

## THE EFFECTS OF CENTRAL ANGIOTENSIN II AND ITS SPECIFIC BLOCKERS ON NOCICEPTION. POSSIBLE INTERACTIONS WITH OXIDATIVE STRESS STATUS

### EFEKTI CENTRALNOG ANGIOTENZINA II I NJEGOVIH SPECIFIČNIH BLOKATORA NA NOCICEPCIJU. MOGUĆE INTERAKCIJE SA STATUSOM OKSIDATIVNOG STRESA

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**Summary:** It has already been demonstrated that a complete brain renin–angiotensin system (RAS) exists distinctly separate from the peripheral system and is implicated in complex functions such as memory, emotional responses and pain. Regarding the implications of angiotensin II (the main bioactive peptide of RAS) in pain, although there are many studies in this area of research, most of the results are controversial. Also, it seems that oxidative stress follows angiotensin II infusion, but the role of AT1 vs. AT2 receptors is not well established. In this context, we were interested in studying the effects of central RAS on nociception, through the intracerebroventricular administration of losartan and PD-123177 (antagonists for the AT1/AT2 receptors), as well as an ACE inhibitor (captopril) and also angiotensin II in rats, which were subsequently tested using the hot-plate task, a well known behavioral test for pain perception. We present here the analgesic effect of angiotensin II administration, as shown by increased latency-time in the hot-plate, as well as a nociceptive effect of angiotensin II blockers like AT1 and AT2 specific antagonists (losartan and PD-123177) and an ACE inhibitor (captopril), as their administration resulted in decreased latency-time. Moreover, we demonstrated a significant correlation between the results of the nociceptive

**Kratak sadržaj:** Do sada je već pokazano da kompletan moždani renin–angiotenzin sistem (RAS) postoji zasebno od perifernog sistema i da je uključen u složene funkcije kao što su memorija, emocionalni odgovori i bol. Što se tiče implikacija angiotenzina II (glavnog bioaktivnog peptida RAS) u bolu, mada u ovoj oblasti istraživanja postoje mnoge studije, rezultati su većinom oprečni. Takođe, izgleda da infuziju angiotenzina II prati oksidativni stres, ali uloga receptora AT1 i AT2 još nije razjašnjena. U tom kontekstu hteli smo da proučimo efekte centralnog RAS na nocicepciju preko intracerebroventrikularne administracije losartana i PD-123177 (antagonista za receptore AT1/AT2) kao i jednog ACE inhibitora (kaptopril) i angiotenzina II kod pacova, koji su potom testirani metodom grejne ploče, poznatim bihevioralnim testom za percepciju bola. Predstavljamo analgetički efekat administracije angiotenzina II pokazan kroz povećanu latenciju na grejnoj ploči, kao i nociceptivni efekat blokatora angiotenzina II kao što su antagonisti specifični za AT1 i AT2 (losartan i PD-123177) i ACE inhibitora (kaptopril), pošto je rezultat njihove administracije bila smanjena latencija. Štaviše, uočili smo značajnu korelaciju između rezultata nociceptivnog bihevioralnog testa i nivoa nekih od glavnih markera oksidativnog stresa. Na taj način obezbeđeni su novi

## Introduction

The discovery that all components of the renin–angiotensin system (RAS) are present in the brain led investigators to postulate the existence of a local brain RAS (1). In this way, it has already been demonstrated that 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 angiotensins (2).

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behavioral task and the levels of some main oxidative stress markers. This provides additional evidence for an analgesic effect of Ang II administration, as well as for a nociceptive effect of Ang II blockers. Moreover, a significant correlation between the nociception and angiotensin II-induced oxidative stress is presented.

**Keywords:** angiotensin II, pain, oxidative stress

Also, it is now generally accepted that the brain RAS with its bioactive peptides, which mainly include angiotensin II (Ang II), is involved not only in cardiovascular functions and body fluid homeostasis (3), but also in the regulation of some superior functions involving the regulation of cognitive functions (learning and memory processes) (4, 5), emotional responses (6, 7) and also nociception (8, 9).

These effects are modulated by specific angiotensin receptors. Numerous studies have led to the identification of two pharmacologically specific angiotensin receptors type 1 (AT 1) and type 2 (AT 2), which are well represented in various brain areas (10). Our group also previously demonstrated that the administration of losartan and PD-123177, which are selective antagonists for the AT 1 and AT 2, results in anxiolytic effects in rats (7). Also, similar effects were reported as the result of angiotensin-converting enzyme (ACE) inhibitors like captopril (7), which is also commonly used as an antihypertensive drug (11).

Regarding the implications of Ang II in pain, although there are many studies in this area of research, most of the results are conflicting. In this way, while some reports stated that Ang II administration resulted in diminishing morphine-induced analgesia (12) and also that specific blockers like spirapril and losartan exert antinociceptive effects (13), other authors demonstrated opposite effects with Ang exhibiting analgesic effects (14) and its blockers (enalapril or losartan) generating increased pain sensitivity (15). Moreover, combined effects were also reported sometimes within the same experiment, as both increased and decreased nociception was observed as a result of various Ang II blockers administration (16).

Additionally, it seems that oxidative stress follows Ang II infusion, but the role of AT 1 vs. AT 2 receptors is not very well established (17). Also, it has been shown that the administration of Ang II facilitates the formation of some free radicals like superoxide ( $O_2^-$ ) (18), while losartan seems to exert antioxidant effects (19). There are also controversies regarding the effects of angiotensin II on oxidative stress, considering that in some experiments losartan significantly decreased angiotensin II-induced oxidative stress, while PD-123319 did not (20).

In this context, in the present paper we were interested in studying the effects of the central RAS on nociception, through the intracerebroventricular

dokazi u korist analgetičkog efekta administracije Ang II, kao i nociceptivnog efekta blokatora Ang II. Uz to, predstavljena je značajna korelacija između nocicepcije i oksidativnog stresa izazvanog angiotenzinom II.

**Ključne reči:** angiotenzin II, bol, oksidativni stres

(icv) administration of losartan and PD-123177 (antagonists for the AT 1/AT 2 receptors), as well as an ACE inhibitor (captopril) and also Ang II in rats, which were subsequently tested in the hot-plate task, one of the most well-known behavioral tests for pain perception. Additionally, we were interested to know if there is a possible correlation between the effects of Ang II on nociception and some oxidative stress markers which were determined from the temporal lobe of rats, considering that this is one of the brain areas most susceptible to oxidative stress (21).

## Material and Methods

### Animals

Sixty male Wistar rats weighing 200–250 g at the start of the experiment were used. 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 on 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 Council Directive of 24 November 1986 (86/609/EEC).

### 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 11° below horizontal zero plane.

Losartan, PD-123177, captopril and Ang II were i.c.v. administered (0.1 µg/kg b.w., Sigma) by freehand through a plastic (silastic) cannula (0.9 mm outer diameter), implanted stereotaxically 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 (22). The cannula was positioned with acrylic dental cement and secured by one stainless steel screw. After surgery the rats were isolated in a separate cage and protected with streptomycin 300 mg/kg bw. The sham-operated rats were injected with saline. The location of the i.c.v. cannulas in lesioned rats was verified by injecting a dye (trypan blue) through each cannula at the end of the experiment. Brains were

removed and cut with a scalpel and the spread of the dye within the ventricles was examined. All cannulas were found to be in the right position. Pain testing was started after 7 consecutive days of treatment.

### Hot-plate

The investigation of pain sensibility was performed using a hot-plate (Hugo Basile). A plastic cylinder is used to confine the rat to the heated surface of the plate which is maintained at 55 °C using a thermostate. The reaction time (the latency time) to two different types of behavior was monitored: licking the paw and jumping (23).

### Tissue collection

After the behavioral tests, all rats were anesthetized, rapidly decapitated, and the whole brain was removed. The temporal lobes were collected. Each temporal tissue sample 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

Regarding the biochemical assessments, we decided to classically determine the main antioxidant enzymes (first line of defense in the way of free radicals) and a lipid peroxidation marker.

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, product number: 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.

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.

Malondialdehyde (MDA) levels were determined by the thiobarbituric acid reactive substances (TBARS) assay. Two hundred microliters 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 (24–26).

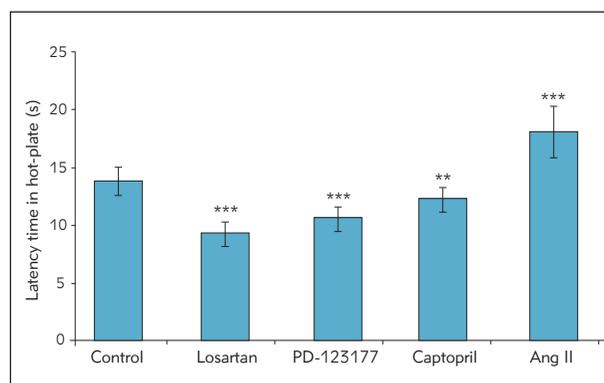
Total protein was measured using the Bradford dye-binding method, with bovine serum albumin as standard (27).

### Statistics

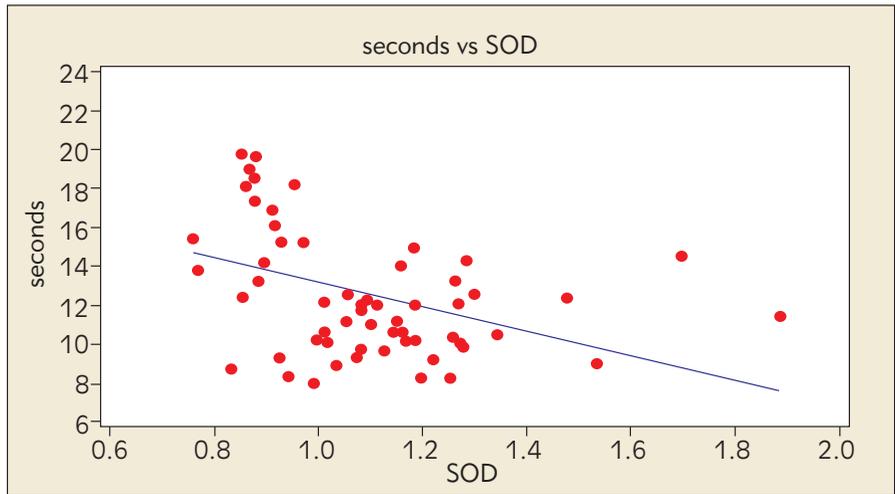
The animal's behavior in the hot-plate task was statistically analyzed using one-way analysis of variance (one-way ANOVA). The results are expressed as mean  $\pm$  SEM. *Post hoc* analyses were performed using Tukey's honestly significant difference test in order to compare losartan, PD-123177, captopril 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 the latency-time in the hot-plate and the central oxidative stress markers.

## Results

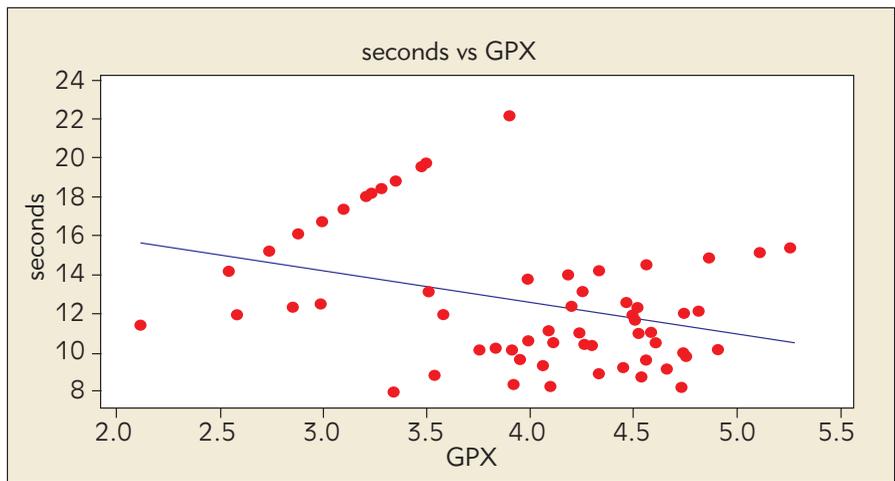
Regarding the effects of RAS components in nociception, as studied in the hot-plate, we report here a significant decrease of the latency time to jump/licking paws in the case of both antagonists administrated: losartan ( $F(1,22)=95$ ,  $p < 0.0001$ ) and PD-123177 ( $F(1,22)=41$ ,  $p < 0.0001$ ), as well as in the case of the angiotensin-converting enzyme inhibitor captopril ( $F(1,22)=18$ ,  $p = 0.003$ ), as compared to the control group (Figure 1).



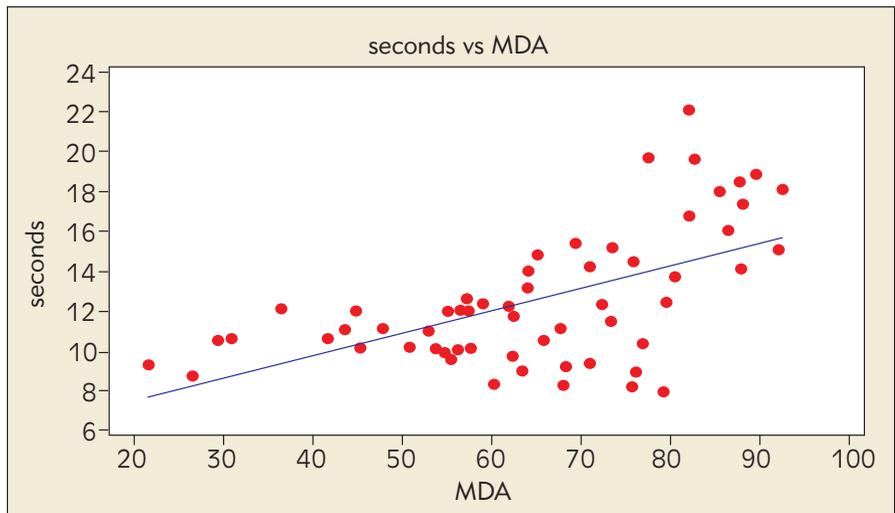
**Figure 1** Effect of angiotensin II on latency time in the hot-plate. The values are mean  $\pm$  S.E.M. ( $n=12$  animals per group). \*\*\* $p < 0.0001$  vs. control group, \*\* $p = 0.003$  vs. control group.



A



B



**Figure 2** Correlations between latency time in the hot-plate and SOD (A), GPX (B) and MDA (C).

Additionally, we observed a significant increase ( $F(1.22)=40$ ,  $p<0.0001$ ) of the latency time in the Ang II administrated group, as compared to control rats (Figure 1).

Also, *post hoc* analysis revealed significant differences between losartan and Ang II ( $p<0.0001$ ), PD-123177 and Ang II ( $p<0.0001$ ), as well as between captopril and Ang II ( $p<0.0001$ ) groups. Still, no significant differences were found between losartan and PD-123177 ( $p=0.1$ ), losartan and captopril ( $p=0.06$ ) and PD-123177 vs. captopril ( $p=0.08$ ) groups.

Moreover, we found significant correlations between the results of the behavioral task, represented by the latency time in the hot-plate, and the levels of some oxidative stress markers, such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and malondialdehyde (MDA). In this way, the Pearson's correlation coefficient and regression analysis showed the following correlations: latency time vs. SOD ( $n=60$ ,  $r=-0.401$ ,  $p=0.001$ ) (Figure 2A), latency time vs. GPX ( $n=60$ ,  $r=-0.343$ ,  $p=0.007$ ) (Figure 2B) and latency time vs. MDA ( $n=60$ ,  $r=0.590$ ,  $p<0.0001$ ) (Figure 2C).

## Discussion

In the present work we demonstrated an analgesic effect of Ang II administration, as shown by increased latency time in the hot-plate, as well as a nociceptive effect of Ang II blockers like AT 1 and AT 2 specific antagonists (losartan and PD-123177) and an ACE inhibitor (captopril), as their administration resulted in decreased latency time in the hot-plate. Moreover, we present here a significant correlation between the results of the nociceptive behavioral task and the levels of some main oxidative stress markers.

As mentioned, previous studies regarding the effects of Ang in nociception resulted in contrasting results with reports stating both analgesic and increased pain sensibility effects (12–16). Similar facts were also reported regarding the various blockers of Ang II, both at the receptor levels, as well as on the ACE blocking level (12–16). Furthermore, there are experiments where the administration of various Ang II blockers, such as saralasin, sarmesin, losartan or PD123319, generated both increased and decreased nociception, depending on the dose (16). Besides the dose used, it seems that the conflicting result described above can also be explained by the different strains of animals used, as well as various behavioral tasks selected for the study of nociception.

These aspects could be very important considering that most of the previous studies regarding various neurotransmitters implicated in the modulation of

pain were especially focused on the opioid peptides. However, considering the integrative aspects which characterize the modulation of nociceptive processes, additional studies regarding other neurochemical systems such as the bioactive peptides of RAS (Ang II) are very significant.

Moreover, in the present work we demonstrated a significant correlation between the nociception expressed as the latency time in the hot-plate and the specific activity of the main antioxidant enzymes (SOD and GPX) and a lipid peroxidation marker (MDA). Actually, there are some authors who previously demonstrated that oxidative stress contributes to persistent pain (28–30). In this way, removal of excessive ROS by free radical scavengers, such as phenyl N-tert-butyl nitron (PBN) and 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL), produced significant analgesic effects in both neuropathic pain (31–33) and inflammatory pain (34).

Furthermore, the number of neurons showing mitochondrial ROS production was significantly increased in the lumbar spinal dorsal horn in spinal nerve ligated neuropathic rats (35). Also, increased levels of extracellular hydrogen peroxide were observed in the spinal trigeminal nucleus after formalin injection into the lip of the rat, and this increase coincided with pain behaviors (36).

Additionally, it was demonstrated that SOD, which converts free-radical superoxide to hydrogen peroxide (37), was very effective in reducing inflammation indicators and hyperalgesia after carrageenan injection into the rat paw (38).

Still, while it is becoming clear that ROS are involved in persistent pain, the mechanisms by which they contribute to pain are still unknown.

We demonstrated here the antinociceptive effects of Ang II administration, as shown by the increased latency time in the hot-plate, as well as a nociception-induced effect of Ang II blockers like losartan, PD-123177 and captopril. Further, we present a significant correlation between the results of the nociceptive behavioral task and the levels of some main oxidative stress markers such as SOD, GPX and MDA.

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## Conflict of interest statement

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

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