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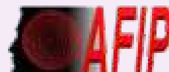

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Effect of immobilization stress on gene expression of catecholamine biosynthetic enzymes in heart auricles of socially isolated rats

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Abstract

Chronic stress is associated with the development of cardiovascular diseases. The sympathoneural system plays an important role in the regulation of cardiac function both in health and disease. In the present study, the changes in gene expression of the catecholamine biosynthetic enzymes tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT) and protein levels in the right and left heart auricles of naive control and long-term (12 weeks) socially isolated rats were investigated by Taqman RT-PCR and Western blot analysis. The response of these animals to additional immobilization stress (2 h) was also examined. Long-term social isolation produced a decrease in TH mRNA level in left auricles (about 70%) compared to the corresponding control. Expression of the DBH gene was markedly decreased both in the right (about 62%) and left (about 81%) auricles compared to the corresponding control, group-maintained rats, whereas PNMT mRNA levels remained unchanged. Exposure of group-housed rats to acute immobilization for 2 h led to a significant increase of mRNA levels of TH (about 267%), DBH (about 37%) and PNMT (about 60%) only in the right auricles. Additional 2-h immobilization of individually housed rats did not affect gene expression of these enzymes in either the right or left auricle. Protein levels of TH, DBH and PNMT in left and right heart auricles were unchanged either in both individually housed and immobilized rats. The unchanged mRNA levels of the enzymes examined after short-term immobilization suggest that the catecholaminergic system of the heart auricles of animals previously exposed to chronic psychosocial stress was adapted to maintain appropriate cardiovascular homeostasis.

Key words: Heart auricles; Tyrosine hydroxylase; Phenylethanolamine N-methyltransferase gene expression; Dopamine-β-hydroxylase

Introduction

Stress exposure activates the sympathoneural system, resulting in catecholamine release. Chronic stress is associated with the development of numerous disorders including cardiovascular diseases such as hypertension and myocardial infarction (1). The sympathoneural system plays an important role in the regulation of cardiac function both in health and disease. Indeed, the sympathetic nervous system has been shown to be a predominant driving force in the cardiovascular response to psychological stress (2). 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 (3). Stranahan et al. (4) reported that individual housing precludes the positive influence of short-term running on adult neurogenesis in the rat hippocampus and, upon additional stress, suppresses the generation of new neurons. Additionally, social isolation has been shown to produce depletion of brain catecholamine stores but no changes in peripheral tissues were observed (5). Previous studies by our group have shown potentiation of the sympatho-adrenomedullary system activity in socially isolated rats exposed to novel stressors (6). The effects of chronic stress are likely to be associated with alterations in gene expression of the catecholamine biosynthetic enzymes tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT). These

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enzymes are present in different types of tissues and their gene expression, protein levels and activity can be changed by various stressors (7,8). Recently, it has been shown that exposure of cold-adapted rats to novel stressors induces exaggerated responses in the expression of the TH gene in the adrenal medulla (9). In exercised rats exposed to shaking stress, TH mRNA levels were also found to be increased (10). Several investigators have reported that social isolation of rats acted by enhancing their responsiveness to stressors (3,11,12). Our previous studies showed that exposure of socially isolated rats to additional immobilization produced exaggerated responses in gene expression of catecholamine biosynthetic enzymes and proteins in the adrenal medulla (13). Since catecholamines are known to be involved in augmenting cardiac function, we investigated the changes in catecholamine biosynthetic enzymes TH, DBH and PNMT gene expression and protein levels by applying TaqMan RT-PCR and Western blot analyses to the right and left heart auricles of naive control and long-term (12 weeks) socially isolated adult rats and the response of these animals to additional immobilization stress (2 h).

Material and Methods

Animals

Adult 11-week-old male Wistar rats maintained under standard laboratory conditions with water and food *ad libitum* were used. Care was taken to minimize the pain and discomfort of the animals according to the recommendations of the Ethics Committee of the “Vinca” Institute, Belgrade, based on the Guide for Care and Use of Laboratory Animals of the National Institute of Health (USA). Before being subjected to stress, the animals were divided into the following groups: 1) control consisting of 4 animals per cage; 2) animals housed individually for 12 weeks; 3) control group (N = 4) exposed to immobilization for 2 h, and 4) rats individually housed for 12 weeks and additionally exposed to 2-h immobilization. After immobilization, the animals were returned to their home cages and decapitated 3 h later. Unstressed controls and long-term isolated rats, which were not exposed to immobilization, were sacrificed immediately after removal from their home cages. The right and left heart auricles were rapidly dissected, frozen in liquid nitrogen and stored at -70°C until the time for analysis.

RNA isolation and cDNA synthesis

Total RNAs were isolated using TRIZOL reagent (Invitrogen, USA). Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Bead (AP, Biotech, UK) and pd $(N)_6$ primer according to the manufacturer 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; AGGACAAGCTCAGG AACTATGCCTC), DBH (Rn00565819_m1; CCAGATGG CACTGCCGAAATCTGGA), and PNMT (Rn01495589_g1; ACAAGGGAGAGTCCTGGCAGGAGAA). 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-µL reaction mixture containing 1X TaqMan Universal Master Mix with AmpErase UNG, 1X Assay Mix (Applied Biosystems) and cDNA template (10 ng 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. The point at which the amplification plot crosses this threshold defined as Ct, represents the cycle number at this point and is inversely proportional to the number of target copies present in the initial sample. Each sample was run in triplicate and the mean value of each Ct triplicate was used for further calculations. 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 cyclophilin A (Rn00690933_m1; TCATGTGCCAGGGTGGTGACTTCAC) expression. For quantification, validation experiments were performed to determine the relevant endogenous control for each target gene. We tested cyclophilin A and demonstrated that the efficiency of amplification was approximately equal for the endogenous control gene and all target genes. Serial dilutions of cDNAs were prepared and amplified by real-time PCR using specific primers and fluorogenic probes for target and endogenous control genes. The reaction mixture for endogenous control gene amplification consisted of 1X TaqMan Universal Master Mix with AmpErase UNG (Applied Biosystems), 1X Assay (6-FAM dye-labeled MGB probes) and cDNA (10 ng RNA converted to cDNA). The levels of expression of cyclophilin A in samples under different treatments were checked by additional experiments, which confirmed that the chosen reference gene was not regulated. Quantification was done using the $2^{-\Delta\Delta Ct}$ method according to Livak and Schmittgen (14). The results obtained were analyzed by the RQ Study Add On software for 7000 v 1.1 SDS instrument (ABI Prism Sequence Detection System) with a confidence level of 95% ($P < 0.05$). The relative expression of the target gene was expressed in relation to the calibrator, i.e., the control sample. Due to individual differences among animals, the sample of the control groups with the expression value close to the mean of all samples in the group and with the lowest measurement error was used as a calibrator. The final result is reported as fold change relative to the calibrator and normalized to cyclophilin A using the equation: $N_{sample} = 2^{-\Delta\Delta Ct}$.

Western blot analysis

Heart auricles were homogenized in 50 mM sodium phosphate buffer, pH 6.65. Subsequently, protein concentration was determined by the method of Stich (15). Fifteen micrograms heart auricle protein extract separated by 10% SDS-polyacrylamide gel electrophoresis was transferred to a supported nitrocellulose membrane (Hybond™ C Extra, Amersham Bioscience, UK) and the membrane was blocked in 5% non-fat dry milk in Tris-buffered saline-Tween (TBST). All subsequent washes and antibody incubations were also performed in TBST at ambient temperature on a shaker. To measure 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, USA) and polyclonal anti-PNMT rabbit primary antibody (dilution 1:1000, Protos Biotech Corporation, USA) were used, respectively. The washed membrane was further incubated with the secondary anti-mouse antibody conjugated with horseradish peroxidase (dilution 1:5000, Amersham Bioscience). The secondary antibody was then visualized with the Western blotting enhanced chemiluminescent detection system (Amersham Bioscience).

Statistical analysis

Data are reported as means ± SEM. The significance of the differences in gene expression levels of the examined catecholamine biosynthetic enzymes in the right and left heart auricles of rats subjected to chronic social isolation and immobilization was estimated by two-way ANOVA. The Tukey *post hoc* test was used to evaluate the differences between groups. Statistical significance was accepted at $P < 0.05$.

Results

Two-way ANOVA showed a significant effect of housed group *vs* housed group exposed to immobilization ($F(1,17) = 10.91$, $P < 0.001$) and a significant effect of social isolation *vs* social isolation and exposure to immobilization ($F(1,17) = 6.57$, $P < 0.05$) on TH mRNA levels. *Post hoc* analysis revealed that social isolation produced a decrease of TH mRNA levels in the left auricles (approximately 70%; $P < 0.05$, Tukey test) compared to control. Expression of the DBH gene was markedly decreased ($P < 0.001$, Tukey test) both in the right (about 62%) and left auricles (about 81%) of socially isolated rats compared to the group-housed controls, while PNMT mRNA levels were unchanged. The exposure of group-housed animals to acute immobilization for 2 h led to a significant 267% increase of TH mRNA ($P < 0.001$, Tukey test), a 37% increase of DBH mRNA, and a 60% increase in PNMT mRNA ($P < 0.001$, Tukey test) only in the right auricles. As shown in Figure 1, additional immobilization of individually housed rats did not affect the levels of gene expression of these enzymes in the right or left auricles. Protein levels of TH, DBH and PNMT remained unchanged in individually housed and short-

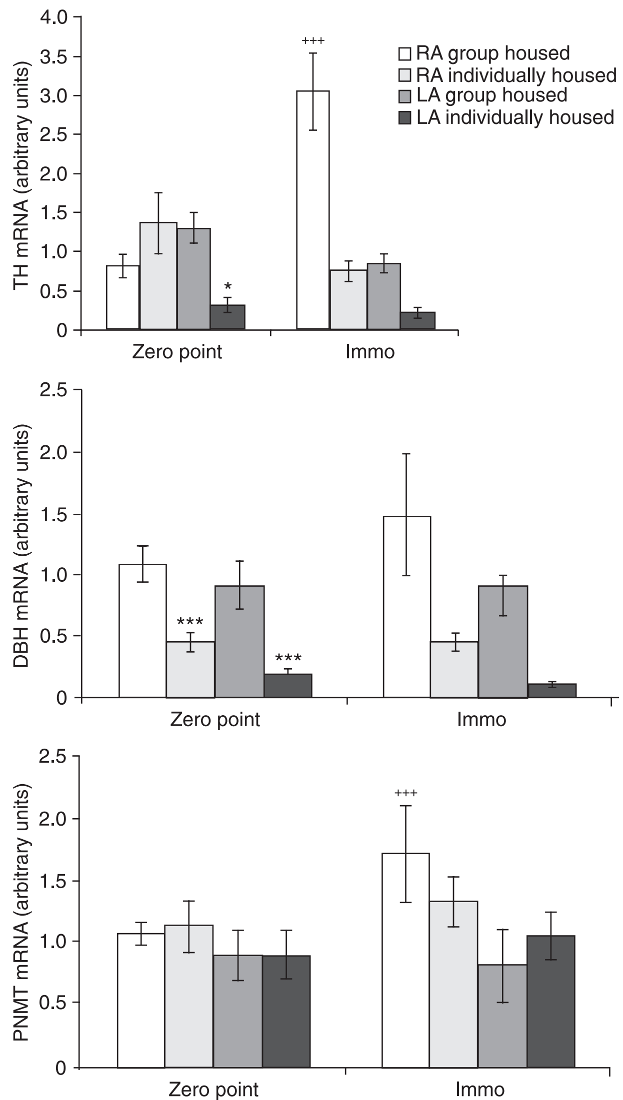
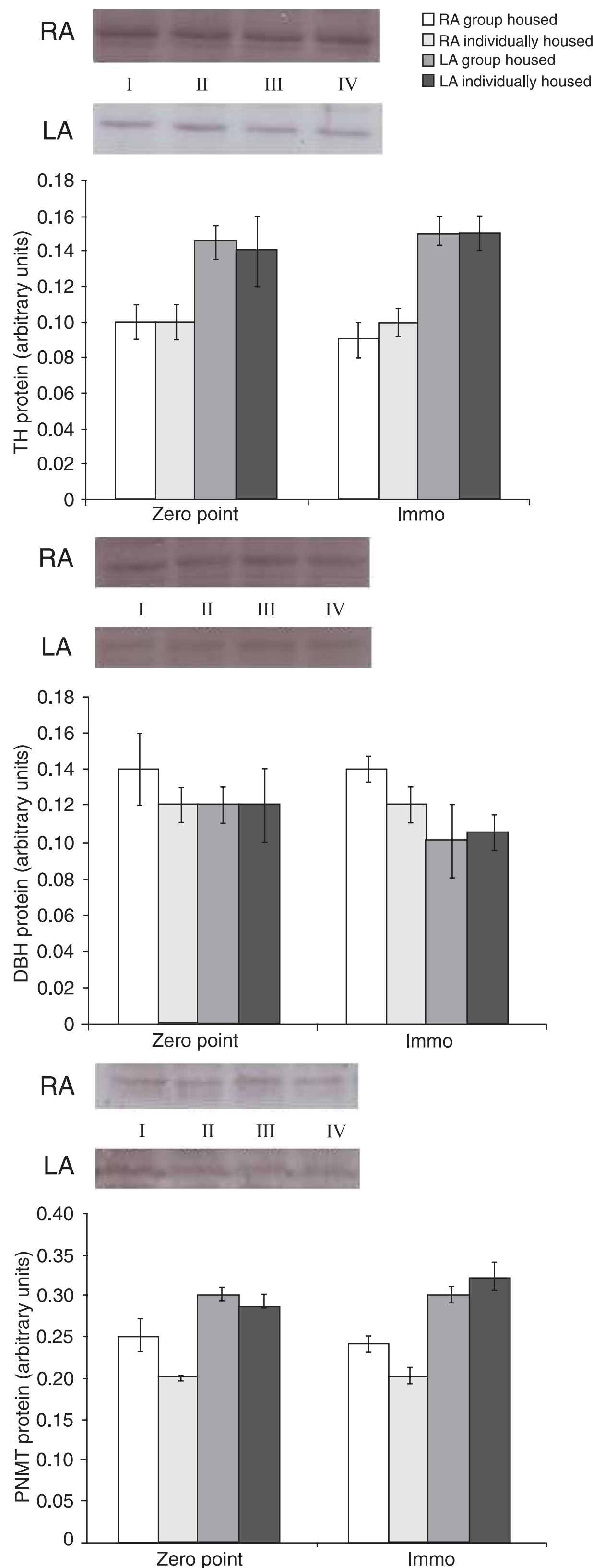


Figure 1. Effects of immobilization stress on tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT) mRNA levels in the right (RA) and left (LA) heart auricles of group- and individually housed adult rat males. The animals were divided into four groups: 1) a control consisting of 4 animals per cage, 2) animals individually housed for 12 weeks, 3) a control group exposed to immobilization for 2 h, and 4) rats individually housed for 12 weeks and additionally exposed to 2-h immobilization. Data are reported as means ± SEM arbitrary units for 5-7 rats. Zero point = the state before immobilization; Immo = immobilization stress. $^{*}P < 0.05$; $^{***}P < 0.001$ for individually housed *vs* group-housed control (Tukey test); $^{+++}P < 0.001$ for 2-h immobilization *vs* group-housed control (Tukey test). The final result is reported as fold change relative to the calibrator and normalized to cyclophilin A.



term (2 h) immobilized rats both in the right and left heart auricles compared to the corresponding group-housed control (Figure 2).

Discussion

It is generally accepted that previous experiences involving stressful events affect the subsequent response to a novel stressor. However, little is known about the effect of novel stressors on gene expression and protein levels of catecholamine-synthesizing enzymes in heart auricles of long-term stressed rats exposed to a novel stressor. In the present study, we investigated changes in gene expression and protein levels of the three catecholamine synthesizing enzymes in the right and left auricles of long-term psychosocially stressed rats exposed to immobilization as an additional novel stressor. The data show that long-term social isolation led to a decreased TH mRNA level in the left auricle compared to the corresponding group-maintained control. Interestingly, the DBH mRNA level was markedly decreased and the PNMT mRNA was unchanged both in the right and left heart auricles. Selective stress-related changes of TH mRNA levels have been previously reported in the literature for catecholamine synthesizing enzymes. McMahon et al. (16) found that immobilization stress acted by elevating the level of TH mRNA without affecting DBH mRNA. Different regulation of the expression of distinct genes has been reported to exist not only in individual organs but also within a single organ, e.g., in the heart. Deindl et al. (17) recently reported that lactate dehydrogenase-B was up-regulated in the right ventricles, but down-regulated in the left ventricles and atria. Also, c-Jun-N-terminal protein kinases (JNKs) were elevated in the right ventricles of rats with chronic intermittent altitude hypoxia, while their expression was down-regulated in the left ventricles (18). Our results showed that chronic social isolation produced a significant decrease of DBH mRNA levels both in the right and left heart auricles. The question here is how this stress-related reduction of DBH mRNA level could be explained. Micutkova et al. (19) reported that the stimulation of sympathetic nerve activity increases norepinephrine production by stimulating both the expression and activity

Figure 2. Effects of immobilization stress on tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT) protein levels in right (RA) and left (LA) heart auricles of group- and individually housed rats. For details about the experimental groups, see the legend to Figure 1. Distribution of TH, DBH and PNMT proteins in the right and left heart auricles of group-housed (I), individually housed (II), group-housed + immobilization (III), and individually housed + immobilization (IV) rats measured by Western blot analysis. Data are reported as means ± SEM arbitrary units for 5-7 rats. Zero point = the state before immobilization; Immo = immobilization stress. The final result is reported in arbitrary units normalized in relation to β-actin.

of DBH. Also, Mann et al. (20) demonstrated that norepinephrine at high concentrations similar to those thought to exist in the myocardium in heart failure exerts a direct toxic effect on cardiac myocytes grown in cell culture, suggesting that chronic cardiac sympathetic nervous activity may cause progressive damage to the myocardium. Indeed, Sabbah et al. (21), applying therapy with nepicastat, a DBH inhibitor, successfully prevented progressive left ventricular dysfunction in dogs with chronic heart failure. It could be hypothesized that the decreased DBH gene expression observed throughout our study in rats exposed to chronic psychosocial stress acts by protecting the heart from extra production of norepinephrine. It should be mentioned also that some investigators detected a significantly lower DBH activity in subjects with greater anxiety (22,23). In addition, we have recently observed anxiety in socially isolated rats (24). It is interesting to emphasize that only PNMT gene expression in the right and left heart auricles of socially isolated animals remained unchanged compared to control group-maintained rats. Several investigators have demonstrated that the stress-induced increase of PNMT mRNA levels in heart auricles fully depends on the presence of glucocorticoids (9,25,26). This agrees with findings showing that 13 weeks of social isolation markedly reduced the plasma corticosterone levels of adult rats (27). The chronic isolation applied in the present study did not affect TH, DBH or PNMT protein levels in the right or left heart auricles. Acute immobilization (2 h) produced a significant enhancement of TH, DBH and PNMT mRNA levels only in the right heart auricles of control group-housed rats, but had no effect on the protein levels of these enzymes. However, additional immobilization of individually housed rats did not induce increased mRNA levels of TH, DBH and PNMT or of the corresponding enzymatic proteins either in the right or left auricles.

Parrish et al. (28) have recently reported increased TH gene expression, norepinephrine synthesis and norepinephrine uptake in stellate ganglia of rats with myocardial infarction, but the rate of norepinephrine synthesis exceeded its uptake. Normal catecholaminergic transmission results from a balance between catecholamine synthesis, release and reuptake. It could be speculated that this balance was not disrupted in the heart auricles of socially isolated rats and that after additional immobilization stress these animals increased the uptake of catecholamines without enhancing the rate or the extent of their biosynthesis. Our hypothesis about heart adaptation to chronic stress is consistent with the results of several studies on adrenergic receptors. Namely, the gene expression of β_2 and α_{1b}, as well as protein levels and receptor binding were found to be reduced in cardiac atria and ventricles of animals subjected to repeated immobilization stress (29,30). Also, Krepsova et al. (31) found significantly decreased gene expression and protein levels of type 1 IP_3 receptors in the left, but not in the right atrium after repeated immobilization. These investigators suggested that repeated immobilization-related down-regulation of the IP_3 receptors in the left atrium was involved in the adaptation of cardiac myocytes to this stimulus. Therefore, after additional immobilization, the catecholaminergic system in the heart auricles of chronic psychosocially stressed rats seems to be adapted to maintain appropriate cardiovascular homeostasis. Immobilization stress is one of the most intensive stimuli. Single or repeated immobilization for 7 days has been shown to produce an increase of PNMT mRNA levels both in atria and ventricles (32,33). Interestingly, the additional immobilization applied in the present study did not affect the gene expression of catecholamine biosynthetic enzymes and enzymatic proteins in the right and left auricles of chronically stressed animals, suggesting that the response to stress depends on prior experience with stressors. These results indicate a possible adaptation of the catecholamine-synthesizing system at the level of gene expression in the heart auricles of long-term isolated rats exposed to additional immobilization stress.

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