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Heart ventricles specific stress-induced changes in β-adrenoceptors and muscarinic receptors

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Abstract. The left and right ventricles fulfill different role in heart function. Here we compare chamber specific changes in local catecholamine concentrations; gene expression and the receptor protein amount of all three β-adrenoceptors (β-AR) in rat right heart ventricles exposed to acute (1 session) and repeated (7 sessions) immobilization stress (IMMO) *vs.* previously observed changes in left ventricles. Density of muscarinic receptors as main cardio-inhibitive receptors was also measured. In the right ventricles, noradrenaline and adrenaline were increased. No β_1-AR changes were observed, in spite of the increased sympathetic activity. On the other hand, we have found a decrease of β_2-AR gene expression (reduction to 30%) after 7 IMMO and protein (to 59%) after 1 IMMO. β_3-AR gene expression was increased after 7 IMMO. Muscarinic receptor density was not changed. When comparing correlation in left and right ventricles, there was strong correlation between adrenaline and β_2-AR gene expression, protein and β_3-AR gene expression in the left ventricles while only correlation between adrenaline and β_2-AR mRNA and protein in the right ventricles was found. Our results show that maintenance of cardiac homeostasis under stress conditions are to a great extent achieved by a balance between different receptors and also by a balanced receptor changes in left *vs.* right ventricles. Taken together, decrease of cardio-stimulating β_2-AR represents a new important mechanism by which β_2-AR contributes to the heart physiology.

Key words: Beta-adrenergic receptors — Binding sites — Catecholamines — Gene expression — Heart — Stress

Introduction

The function of the heart is regulated *via* sympathetic and parasympathetic nerves. The effects of respective neurotransmitters (i.e. adrenaline/noradrenaline and acetylcholine) are mediated *via* almost antagonistic G-protein-coupled receptors: adrenoceptors and muscarinic receptors (Burnstock 2009). In the heart, the main subtypes of receptors that mediate parasympathetic action are M_2 muscarinic receptors (Gomeza et al. 1999; Burnstock 2009). As there is need for the homeostatic balance between sympathetic and parasympathetic nerve tonus, there is also need of balance between the amount of muscarinic receptors (MR) and β-adrenoceptors (β-AR) as well as the functional output of these receptors. These premises have been proved repeatedly (Myslivecek et al. 1996; Garofolo et al. 2002; Brodde and Leineweber 2004; Stavrakis et al. 2011).

Physiological effects of catecholamines in the heart are mediated mainly *via* β-AR (Brodde and Michel 1999; Brodde et al. 2006). β_1-AR are the predominant subtypes expressed in the heart and their stimulation produces positive inotropic and chronotropic effects. In addition to this adrenoceptor subtype, β_2-AR are also expressed in the heart with partly similar function as β_1-AR, i.e. they are cardio-stimulative (Bernstein

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et al. 2011) but with important differences in pathological circumstances like heart failure (Bernstein et al. 2011).

All β-AR are members of the G-protein-coupled receptor family. β_1-AR and β_2-AR couples to G_s proteins, stimulates adenylyl cyclase (AC), which subsequently leads to increase in cAMP intracellular levels. The sufficiency of cAMP is the key point in activation of protein kinase A (PKA), which phosphorylates also the L-type Ca^{2+} channels and thus promotes calcium influx and enhances cardiomyocytes contraction. Accumulated evidence indicates that, at least in the rat and murine hearts, stimulation of β_1-AR causes not only positive inotropic and chronotropic effect, but can also promote apoptosis of cardiomyocytes (Xiao et al. 2006). Persistent β_1-AR stimulation changes the receptor signaling pathway from PKA to Ca^{2+}/calmodulin-dependent protein kinase II predominance, leading to myocyte maladaptive remodeling (Xiao et al. 2006). On the other hand, prolonged β_2-AR activation switches the receptor G-protein coupling from G_s to G_i and thereby possesses antiapoptotic effect resulting in cardioprotection (Communal et al. 1999; Xiao et al. 2006).

On the other hand, the role of β_3-AR in the heart remains controversial and contradictory data were published. Shortly, different expression/role/function was found in specific heart chambers (i.e. in atria and ventricles (Brixius et al. 2004; Spasojevic et al. 2011) and in different species (Gauthier et al. 2000; Skeberdis et al. 2008)). Some authors found the β_3-AR to be antagonistic to β_1- and β_2-AR (Rozec and Gauthier 2006) – following prolonged activation by the sympathetic nervous system, the β_3-adrenergic response is preserved, while the β_1- and β_2-adrenergic responses are diminished; some authors found that β_3-AR increase contractility (Skeberdis et al. 2008) but this can be also the question of different heart chambers. Moreover, metabolic effects were also demonstrated for β_3-AR (Arch and Wilson 1996), these effects are mediated *via* different pathways (cAMP-dependent pathway, NO-cGMP-mediated pathway, activation of ion channels and many others), what also enlarge the functional consequences of β_3-AR activation. Importantly, some authors also claimed no β_3-AR agonist-induced effects in the heart (Molenaar et al. 1997; Kaumann and Molenaar 2008).

In the heart, the coupling of β_3-AR is proposed to be coupled to the G_i protein. In human ventricles activation of G_i proteins causes an activation of endothelial NO (eNOS) synthase (Gauthier 1998; Schulz et al. 2005). The NO production induces an increase in intracellular cyclic guanosine monophosphate (cGMP). Alternatively, NO can act *via* cGMP-independent way through the modification of cytochrome c oxidase or L-type calcium channel (for review see (Rozec and Gauthier 2006). It's supposed that β_3-AR/NO pathway could act as a negative feedback mechanism opposing the positive inotropic influences of catecholamines in the heart. In full contrast with the other β-AR subtypes, stimulation of the β_3-AR produced a marked decrease in cardiac contractility in human heart (Rozec and Gauthier 2006). Negative inotropic effects and changes in β_3-AR gene expression were noted in many cases, when β_3-AR agonists were used (Gauthier 1998; Gauthier et al. 1999; Tavernier et al. 2003) . Compared to β_1- and β_2-AR, the β_3-AR presents a relative *in vitro* and *in vivo* lack of desensitization following activation with agonists (Nantel et al. 1993).

Recently, we have shown important changes in β_3-AR and other AR in stress (Laukova et al. 2013). Shortly, the concentration of noradrenaline in the left ventricles decreased while adrenaline increased, especially after repeated immobilization (IMO). The mRNA and protein levels and binding sites of β_3-AR significantly rose following chronic IMO while all parameters for β_2-AR dropped after single as well as repeated exposure. Similarly, the mRNA levels and binding sites for β_3-AR increased in the left atrium as a consequence of chronic IMO. The rise in β_3-AR and a drop in β_2-AR resulted in inhibition of adenylyl cyclase activity within the left ventricles.

Therefore, this study was focused on the investigation of single and repeated stress effects on mRNA and protein levels of β_1-, β_2-, and β_3-AR, as well as on total muscarinic receptor binding sites (as virtually only receptor subtype in the heart is M_2 muscarinic receptors) in right cardiac ventricles and on comparison of right/left ventricles stress-induced changes.

Materials and Methods

Animals and tissue preparation

Male Sprague-Dawley rats (250–300 g, Charles River, Suzfeld, Germany) were used in our experiments. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by The Ethic Committee of the Institute of Experimental Endocrinology (Slovak Academy of Sciences, Bratislava, Slovakia). Prior to experiments, animals were housed for at least 1 week, four animals *per* cage in a controlled environment (22 ± 2°C, 12 h light/dark cycle, lights on at 6 a.m.). Food and water were available *ad libitum*. Immobilization stress was performed as described previously (Kvetnansky and Mikulaj 1970): by taping (with adhesive tape) all four legs, in the prone position, to a fixed board. In the process of a single immobilization, rats were restrained once for 2 h and decapitated 3 h after the termination of the immobilization stimulus. Repeated stress was achieved by immobilizing animals for the 7 consecutive days (2 hours each), with decapitation after the last, seventh immobilization (3 h after the termination of last immobilization). The rats endured the stress procedure well and all survived the seven-fold exposure. All animals were sacrificed without anaesthetic. Heart was rapidly removed,

ventricles (left and right) separated, and immediately frozen in the liquid nitrogen and stored at –70°C until the assay.

Determination of tissue catecholamines (noradrenaline and adrenaline)

Catecholamine concentration was determined by a modified method of Peuler and Johnson (Peuler and Johnson 1977). Frozen tissues were weighted and immediately homogenized in 0.1 mol/l $HClO_4$. Homogenate was centrifuged at 10,000 × *g* for 15 min. An aliquot of supernatant was taken and diluted to the final concentration 1 mg of tissue *per* 50 μl. Rest of the method was the same as for plasma catecholamine quantification (Peuler and Johnson 1977). Briefly, tritiated neurotransmitter metabolites production was measured using catechol-O-methyl-transferase-catalyzed radioenzymatic assay. The assay was sensitive similarly to the values reported originally by Peuler and Johnson (1977), i.e. 1 pg (20 pg/ml of plasma) for both neurotransmitters.

RNA isolation and relative quantification of mRNA levels by reverse transcription with subsequent polymerase chain reaction (RT-PCR)

Total RNA from frozen heart tissue was isolated by TRI Reagent (MRC Ltd.) according to the manufacturer's instructions. Concentration and purity of RNA was determined spectrophotometrically on a GeneQuant Pro (Amersham Bioscience). PCR specific for adrenoceptors, primers and PCR conditions were carried out as described previously (Myslivecek et al. 2006). Reverse transcription was performed using Ready-To-Go You-Prime First-Strand beads and pd(N)6 primer. The initial denaturation was performed at 95°C for 5 minutes followed by denaturation and annealing (different temperature and time) and followed by polymerization (at 72°C in all cases). The final polymerization lasting 7 minutes was performed at 72°C in all cases.

PCR products were analyzed on 2% agarose gels and visualized using ethidium bromide. Intensity of individual bands (OD/mm^2) was evaluated by PCBAS 2.08e software (Raytest, GmbH). As a control, the housekeeper glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used. For semiquantitative evaluation the values were normalized to the signal obtained with GAPDH.

Protein isolation and relative quantification of protein levels by Western blot analysis

Membrane proteins were isolated by modified method described in Barbier et al. (2004). Heart tissue was homogenized in 20 mmol/l Tris (pH 7.0), 0.3 mol/l sucrose and proteases inhibitors cocktail (Pefabloc SC and Complete (EDTA free); ROCHE Diagnostic), then centrifuged for 15 min at 1000 × *g* and 4°C. Supernatant was taken out and centrifuged at 45,000 × *g* and 4°C for 45 min. The pellet was dissolved in homogenization solution, and protein concentration was determined according to Bradford (1976). Western blot analysis was carried out as described previously (Krizanova et al. 2001). Briefly, protein extract from the cardiac ventricles (50–180 mg) was separated by SDS-PAGE and then transferred to a supported nitrocellulose membrane (Hybond-ECL, AP Biotech) using semidry blotting. The membrane was blocked in 5% nonfat dry milk in Tris-buffered saline-Tween and incubated with the rabbit polyclonal antibody against specific receptors (see below). After the membrane was washed three times, it was incubated in the secondary anti-rabbit antibody conjugated to horseradish peroxidase (dilution 1:5,000). Secondary antibody was visualized by enhanced chemiluminiscence (AP Biotech). Levels of the β_1-, β_2- and β_3-AR proteins were determined using a specific polyclonal primary antibody (sc-568, sc569, sc-1473, all were targeted to C-terminus; Santa Cruz Biotechnology). Every protein was quantified using GAPDH protein as housekeeping gene standard. All protein levels were normalized using GAPDH standard (measured as optical density) which level did not differ between blots for different types of receptors and between experiments. The scatter was less than 10% in every case.

Radioligand binding experiments

The tissue sample was weighted and homogenized in ice cold Tris-EDTA buffer (Tris-HCl 50 mmol/l, EDTA 2 mmol/l, pH adjusted to 7.4). Membranes were prepared as was previously reported (Fischer et al. 2008). Amount of muscarinic receptor binding sites was determined as previously reported (Myslivecek et al. 2006), using 65–2000 pmol/l [^{3}H]QNB (^{3}H-(±)-Quinuclidinyl[phenyl-4-3H] benzilate, As = 1.35 TBq/mmol)), non-specific binding was determined with 5 μmol/l atropine (incubation for 120 min at 38°C). All incubation times were adjusted previously (Myslivecek et al. 2006). The amount of binding sites (B_{max}) *per* mg protein was determined using BCA (bicinchoninic acid) method kit (Sigma) and the affinity constant (K_D) was computed by non-linear regression using GraphPad Prism 5.01 program (GraphPad Software). K_D were used for the "single-point" measurement in order to save the amount of tissue needed, using following saturating concentrations of radioligand (1500 pmol/l [^{3}H]QNB) using the equation as previously (Fischer et al. 2008).

Statistical analysis

Results are presented as mean ± S.E.M. and each group represents an average of 6 animals. Statistical differences among groups were determined by one-way or two-way

ANOVA, and for multiple comparisons an adjusted *t*-test modified by Bonferroni's or SNK (Student-Newman-Keules) correction was used. Subsequently, the values (catecholamine concentration *vs.* mRNA, protein, binding) were plotted against each other. The correlations were analyzed using Pearson's correlation coefficient (r) and the corresponding p-values (using GraphPad software). In all cases, a value of $p < 0.05$ was considered as statistically significant.

Results

Catecholamine levels in right cardiac ventricles of rats exposed to single or repeated immobilization

Noradrenaline concentration significantly increased in repeatedly (7×IMMO) stressed rats (Fig. 1A). Adrenaline concentration was much lower compared to noradrenaline. In spite of the low concentration, adrenaline levels were increased in right ventricles of both singly (1×IMMO) and repeatedly (7×IMMO) stressed rats.

Effect of single and repeated immobilization on β-AR's gene expression, and protein levels in right heart ventricles

No significant changes of β_1-AR mRNA levels of singly (1×IMMO) or repeatedly (7×IMMO) stressed rats were found (Fig. 1B). On the other hand, significant changes were observed in β_2-AR gene expression in repeatedly (7×IMMO) stressed rats. Similarly, mRNA levels of β_3-AR were elevated in repeatedly (7×IMMO) stressed rats.

Protein levels of β_1-AR were also unchanged in 1× and 7×IMMO rats (Fig. 1C). Protein levels of β_2-AR were found significantly decreased after the single immobilization. Protein levels of β_3-AR were not changed.

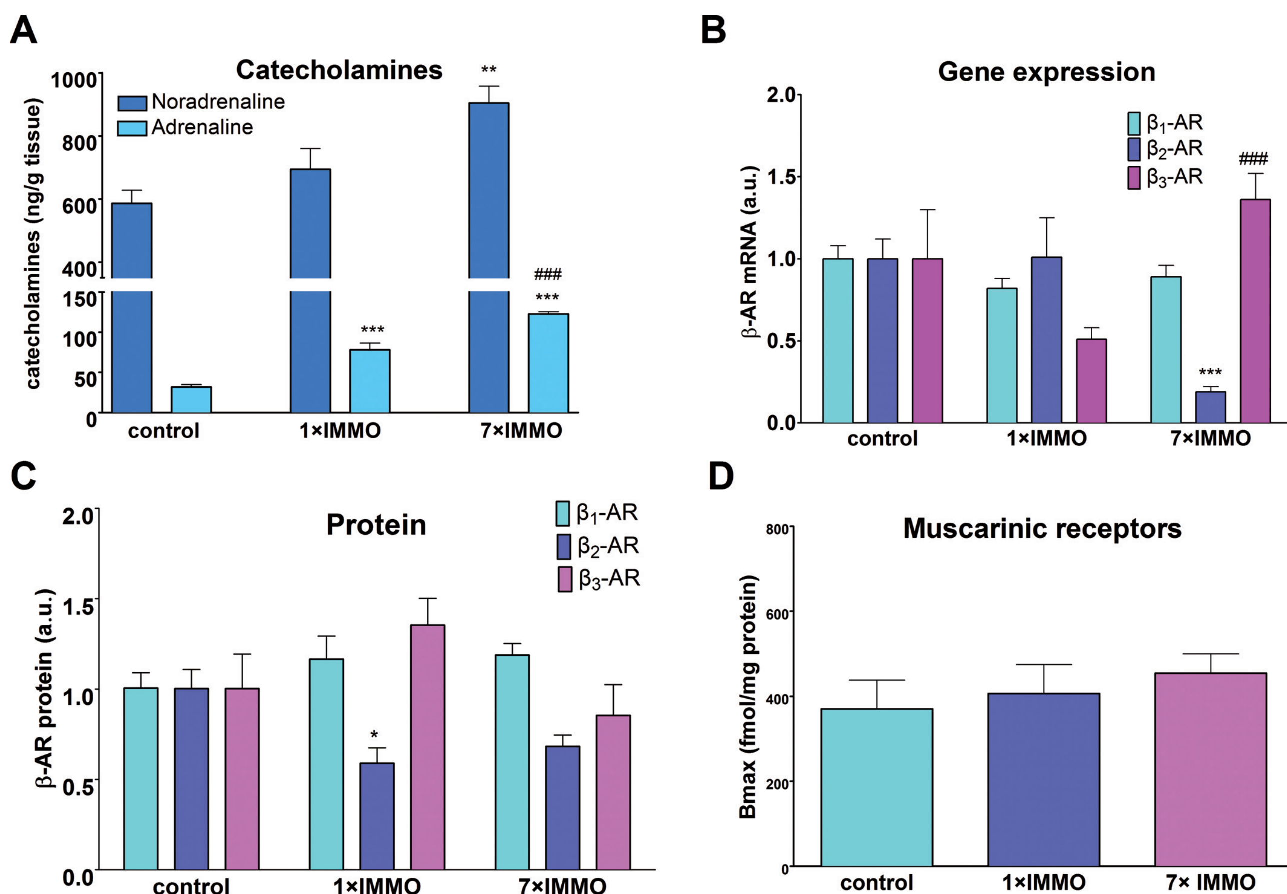

Figure 1. Noradrenaline and adrenaline concentrations (**A**), changes in gene expression (**B**), protein levels of β-adrenoceptors (**C**) and binding to muscarinic receptors (**D**) in the right cardiac ventricles of rats exposed to a single (1×IMMO) or repeated (7×IMMO) immobilization stress. Results are presented as means ± S.E.M of 5–10 animals. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to control group; ### $p < 0.001$ compared to 1×IMMO. β-AR, β-adrenoceptors.

Effect of single and repeated immobilization on muscarinic receptor binding sites

In order to ascertain how stress disrupts equilibrium between β-AR (stimulators of adenylyl cyclase), and muscarinic receptors (MR; main cardiac receptors inhibiting adenylyl cyclase), we measured the number of MR in cardiac ventricles. No differences in receptor number between control and stressed animals were observed in right ventricles (Fig. 1D).

Correlation between catecholamine levels, adrenoceptor mRNA, proteins and MR binding

There was variance in the stress effects on left and right ventricles (see Fig. 2). While in left ventricles the adrenaline levels correlated with β_2-AR mRNA, β_2-AR protein and β_3-AR mRNA, in right ventricles we were able to find correlation between adrenaline and β_2-AR mRNA and protein only. All other variables were not dependent. It can be deduced from Figure 2 that β_2-AR mRNA correlated negatively with adrenaline levels in left ventricles. This correlation (adrenaline-β_2-AR mRNA), however, is stronger in left ventricles ($r = -0.7156$, $p < 0.001$) than in right ventricles ($r = -0{,}4998$, $p < 0.05$). Similarly, left ventricles β_2-AR protein also revealed negative correlation with adrenaline levels. On the other hand, β_3-AR mRNA correlated positively with adrenaline levels in left ventricles. Likewise in left ventricles, β_2-AR protein and mRNA in right ventricles also correlated negatively to adrenaline levels.

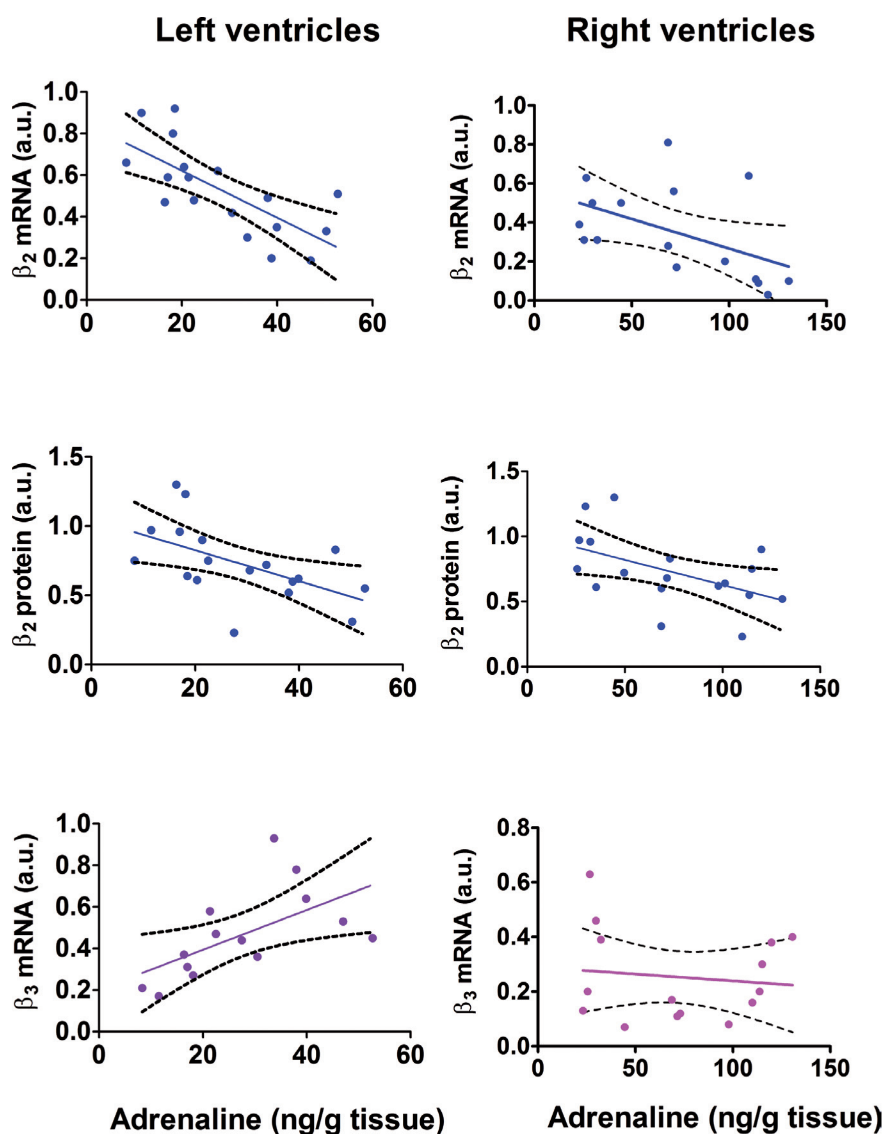

Figure 2. Correlation between adrenaline levels (expressed as ng/g tissue) and β_2-AR mRNA (a.u.), β_2-AR protein (a.u.), and β_3-AR mRNA (a.u.) in left ventricles (left panel) and right ventricles (right panel). The correlation was significant in all case except β_3-AR mRNA *vs.* adrenaline in right ventricles. $p < 0.001$ in left ventricles, β_2-AR mRNA *vs.* adrenaline; $p < 0.05$ in all other cases. a.u., arbitrary units.

Discussion

Our results have shown that immobilization stress (acute or repeated) increased adrenaline and noradrenaline concentration in right ventricles. These results are in good agreement with previous observations (Kvetnansky and Torda 1985). Increased adrenaline levels in ventricles are consequence of stress-induced uptake from circulation or of increased endogenous adrenaline synthesis directly in the heart (Axelrod 1962; Krizanova et al. 2001). These data show specific elevation of catecholamines in the heart chamber and allow correlating these changes with changed protein and gene expression, as shown in Fig. 2, where local catecholamine levels, β-AR subtypes gene expressions and protein levels are compared. There were less pronounced changes in the right ventricles compared to the left ventricles, which revealed broad changes (Laukova et al. 2013). More specifically, in the right ventricles, a decrease of β_2-AR gene expression (together with increase in β_3-AR gene expression) was observed after repeated immobilization. Moreover, MR changes occurred in left ventricles only (Laukova et al. 2013). The main MR subtype is M_2 and we can therefore conclude that these changes occur therefore mainly for M_2 MR.

It is generally accepted that in the failing human heart, β_1-AR are decreased, β_2-AR may or may not be decreased but are uncoupled from the effector system adenylyl cyclase (Brodde 1991; Brodde and Leineweber 2004). Our results, in contrast, showed unchanged β_1-AR and decreased β_2-AR during immobilization stress. In experiments with animals exposed to a long-term cold stress (28 days at 4°C) we did not find any changes in β_1-AR gene expression as well (Tillinger et al. 2008), in spite of huge activation of sympathoneural system and elevation of circulating noradrenaline levels. These findings could be related to the higher resistance of β_1-AR to agonist-induced down-regulation (Liang et al. 2003).

Stress-induced changes of cardiac β_1-AR and β_2-AR were first described in 80´s (U'Prichard and Kvetnansky 1980; Torda et al. 1981, 1985). Further, the effetcs of foot-shock stress on β_2-AR were studied in SHR and WKY rats (Kirby and Johnson 1990) and β_2-AR were shown to be activated during acute stress in SHR but not in WKY. Another authors (Bassani and Bassani 1993) reported the down-regulation of the pacemaker β_1-AR but not the increased participation of β_2-AR in the response of the rat sinoatrial node to catecholamines after repeated foot-shock. Also, different stress types, like neonatal stress, restriction stress or restricted food intake induced a decrease in the density of binding sites or down-regulation of β-AR (Gudbjarnason and Benediktsdottir 1996). Another study tried to simulate oxidative stress condition in ischemia-reperfusion model and showed that H_2O_2 may depress the β_1-AR, G_s proteins and catalytic subunit of AC and thus may play an important role in attenuating the β-AR linked signal transduction due to ischemia-reperfusion injury (Persad et al. 1998). Important differences were shown between oestrus phase after inescapable foot-shock stress (Spadari-Bratfisch et al. 1999). Moreover, the stress-induced response was shown to be mediated by the β_2-AR subtype. In another paper of this group (Santos and Spadari-Bratfisch 2001), the chronotropic effect of (+/-)-CGP12177 were studied and it was shown that are mediated by β_{1L}-AR. In constrast with β_1-AR and (or) β_2-AR, these receptors were resistant to the effects of foot-shock stress. In general, the effects of stress on heart β_1-AR and β_2-AR receptors differ (Santos and Spadari-Bratfisch 2006). Restraint stress (acute, 1 hour) did not changed β_1-AR or β_2-AR (Penna et al. 2007) while acute immobilization for 120 minutes changed β_2-AR (Myslivecek et al. 2004, 2008). Cold stress induced decrease of β_1-AR and β_2-AR and increased the number of β_3-AR (Benes et al. 2012).

Considering the effects of stress, it is also necessary to consider the regulatory role of CNS (Fortaleza et al. 2012a, 2012b).

Correlation between catecholamine levels and receptor gene expression and protein

Our results showed variance in the stress effects on left and right ventricles. Adrenaline, but not noradrenaline levels correlate negatively with β_2-AR mRNA and protein both in left and right ventricles (Fig. 2). That means while the adrenaline level increases the amount of mRNA and protein decreases. This correlation (adrenaline-β_2-AR mRNA), however, is stronger in left ventricles than in right ventricles. That means that the decrease in β_2-AR mRNA is more susceptible to changes in left ventricles than in right ventricles. On the other hand, the protein was similarly decreased as a result of increased adrenaline levels in both left and right ventricles. Moreover, β_3-AR mRNA correlated positively with adrenaline levels in left ventricles but not in right ventricles showing that balance between β-AR receptor subtypes is more important in left than in right ventricles.

A balance of receptor functions achieves needed homeostasis in the heart. Our results have shown that a single and repeated immobilization stress causes complex changes in receptor proteins or binding in left ventricles (Laukova et al. 2013) but present results show only modest changes in right ventricles. On the other hand, β_2-AR mRNA and protein correlates with adrenaline level in both ventricles.

Taken together, decrease of cardio-stimulating β_2-AR represents a new important mechanism by which β_2-AR contributes to the heart physiology.

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