



Treadmill exercise does not change gene expression of adrenal catecholamine biosynthetic enzymes in chronically stressed rats

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ABSTRACT

Chronic isolation of adult animals represents a form of psychological stress that produces sympatho-adrenomedullary activation. Exercise training acts as an important modulator of sympatho-adrenomedullary system. This study aimed to investigate physical exercise-related changes in gene expression of catecholamine biosynthetic enzymes (tyrosine hydroxylase, dopamine- β -hydroxylase and phenylethanolamine N-methyltransferase) and cyclic adenosine monophosphate response element-binding (CREB) in the adrenal medulla, concentrations of catecholamines and corticosterone (CORT) in the plasma and the weight of adrenal glands of chronically psychosocially stressed adult rats exposed daily to 20 min treadmill running for 12 weeks. Also, we examined how additional acute immobilization stress changes the mentioned parameters.

Treadmill running did not result in modulation of gene expression of catecholamine synthesizing enzymes and it decreased the level of CREB mRNA in the adrenal medulla of chronically psychosocially stressed adult rats. The potentially negative physiological adaptations after treadmill running were recorded as increased concentrations of catecholamines and decreased morning CORT concentration in the plasma, as well as the adrenal gland hypertrophy of chronically psychosocially stressed rats. The additional acute immobilization stress increases gene expression of catecholamine biosynthetic enzymes in the adrenal medulla, as well as catecholamines and CORT levels in the plasma.

Treadmill exercise does not change the activity of sympatho-adrenomedullary system of chronically psychosocially stressed rats.

Key words: adrenal medulla, acute immobilization stress, catecholamine, chronic social isolation, gene expression, treadmill exercise.

INTRODUCTION

A number of diseases and pathological conditions are related to the long-term adaptive response to stress, particularly under conditions of chronic stress when allostasis can shift from a healthy toward a pathological state. Chronic individual housing of rats, frequently termed “isolation stress”, represents a

very strong psychosocial stress (Bartolomucci et al. 2003, Vöikar et al. 2005). It is known that social isolation is a risk factor for human depression (Ishida et al. 2003). The literature data indicate that exercise training reduces the risk of developing diseases related to chronic stress. In humans, regular exercise has a beneficial impact on depression (Dunn et al. 2005). Changes in catecholamine biosynthesis represent one of the mechanisms by which exercise training

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may diminish circulating catecholamine levels. The exercise training lowers plasma noradrenaline (NA) concentration in elderly patients with elevated baseline levels (Seals et al. 1994). Fleshner (2000) found that 4 weeks of freewheel running attenuates the elevation of plasma noradrenaline produced by acute exposure to inescapable tail shock stress. Although a number of markers are frequently used to assess the involvement of the sympatho-adrenal response (plasma and tissue noradrenaline and adrenaline levels), it is important to examine more specific variables such as gene expression of key enzymes involved in catecholamine biosynthesis. Tyrosine hydroxylase (TH), as the “rate limiting” enzyme in the biosynthesis of catecholamines, is localized in all cells that produce catecholamines (Kvetnansky et al. 2004). Dopamine- β -hydroxylase (DBH) is another important catecholamine biosynthetic enzyme that converts dopamine (DA) into NA. During repeated immobilization stress DBH may become “rate limiting” when dopamine formation is markedly accelerated (Kvetnansky et al. 1971). In addition, Scatton et al. (1984) showed that under conditions of neural activation DBH becomes “rate limiting” enzyme. Phenyl ethanolamine N-methyltransferase (PNMT) is considered as the “rate limiting” enzyme for the synthesis of adrenaline (A) (Kvetnansky et al. 2004).

Our earlier studies showed that long-term social isolation (12 weeks) of adult rat males produced a decreased gene expression of catecholamine biosynthetic enzymes in the adrenal medulla (Gavrilović et al. 2008), and increased concentrations of catecholamines in the plasma (Gavrilović et al. 2010). In this study we wanted to investigate whether treadmill exercise changed the activity of sympatho-adrenomedullary system of chronically psychosocially stressed rats. We applied a combined model of chronic social isolation and treadmill running (CSITR) in rats. One of the key questions in stress research is how the same stressor can elicit a variant or altered response depending on prior experience with the current or different stressor. Immobilization

is frequently used as an additional acute stressor and considered as one of the most intensive stressors that significantly changes gene expression (Kvetnansky et al. 2009). Our previous data showed that exposure of socially isolated rats to additional immobilization produced exaggerated responses in gene expression of catecholamine biosynthetic enzymes in the adrenal medulla (Gavrilović et al. 2008). The response to novel additional acute immobilization stress might reveal the detailed mechanisms underlying the gene expression of catecholamine biosynthetic enzymes in the adrenal medulla in specific stress conditions.

This study aimed to investigate physical exercise-related changes in gene expression of catecholamine biosynthetic enzymes tyrosine (TH, DBH and PNMT) and expression of cAMP response element-binding protein (CREB) in the adrenal medulla, concentrations of catecholamines and morning corticosterone (CORT) in the plasma and the weight of adrenal glands of chronically psychosocially stressed adult rats exposed daily to 20 min treadmill exercise for 12 weeks. In addition, we examined the impact of the additional acute immobilization stress (CSITR+IMM) on the mRNA and protein levels of catecholamine biosynthetic enzymes in the adrenal medulla, as well as concentrations of NA, A and CORT in the plasma of rats. Levels of CORT were measured in the morning because previous findings had shown that light is capable of eliciting a rapid CORT response in rats (Mohawk et al. 2007), humans (Leproult et al. 2001) and mice (Ishida et al. 2005).

Detecting regulatory physiological mechanism for catecholamine synthesis in the adrenal medulla in conditions provoked by prolonged social isolation stress and physical activity is extremely important in the prevention of diseases caused by chronic stress in sports medicine and pathophysiology.

MATERIALS AND METHODS

ANIMALS AND STRESS MODELS

In this study Wistar male rats 11-week-old were used. Animals were under standard laboratory conditions

with water and food *ad libitum* and kept three to four per cage. The care was taken to minimize the pain and discomfort of the animals according to the recommendations of the Ethical Committee of the Vinča Institute of Nuclear Sciences, Belgrade, Serbia, which are in accordance with the Guide for Care and Use of Laboratory Animals of the National Institute of Health, Bethesda, MD, U.S.A. The experiment is complied with the current laws of the Republic of Serbia.

Animals were divided into four groups. The **control group** (n = 10) was not exposed to any treatment. **CSITR group** (n = 10) consisted of animals exposed to treatment of chronic combined social isolation and treadmill running. CSITR treatment was achieved by exposing the individually housed rats to the daily treadmill running for a period of 12 weeks. The duration and speed of running was gradually increased from week to week, from the initial 10 minutes-10m/min up to 20 minutes-20m/min at 0° incline. The treadmill training protocol used in this study involves a gradual increase in running intensity and is commonly used in the similar studies (Tümer et al. 2001, Erdem et al. 2002). **IMM group** (n = 40) consisted of animals exposed to acute stress immobilization, for a period of 2 hours. Immobilization stress was provoked as described by Kvetnansky and Mikulaj (1970). **CSITR+IMM group** (n = 40) consisted of animals exposed to CSITR treatment for a period of 12 weeks and, after chronic treatment, these animals were exposed to additional acute IMM stress for a period of 2 hours. Groups IMM and CSITR+IMM contained 40 animals. These groups were divided into four subgroups (n = 10), as we examined changes in catecholamine biosynthetic enzymes in four different time periods after the cessation of immobilization. The animals were sacrificed after CSITR treatment, immediately after the cessation of acute immobilization and 3, 6 and 22 hours after the acute immobilization. Literature data show that in these periods, changes in gene expression of

catecholamine biosynthetic enzymes in the adrenal medulla are expected (Kvetnansky et al. 2009, Wong and Tank 2007). Samples of blood were collected and both adrenal glands were isolated and weighed. Separation of cortex and medulla was performed as previously described (Fliedner et al. 2010). The tissue was kept frozen on dry ice at all times. The adrenals were cut centrally, perpendicular to the longest axis. The surface cut was visually inspected for the presence of adrenal medulla. The border between the cortex and medulla was easily identified by the red/brown color of the cortical zona reticularis. The tissue was again cut perpendicular to the longest axis at a distance of 1 cm from the first cut, until an area with a wider section of medulla was found. The tissues were examined under the dissection microscope, and the gray/pink medulla was carefully cut out of the surrounding cortex with size 11 scalpel blades. The adrenal medullas were stored in liquid nitrogen until analyzed. The morning CORT was measured to determine whether CSITR affects the diurnal rhythm of CORT in this experiment.

RNA ISOLATION AND CDNA SYNTHESIS

Total RNAs were isolated using TRIZOL reagent (Invitrogen, USA). After the isolation of mRNA, DNA-ase treatment was applied with DNase I (Fermentas, Lithuania). Concentration of total mRNA was measured in triplicates on a spectrophotometer. Quality of mRNA was checked on agarose gel. Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Bead (Amersham Biosciences, UK) and pd(N)₆ Random Hexamer (Amersham Biosciences, UK) primer according to the manufacturer's protocol.

REAL-TIME RT-PCR

TaqMan PCR assays were carried out using Assay-on-Demand Gene Expression Products (Applied Biosystems, USA) for TH (Rn00562500_m1), DBH (Rn00565819_m1), PNMT (Rn01495589_g1) and

CREB (Rn01441386_g1). The gene expression assays contained primers for amplification of the target gene and the TaqMan MGB (Minor Groove Binder) probe 6-FAM dye-labeled for the quantification. Reactions were performed in a 25 μ L reaction mixture containing 1x TaqMan Universal Master Mix with AmpErase UNG, 1x Assay Mix (Applied Biosystems, USA) and cDNA template (10 ng of RNA converted to cDNA). PCR was carried out in the ABI Prism 7000 Sequence Detection System at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The experimental threshold was calculated based on the mean baseline fluorescence signal from cycle 3 to 15 plus 10 standard deviations. Each sample was operated in triplicates and the mean value of each Ct triplicate was used for further calculations. The reference gene (endogenous control) was included in each analysis to correct for the differences in the inter-assay amplification efficiency and all transcripts were normalized to cyclophyline A (Rn00690933_m1) expression. The reaction mixture for endogenous control gene amplification consisted of 1x TaqMan Universal Master Mix with AmpErase UNG (Applied Biosystems, USA), 1x Assay (6-FAM dye-labeled MGB probes) and cDNA (10 ng of RNA converted to cDNA). The levels of expression of cyclophyline A in samples under different treatments were checked by additional experiments that confirmed that the chosen reference gene was not regulated. Before quantification, validation experiments were performed to determine the similar amplification efficiency of endogenous control and each target gene. We tested cyclophyline A and demonstrated that its efficiency of amplification was approximately equal to all assays used for target genes. Briefly, serial dilutions of cDNA were prepared and amplified by real-time PCR using specific primers and fluorogenic probes for target and endogenous control gene.

Quantification was done using the $2^{-\Delta\Delta Ct}$ method according to Livak and Schmittgen (2001). The results obtained were analyzed by the RQ Study Add On software for 7000 v 1.1 SDS instrument

(ABI Prism Sequence Detection System, Applied Biosystems, USA) with a confidence level of 95% ($P < 0.05$). The relative expression of the target gene was normalized to cyclophyline A and expressed in relation to the calibrator, i.e. the control sample. Due to individual differences among animals, one sample from control group with the expression value closest to the mean of all samples in this group and with the lowest measurement error was chosen as a calibrator. The results are reported as a fold change relative to the calibrator and normalized to cyclophyline A using the equation: $N_{\text{sample}} = 2^{-\Delta\Delta Ct}$.

WESTERN BLOT ANALYSIS

The adrenal medullas were homogenized in 0.05 M sodium phosphate buffer (pH 6.65). Subsequently, the protein concentration was determined using BCA method (Pierce, USA), described by Stich (1990). The samples were boiled in denaturing buffer according to Laemmli (1970), for 5 min at 95°C. Fifteen micrograms of protein extract from adrenal medulla were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to a supported nitrocellulose membrane (Hybond™ C Extra, Amersham Biosciences, UK). The membrane was blocked in 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST). All following washes and antibody incubations were also carried out in TBST at room temperature on a shaker. The antibody incubation time was 1 hour. Protein molecular mass standards (PageRuler™ Plus Prestained Protein Ladder, Fermentas) were used for calibration. Antibodies used for quantification of specific proteins were as follows: for TH the monoclonal primary antibody against mouse TH (monoclonal antibody against TH from mouse-mouse hybrid cells, clone 2/40/15, dilution 1:5000, Chemicon International, USA); for DBH the anti-dopamine- β hydroxylase (N-terminal) antibody, sheep (dilution 1:5000, Sigma, USA); for PNMT the polyclonal ant-PNMT primary antibody, rabbit (dilution 1:1000, Protos Biotech Corporation, USA); and for β -actin the rabbit

polyclonal anti- β -actin (ab8227, dilution 1:5000, Abcam, USA). After washing, the membranes were incubated in the secondary anti-mouse, anti-rabbit (dilution 1:5000, Amersham ECLTM Western Blotting Analysis System, UK) and anti-sheep (dilution 1:5000, Calbiochem, Germany) antibodies conjugated to horseradish peroxidase. A secondary antibody was then visualized by the Western blotting enhanced chemiluminiscent detection system (ECL, Amersham Biosciences, UK). The membranes were exposed to ECL film (Amersham Biosciences, UK). Densitometry of protein bands on ECL film was performed by Image J analysis PC software. The result was expressed in arbitrary units normalized in relation to β actin.

CATECHOLAMINE AND CORT MEASUREMENTS

Plasma catecholamines were measured by a standard radioenzymatic assay described previously by Peuler and Johnson (1977) and the values were expressed as pg/mL plasma. Catecholamines present in the plasma aliquots were converted to their labeled O-methylated derivatives by S-(3H) adenosylmethionine (Lacomed, Czech Republic) and the lyophilized catechol-O-methyl transferase isolated from the rat liver. The O-methylated derivatives of the amines were then extracted along with unlabeled carrier compounds.

Plasma CORT was measured upon prior extraction directly, using RIA commercial kits (MP Biomedicals, Germany) and the values were expressed as ng CORT/mL plasma.

DATA ANALYSIS

The data are presented as means \pm S.E.M. Differences of gene expression (mRNA and protein levels) of catecholamine biosynthetic enzymes (TH, DBH, PNMT) and level of CREB mRNA in the adrenal medulla and concentration of NA, A and CORT in the plasma, as well as weight of adrenal gland were analyzed by One-way ANOVA. The effects of chronic social isolation and treadmill running (CSITR) and

acute immobilization stress (IMM) compared to control, as well as the effects of additional acute immobilization stress after chronic social isolation and treadmill running (CSITR+IMM) compared to chronic social isolation and treadmill running (CSITR), were tested by Tukey post-hoc test.

The correlation between PNMT protein level and A level of animals exposed to CSITR was analyzed by the Spearman test, using the Sigma Plot v10.0 (with SigmaStat integration).

Statistical significance (p) was set to 0.05, statistical power ($1-\beta$) exceeded 82%. Statistical power confirms that the number of animals ($n = 10$) was sufficient for this experiment. Reliability test was designed so we did three repeated measurements of the level of gene expression of TH, DBH, PNMT and CREB. The calculated value of the ICCR test of >0.80 was considered to be satisfactory and it proves the reliability of the applied methods. Statistical analysis was carried out using the SPSS.

RESULTS

One-way ANOVA analysis revealed significant changes of TH ($F = 10.19$; $p < 0.01$), DBH ($F = 7.22$; $p < 0.05$), PNMT ($F = 11.78$; $p < 0.05$), CREB ($F = 12.29$; $p < 0.01$) mRNA levels, and TH ($F = 8.18$; $p < 0.01$), DBH ($F = 7.12$; $p < 0.05$), PNMT ($F = 16.20$; $p < 0.01$) protein levels in the adrenal medulla, as well as NA ($F = 19.28$; $p < 0.01$), A ($F = 21.7$; $p < 0.01$) and CORT ($F = 24.7$; $p < 0.01$) plasma concentrations and the weight of adrenal glands ($F = 11.07$; $p < 0.05$) under examined treatments.

CHANGES IN THE PLASMA CONCENTRATIONS OF NA, A, CORT AND THE WEIGHT OF ADRENAL GLANDS

CSITR treatment

CSITR treatment significantly increased the plasma concentrations of NA by 78% ($p < 0.01$, Tukey test, Fig. 1a) and A by 87% ($p < 0.01$, Tukey test, Fig. 1b), decreased CORT concentration by 73% ($p <$

0.01, Tukey test, Fig. 1c) and increased weight of adrenal glands by 26% ($p < 0.05$, Tukey test, Fig. 1d), compared with control animals.

IMM and CSITR+IMM treatments

The exposure of the control animals to acute immobilization stress significantly increased NA concentration by 185% ($p < 0.01$, Tukey test, Fig. 1a) and A concentration by 112% ($p < 0.01$, Tukey test, Fig. 1b), while the additional acute immobilization of CSITR animals increased NA concentration by 20% ($p < 0.05$, Tukey test, Fig. 1a) and A concentration by 20% ($p < 0.05$, Tukey test Fig. 1b) 3 hours after the cessation of immobilization. After 22 hours, the plasma NA in the control group was reduced by 40% when comparing to 3 hours. However, after 22 hours the NA plasma level in CSITR group was higher by 26% when compared to 3 hours. Also, we found that

acute IMM increased CORT concentration by 66% ($p < 0.05$, Tukey test, Fig. 1c), while the exposure of CSITR animals to additional acute immobilization stress led to increased CORT concentration by 212% ($p < 0.01$, Tukey test, Fig. 1c) in the plasma immediately after the cessation of immobilization. After 3 hours, the plasma CORT in the control group returned to the basal value, while in CSITR group was reduced by 40% when comparing to the value immediately after the cessation of immobilization.

CHANGES OF THE TH, DBH, PNMT AND CREB MRNA LEVELS IN THE ADRENAL MEDULLA

CSITR treatment

The animals exposed to CSITR showed a decreased levels of TH mRNA by 10% ($p < 0.05$, Tukey test, Fig. 2a), DBH mRNA by 11% ($p <$

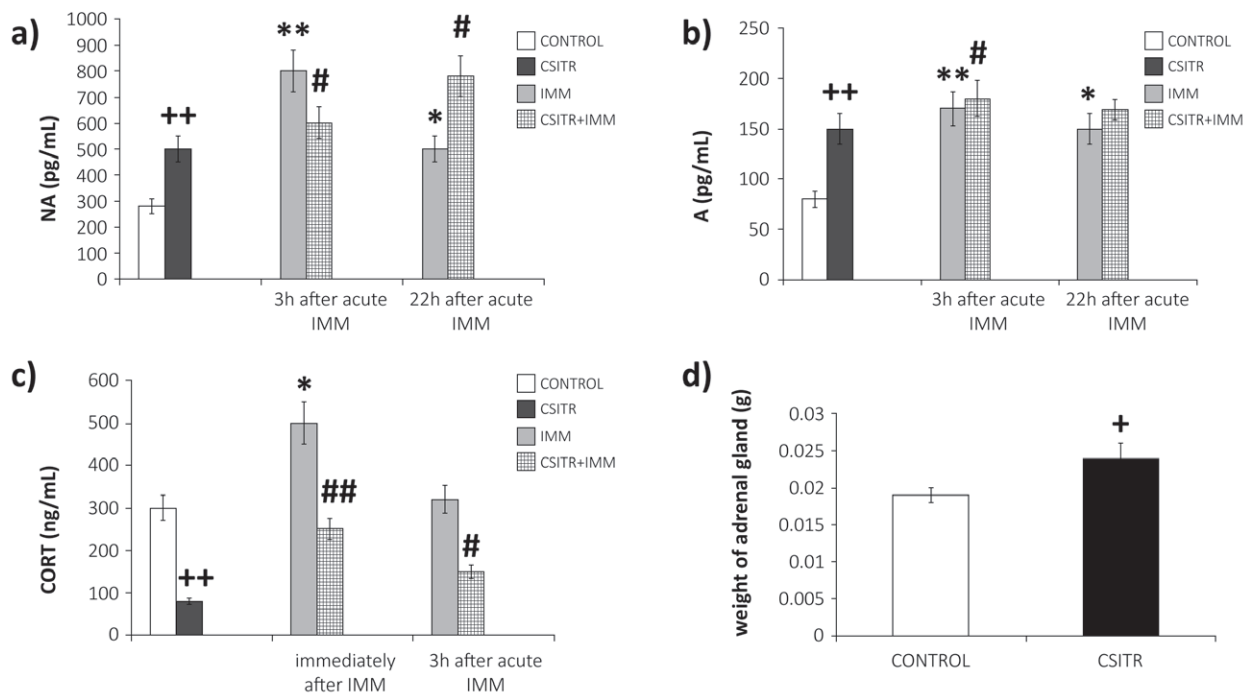


Figure 1 - Effects of chronic social isolation and treadmill running and additional acute immobilization stress on the concentration of noradrenaline (NA) [a], adrenaline (A) [b] and corticosterone (CORT) [c] in the plasma and the weight of adrenal gland [d]. The values are means \pm S.E.M. of 10 rats. Statistical significance: + $p < 0.05$, ++ $p < 0.01$ animals exposed to chronic social isolation and treadmill running vs. control animals (Tukey test); * $p < 0.05$, ** $p < 0.01$ animals exposed to acute 2h immobilization vs. control animals (Tukey test); # $p < 0.05$, ## $p < 0.01$ animals exposed to additional acute 2h-immobilization stress after chronic social isolation and treadmill running vs. animals exposed to chronic social isolation and treadmill running (Tukey test).

0.05, Tukey test, Fig. 2b), PNMT mRNA by 20% ($p < 0.05$, Tukey test, Fig. 2c) and CREB mRNA by 50% ($p < 0.01$, Tukey test, Fig. 2d), compared with control animals.

IMM and CSITR+IMM treatments

IMM treatment significantly increased the level of TH mRNA by 110% ($p < 0.01$, Tukey test) 3 hours after the cessation of immobilization and by 100% ($p < 0.01$, Tukey test) 6 hours after the cessation of immobilization, compared to the control group (Fig. 2a). The additional acute immobilization of CSITR animals increased mRNA levels of TH by 111% ($p < 0.01$, Tukey test, Fig. 2a), DBH by 46% ($p < 0.05$, Tukey test, Fig. 2b) 3 hours after the cessation of immobilization and PNMT by 14% ($p < 0.05$,

Tukey test, Fig. 2c) 6 hours after the cessation of immobilization, compared to the CSITR group.

CHANGES OF THE TH, DBH AND PNMT PROTEIN LEVELS IN THE ADRENAL MEDULLA

CSITR treatment

CSITR provoked a decreased protein levels of TH by 64% ($p < 0.01$, Tukey test, Fig. 3a) and of DBH by 20% ($p < 0.05$, Tukey test, Fig. 3b); however, it induced the increase of PNMT protein level by 18% ($p < 0.05$, Tukey test, Fig. 3c), compared with the controls. The significant positive correlation was found between the levels of PNMT protein in the adrenal medulla and A concentration in the plasma of animals exposed to CSITR (Spearman $\rho = 0.904$, $P < 0.0005$, Fig. 4).

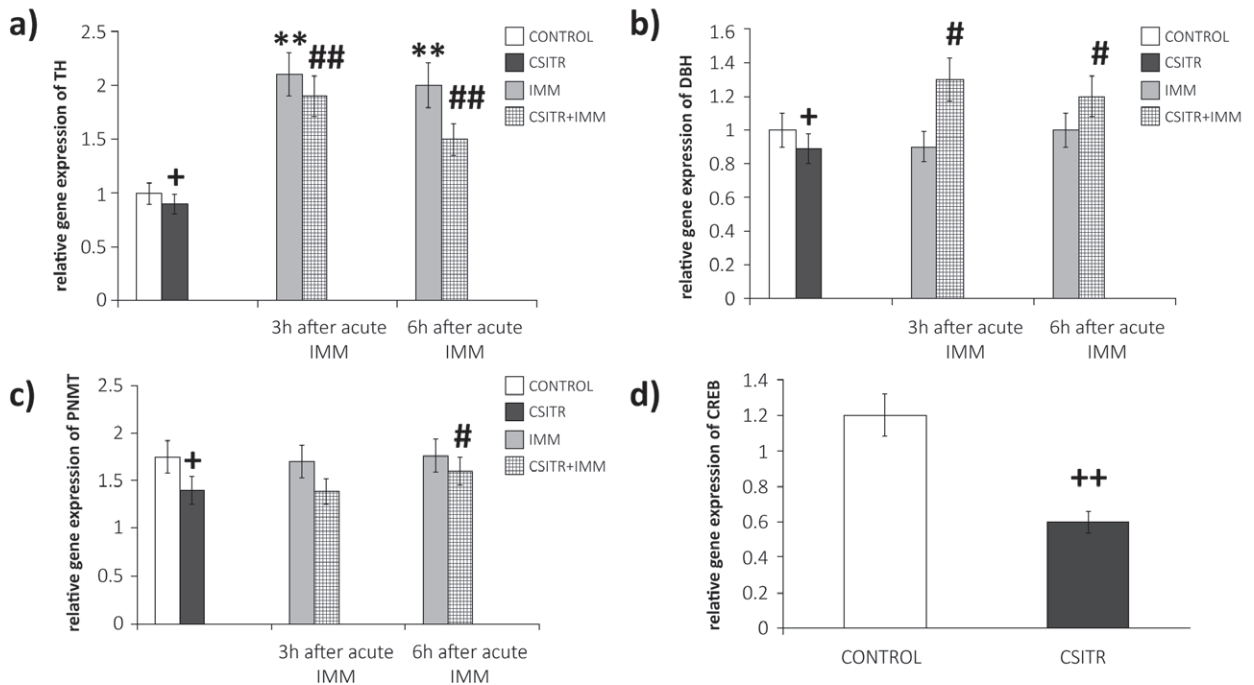


Figure 2 - Effects of chronic social isolation and treadmill running and additional acute immobilization stress on tyrosine hydroxylase (TH) [a], dopamine-β-hydroxylase (DBH) [b], phenylethanolamine N-methyltransferase (PNMT) [c] and cAMP response element-binding (CREB) [d] mRNA levels in the adrenal medulla. The values are means ± S.E.M. of 10 rats. Statistical significance: + $p < 0.05$, ++ $p < 0.01$ animals exposed to chronic social isolation and treadmill running vs. control animals (Tukey test); ** $p < 0.01$ animals exposed to acute 2h immobilization vs. control animals (Tukey test); # $p < 0.05$, ## $p < 0.01$ animals exposed to additional acute 2h immobilization stress after chronic social isolation and treadmill running vs. animals exposed to chronic social isolation and treadmill running (Tukey test). The final result was expressed as fold change relative to the calibrator and normalized to cyclophylina A.

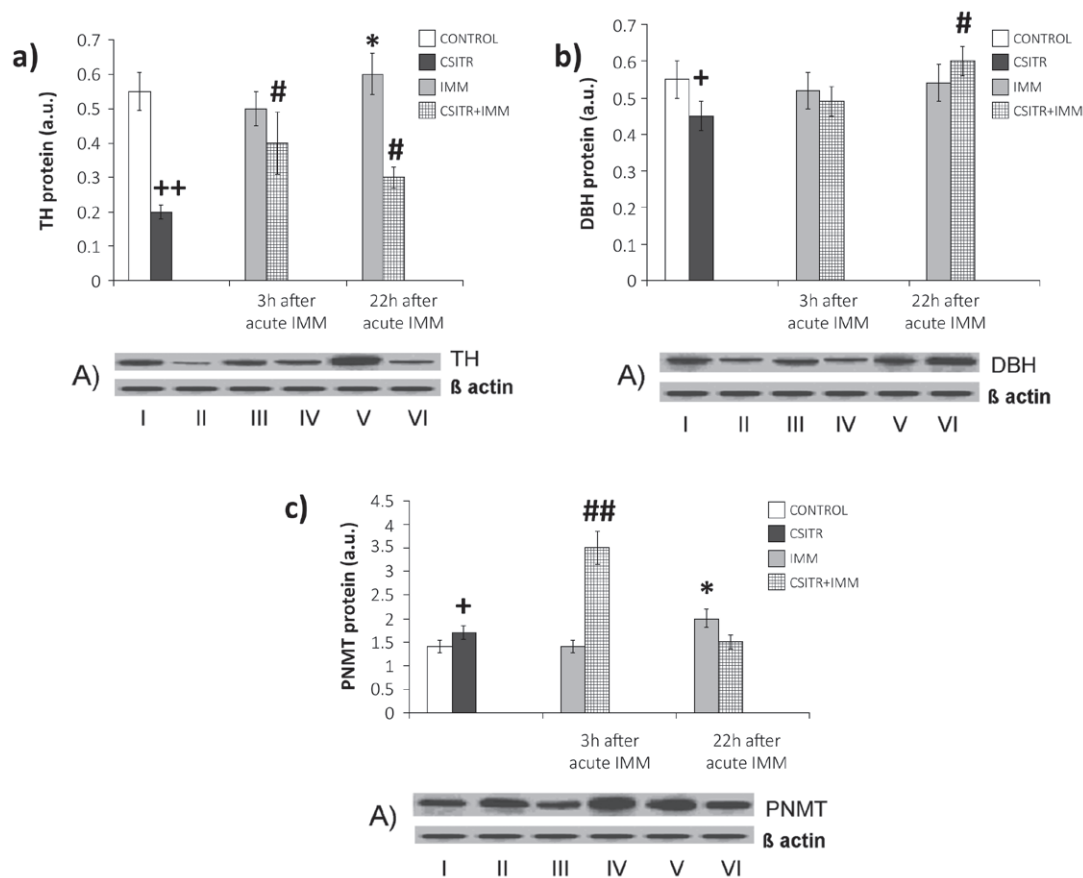


Figure 3 - Effects of chronic social isolation and treadmill running and additional acute immobilization stress on tyrosine hydroxylase (TH) [a], dopamine- β -hydroxylase (DBH) [b] and phenylethanolamine N-methyltransferase (PNMT) [c] protein levels in the adrenal medulla. The values are means \pm S.E.M. of 10 rats. Statistical significance: + $p < 0.05$, ++ $p < 0.01$ animals exposed to chronic social isolation and treadmill running vs. control animals; (Tukey test); * $p < 0.05$ animals exposed to acute 2h immobilization vs. control animals (Tukey test); # $p < 0.05$, ## $p < 0.01$ animals exposed to additional acute 2h immobilization stress after chronic social isolation and treadmill running vs. animals exposed to chronic social isolation and treadmill running (Tukey test). The result was expressed in arbitrary units normalized in relation to β actin.

(A) Distribution of TH, DBH, PNMT and β -actin proteins in the adrenal medulla of control animals [I], animals exposed to CSITR [II], animals exposed to IMM (3h after immobilization) [III], animals exposed to CSITR+IMM (3h after immobilization) [IV], animals exposed to IMM (22h after immobilization) [V] and animals exposed to CSITR+IMM (22h after immobilization) [VI].

IMM and CSITR+IMM treatments

Acute IMM increased the protein levels of TH by 10% ($p < 0.05$, Tukey test, Fig. 3a) and PNMT by 42% ($p < 0.05$, Tukey test, Fig. 3c) 22 hours after the cessation of immobilization, while the additional exposure of CSITR animals to acute immobilization stress led to increased protein levels of TH by 100% ($p < 0.05$, Tukey test, Fig. 3a), and PNMT by 133% ($p < 0.01$, Tukey test Fig.

3c) 3 hours after the cessation of immobilization and DBH by 33% ($p < 0.05$, Tukey test, Fig. 3b) 22 hours after the cessation of immobilization.

DISCUSSION

CHANGES IN THE PLASMA CONCENTRATIONS OF NA, A AND CORT

When the sympathoadrenal system is activated repeatedly over a long period of time, the response is

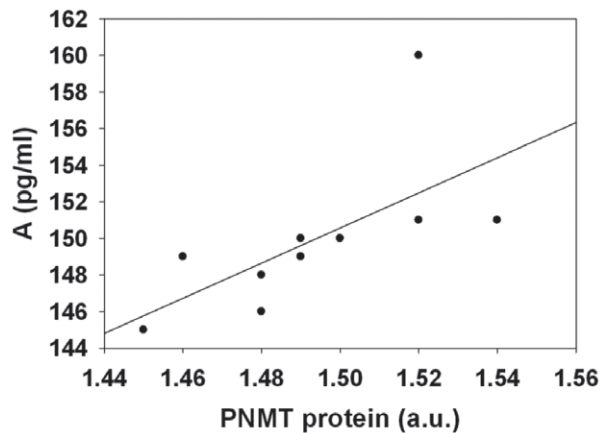


Figure 4 - The correlation between PNMT protein level in the adrenal medulla and A level in the plasma of animals exposed to chronic social isolation and treadmill running (Spearman).

not only adaptive, but also maladaptive (Micutkova et al. 2003). It is known that catecholamine hyperactivity and glucocorticoid dysregulation are the biological consequences of chronic stress. In our previous study (Gavrilović et al. 2010), we found that long-term isolation stress caused the increased concentration of catecholamines in the plasma. Although we expected positive adaptations in this study, we observed that CSITR produced adaptive physiological changes, indicating chronic stress. The potentially negative physiological adaptations after CSITR were recorded as increased concentration of catecholamines and decreased morning CORT concentration in the plasma, as well as the adrenal gland hypertrophy. Treadmill running does not change the activity of sympatho-adrenomedullary system of chronically psychosocially stressed rats. According to the literature data, treadmill running is forced exercise and it is a combination of hard physical and psychological stressors. Moraska et al. (2000) found that the treadmill training for a period of 8 weeks may cause physiological adaptations indicative of chronic stress. Also, the treadmill running stimulated concomitantly peripheral catecholamines secretion and central noradrenergic activity, i.e. NA turnover and release (Pagliari

and Peyrin 1995). In this study, we observed that CSITR provoked a decreased morning CORT concentration in the plasma. Zarković et al. (2003) showed that chronic stress is associated with a transient suppression of the HPA axis, manifested by the lower morning cortisol and the reduced adrenal cortisol response to ACTH stimulation. This HPA response pattern is manifested by decreased morning plasma or urinary cortisol, especially in the subjects with post-traumatic stress disorder (PTSD) (Yehuda et al. 2002). It is interesting to note that reduced concentration of CORT in the plasma was recorded in depressed conditions (Malkesman et al. 2006). Adrenal hypertrophy found in our experiments may be interpreted as the consequence of stress conditions. The weight of the adrenal gland may be a reliable criterion of the experienced chronic stress and may serve as the evidence of depressive conditions (Westenbroek et al. 2003). Hypertrophy of the adrenal glands has also been found in depressed patients (Nemeroff et al. 1992), indicating that adrenal size provides a good measure of the stress perception over periods of time.

Response to the acute IMM stress is characterized by the activation of the sympatho-adrenomedullary system and the HPA axis. Our results show that the acute IMM stressor triggers an exaggerated elevation of the plasma catecholamines. However, heterotypic novel additional acute IMM stressor does elevate the plasma catecholamines but not excessively in the animals previously exposed to CSITR. This finding might be explained by the quality and especially intensity of the stressor used. Immobilization is considered as one of the most intensive stressors. The novel stressors elicit exaggerated responses in prestressed animals, when the novel stressor is of equal or greater intensity or duration and/or it is repeated (Kvetnansky et al. 2009). In this work, animals exposed to CSITR are already prepared to manage the new situation evoked by a novel stressor and the exaggerated response is not necessary. Our results, together with

above mentioned data, show that the CSITR induces adaptations that are indicative of chronic stress.

CHANGES OF THE TH, DBH AND PNMT GENE EXPRESSION IN THE ADRENAL MEDULLA

In our previous study we showed that long-term isolation had induced a reduction gene expression of catecholamine biosynthetic enzymes in the adrenal medulla (Gavrilović et al. 2008). Also, in the present study we have found that the CSITR has induced a reduction of TH and DBH mRNAs and protein levels in the adrenal medulla. Treadmill exercise did not result in further modulation of gene expression of catecholamine synthesizing enzymes in the adrenal medulla of chronically psychosocially stressed adult rats. In the adrenal medulla, the DNA-binding activities of AP-1 and CREB play a major role in regulating the expression of TH and DBH genes during forced exercise (Erdős et al. 2007). Transcription factor CREB may be important in establishing the stress-induced patterns of gene expression. For these reasons, we analyzed the transcription factor involved in the down regulation of TH and DBH gene expression. Our results demonstrate that reduced level of CREB mRNA coincide with the reduced TH and DBH mRNA levels. Many authors have confirmed that chronic stress is associated with the reduction of phospho-CREB expression. Trentani et al. (2002) showed that in male rats chronically exposed to a mild electrostimulation, phospho-CREB expression was reduced, especially in the subcortical and cortical region. Wang et al. (2006) observed that chronic stress significantly reduces the expression of cAMP dependent kinase A (PKA) and phospho-CREB in the hippocampus of rats. However, in stressed rats treated with fluoxetine, the expression of phospho-CREB was significantly increased, indicating that chronic stress can affect the PKA and phospho-CREB expression and that the antidepressant is an antagonist. In addition to that, the decreased TH and DBH gene expression may be the consequence

of the adrenomedullary cell desensitization. The excessive stimulation by chronic stress could provoke the adrenomedullary cell desensitization and depress the catecholamine synthesis pathway. It is known that the glucocorticoids regulate the expression and enzyme activity of TH and DBH genes (Núñez et al. 2009, Hwang and Joh 1993, McMahon and Sabban 1992). We found that CSITR induces the decrease of CORT concentration in the plasma, which coincides with the decrease of TH and DBH mRNA levels in the adrenal medulla.

A significant result in this work is that CSITR does not affect *de novo* synthesis of TH enzyme, but increases the concentration of NA in the plasma. Many factors can affect the activity of TH enzyme without changing its expression. One of the answers may be in the intracellular level of tetrahydrobiopterin, which can be altered by stress and sympathetic nervous activity and thus, it may affect the activity of TH without changing the level of the enzyme (Baruchin et al. 1990). Many studies have shown that noncholinergic neurotransmitters affect the biosynthesis of catecholamines. Bobrovskaya et al. (2007) observed that the amount of TH enzyme is regulated by pituitary adenylate cyclase-activating peptide (PACAP). Specifically, prolonged activation of TH enzyme resulting from phosphorylation of TH at Ser 40 can maintain the synthesis of catecholamine without synthesis of TH enzyme. Although expression of TH and DBH gene is decreased in the adrenal medulla after CSITR treatment regimes, this treatment may lead to continuous increased biosynthesis of NA as well as increased releasing of NA in plasma, which might represent an adaptation on applied stress regime. Also, it is important that the sympathetic nervous system (stellate ganglia) may be a source of NA in the circulation after chronic stress. In our previous work (Gavrilović et al. 2009), we found that chronic stress causes the increase of TH and DBH gene expressions in stellate ganglia. Increased synthesis of TH and DBH enzymes in

stellate ganglia causes the increase in NA plasma levels, which is in accordance with the reports of Sabban et al. (2004).

Glucocorticoids are important regulators of PNMT gene expression. In this work, we observed that CSITR induces the decrease of CORT concentration in the plasma, coinciding with the decrease of PNMT mRNA level in the adrenal medulla. Studies on the hypophysectomised rats have shown that reduced amounts of corticosteroids cause the reduction of level of PNMT mRNA level (Evinger et al. 1992, Wong et al. 1995, Krizanova et al. 2001). A significant result in this study is that CSITR induces the increase of PNMT protein level in the adrenal medulla, with the consequent increase of A level in the plasma. We found a significant positive correlation between the levels of PNMT protein in the adrenal medulla and A in the plasma of the animals exposed to CSITR. It is interesting to note that although PNMT mRNA is decreased after CSITR treatment regimes, this treatment may lead to continuous accumulation of its proteins as an adaptation on applied stress regime. This adaptive response is necessary to maintain the A biosynthetic capacity in the adrenal medulla during periods of sustained A secretion. Long term stress induces appropriate translational mechanisms (Xu et al. 2007). During CSITR treatment the increased synthesis of PNMT protein affects the sustained increase of A secretion. Our results confirm that the CSITR shows adaptations that are indicative of chronic stress.

In this study, we showed that acute immobilization stress induces the increase of TH and PNMT protein levels in the adrenal medulla and levels of catecholamines in the plasma. However, acute immobilization does not change the levels of DBH and PNMT mRNAs, as well as the protein levels of DBH. Nankova et al. (1999) found that the DBH gene expression changes are caused by prolonged or repeated stress. A significant result in this work is that chronically stressed animals

(CSITR) have statistically more significant expression of TH, DBH and PNMT genes after additional acute immobilization stress compared with the animals exposed to acute immobilization stress. Wong et al. (2002) reported that PNMT protein and enzymatic activity change require additional time of approximately 18–20 hours to reach maximum stimulated levels. Kvetnansky et al. (2006) showed that in rats exposed repeated immobilization stress (7 days), 3 hours after a termination of immobilization stimulus PNMT level in the adrenal medulla was significantly increased. We found significantly elevated levels of TH and PNMT proteins 3 hours after, and DBH proteins 22 hours after the cessation IMM in chronically stressed animals. This data suggest the possibility of increased catecholamine synthesis in the adrenal medulla of chronically stressed animals after novel immobilization stress. This could mean that prior experience may condition physiological systems to “expect” a problem and therefore, be more ready to respond to a novel additional acute stressor. The readiness of the organism prolongly exposed to homotypic stressor to respond to a heterotypic stressor by an exaggerated expression of catecholamine biosynthetic enzymes that is considered to be an important adaptive phenomenon of the sympatho-adrenomedullary system in rats (Kvetnansky et al. 2009).

Our results confirm that the CSITR shows adaptations that are indicative of chronic stress. We found that treadmill running did not result in modulation of gene expression of catecholamine synthesizing enzymes in the adrenal medulla of chronically psychosocially stressed adult rats. A significant result in this study is that during CSITR treatment the increased synthesis of PNMT protein in the adrenal medulla affects the sustained increase of A secretion. The potentially negative physiological adaptations after CSITR were recorded as increased concentrations of catecholamines in the plasma. It is known that

increased level of catecholamines in the plasma after chronic stress is the allostatic load that may provoke disease indicative of sports medicine and pathophysiology. The results presented here confirm that the chronic treadmill running in rats is forced exercise. An alternative to forced exercise paradigms would be to allow experimental animals free access to run wheels and to allow voluntary exercising for extended periods of time. Using this method, the stressor effects of forced training schedules could be avoided.

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RESUMO

O isolamento crônico de animais adultos representa uma forma de estresse psicológico que produz ativação do sistema simpático adrenomedular. O treinamento físico atua como um importante modulador do sistema simpático adrenomedular. Este estudo tem como objetivo investigar mudanças relacionadas ao exercício físico na expressão dos genes de enzimas envolvidas na biossíntese de catecolaminas (tirosina hidroxilase, dopamina-B-hidroxilase e feniletanolamina N-metiltransferase) e da proteína de ligação ao elemento de resposta ao monofosfato cíclico de adenosina (CREB) na medula adrenal, concentrações de catecolaminas e corticosterona (CORT) no plasma e o peso das glândulas adrenais de ratos adultos psicologicamente estressados por exposição diária a 20 min de corrida em esteira durante 12 semanas. Além disso, examinamos como o estresse adicional por imobilização aguda altera os parâmetros mencionados. O treinamento em esteira não resultou na modulação da expressão dos genes de enzimas que sintetizam catecolaminas, mas houve uma diminuição dos níveis de RNAm de CREB na medula

adrenal de ratos adultos com estresse psicossocial crônico. As adaptações psicológicas potencialmente negativas após corrida na esteira foram registradas como um aumento na concentração de catecolaminas e diminuição matinal nas concentrações de CORT no plasma, bem como na hipertrofia da glândula adrenal de ratos psicossocialmente estressados. O estresse adicional por imobilização aguda aumenta a expressão dos genes de enzimas da via de biossíntese de catecolaminas na medula adrenal, bem como os níveis de catecolaminas e CORT no plasma. O exercício em esteira não altera a atividade do sistema simpático adrenomedular de ratos com estresse psicossocial crônico.

Palavras-chave: medula adrenal, estresse por imobilização aguda, catecolaminas, isolamento social crônico, expressão gênica, exercício em esteira.

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