Effect of Two Distinct Stressors on Gene Expression of the Type 1 IP_3 Receptors

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Abstract. Inositol 1,4,5-trisphosphate (IP_3) is one of the second messengers, which triggers calcium release from intracellular pools *via* IP_3 receptors. Previously we have shown that single immobilization stress increased gene expression of both, the type 1 and type 2 IP_3 receptors (IP_3R1 and IP_3R2, respectively). In this study we evaluated whether long-term exposure to softer stressor (cold exposure to 4 ℃) can affect the response to single immobilization stress. We examined modulation of the type 1 IP_3 receptor gene expression by each stressor separately, and then in their combination. Rats were immobilized for 30 and 120 min and were decapitated immediately or 3 h after immobilization. Cold stress was performed by exposure of animals to 4 ℃ temperature for 1, 7 and 28 days. To determine the effect of both stressors in combination, animals exposed to cold for 28 days were afterwards exposed to immobilization for 120 min and decapitated 3 h after the end of stressful stimulus.

Our results verify that single immobilization increases the IP_3R1 gene expression in left atria of rat heart, while cold stress elevates the level of gene expression only after the exposure to cold for 7 days. The exposure to cold for 28 days did not increase the gene expression of the type 1 IP_3 receptor compared to control. Application of both stressors (28 days of cold exposure followed by 120 min of immobilization with subsequent 3 h rest) showed the tendency of increased IP_3R1 gene expression compared to absolute, nonstressed control, but level of the type 1 IP_3 receptor mRNA was significantly lower compared to mRNA levels of solely immobilized animals. Thus, cold exposure affects the response of the gene expression of the type 1 IP_3 receptor to immobilization stress.

Key words: Type 1 IP_3 receptor — Immobilization stress — Cold stress — Left cardiac atria

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Introduction

One of the mechanisms of cell activation depends on receptor-stimulated hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2) *via* G protein-dependent phospholipase C (Berridge and Irvine 1989). Hydrolysis of PIP_2 results in two intracellular second messengers, diacylglycerol and inositol-1,4,5-trisphosphate (IP_3). In cardiac muscle, several plasma membrane receptors (α1-adrenergic, muscarinic, endothelin, angiotensin II) are coupled to PIP_2 turnover (Brown et al. 1985; Renard and Poggioli 1987; Kentish et al. 1990; Vites and Pappano 1990; Yorikane et al. 1990; Eckel et al. 1991; Kern et al. 1991), and different metabolic pathways lead to IP_3 production (Brown et al. 1985; Renard and Poggioli 1987). The mRNA encoding the intracellular IP_3 receptors (Ca^{2+} channels) has been detected in low amounts in the heart of various species by Northern blot analysis (Furuichi et al. 1990; Mignery et al. 1990; Nakagawa et al. 1991). Lencesova and co-workers (2002) using RT-PCR methodology identified type 1 and type 2 IP_3 receptor (IP_3R1 and IP_3R2) mRNA in the atria and ventricles, with the highest predominance in the left atrium.

Physiological role of the Ca^{2+}-mobilizing messenger IP_3 in heart is still vague, although many hormones activate IP_3 production in cardiomyocytes and some of their inotropic, chronotropic and arrhythmogenic effects may be due to Ca^{2+} release mediated by IP_3 receptors (Nosek et al. 1986; Shah 1996; Jacobsen et al. 1997).

Stress is one of the major contributors to the development of cardiovascular disorders and psychiatric illnesses. Immobilization stress is one of the severe stressors, since it activates both pathways of sympathoadrenal system. We have already shown that single immobilization for 2 h increases significantly the gene expression of IP_3 receptors. This increase was glucocorticoid-dependent, probably through the glucocorticoid responsive element (Lencesova et al. 2002). Exposure to cold results in sympathetic and neuroendocrine activation. In brown adipose tissue, mRNA levels of both the type 1 and 2 IP_3 receptors were found to be remarkably elevated when rats were exposed to cold (Kajimoto et al. 2003). Both continuous and intermittent cold stress (1.5 days) have been shown to regulate adrenal tyrosine hydroxylase activity (Fluharty et al. 1983) and increase plasma norepinephrine levels (Fukuhara et al. 1996). In addition, chronic cold also results in vascular remodeling (Illyes et al. 2000). Furthemore, the rate of body weight gain is reduced (Finlay et al. 1997) as animals presumably utilize energy for thermogenesis. Thus, cold stress not only activates physiological homeostatic mechanisms, but also seems to impact upon brain nuclei associated with the integration of emotional stressors (e.g. Gresch et al. 1994; Finlay et al. 1997; Pardon et al. 2003). Notably, chronic cold appears to cause long-term changes to central catecholamine system, particularly noradrenergic pathways.

In the present study we tested, whether long-term exposure to cold can alter the immobilization-induced increase of the gene expression of the type 1 IP_3 receptors in the rat left atrium.

Materials and Methods

Animals

Three-month-old male Sprague-Dawley rats (280 to 320 g) were used in the experiments. Before initiation of experimental procedures, animals were housed 3 to 4 *per* cage for at least 7 days. Room temperature was held at 22 ± 2 °C, and periodic 12 h alteration of the light and dark was maintained during the entire experiment. Food and water were available *ad libitum*. The Animal Care Committee of the Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovakia approved the experimental protocol. Immobilization stress was performed as described by Kvetnansky and Mikulaj (1970). Briefly, animals were immobilized tighly to a board for 30 or 120 min, afterwards transferred to their home cages and decapitated immediately after the immobilization or 3 h after the end of immobilization.

Cold exposure was performed as follows: animals were transferred into the cold chamber, with temperature 4 °C and kept in the chamber for 1, 7 or 28 days. When both stressors were administered subsequently, animals exposed to cold for 28 days were immobilized afterwards for 120 min and decapitated after 3 h of rest. Hearts were dissected; left atria were withdrawn and frozen in liquid nitrogen for further analysis.

RNA isolation and relative quantification of mRNA levels by RT-PCR

Total RNA was isolated by TRI Reagent (MRC Ltd.). Briefly, tissue samples were homogenized by tissue homogenizer (Biospec Products Inc.) in TRI Reagent and after 5 min the homogenate was extracted by chloroform. RNAs in the aqueous phase were precipitated by isopropanol. RNA pellet was washed with 75 % ethanol and stored under 96 % ethanol at −70 °C. The purity and integrity of isolated RNAs was checked on GeneQuant Pro spectrophotometer (Amersham Biosciences). Reverse transcription was performed using 1.5 μg of total RNAs and Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences) with pd(N_6) primer. PCR specific for the IP_3R1 was carried out afterwards using following primers: IP_3R1A: 5′-GTG GAG GTT TCA TCT GCA AGC-3′ (position 70–90) and IP_3R1B: 5′-GCT TTC GTG GAA TAC TCG GTC-3′ (position 456–476, *Rattus norvegicus* GI: 1055286) giving a 410 bp fragment as described in Genazzani et al. (1999). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Terada et al. 1993) was amplified with primers GA1: 5′-AGA TCC ACA ACG GAT ACA TT-3′ (position 795–814) and GA2: 5′-TCC CTC AAG ATT GTC AGC AA-3′ (position 506–525) giving 309 bp fragment. GAPDH was used as a housekeeper gene for semi-quantitative evaluation of PCR. Each PCR program started with initial denaturation at 94 °C for 5 min, followed by 35 (for IP_3R1) or 30 (for GAPDH) cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and polymerization at 72 °C for 1 min. PCRs were terminated by final polymerization at 72 °C for 7 min. All PCR products were analyzed on 2 % agarose gels.

Statistical analysis

Each value represents the average for 5–10 animals. Results are presented as means ± S.E.M. Statistical differences among groups were determined by one-way analysis of variance (ANOVA). Statistical significance $p < 0.05$ was considered to be significant. For multiple comparisons, an adjusted t-test with p values corrected by the Bonferroni method was used (Instat, GraphPad Software, USA).

Results

Single immobilization stress for 30 min did not increase level of the type 1 IP_3 receptor in left ventricle compared to absolute control (Figure 1; I30: 6.78 ± 0.74 a.u. *vs.* AC: 5.36 ± 0.9 a.u.). On the other hand, both single immobilization stress for 120 min and single immobilization stress for 120 min followed by 3 h rest caused significant upregulation of gene expression for type 1 IP_3 receptor compared to absolute control (Figure 1; I120: 8.8 ± 0.66 a.u. *vs.* AC: 5.36 ± 0.9 a.u. and I120+3: 9.66 ± 1.5 a.u. *vs.* AC: 5.36 ± 0.9 a.u.).

Animals exposed to cold for 1 day did not show any significant difference in the gene expression of the IP_3R1 compared to controls. After 7 days of cold expo-

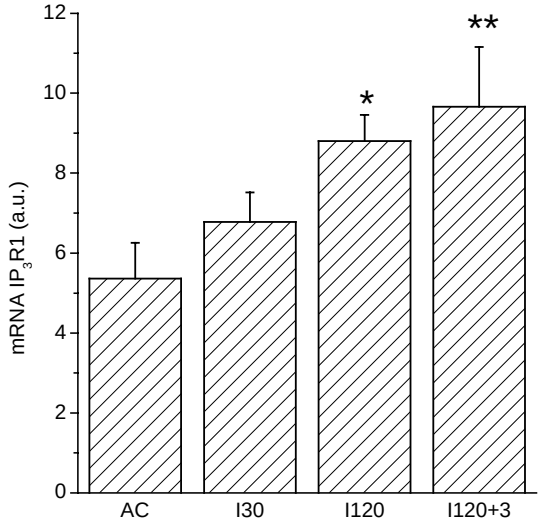

Figure 1. The IP_3R1 mRNA levels in the left atrium of immobilized rats. Immobilization administered for 30 min (I30) did not affect the gene expression of type 1 IP_3 receptor in left atria of rat heart while 120 min of immobilization (I120) increased the expression significantly compared to absolute control (AC) even when measured after 3 h of rest (I120+3). Statistical significances between control and immobilized rats were considered as * $p < 0.05$ and ** $p < 0.01$. Each value represents an average of 5 animals and is expressed as mean ± S.E.M.

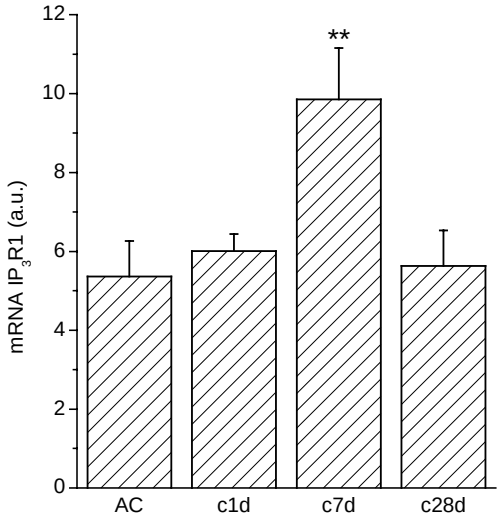

Figure 2. The IP_3R1 mRNA levels in the left atrium of rats exposed to cold stress. Exposure of animals to cold for 1 day (c1d) did not affect the gene expression of the type 1 IP_3 receptors in left atria compared to absolute controls (AC), while after 7 days of exposure (c7d), the gene expression of type 1 IP_3 receptors was significantly upregulated. Animals exposed to cold for 28 days (c28d) became cold-adapted and did not show any change in type 1 IP_3 receptor mRNA level compared to AC. Statistical significances between control rats and rats exposed to cold for 7 days were considered as ** $p < 0.01$. Each value represents an average of 5 animals and is expressed as mean ± S.E.M.

sure to 4℃, the levels of mRNA of IP_3R1 in left atria were significantly affected: animals showed almost 100 % elevation of the type 1 IP_3 receptor mRNA expression level compared to absolute control (Figure 2; c7d: 9.85 ± 1.31 a.u. *vs.* AC: 5.36 ± 0.9 a.u.). Level of the IP_3R1 mRNA after 28 days of exposure to cold was not changed compare to absolute control level (Figure 2; c28d: 5.63 ± 0.9 a.u. *vs.* AC: 5.36 ± 0.9 a.u.). Finally, both absolute controls and animals exposed to cold for 28 days (cold-adapted) were immobilized for 120 min with following rest for 3 h. Absolute control animals and cold-adapted animals expressed similar levels of type 1 IP_3 receptor mRNA (Figure 3; AC: 5.36 ± 0.9 a.u. and c28d: 5.63 ± 0.9 a.u.) and both served as control groups for immobilization procedure (Figure 3). In the group of control animals, immobilization significantly upregulated the IP_3R1 gene expression compared to absolute control (Figure 3; I120+3: 9.66 ± 1.5 a.u. *vs.* AC: 5.36 ± 0.9 a.u.). Any significant elevation of mRNA level was observed in the group of immobilized cold-adapted animals when compared to cold-adapted animals that were not subjected to immobilization (Figure 3; c28I: 7.06 ± 0.47 a.u. *vs.* c28d: 5.63 ± 0.9 a.u.). When comparing effect of immobilization in group of control animals and animals previously exposed to cold we can summarize that previous exposure to cold significantly suppressed responsiveness of the type 1 IP_3 receptor

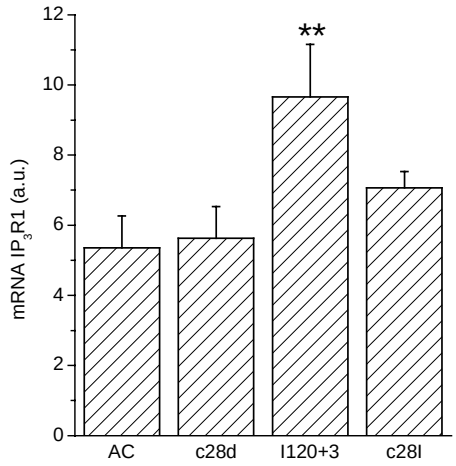

Figure 3. The effect of both cold and immobilization stress on gene expression of the IP_3R1 receptors. Both absolute control (AC) animals and animals exposed to cold for 28 days (c28d) (cold-adapted) were divided into two parts and one part was immobilized for 120 min with following rest for 3 h (I120+3 and c28I). Absolute control animals and cold-adapted animals expressed similar levels of type 1 IP_3 receptor mRNA. Immobilization significantly upregulated the type 1 IP_3 receptor gene expression of control animals but no significant elevation of mRNA level was observed in the group of immobilized cold-adapted rats (c28I). Statistical significances between control rats and immobilized control rats were considered as ** $p < 0.01$. Each value represents an average of 5 animals and is expressed as mean ± S.E.M.

gene expression caused by immobilization stress (Figure 3; I120+3: 9.66 ± 1.5 a.u. *vs.* c28I: 5.63 ± 0.9 a.u.).

Discussion

In this report we showed that besides immobilization stress, exposure to cold also significantly changes gene expression of the type 1 IP_3 receptor. While immobilization significantly upregulates the gene expression after single exposure for 120 min, cold significantly changes the level of expression of the type 1 IP_3 receptor mRNA after 7 days of exposure. Animals exposed to cold for 28 days did not respond to this stressor anymore and presumably became cold adapted. When these cold-adapted animals were exposed to additional stress, immobilization for 120 min, level of the gene expression of the type 1 IP_3 receptor was not increased to the same values as it is in the group of immobilized rats. However, a tendency of elevation occurred in the group of rats exposed to both stressors, compared to their cold-adapted controls that were not subjected to immobilization.

The physiological responses to stress are initiated by the activation of the sympatho-adrenomedullary system (SAS) and the hypotalamo-pituitary-adrenal

(HPA) axis, resulting in the release of catecholamines and stress hormones, such as glucocorticoids from the adrenal gland (Von de Kar and Blair 1999; Lapiz et al. 2000; McEwen et al. 2000). Although all stressors activate HPA and SAS, the degree of their activation depends on stress duration, type and intensity. Immobilization represents one of the most potent stressors, whereas exposure to cold is assumed to be a moderate stress. It has been shown that immobilization stress resulted in increased levels of plasma catecholamines and corticosterone accompanied by the activation of both sympatho-adrenomedullary and adrenocortical systems (Pacak et al. 1998; Dronjak et al. 2001). Chronic cold can result in long-term neuroadaptation that may relate to sensitization of stress-sensitive circuitry. Persistent, stress-induced sensitization of noradrenaline (NA) release has been demonstrated in rats previously exposed to cold (5 ℃) for 2–4 weeks (Jedema et al. 1999), as evidenced by a greater increase in extracellular NA in the hippocampus and medial prefrontal cortex in response to subsequent, acute tail shock (Gresch et al. 1994; Zigmond et al. 1995; Finlay et al. 1997). While the locus coerulus is considered as a potential focus for central sensitivity to cold, the potential involvement of other catecholaminergic neurons cannot be discounted. As indicated by Pardon et al. (2003), in Wistar-Kyoto rats, previous cold exposure resulted in an exaggerated release of NA following acute immobilization stress. Collectively, this suggests that exposure to cold may also sensitize subcortical noradrenergic pathways, possibly arising from the adrenal medulla. Previous exposure of rats to cold may increase the susceptibility of the rats to a subsequent stressor (Featherby and Lawrence 2004).

Short-term exposure to cold for 24 h did not affect the gene expression of the type 1 IP_3 receptors. We have observed significant increase in the mRNA levels of the type 1 IP_3 receptors after 7 days exposure to cold, but after 28 days, the increase in mRNA levels of these receptors completely diminished. We proposed adaptation to this stressor. Kajimoto and co-workers (2003) exposed rats to cold and also observed significant increase in the gene expression of the type 1 IP_3 receptors in the brown adipose tissue.

We have already shown that in rat atria, gene expression of the type 1 and 2 IP_3 receptors was upregulated by single immobilization stress for 2 h (Lencesova et al. 2002). This increase was regulated by glucocorticoids, possibly through the glucocorticoid responsive element (Krizanova et al. 2001). No effect of glucocorticoids was observed on basal levels of the IP_3 receptor's mRNA (Lencesova et al. 2002). Interestingly, after the repeated immobilization (2 h daily, for 7 days) both gene expression and protein of the type 1 IP_3 receptors decreased significantly in the left, but not the right atrium (Krepsova et al. 2004). Decrease in the gene expression of the IP_3 receptors is consistent with observations of this type of IP_3 receptors in renal medulla (Zacikova et al. 2000) and stellate ganglium (Micutkova et al. 2003).

The immobilization, when administered as an additional stressor, produces a much more pronounced response of SAS and a less pronounced response of HPA axis (Dronjak et al. 2004). This might be the reason, why subsequent exposure to immobilization stress in cold exposed rats did not result in the same increase in

the mRNA levels of the type 1 IP_3 receptors as in the group of purely immobilized rats.

The obtained results show that both immobilization and cold stress significantly upregulates the type 1 IP_3 receptor gene expression, although cold is considered as moderate stressor. Exposure of animals, previously exposed to chronic cold, to single imobilization stress for 120 min elevates the mRNA level of type 1 IP_3 receptor which is suggesting sensitization of subcortical noradrenergic pathways.

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