CHANGES IN ANGIOTENSIN AT_1 RECEPTOR MRNA LEVELS IN THE RAT BRAIN AFTER IMMOBILIZATION STRESS AND INHIBITION OF CENTRAL NITRIC OXIDE SYNTHASE

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Objective. To study functional interactions between angiotensin II AT_1 receptors and nitric oxide (NO) activity in different brain areas in rats exposed to immobilization stress.

Methods. Central inhibition of nitric oxide synthase (NOS) was provided by intracerebroventricular (i.c.v.) administration of (N-omega-nitro-L-arginine-methylester) L-NAME and analysis of AT_1 receptor mRNA was performed using semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) technique. The immobilization in prone position lasted 2 hrs and the rats were sacrificed 24 hr later. The hypothalamus, hippocampus, thalamus, and cortex were isolated from fresh brains.

Results. In the cortex, gene expression of AT_1 receptors was unaffected either by L-NAME treatment, or by a single exposure to immobilization stress for 2 hours followed by 24 hours of rest. In the hippocampus, the repeated treatment with L-NAME increased mRNA levels of AT_1 receptors approximately 9-times compared to those in the control (untreated) group. Immobilization also increased AT_1 receptor mRNA levels in the hippocampus which was similar to that induced by the L-NAME.The increase of AT_1 receptor mRNA levels in the hippocampus of immobilized rats was not further altered when the animals were pretreated with L-NAME. In control rats, exposure to immobilization resulted in a significant rise in mRNA levels coding for AT_1 receptors in the hypothalamus, but not in the thalamus. L-NAME treatment showed a tendency of increase in AT_1 receptor mRNA levels in the hypothalamus. Moreover, when animals treated with L-NAME were subjected to immobilization, a further increase in AT_1 receptor mRNA levels was observed in the hypothalamus in comparison with corresponding controls.

Conclusions. The present data indicate that a single immobilization stress results in increased gene expression of AT_1 receptors in the hypothalamus and hippocampus. The rise in AT_1 mRNA levels in the same brain structures after repeated treatment with L-NAME allow to suggest an interaction between the central angiotensin II and nitric oxide.

Key words: Nitric oxide synthase – AT_1 receptors – Gene expresssion – Hypothalamus – Hippocampus –Immobilization stress – Rat

Likewise circulating angiotensin II, brain angiotensin II exerts its function through two types of receptors, the type-1 (AT_1) and the type 2 (AT_2) receptors (Wright and Harding 1995; Lenkei et al.1997). The AT_1 receptors are functionally well defined and they mediate the majority of classic angiotensin II functions concerned with regulation of fluid electrolyte homeostasis, cardiovascular, and neuroendocrine controls (Bunneman et al. 1992; Lenkei et al. 1995). Mapping of AT_1 and AT_2 receptor mRNA levels in adult rat brains revealed a detailed distribution pattern of these receptors (Lenkei et al. 1997; Krizano-

va et al. 2001). In most of the nuclei in the adult rat brain a good correlation was observed between the distribution of angiotensin binding sites and angiotensin receptor mRNA levels (Allen et al. 1992). Moreover, a significant correlation was found between brain NO synthase and AT_1 receptor mRNA, but not with mRNA of AT_2 receptors (Krizanova et al. 2001). Parallel distribution of mRNAs coding for bNOS and AT_1 receptors in several brain structures suggests a possible interaction between brain angiotensin II and nitric oxide. Nevertheless, this speculation remains to be experimentally approved.

Both AT_1 receptor mRNA and bNOS mRNA have been shown to be abundant in many areas of the rat brain including cerebellum, amygdala, thalamus, pons, medulla and hypothalamus (Lenkei et al. 1998; Krizanova et al. 2001). Central NO has been shown to be involved in the regulation of plasma ACTH response to physico–emotional stressors (Kim and Rivier 2000) and NO has been shown to be elevated in the hippocampus after nicotine treatmnent (Pogun et al. 2000).

The present study was designed to test the hypothesis that the parallel occurrence of central ANG II and NO system components may lead to mutual functional interactions in animals under stress. The central NOS activity was blocked repeatedly by i.c.v. administration of L-NAME for four days. Afterwards, the animals were exposed to a single immobilization stress and the hippocampus, hypothalamus, thalamus and frontal cerebral cortex were dissected 24 hours later. Levels of AT_1 receptors were estimated by RT-PCR and quantified relatively to the housekeeper gene GAPDH.

Materials and Methods

Animals. Adult male Wistar rats weighing 250-270 g kept under conditions of 12 h light-dark cycle (light on from 06.00 h), constant temperature (22-24 °C), and free access of food and water were used. The experiment was performed between 08.00 and 12.00 h.

Treatments. Rats were stereotaxically installed with a stainless steel intracerebroventricular (i.c.v.) guide cannulae (23 G) into the lateral brain ventricle (4.5 mm deep and 1.5 mm lateral and 0.5 mm caudal from the bregma, according to the atlas of Paxinos and Watson) under pentobarbital anaesthesia. Animals were individually caged to avoid disruption of the cannulae and handled daily for 13 days to prevent from manipulation stress. After 13 days 150 ug of L-NAME (Sigma, St.Louis, USA) or saline were injected in volume of 5 ul through the i.c.v. cannula to freely moving animals using a stainless steel needle (30 G) connected to Hamilton syringe via a polyethylene tube (PE-10). The L-NAME or saline were injected twice a day (09.00 and 16.30 h) for 4 days.

Stress exposure. Randomly selected groups of animals were exposed to 120 min of restraint 60 min after L-NAME or vehicle i.c.v. administrations and sacrificed 24 h later. The immobilization was performed by taping all four limbs of animals to metal holders by an adhesive tape After decapitation the brains were quickly removed and selected areas including whole thalamus, hippocampus, hypothalamus and part of the frontal cerebral cortex were isolated and kept frozen at –70 °C until analyzed.

Isolation of total RNA. RNA was isolated by QuickPrep®. Total RNA Extraction Kit from Pharmacia Biotech (Piscatawy, NJ, USA)according to manufacturer's protocol. Briefly, the brain tissue was extracted by homogenization in a buffered solution containing high concentration of guanidium thiocyanate. This ensured rapid inactivation of endogenous RNAse activity and complete dissociation of cellular components from the RNA. After further homogenization in the presence of lithium chloride, the extract was centrifuged in a solution of CsTFA. Upon centrifugation, the RNA formed a pellet on the bottom of the tube while the proteins formed a coat at the top of the tube and the DNA remained in the liquid phase. Concentration and purity of RNA were determined spectrophotometrically using Shimadzu UV-3000 (Kyoto, Japan).

Relative quantification of mRNA levels by RT-PCR. Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Beads (Pharmacia Biotech, Piscatawy, NJ, USA), and $pd(N)_6$ primer. PCR for AT_1 receptors was done according to Llorens-Cortes et al. (1994) with primers AT_1 5'-GCA CAA TCG CCA TAA TTA TCC-3' and AT_2 5'-CAC CTA TGT AAG ATC GCT TC-3'. Amplification using annealing 54 °C for 30 cycles displayed 444 bp fragment. As a control for quantitative eval-

uation of PCR primers for the housekeeper glyceraldehyde 3-phosphate dehydrogenase (GAPDH) – (GPH1: 5'-AGA TCC ACA ACG GAT ACA TT-3'; GPH2: 5'-TCC CTC AAG ATT GTC AGC AA-3') were used to amplify 309 bp fragment from each first strand sample. After denaturation at 94 °C for 5 min, 30 cycles of PCR at 94 °C, 60 °C and 72 °C for 1 min were performed (TERADA et al. 1993). PCR products were analyzed on 2 % agarose gels. Before quantification of PCR products it was verified whether under described conditions the PCR amplification of each fragment is still in the linear range. Intensity of the individual bands was measured by Kodak camera and quantified using IMAGE software and compared to GAPDH. As a negative control, amplification from mRNA omitting reverse transcription was performed.

Statistical evaluation. Data are presented as mean ± S.E.M. Each value represents an average from 4 measurements. Statistical differences among groups were determined by one-way analysis of variance (ANOVA). A value of $P<0.05$ (two-tailed test) was considered statistically significant. For multiple comparisons, an adjusted t-test with P values corrected by the Bonferroni method was used (Instat, GraphPad Software, USA).

Results

In the cortex, gene expression of AT_1 receptors was unaffected either by L-NAME treatment, or by a single exposure to immobilization stress for 2 hours followed by 24 hours of rest (Fig. 1). In the hippocampus, four days of i.c.v. treatment with L-NAME increased mRNA levels of AT_1 receptors approximately 9-times (from 100±9 to 903±90 %) compared with control (untreated) group (Fig. 1). Exposure to immobilization increased mRNA levels of AT_1 receptors in the hippocampus (to 909±90 %) to the same extend as did L-NAME treatment (Fig. 1). When the animals pretreated with L-NAME were subjected to immobilization, no further increase in AT_1 receptor mRNA levels (858±70 %) was observed (Fig. 1).

Gene expression of AT_1 receptors was also measured in the thalamus and hypothalamus (Fig. 2) of vehicle and L-NAME treated rats under control and stress conditions induced by exposure of animals to immobilization. In control rats, exposure to immobilization resulted in a significant rise in mRNA levels coding for AT_1 receptors in the hypothalamus (from 100 ± 22 % to 458±28 %), but not in the thal-

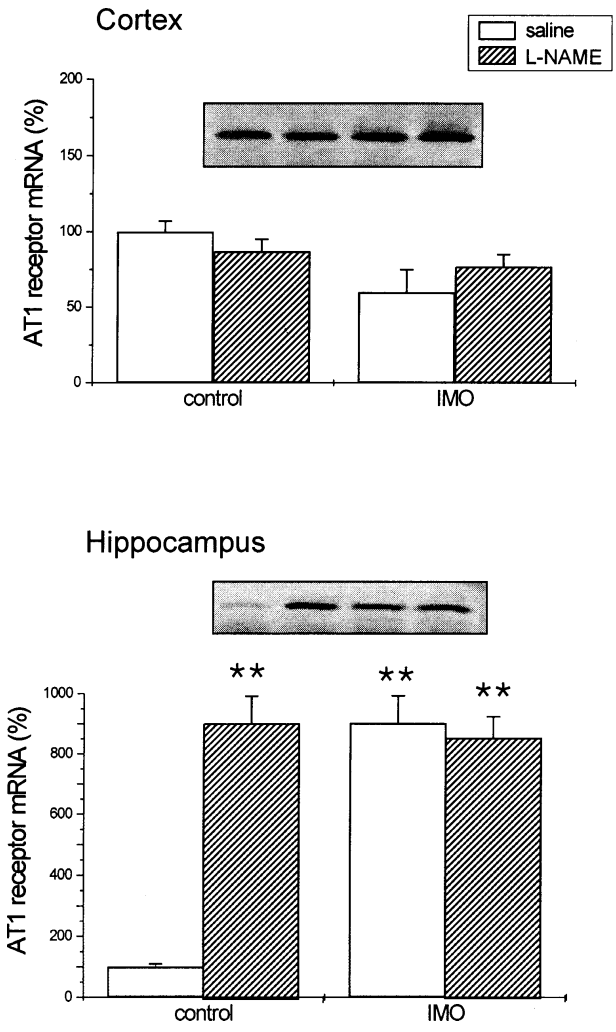

Figure 1
Effect of L-NAME treatment on mRNA levels of AT_1 receptors in the cortex and hippocampus of intact and immobilized rats.
Marked increase in AT_1 receptor mRNA was observed in the hippocampus but not in the frontal cortex of rats after i.c.v. injection of L-NAME (150 µg per 5 µl of saline, administered twice a day for 4 days) and immobilization stimulus for 2 hours (animals were sacrificed 24 later). Upper part of the graphs shows representative example of the gel. Results are displayed as means ± S.E.M. and each column represents an average of four measurements (P< 0.01 vs saline control).**

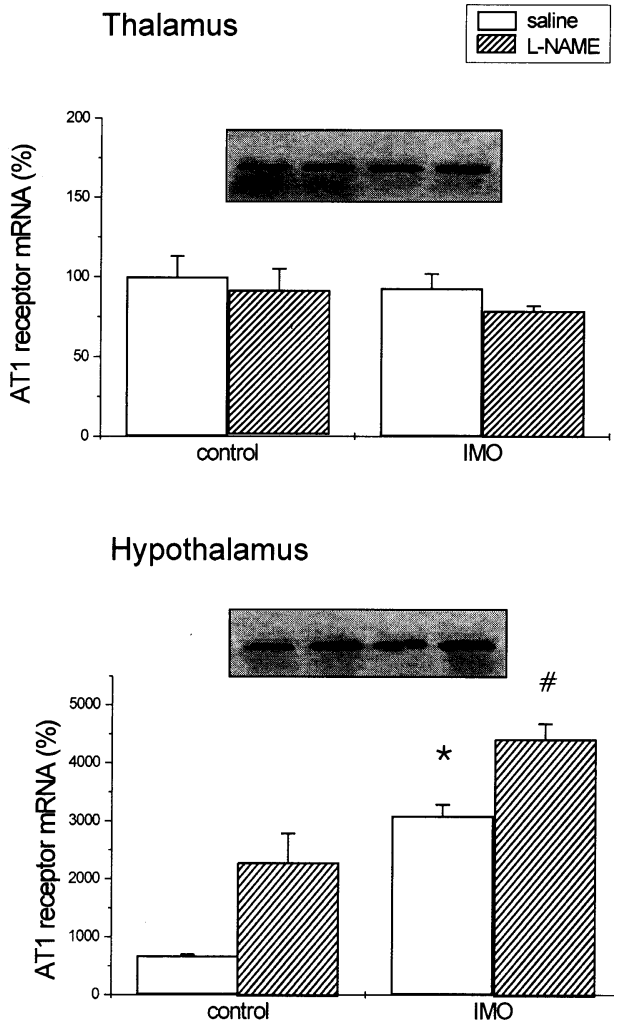

Figure 2
Effect of L-NAME treatment on mRNA levels of AT_1 receptors in the thalamus and hypothalamus of intact and immobilized rats.
Immobilization for 2 hours (animals were sacrificed 24 hours later) significantly while the i.c.v. L-NAME administration (150 μg per 5 μl of saline, administered twice a day for 4 days) markedly but not significantly elevated the AT_1 receptor mRNA levels in the hypothalamus of rat. L-NAME treatment in combination with the immobilization stress potentiated the increase of AT_1 receptor mRNA levels in the hypothalamus in comparison with corresponding controls. No effect was either after immobilization or the administration of L-NAME observed in the thalamus Upper part of the graphs shows representative example of the gel. Results are displayed as mean ± S.E.M. and each column represents an average of four measurements (*P< 0.05 vs saline control; # P< 0.05 vs L-NAME control).

amus. L-NAME treatment induced a rise of AT_1 receptor mRNA in the hypothalamus which , however, failed to be statistically significant (Fig. 2). Moreover, when animals treated with L-NAME were subjected to immobilization (Fig. 2), a further increase in AT_1 receptor mRNA levels was observed in comparison with corresponding controls (673±39 % vs. 351±75 %).

Discussion

The present study revealed selective changes of AT_1 mRNA levels in the brain in response to single exposure to immobilization stress. Moreover, central inhibition of NOS induced by i.c.v. administration of L-NAME resulted in a rise in AT_1 mRNA levels in the brain structures, which were influenced by stress exposure, namely in the hippocampus and the hypothalamus.

Brain renin-angiotensin system has been suggested to participate in the stress response (Aguilera et al. 1995a). We have shown previously that central blockade of AT_1 receptors inhibits stress-induced rise in corticoliberin (CRH) mRNA concentrations in hypothalamic paraventricular nucleus (Jezova et al. 1998). In this brain region, AT_1 receptor gene expression was found to be increased in response to stress exposure as measured by *in situ* hybridization (Aguilera et al. 1995b). The rise in AT_1 receptor mRNA levels in the hypothalamus observed in present experiments using semiquantitative RT-PCR technique are in agreement with the above mentioned data and allow to suggest a role of AT_1 receptors in the control of neuroendocrine response in stress.

No information is available on gene expression of AT_1 receptors during stress in other brain structures. The present results document that single stress exposure failed to modify AT_1 receptor mRNA concentrations in the thalamus or brain cortex, but resulted in a pronounced rise of the message in the hippocampus. The role of AT_1 receptors in the hippocampus during stress is unclear. However, the hippocampus was found to be very vulnerable to stress exposure (Watanabe et al. 1992) and stress-induced changes of gene expression of other neuromediator receptors have been reported (Schwendt and Jezova 2000).

Our recent experiments revealed a similar distribution of AT_1 receptor mRNA and brain NO synthase mRNA levels in several brain regions, including hippocampus and hypothalamus (Krizanova et al. 2001). A significant correlation was found between brain NO synthase and AT_1 receptor mRNAs, but not with mRNA of AT_2 receptor, angiotensin converting enzyme and renin. Gene expression in the same brain regions may represent a potential basis for the interaction of NO and angiotensin II at the level of AT_1 receptors. Indeed, the present study demonstrates an enhanced AT_1 receptor gene expression after inhibition of NO synthase by central administration of L-NAME in the hippocampus. Angiotensin II is thought to be involved in L-NAME hypertension (Melaragno and Fink 1996) and the renin-angiotensin system was found to participate also on blood pressure responses to intracerebroventricular administration of L-NAME (Moriguchi et al. 1998; Liu et al. 1998). It is not very likely that these effects are mediated via the hippocampus. Moreover, central blockade of NO synthase failed to modify gene expression of AT_1 receptor in brainstem structures, such as pons and medulla (unpublished observations). It may be speculated that an interaction between NO and AT_1 receptors in the hippocampus is related to learning, as central injection of L-NAME blocked the memory formation and the increased expression of cFos in this brain region of rats subjected to training (Qiang et al. 1999).

Stress-induced rise in AT_1 receptor mRNA levels in the hippocampus and the hypothalamus were differentially influenced by pretreatment with L-NAME. As to the hypothalamus, L-NAME pretreated rats showed a tendency to increase AT_1 receptor gene expression with an additional rise in response to stress exposure. On the other hand, immobilization stress failed to induce an additional rise in AT_1 receptor mRNA levels in the hippocampus. Thus, similar mechanisms in stress-induced and L-NAME-induced rise in AT_1 receptors may be suggested in the hippocampus but not in the hypothalamus.

In conclusion, the present findings allow to suggest that enhanced gene expression of central AT_1 receptors plays a role in the stress response as well as in the interaction between brain angiotensin II and nitric oxide. The main brain regions involved appear to be the hippocampus and the hypothalamus.

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