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GENE EXPRESSION OF CATECHOLAMINE SYNTHESIZING ENZYMES IN STELLATE GANGLIA OF STRESSED RATS

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Enhanced activation of sympathetic neurons during stress results in an increased cardiovascular function. Social isolation is a psychological stress which has deleterious effects on health and represents the most relevant cause of diseases in mammalian species. In this study we investigated the changes in catecholamine biosynthetic enzymes tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT) gene expression and protein levels in the stellate ganglia of naive controls and chronically socially isolated (12 weeks) adult rats and the response of these animals to additional immobilization stress (2 h) by applying TaqMan RT-PCR assay and Western blot analysis. Psychosocial stress produced a significant increase of both TH mRNA ($p<0.05$) and DBH mRNA ($p<0.05$) levels in stellate ganglia. The exposure of control rats to acute immobilization significantly increased TH mRNA ($p<0.001$) and DBH mRNA ($p<0.01$) levels, while additional immobilization of chronic psychosocially stressed rats expressed no effect on gene expression of these enzymes. Protein levels of TH, and DBH remained unchanged in control and chronic social isolation rats and also after short-term immobilization. The results presented here suggest that psychosocial stress-induced an increase in gene expression of catecholamine biosynthetic enzymes in stellate ganglia and thus may be connected to the increased risk of cardiovascular disease.

Key words: stellate ganglia, psychosocial stress, catecholamine biosynthetic enzymes, gene expression

INTRODUCTION

Stress disturbs homeostasis and may induce various disorders. Crucial mediators of the stress response are catcholamines released from the adrenal medulla and sympathetic nerves. Sympathetic neurons are major contributors to the huge rise in circulating norepinephrine level in response to stressful stimuli. Recently, Blumenthal *et al.* (2002) presented a convincing evidence on stress-related risk increase of myocardial ischaemia and hypertension. Social defeat

represents an important event in the life of numerous animal species and a part of the process of social control. Adaptation to social defeat is thus an intrinsic part of social homeostasis and mal-adaptation has pathological sequelae as claimed by Martinez *et al.* (2002). Social isolation is a psychological stress which has deleterious effects on health, thus being regarded as one of the most relevant causes of diseases in mammalian species (Bartolomucci *et al.,* 2003). Stranahan *et al*. (2006) reported that individual housing precludes positive influence of short-term running on adult neurogenesis in the rat hippocampus and upon additional stress, supresses the generation of new neurons. Our earlier studies showed that social isolation of adult rat males produced a depletion of brain catecholamine stores, but no changes in heart auricles and adrenal glands were observed (Gavrilović *et al.,* 2005). Also, we recorded a potentiation of the sympatho-adrenal system activity in socially isolated rats upon exposure to novel stressors (Dronjak and Gavrilović, 2005). Stellate ganglia represent the main sympathetic input to the heart and affect catecholamine levels in this tissue (Pardini *et al*., 1990; Cavalcanti *et al.*, 2009). Thus, the levels of mRNAs of catecholamine-synthesizing enzymes in stellate ganglia and their modulation by stress could have a direct impact on cardiac function. Tyrosine hydroxylase (TH), a rate-limiting enzyme of catecholamine biosynthesis, dopamine-β-hydoxylase (DBH) that converts dopamine to norepinephrine and phenylethanolamine N-methyltransferase (PNMT) catalyzing the conversion of norepinephrine to epinephrine are presented in different types of sympatetic ganglia (Kiran and Ulus, 1992; Nankova *et al.*, 1996). The impact of stress on neurotransmitter gene expression in adrenal medula has been intensively studied (Kvetnansky *et al.,* 2002). Much less is known regarding the mechanisms of stress-triggered activation of neurotransmitter gene expression in sympathetic nerves, which are the major sources of circulating noradrenaline. These data together with our recent results showing that social isolation led to decreased levels of catecholamine biosynthetic enzymes in the adrenal medulla of adult rats (Gavrilović *et al*., 2008) prompted us to investigate the changes in gene expression and protein levels of TH, DBH and PNMT in the stellate ganglia of naive controls and chronic (12 weeks) socially isolated adult rat males and their response to additional immobilization stress (2h). With this in mind TaqMan RT-PCR assay and Western blot analysis were applied.

MATERIAL AND METHODS

Animals

Male 11-week-old (260-330 g) Wistar male rats maintained under standard laboratory conditions with water and food *ad libitum* in groups of four individuals *per* cage were used. Care was taken to minimize the pain and discomfort of the animals according to the recommendations of the Ethical Committe of the "Vinča" Institute, Belgrade based on the Guide for Care and Use of Laboratory Animals of the National Institute of Health (Bethesda, MD, U.S.A). One group of animals was subjected to social isolation with a single animal *per* cage for 12 weeks. After that, naive group-housed controls and the rats that suffered chronic isolation were

exposed to acute immobilization stress for 2 h (Kvetnansky and Mikulaj, 1970). After 12 weeks of individual housing or 3 h after the termination of immobilization, the animals were decapitated, the stellate ganglia rapidly dissected, frozen in liquid nitrogen and stored at -70 ^{o}C until analyzed.

Real-time RT-PCR

Total RNAs were isolated using TRIZOL reagent (Invitrogen, CA, U.S.A.). Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Bead (AP, Biotech) and pd $(N)_6$ primer according to manufacturer's protocol. Real-Time RT-PCR assay was done exactly as previously described by Gavrilović *et al.* (2008). PCR reactions were performed in the ABI Prism 7000 Sequence Detection System at 50 ^{o}C for 2 min, 95 ^{o}C for 10 min, followed by 40 cycles at 95 ^{o}C for 15 sec and 60 ^{o}C for 1 min. A reference, endogenous control, was included in each analysis to correct the differences in the inter-assay amplification efficiency and all transcripts were normalized to cyclophyline A (ID:Rn 00690933) expression.

Western blot analysis

Heart auricles were homogenized in 0.05 M sodium phosphate buffer pH 6.65. Subsequently, the protein concentration was determined according to Stich (1990). Fifteen μg of heart auricle protein extract separated by 10% SDS-polyacrylamide gel electrophoresis were transferred to a supported nitrocellulose membrane (Hybond^{TM} C Extra, Amersham Bioscience). The membrane was blocked in 5% non-fat dry milk in Tris-buffered saline-Tween (TBST). All following washes and antibody incubations were also performed in TBST at room temperature on a shaker. For measuring TH, DBH and PNMT protein levels, a monoclonal primary antibody against mouse TH (monoclonal antibody against TH from mouse-mouse hybrid cells, clone 2/40/15, dilution 1:5000), anti-dopamine-β hydroxylase (N-terminal) antibody, human (dilution 1:1000, Sigma) and polyclonal anti-PNMT primary antibody, rabbit (dilution 1:1000, Protos Biotech Corporation, New York, U.S.A.), respectively, were used. The washed membrane was further incubated in the secondary anti-mouse antibody conjugated to horseradish peroxidase (dilution 1:5000, Amersham Bioscience). Secondary antibody was then visualized by the Western blotting enhanced chemiluminiscent detection system (ECL, Amersham Bioscience).

Statistics

The results are reported as means $\pm$ S.E.M. Significance of the differences in gene expression levels of the examined catecholamine biosynthetic enzymes in the stellate ganglia of rats subjected to chronic social isolation and immobilization were estimated by one-way ANOVA test. The Tukey *post-hoc* test was used to evaluate the differences between the groups. Statistical significance was accepted at $p<0.05$.

RESULTS

One-way ANOVA test revealed significant variations of TH mRNA ($F=8.47$, $p<0.001$) and DBH mRNA ($F=2.5$, $p<0.01$) levels under the examined stress conditions. *Post-hoc* analysis (Tukey test) demonstrated a significant increase in the level of TH mRNA (1.8-fold, $p<0.05$) and DBH mRNA (2-fold, $p<0.05$) level in stellate ganglia of the rats exposed to chronic psychosocial stress. The exposure of control animals to acute immobilization significantly increased TH mRNA level (5.1-fold, $p<0.001$) and DBH mRNA level (7.2-fold, $p<0.01$). However, additional immobilization of chronic psychosocially stressed rats did not affect mRNA level of these enzymes (Fig.1). The presence of PNMT mRNA was also detected in the

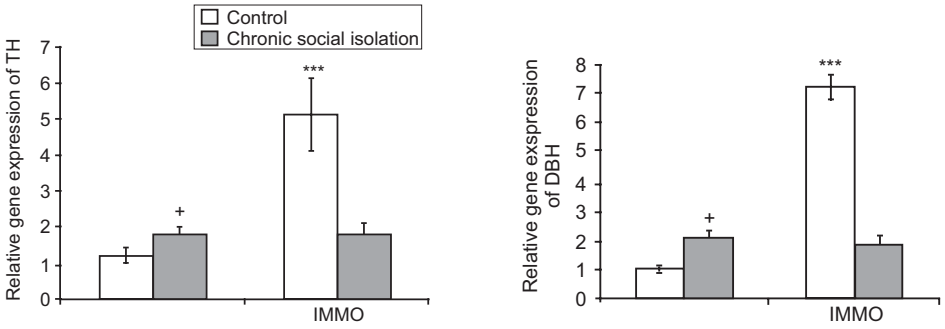

Figure 1. Effects of immobilization stress on tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH) mRNA levels in stellate ganglia of control and chronic social isolation adult rat males. The values are means ±S.E.M. of 5-7 rats. Statistical significance: +$p<0.05$ chronic social isolation *vs.* control (Tukey-test); ***$p<0.001$ 2 h immobilization *vs.* control (Tukey-test); The final result was expressed as fold change relative to the calibrator and normalized to cyclophyline A

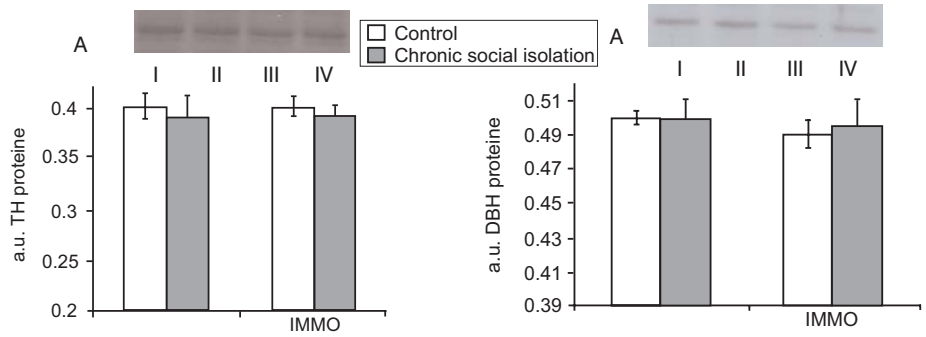

Figure 2. Effects of immobilization stress on tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH) protein levels in stellate ganglia of control and chronic social isolation adult rat males. Distribution of TH and DBH proteins in the stellate ganglia of control I, chronic social isolation II, control+immobilization III and chronic social isolation+immobilization IV rats measured by Western blot analysis (A). The values are means ±S.E.M. of 5-7 rats. The final result was expressed in arbitrary units normalized in relation to β actine

stellate ganglia, but its extremely low amount made its quantification impossible. Protein levels of TH, and DBH remained unchanged in control and chronic social isolation rats and also after exposing these animals to short-term (2 h) immobilization (Fig. 2).

DISCUSSION

The sympathetic nervous system has a profound influence on myocardial function as reported by Dae *et al.* (1995) and Gorbunova (2000). Besides, enhanced activation of the sympathetic neurons during stress was shown to lead to an increased cardiovascular function and catecholamine release (Jansen *et al.,* 1995). In the present study, we found that chronic psychosocial stress produced a significant elevation of TH mRNA and DBH mRNA levels in stellate ganglia of adult rat males. Elevated gene expression of the examined catecholamine biosynthetic enzymes may designate a greater increase of norepinephrine synthesis and this catecholamine exerts direct toxic effects on cardiac sympathetic nerves. A direct demonstration of norepinephrine-induced sympathetic denervation has been shown in the canine lateral saphenous veins. Teixeira *et al*. (1989) speculated that probably norepinephrine-derived free radicals acted by damaging sympathetic nerve endings. Recently, Lai *et al.* (2008) revealed cytotoxic and apoptotic effects of a high norepinephrine dose on cardiac fibroblasts in cell culture and both non-selective and selective adrenergic receptor antagonists were not capable to inhibit the latter effect of this catecholamine. Likewise, Kassim *et al.* (2008) reported that catecholamines and their oxidation products expressed a direct toxic effect on the myocardium and that catecholamine-mediated myocardial stunning has been implicated in the pathogenesis of stress-induced cardiomyopathy. The levels of mRNAs of norepinephrine-synthesizing enzymes in stellate ganglia and their modulation by stress could have a direct impact on cardiac function. Also, Swissa *et al*. (2008) demonstrated that continuous sub-threshold electrical stimulation to the left stellate ganglion induced both sympathetic and parasympathetic hyperinnervation in right and left atria and ventricles of dogs. It is worth mentioning that increased TH mRNA level in stellate ganglia was observed in rats with myocardial infraction (Parish *et al*., 2008) and in experimental rat heart failure model (Kristen *et al*., 2006). It could be speculated that the observed increase of the TH and DBH gene expression in the observed stellate ganglia in the present study may have an impact on different pathophysiological processes. Chronic isolation applied in the present study did not affect TH and DBH protein levels in the stellate ganglia. Our results showed that acute immobilization produced a significant elevation of TH mRNA and DBH mRNA levels in stellate ganglia of control rats. This is in line with the data of Kvetnansky *et al.* (2004) who observed that single and repeated immobilization stress acted by increasing gene expression of catecholamine biosynthetic enzymes. However, exposure of chronic psychosocially stressed rats to a novel, immobilization stress did not result in a further increase of mRNA levels of the examined enzymes in the rat stellate ganglia. The absence of response of TH mRNA and DBH mRNA levels in the stellate ganglia to a novel immobilization

stress is unclear at the moment. In the present study mRNA levels were measured 3 h after the termination of immobilization. So, it could be hypothesized that the process of gene expression of these enzymes in animals exposed to chronic psychosocial stress required a longer period of time, *i.e.* that the transcription of the genes started in some later experimental point. Also, unchanged gene expression of the examined enzymes could be due to a prolonged half-life or stability of the mRNAs in the stellate ganglia of long-term psychosocially stressed rats after the application of a novel stressor. The results presented here differ from our previous observations on the response of rat adrenal medulla to chronic social isolation (Gavrilović *et al.*, 2008). Namely, we have found reduced levels of both TH and DBH mRNAs in the adrenal medulla of these rats, and their exaggerated increase after additional immobilization stress. It could be hypothesized that chronic psychosocial stress differently affects gene expression of catecholamine synthesizing enzymes in two components of the sympatho-adrenal system. The results presented here suggest that the enhancement in the expression of catecholamine biosynthetic enzymes in rat stellate ganglia may be involved in the increased risk of cardiovascular disease connected to psychosocial stress.

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Gavrilović Ljubica *et al*.: Gene expression of catecholamine synthesizing enzymes in stellate ganglia of stressed rats

EKSPRESIJA GENA BIOSINTETSKIH ENZIMA KATEHOLAMINA U STELATNOJ GANGLIJI KOD STRESIRANIH PACOVA

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i DRONJAK SLAĐANA

SADRŽAJ

Povećana aktivacija simpatičkih neurona u toku stresa dovodi do povećanja kardiovaskularne funkcije. Socijalna izolacija je psihosocijalni stres koji je najzastupljeniji kod sisara i ima štetno dejstvo na zdravlje. U ovom radu izučavali smo promene u ekspresiji gena za sintezu enzima koji učestvuju u biosintezi kateholamina: tirozin hidroksilaze (TH), dopamin beta hidroksilaze (DBH) i feniletanolamin N-metiltransferaze (PNMT) i nivo proteina u stelatnoj gangliji kod kontrola i hronično socijalno izolovanih (12 nedelja) odraslih pacova kao i odgovor ovih životinja na dodatni stres imobilizacije (2 časa), korišćenjem Taq-Man RT-PCR eseja i Western blot analize. Psihosocijalni stres dovodi do značajnog povećanja kako TH iRNK ($p<0,05$) tako i DBH iRNK ($p<0,05$) u stelatnoj gangliji. Izlaganje kontrolne grupe životinja akutnoj imobilizaciji značajno povećava nivo TH iRNK ($p<0,001$) i DBH iRNK ($p<0,01$), dok dodatni stres imobilizacije kod hronično psihosocijalno stresiranih pacova ne dovodi do povećanja ekspresije gena za sintezu ovih enzima. Sinteza proteina ostaje nepromenjena kako kod kontrola i hronično socijalno izolovanih pacova, tako i nakon izlaganja ovih životinja kratkotrajnom stresu imobilizacije. Dobijeni rezultati ukazuju da povećana ekspresija gena biosintetskih enzima kateholamina, kod psihosocijalno stresiranih pacova može biti povezana sa povećanim rizikom od nastanka kardiovaskularnih oboljenja.