Endocr (2010) 37:425–429
DOI 10.1007/s12020-010-9325-5

ORIGINAL ARTICLE

Subsequent stress increases gene expression of catecholamine synthetic enzymes in cardiac ventricles of chronic-stressed rats

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Received: 19 January 2010 / Accepted: 9 March 2010 / Published online: 25 March 2010

Abstract Since previous experience of stressful situation profoundly affects response to a subsequent novel stressor, we examined changes in gene expression and protein levels of catecholamine biosynthetic enzymes in cardiac ventricles after exposure of chronic psychosocially isolated adult Wistar male rats to short-term immobilization stress. Chronic social isolation did not affect gene expression of tyrosine hydroxylase (TH) in either right or left ventricle. Subsequent immonilization of these animals produced an elevation of TH mRNA level in right and left ventricles. The levels of dopamine-β-hydroxylase (DBH) mRNA were detectable only after immobilization both in right and left ventricles of control and chronically isolated rats. Chronic isolation stress increased phenylethanolamine *N*-methyltransferase (PNMT) mRNA levels in the right ventricle. Immobilization led to an elevated PNMT mRNA level in right and left ventricles of both control and chronically stressed animals. Protein levels of TH, DBH, and PNMT in right and left ventricles of socially isolated rats were increased after subsequent immobilization. Taking into consideration the role of cardiac catecholamines in physiological and pathophysiological processes, it could be hypothesized that increased catecholamine synthesis in the ventricles after novel immobilization stress could point to the susceptibility of the heart to subsequent stress.

Keywords Catecholamine biosynthetic enzymes · Stress · Gene expression · Ventricles

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Introduction

The sympathetic nervous system is known to play an important role in the regulation of cardiac function. Enhanced peripheral sympathetic outflow has been found in a variety of cardiovascular diseases such as heart failure and hypertension [1]. On the other hand, catecholamines play a crucial role in response to stress. Stress involves neuroendocrine and behavioral responses elicited by different stimuli. When stress response is maintained because threat has not been diminished or because mechanisms underlying the endogenous stress response are dysregulated, the response itself can become maladaptive [2]. The question arises how the cardiovascular system responds to stress. While a short-term acute stress causes time-limited homeostatic changes of the cardiovascular system, long-term chronic stress leads to permanent alterations in cardiac gene expression resulting in persistent homeostatic disorders that could be deleterious. The effects of chronic stress are likely to be associated with alterations in gene expression of catecholamine biosynthetic enzymes: tyrosine hydroxylase (TH) a rate-limiting enzyme of catecholamine biosynthesis, dopamine-β-hydroxylase (DBH) that converts dopamine to noradrenaline and phenylethanolamine *N*-methyltransferase (PNMT) catalyzing conversion of noradrenaline to adrenaline. These enzymes are present in different types of tissues and their gene expression, protein levels, and activity can be changed by various stressors [3, 4]. Kvetnansky et al. [5] and Micutkova et al. [6] found that single or repeated 7-day-immobilization of adult rats produced an increase in PNMT mRNA levels both in atria and ventricles. The response to stress depends on prior experience with same or different stressors. Earlier, Bhatnagar et al. [7] demonstrated that cardiovascular response could be affected by prior stress

exposure. These authors observed a greater tachycardia upon acute formalin injection to Sprague–Dawley rats previously exposed to repeated cold stress. Social isolation is a psychological stress which has deleterious effects on endocrine and immune systems [8]. In our earlier studies an exaggerated response of gene expression of catecholamine biosynthetic enzymes and increased enzymatic protein levels in adrenal medulla of socially isolated rats exposed to additional immobilization stress have been recorded [9].

Considering that catecholamines are known to be involved in augmenting cardiac function, we have found it of interest to investigate changes in gene expression of catecholamine biosynthetic enzymes and the corresponding enzymatic proteins in right and left cardiac ventricles of chronic psychosocially stressed rats subsequently exposed to a novel immobilization stress.

Materials and methods

Animals

11-week-old Wistar male adult rats maintained under standard laboratory conditions with water and food ad libitum in the 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 Committee of the "Vinca" Institute, Belgrade, based on the Guide for Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). In these experiments, 32 rats were used. 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 short-term immobilization stress for 2 h [10]. Upon 12 weeks of individual housing or 3 h after the termination of immobilization, the animals were decapitated, right and left cardiac ventricles rapidly dissected, frozen in liquid nitrogen, and stored at −70°C until analyzed.

Real-time RT-PCR

Total RNAs were isolated using TRIZOL reagent (Invitrogen, CA, USA). Reverse transcription was performed employing 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 as previously described [9]. PCR reactions were performed 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. 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

Cardiac ventricles were homogenized in 0.05 M sodium phosphate buffer (pH 6.65). 15 μg of protein extract from ventricle was separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to a supported nitrocellulose membrane (HybondTM C Extra, Amersham Bioscience, Buckinghamshire, UK). The membrane was blocked in 5% non-fat dry milk in *Tris*-buffered saline-Tween (TBST). All the following washes and antibody incubations were also carried out in TBST at room temperature on a shaker. For measuring TH, DBH, and PNMT protein levels, a monoclonal primary antibody against mouse TH (from mouse–mouse hybrid cells, clone 2/40/15, dilution 1:5000), anti-DBH (N-terminal antibody, human, dilution 1:1000, Sigma, St. Louis, USA), and polyclonal anti-PNMT primary antibody, rabbit (dilution 1:1000, Protos Biotech Corporation, New York, USA), respectively, were used. Western blot analysis was performed as previously described [9].

Statistical analyses

The results are reported as means ± SEM. Significance of the differences in gene expression levels of the examined catecholamine biosynthetic enzymes in rats' right and left cardiac ventricles subjected to chronic social isolation and immobilization were estimated by a two-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

The two-way ANOVA test showed a significant effect of ventricle position (left or right) $F(1.6) = 276.8$, $P < 0.001$ and social isolation and immobilization $F(1.6) = 49.7$, $P < 0.01$ on TH mRNA levels and a significant effect of ventricle position $F(1.17) = 8.8$, $P < 0.05$, and social isolation and immobilization $F(1.7) = 4.5$, $P < 0.05$ on PNMT mRNA levels. Subsequent immobilization of chronic socially isolated rats produced 2.1-fold elevation ($P < 0.01$, Tukey test) of TH mRNA level in left ventricles, whereas mRNA level of this enzyme was elevated 1.3-fold ($P < 0.05$) in right ventricles (Fig. 1a). Post-hoc analysis revealed that 2-h immobilization led to an elevated PNMT mRNA level in right ventricles (1.3-fold, $P < 0.05$, Tukey test), as well as in left ventricles (2.7-fold, $P < 0.01$,

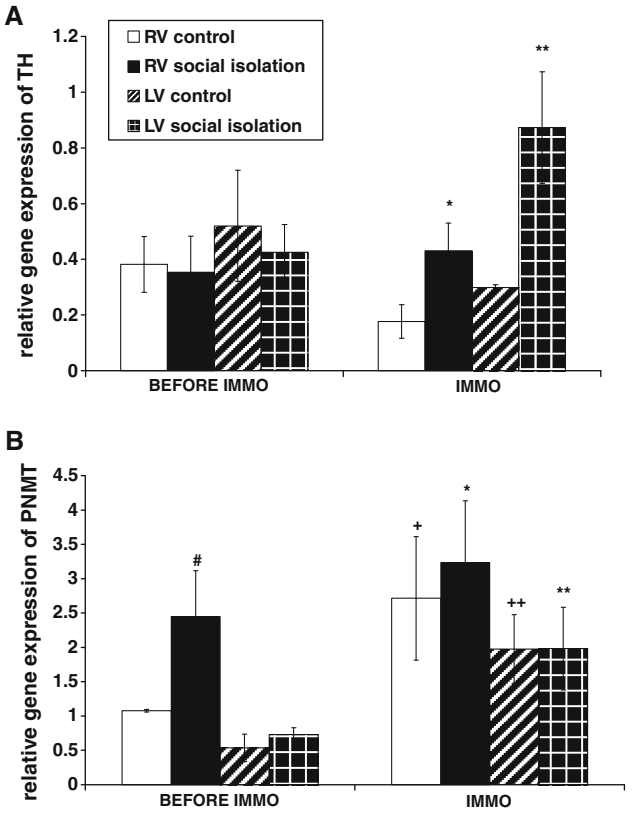

Fig. 1 Effects of immobilization stress on **a** TH and **b** PNMT mRNA levels in right (*RV*) and left (*LV*) heart ventricles of control and chronic social isolated adult male rats. The values are means ± SEM of five to seven rats. Statistical significance: $^{\#}P < 0.05$ chronic social isolated versus control rat (Tukey-test); $^{+}P < 0.05$; $^{++}P < 0.01$ 2 h immobilization versus control rat (Tukey-test); $^{*}P < 0.05$; $^{**}P < 0.01$ 2 h immobilization versus chronic social isolated rat (Tukey-test). The final result was expressed as fold change relative to the calibrator and normalized to cyclophyline A

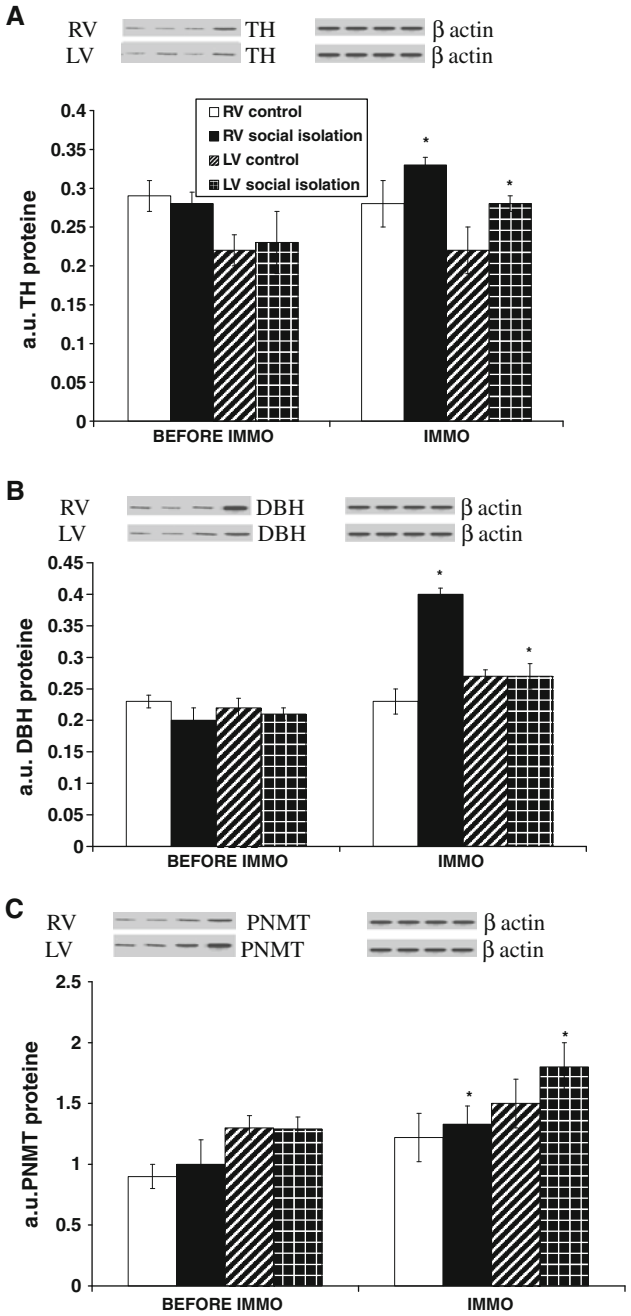

Fig. 2 Effects of immobilization stress on TH, DBH, and PNMT protein levels in right (*RV*) and left (*LV*) heart ventricles of control and chronic social isolated adult male rats. Distribution of TH, DBH, and PNMT proteins in the right and left heart ventricles of control I, chronic social isolation II, control + immobilization III, and chronic social isolation + immobilization IV rats measured by western blot analysis for TH (**a**), DBH (**b**), and PNMT (**c**). The values are means ± SEM of five to seven rats. $^{*}P < 0.05$ 2 h immobilization versu chronic social isolated rat (Tukey-test). The final result was expressed in arbitrary units normalized in relation to β-actin

Tukey test) of both controls and rats that suffered chronic social isolation (Fig. 1b). The levels of DBH mRNA were detectable only after immobilization in both right and left ventricles of control and chronically isolated rats. However, DBH protein was also detectable before immobilization (Fig. 2b).

As seen from Fig. 2, protein levels of TH, DBH, and PNMT in right and left ventricles of chronic socially isolated rats were increased after short-term immobilization stress ($P < 0.05$, Tukey test), whereas the levels of these proteins were unchanged in control animals exposed to immobilization.

Discussion

In this study, we investigated changes in gene expression and protein levels of the three catecholamine synthesizing enzymes in right and left ventricles of chronic psychosocially stressed rats exposed subsequently to a novel stressor, immobilization. The application of the quantitative RT-PCR assay with TaqMan probes enabled

us to detect the TH mRNA in rats' cardiac ventricles. What would be the significance of TH mRNA expression and catecholamine synthesis in cardiac tissues? Since previous data [11] show that only PNMT mRNA was detected in cardiac tissues, it was hypothesized that cardiomyocytes may uptake noradrenaline and convert it by PNMT to adrenaline, which may affect heart function. However, the detection of TH gene expression in cardiac ventricles suggests synthesis of noradrenaline directly in the heart. These data show that TH mRNA and protein levels in ventricles of chronically stressed rats remain unchanged compared with controls, but after exposure to subsequent short-term immobilization stress, they were significantly elevated. Acute immobilization did not influence TH mRNA and protein levels either in right or in left ventricles of naive controls. We suggest that during psychosocial chronic stress, cardiac noradrenaline might only be released from sympathetic nerves, while after additional immobilization stress, synthesis of noradrenaline restarts in cardiomyocytes. The data from the available literature showed that TH mRNA was detected in intrinsic cardiac adrenergic cells of fetal rat hearts before sympathetic innervation and these cells may provide an alternate adrenergic supply [12, 13]. To our knowledge, our study is the first report on TH mRNA presence in the ventricles of adult rats. Recently, we have found that chronic psychosocial stress produced increases in TH and DBH mRNAs in stellate ganglia which innervate the heart [14].

High catecholamine levels in the myocardial interstitium may cause a progressive damage of the myocardium. Mann et al. [15] found that noradrenaline in high concentrations, believed to exist in the myocardium in heart failure, exerts a direct toxic effect on cardiac myocytes grown in cell culture. These findings could be related to recent data of Nef et al. [16] that demonstrated a significant contribution of oxidative stress to pathomechanism of Tako-Tsubo cardiomyopathy, possibly triggered by catecholamine excess. Also, it is worth mentioning that noradrenaline treatment resulted in left ventricular hypertrophy [17]. Recently, Lai et al. [18] revealed cytotoxic and apoptotic effects of a high noradrenaline dose on cardiac fibroblasts in cell culture and both non-selective and selective adrenergic receptor antagonists were not capable of inhibiting the latter effect of this catecholamine. Collectively, these data suggest that the observed increases in TH mRNA and protein expression in cardiac ventricles could have an impact on different pathophysiological processes.

Normal catecholaminergic transmission results from an equilibrium among catecholamine synthesis, release, and reuptake. Parrish et al. [19] have observed increased TH gene expression, noradrenaline synthesis, and uptake in stellate ganglia of rats with myocardial infarction, but noradrenaline synthetic rate exceeded its uptake. Based on these data, we can hypothesize that the equilibrium among catecholamine synthesis, release, and reuptake was disrupted in ventricles of chronic socially isolated rats subsequently subjected to immobilization stress. Perhaps exposure of these animals to a short-term novel stress leads to a decreased catecholamine uptake and enhanced rate of their biosynthesis. This hypothesis is supported by the study of Li et al. [20] on noradrenaline transporter (NET), a protein synthesized in neuronal cells situated distant from the heart and transported into cardiac nerve endings via axonal transport. These authors demonstrated a significantly greater NET expression level in right ganglion than in the left one, rendering the greater capacity of noradrenaline uptake in right ventricle, a fact which may contribute to the maintenance of right ventricular function under pathologic state.

Interestingly, DBH mRNA level was recorded in right and left ventricles only after short-term immobilization (2 h) of both control and chronically stressed rats, whereas DBH protein levels were detected even in chronic stress situation. Our data demonstrated that DBH mRNA levels are not correlated with the enzymatic protein levels. One explanation for this finding could be attributed to DBH itself. DBH is the only catecholamine biosynthetic enzyme that has membrane-bound forms, suggesting that its turnover, i.e., half-life may be longer comparing to other enzymes of this group [21]. It could be hypothesized that once DBH was translated during chronic stress, further gene expression of DBH might be unnecessary until exposure of animals to a subsequent stress. Chronic isolation stress increased PNMT mRNA levels in the right ventricles, whereas the increase in this variable in the left ventricles was statistically insignificant in relation to naive control. Immobilization stress produced a significant elevation of PNMT mRNA levels in left and right ventricles of both naive control and chronic socially isolated rats. Immobilization stress is one of the most intensive stimuli. Taking into account the fact that chronic psychosocial isolation produced an enhancement of PNMT mRNA level in cardiac ventricles, the question arises whether further increase after subsequent stressor might have a pathophysiological impact? It has been observed that myocardial ischemia evokes an excessive noradrenaline and adrenaline accumulation in the myocardial interstitial space and on the other hand, ischemia may promote adrenaline synthesis and release by a high noradrenaline accumulation via cardiac PNMT activity [22]. It is also possible that cardiac release of adrenaline in the failing human heart is accompanied by a parallel increase in noradrenaline release from the cardiac sympathetic nerves, thus contributing to catecholamine myocardial toxicity and the development of ventricular arrhythmia as previously suggested by Kaye et al. [23].

Acute immobilization applied in this study did not affect TH, DBH, and PNMT protein levels either in right or left ventricles of control animals. In spite of highly elevated level of mRNAs in the ventricles after immobilization stress, enzymatic protein levels were not significantly changed. This could be explained by the fact that the process of catecholamine enzymes translation requires a prolonged stress stimulation and longer period of time [24, 25]. Wong et al. [26] reported that PNMT protein and enzymatic activity changes require additional time of approximately 18–20 h to reach maximum stimulated levels. In this study, we measured protein levels of catecholamine synthesizing enzymes 3 h after the end of immobilization and therefore, it is possible that a longer time was required to detect any protein changes. However, this period of stress significantly elevated TH, DBH, and PNMT protein levels in chronically stressed rats. These data suggest the possibility of increased catecholamine synthesis in the cardiac ventricles of chronically stressed animals after novel immobilization stress and together with the proposed role of elevated cardiac catecholamines in pathophysiological processes, pointing the susceptibility of the heart to subsequent stress.

Acknowledgment This study was supported by the Ministry of Science and Technological Development of the Republic of Serbia, under the Contract No. 143044B.

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