correlation between the time spent by rats in the open arms of the elevated plus maze and oxidative stress markers from the temporal lobe, which is known to be the cortical area most susceptible to reactive oxygen species (12).

Previous studies have suggested that blocking Ang II by administrating ACE inhibitors could facilitate mental functioning and well-being in both animals and human patients. In this way, captopril was reported by many authors to have a beneficial effect on cognition, attention and emotional functions (5–7, 16). Also, it was reported that icv administration of Ang II results in increased anxiety-like behavior in rats (17). In addition, captopril has been suggested to possess antidepressant activity, as shown by specific tests like forced-swimming and learned helplessness (6, 18).

Still, there are studies which failed to find any effects of captopril on cognition, subjective feeling or mood (8) in human patients. Also, some studies reported no cognitive or mood effects in rats as a result of captopril administration (9).

These differences could be explained by the different way of administration, the strain of animal used or the time of testing after drug administration (5). Regarding the time-dependent response, it was previously demonstrated using open-field behavior that angiotensin II initially increases anxiety-like behavior (e.g. after 5 minutes), and then a rebound anxiolytic effect can be observed 15 minutes after administration (19).

Hence, the mechanisms of action for Ang II mediated effects on anxiety-like behavior are still unknown. It seems that the respective mechanisms reach beyond ACE inhibition (5, 6).

There is increasing evidence that oxidative stress and consecutively the production of free radicals may play an important role in Ang II mediated effects. In this way, experimental data demonstrated that increased reactive oxygen species generation through the activation of NAD(P)H oxidase is an obligatory step in Ang II effects (10, 20). It has also been reported that the inhibition of Ang II with specific receptor blockers, but mainly with ACE inhibitors (11), results in decreased oxidative stress status. This is generally expressed by an increased activity of anti-oxidant enzymes like SOD or catalase and a decrease of some peroxidation products like malondialdehyde

(11, 21). Similar results were also observed in our experiment, where we report an increase of SOD and GPX specific activities and a decrease of MDA levels, as a result of captopril administration.

This could be an important aspect considering the implications of oxidative stress in different neuro-psychiatric disorders and/or aging (22–24). Along these lines, it was demonstrated that mice develop anxious behavior during aging (25), likely due to the accumulation of reactive oxygen species, which is a characteristic of the aging process in animals. It was also reported that ACE inhibitors reduce anxiety and improve behavioral and motor performance in the aged rat (26) and that the administration of captopril results in a significant prolongation of life span in rats (20). This protective effect could be related to the antioxidant action of RAS inhibitors and a reduced formation of reactive oxygen species.

Additionally, in the present study we demon-strated a significant correlation between anxiety-re-lated behavior and oxidative stress markers. A causal link between these two was also recently noted by several authors (27–29), but the underlying mecha-nism of this relationship is still waiting to be estab-lished.

Our results showed that the inhibition of central Ang II by an ACE inhibitor (captopril) results in significant decrease in anxiety state and a decrease in the oxidative stress status in rats. Moreover, we de-monstrated a significant correlation between the anxiety-related behavior and central oxidative stress markers. This could raise some important therapeutic issues regarding the anxiolytic effects of some ACE inhibitors used primarily for hypertension, such as captopril.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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