Corticotropin-releasing Hormone Receptor Subtypes in the Rat Anterior Pituitary after Two Types of Restraint Stress

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The stress response in anterior pituitary (AP) is mediated by corticotropin-releasing hormone (CRH) acting through CRH-R1 and -R2, however, the function of CRH-R2 in AP is still not fully elucidated. We used 1-h long restraint (IMO) as well as restraint combined with water immersion (IMO+C). Using real-time PCR we quantified mRNA expression of CRH-R1, CRH-R2α-soluble and -insoluble, and cAMP response element binding (CREB), with reference gene GAPDH. In control AP, CRH-R1 mRNA was up to 20-fold higher than levels of CRH-R2α-soluble or CRH-R2α-insoluble mRNA. IMO reduced CRH-R1 mRNA to 47% and 63% of control levels 1 and 2 h after the onset of stressor, respectively, while IMO+C did not produce significant changes. Our data demonstrated that these stressors did not change CRH-R2α mRNA, unlike the very significant response of CRH-R1 to IMO.

Key words: **anterior pituitary; CRH receptor subtypes; gene expression; immobilization; restraint; stress; real-time PCR; Wistar rats**

Introduction

Corticotropin-releasing factor or hormone (CRH) has a wide range of behavioral and physiological effects. It acts as a neurohormone in the anterior pituitary (AP) and as a neurotransmitter in the brain. The primary action of CRH is promoting the synthesis and secretion of pituitary corticotropin (ACTH), which triggers the endocrine response to stress. CRH is known as a central mediator of stress-related responses.[1–3] The widespread expression of CRH-R mRNA was identified in AP and was preferentially associated with corticotropes. Thus CRH-R transcripts in anterior pituitary are fully compatible with the principal neuroendocrine action of CRH.[4] CRH initiates its biological effects through a CRH receptor (CRH-R), and two major receptors activated by CRH have been cloned and functionally characterized in the brain and AP—type CRH-R1[5] and CRH-R2[6,7] receptors. CRH-R1 has been cloned from human and mouse pituitary.[5,8] Distribution of CRH-R1 mRNA suggests that this receptor is the primary neuroendocrine pituitary CRH receptor and plays dominant role. The CRH-R2 exists in at least two isoforms.[7,9] Two splice variants differ in their N-terminal domains.[10] CRH-R2α was cloned from the rat brain[7] and CRH-R2β was cloned from the mouse heart.[6]

Recent investigations demonstrated the existence of stressor-specific responses in laboratory animals. In our investigations we have demonstrated differences in the responses of rats to two different stressors, immobilization (IMO) and immobilization combined with water immersion (IMO+C).[11–13] These differences can be attributed to their diverse influences on such brain factors as CRH, vasopressin, and oxytocin. Molecular biological studies enable the investigation of brain functions on the level of

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genes. One suitable method for these studies is quantification of mRNA using real-time polymerase chain reaction (PCR). The stress response in anterior pituitary is mediated mainly by CRH acting through CRH-R1, but the function of the CRH-R2 system in AP is still not fully elucidated.

In this study we were interested in the expression of mRNA for CRH receptor subtypes in the rat anterior pituitary under control conditions as well as after IMO and IMO+C.

Materials and Methods

Animals and Stress Procedure

Male Wistar rats, with starting body weight 240–260 g, were purchased from Velaz, Prague, Czech Republic. Animals had free access to a standard pellet food and water. Rats were housed in a room with controlled light regime maintained on a 12 h light/dark cycle (lights on 06.00–18.00 h), temperature (21 ± 1°C). Tests were performed from 08.00 h to 13.00 h. Treatment of animals was in accordance with the Declaration of Helsinki Guiding Principles on Care and Use of Animals (DHEW Publication, NHI 80-23).[11,12]

Stress Procedure

Rats were exposed to two types of restraint/IMO stressors for 1 h.[11–13] First, IMO alone was applied by fixing the forelimbs and hindlimbs of the rat; then the animal was restrained in a snug-fitting plastic mesh. This mesh was bent to conform to the size of individual animal, and a bandage fixed this shape of the mesh. Second, half of the immobilized rats were immersed in the water bath (21°C) in such a way that the upper 1/4 of the animal was outside of water (IMO+C). After the exposure to either of the two stressors, the animals were either immediately decapitated [IMO(1); IMO+C(1)] or executed after 1 h pause in the home cage [IMO(2); IMO+C(2)]. Thus, one group of animals was decapitated 1 h and the other 2 h after the beginning of the 60-min stress procedure. Control animals remained untreated. For the stress procedure, the rats were transferred to a separate room.

Analyzed Tissue

After rat execution the brains were removed and neurohypophyses were separated from the anterior pituitary glands. Tissues were frozen on dry ice and kept at −80°C until further processed.

Real-Time PCR

Total mRNA was isolated from the tissue by the commercial kit Chemagic mRNA Direct (Chemagen, Brno, Czech Republic) using magnetic particles and converted to cDNA using RT kit (Amplimedical, Milan, Italy), which uses random hexamers.

Primers and probes for genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ("house-keeping gene"), CREB, CRH, and its receptors, CRH-R1, CRH-R2α-insoluble (CRH-R2α-in), and CRH-R2α-soluble (CRH-R2α-so) were designed in the Laboratory of Neurobiology and Molecular Psychiatry using the software "Primer Express" from sequences freely available in the database GeneBank. TaqMan probes with 6-carboxyfluorescein (FAM) or Yakima Yellow on 5′ end and ECLIPSE on 3′ end were used.

The sequences for individual genes were as follows. CRH-R1: Fprimer TCCACC TCCCTTCAGGATCA (exon 2), Rprimer TGCAGGCCAGAAACATTGC (exon 2/3), TaqMan probe: CGCTGTGA-GAACCTG TCCCTGACC (exon 2); CRH-R2α-insoluble: Fprimer TTTGGATGACAAG-CAGAGGA AGT (exon 5/6), Rprimer GCACTAG GAAAAGCAGGAAAGC (exon 6), TaqMan probe: TGACCTGCATTACCGAA TCGCCC (exon 6); CRHR2alfa (soluble): Fprimer CCCATTTTGGATGACAAGGA GTA (exon 5/7), Rprimer GGATGAAGGTG-GTGATGAGGTT (exon 7), TaqMan probe TGCCTGCGGAATGTGATCCACTG (exon 7).

Amplification reactions were carried out in a volume of 25 μL, containing 12.5 μL of Taq-Man Universal Master Mix (Applied Biosystems, Foster City, CA), 1 μL of cDNA, 0.5 μL of each of the primers (10 mM solution), and 0.5 μL of probe (0.5 mM solution). The detection of each gene was carried out in a separate microtube. After initial denaturation at 95°C for 10 min, DNA was amplified in three-step cycles as follows: denaturation at 95°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 32 s.

DNA was amplified by PCR. For real-time PCR, we used the instrument Applied Biosystems RealTime system 7300 (ABI7300 instrument).

The results were calculated by the normalized expression ratio $2^{-\Delta\Delta Ct}$ by using the equation of Pfaffl,[14] where mRNA of tested genes is related to house-keeping gene GAPDH. Then the results of relative expression of individual genes were expressed as percent of controls.

Statistical Analysis

Results are presented as mean ± SEM for 5–7 animals. Statistical differences among groups were calculated by *t*-test or one-way ANOVA with post hoc tests (Newman-Keuls multiple comparison test). All statistical tests were conducted using the Systat 10 software (SPSS Inc., Chicago, IL). Statistical significance was accepted when $P \leq 0.05$.

Results

Using real-time PCR we quantified mRNA of CRH-R1, CRH-R2α-in, and CRH-R2α-so. Relative quantification of their expression was compared to mRNA expression of CREB and normalized to a reference gene GAPDH.

Figure 1 shows that the expression of CREB mRNA in AP decreased stepwise with time after both stresses. One-way ANOVA showed very strong differences between groups. The decrease of mRNA expression was larger in IMO+C than in IMO and in groups where the stress was followed by 1 h pause (IMO[2] and IMO+C[2]).

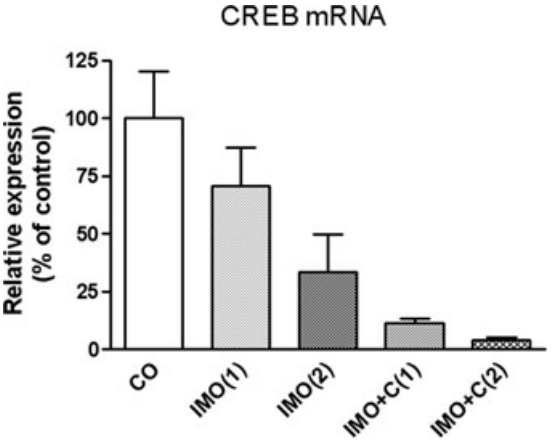

Figure 1. Effect of single 1-h long immobilization (IMO) and immobilization combined with immersionin water (IMO+C) on CREB mRNA relative expression in rats, estimated by real-time PCR (see Methods). Results are normalized relative to the housekeeping gene GAPDH. Rats in groups marked with (1) were decapitated immediately after stress, while (2) indicates animals that were decapitated 1 h after the end of the 1-h stress, that is, 2 h after the onset of stress exposure; this design has been used in previous behavioral tests. One-way ANOVA revealed very significant differences ($P < 0.001$) among the groups.

When the relative expression of CRH-R1 in controls was compared to the expression of mRNA of CRH-R2α- insoluble and -soluble, we found that the expression of the latter two receptors were at least 25 times lower than that of CRH-R1 ($P < 0.001$; $F = 147.8$) (Fig. 2).

Figure 3 demonstrates that the expression of mRNA of CRH-R1 is very sensitive to the effects of both stressors ($P < 0.001$; $F = 7.69$). When compared to controls, IMO significantly reduced this expression of CRH-R1 mRNA to 47% and 63% of the control level 1 h and 2 h after the onset of the stressor, respectively. IMO+C(1) reduced mRNA most potently to 36% ($P < 0.001$), while in the IMO+C(2) group no significant decrease from control was observed (87%).

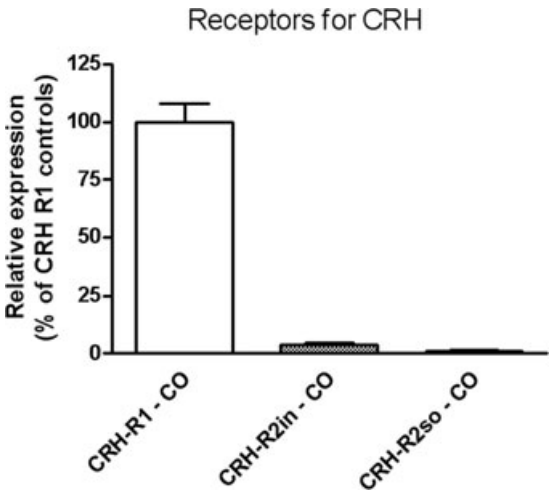

Figure 2. Relative control mRNA expression of receptor subtypes R1 and R2α, soluble and insoluble, estimated by real-time PCR. Results are normalized to the house-keeping gene GAPDH and expressed in percent of CRH-R1. One-way ANOVA revealed very significant differences among the groups (see text).

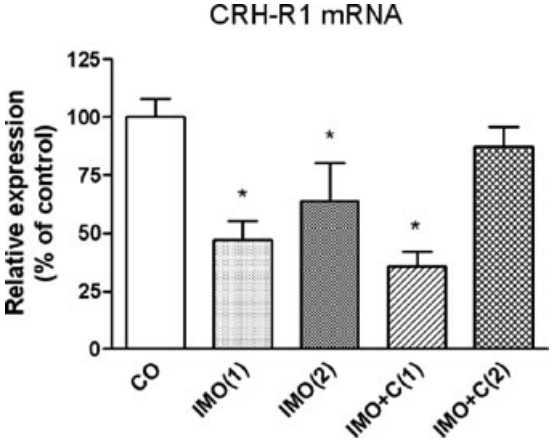

Figure 3. Effect of IMO and IMO+C on CRH-R1 mRNA relative expression. For explanation of abbreviations see text and Figure 1. One-way ANOVA revealed significant differences among groups (see text); *indicates significance versus controls at $P < 0.05$.

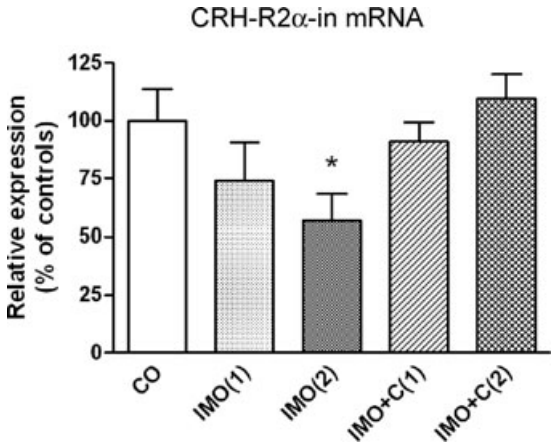

Figure 4. Effect of IMO and IMO+C on CRH-R2α-insoluble mRNA relative expression. For explanation of abbreviations see text and Figure 1. One-way ANOVA revealed significant differences among groups (see text); *indicates significance versus controls at $P < 0.05$.

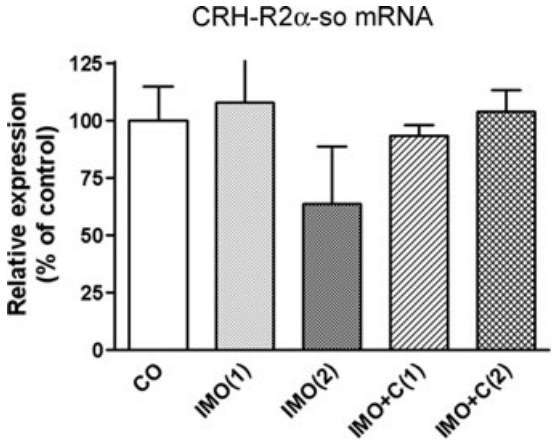

Figure 5. Effect of single 1-h long immobilization (IMO) and immobilization combined with immersion in water (IMO+C) on CRH-R2α-soluble mRNA relative expression (see Methods). Rats in groups marked with (1) were decapitated immediately after stress while (2) indicates animals that were decapitated 2 h after the onset of 1-h stress.

Figures 4 and 5 show the expression of mRNA of CRH-R2α-insoluble and -soluble, respective. For CHR-R2α-insoluble, the two-tailed P value for control versus IMO(2) was significant ($P < 0.05$; $t = 2.26$). Other changes were not significant. A similar trend was observed in the experiment with the expression of mRNA for CRH-R2α-so, but no statistically significant differences were found.

Discussion

CRH is a critical factor in the regulation of hypothalamo-pituitary-adrenal (HPA) axis

activity and is responsible for the release of ACTH from the anterior pituitary, where it acts through two receptor subtypes, CRH-R1 and CRH-R2.[15,16]

The stress response in AP is known to be mediated by CRH acting through CRH-R1, but the function of the CRH-R2 system in AP is still not fully elucidated. CRH-R2 mRNA has also been reported to be present in the rat AP.[17] Several studies have demonstrated the decrease of CRH-R2mRNA in this tissue after restraint stress and immune challenge (see Ref. 17). Since we are using two different immobilization stressors, IMO and IMO+C, which reveal strong differences in the behavioral responses[11,12] and which may differ in their emotional and physical components of action, we decided to test their effects on the expression of mRNA for CRH receptors subtypes. Since we know that the expression of early genes has significantly different time dynamics from the onset of the stressors,[18,19] we decided to test both IMO and IMO+C immediately after stress termination and after a 1 h pause, which corresponds to our experimental protocol used in many our behavioral studies.[11,12]

In this study we tested two different 1-h long stressors and observed the mRNA of CRH receptor subtypes 1 or 2 h after the onset of the application of stress. Since it was demonstrated that CREB protein may be involved in stress-induced CRH gene expression,[20] we decided to compare the time dynamics and stress effects on both CREB mRNA and CRH receptor subtypes mRNA responses to two different stressors. CREB mRNA expression was reduced more after IMO+C than after IMO alone, and more after longer interval from the onset of stressor. These results are in good agreement with the hypothesis that stress with an emotional component dominant (IMO) elicits a weaker response than restraint combined with the more physical stress of immersion in water, in which catecholamine release may also participate in the overall effects. Unlike the expression of c-fos, where the effect declines during the pause following the stress,[18] CREB mRNA continued to decline even when the stress was discontinued; this is situation, which we found i.e. in arc expression.[19]

Similarly to other authors,[17] we have found (Fig. 2) that under control conditions there is higher mRNA expression of CRH-R1 than the sum of CRH-R2α subtypes. It was demonstrated that CRH-R2α in the anterior pituitary is present in gonadotropes in the AP and might be involved in the regulation of gonadal functions under stress.[17]

The expression of CRH-R1 mRNA in AP was decreased nearly equally by IMO(1) and IMO+C(1), but stronger than in groups where the stress was followed by the 60 min pause (Fig. 3). Thus, the decrease of CRH-R1 mRNA expression has a different pattern than that for CREB where the effect of stressors is enhanced during the pause following stress application. Moreover, the effect of IMO+C(2) is weaker than that of IMO(2); under these conditions IMO+C(2) produced much stronger behavioral deteriorations than IMO(2).[11,12] Which factors, such as glucocorticoids, catecholamines, or others, influence these responses should be investigated in further studies.

From our studies it is evident that expression of mRNA of both CRH-R2α isoforms (soluble and insoluble) was decreased only in the IMO(2) group. This decrease is in accordance with the findings of others.[17] The reason why expression of CRH-R2α mRNA is relatively stable is not yet known.

The present study demonstrated that exposure to IMO and IMO+C stressors did not change expression of CRH-R2α mRNA, in contrast to the very significant response of CRH-R1 mRNA to IMO and IMO+C. Although the CRH-R1 is essential for ACTH responses to stress, the changes in expression of CRH-R1 mRNA do not correlate with the behavioral actions of the stressors used in this experiment. This finding is in accordance with the conclusion of Aguilera and colleagues[15] that the translation of receptors for CRH is more important for CRH function than the gene expression alone. Moreover, it is likely that

post-transcriptional regulatory mechanisms are the most important factors in CRH receptor activity.[15]

Acknowledgments

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Conflicts of Interest

The authors declare no conflicts of interest.

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