BRAZILIAN JOURNAL
OF MEDICAL AND BIOLOGICAL RESEARCH

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Abstract

The regulatory function of α_{1B}-adrenoceptors in mammalian heart homeostasis is controversial. The objective of the present study was to characterize the expression/activity of key proteins implicated in cardiac calcium handling (Na^+/K^+-ATPase and Ca^{2+}-ATPases) and growth (ERK1/2, JNK1/2 and p38) in mice with cardiac-selective overexpression of constitutively active mutant α_{1B}-adrenoceptor (CAMα_{1B}-AR), which present a mild cardiac hypertrophy phenotype. Immunoblot assays showed that myocardial plasma membrane Ca^{2+}-ATPase (PMCA) expression was increased by 30% in CAMα_{1B}-AR mice (N = 6, P < 0.05), although there was no change in sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2) expression. Moreover, total Ca^{2+}-ATPase activity was not modified, but a significant increase in the activity of the thapsigargin-resistant (PMCA) to thapsigargin-sensitive (SERCA) ratio was detected. Neither Na^+/K^+-ATPase activity nor the expression of α_1 and α_2 subunit isoforms was changed in CAMα_{1B}-AR mouse hearts. Moreover, immunoblot assays did not provide evidence for an enhanced activation of the three mitogen-activated protein kinases studied in this stage of hypertrophy. Therefore, these findings indicate that chronic cardiac α_{1B}-AR activation *in vivo* led to mild hypertrophy devoid of significant signs of adaptive modifications concerning primary intracellular calcium control and growth-related proteins, suggesting a minor pathophysiological role of this adrenergic receptor in mouse heart at this stage of development.

Key words: α_{1B}-adrenoceptors; Ca^{2+}-ATPases; Cardiac hypertrophy; Na^+/K^+-ATPase; Mitogen-activated protein kinases

Introduction

In mammalian heart, adrenoceptors (AR) mediate myocyte contraction and growth induced by the sympathetic nervous system. The β_1-AR is the most abundant cardiac AR subtype and its functional importance has been extensively studied (1). However, other AR subtypes are present in the heart and their relevance to pathophysiological conditions has been studied in recent years (2,3). The α_1-AR, which primarily activates Gαq protein-coupled phospholipase Cβ signal transduction, has been implicated

mostly in cardiomyocyte growth *in vitro* (2-5). *In vivo*, however, unbiased myocardial responses are impossible to evaluate conclusively because of the vasoactive effect of α_1-AR agonists. In addition, the restricted number of appropriate subtype-selective ligands, especially for α_{1B}-AR (1,2), makes the elucidation of specific effects of different α_1-AR particularly challenging.

In order to overcome these drawbacks, transgenic mouse models overexpressing distinct myocardial-targeted

α$_1$-AR subtypes have been generated, i.e., either the wild-type (WT) or the agonist-independent constitutively active α$_1$-AR (6). In the case of α$_{1B}$-AR, these models exhibited a certain degree of cardiac dysfunction/hypertrophy (7-11), although some studies have found clear-cut cardiac hypertrophy just at an advanced age (9-11). Interestingly, cardiac hypertrophy was also reported in systemic overexpression of constitutively active or WT α$_{1B}$-AR, a phenomenon not due to pressure load since these animals presented a normotensive (WT α$_1$-AR) or even hypotensive (constitutively active α$_1$-AR) phenotype (12,13). In contrast, other studies have not detected any sign of cardiac hypertrophy in these models (14-17) or have observed them only after marked hypertrophic stimuli (16,18), indicating that the function of α$_{1B}$-AR in murine heart is still a debatable issue.

P-type ATPases are membrane-bound ion-transporting proteins that are vital for cell function and are particularly involved in myocardial ion homeostasis. It is well known that some members, such as Na$^+$/K$^+$-ATPase α$_2$ isoform and sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2), are down-regulated by cardiac stress associated with adrenergic stimulation and may progress to hypertrophy and failure (19,20). For cardiac plasmalemmal Ca^{2+}-ATPase (PMCA), an early expression increase and further decline apparently reflect an initial adaptation to facilitate calcium removal and are the basis of more persistent cardiac dysfunction, respectively (21).

Stress-induced changes mediated by persistent stressors may contribute to cardiac hypertrophy. In fact, an increasing number of intracellular signaling pathways have been described as important transducers of the hypertrophic response, such as the mitogen-activated protein kinases (MAPKs) (22). Recent studies indicate that α$_1$-AR may signal through MAPK pathways to stimulate growth responses in rat but not mouse cardiomyocytes (23). More importantly, MAPKs are also able to cross-talk and regulate intracellular calcium-handling proteins (24).

Because of the controversial role of cardiac α$_{1B}$-AR and the absence of information regarding the specific regulatory function of this receptor on proteins involved in cardiac calcium homeostasis *in vivo*, we used transgenic mice that overexpress myocardial-targeted constitutively active mutant α$_{1B}$-AR (CAMα$_{1B}$-AR mice) (7) in order to evaluate the activity and expression of cardiac ion transport ATPases (Na$^+$/K$^+$-ATPase, SERCA and PMCA) and MAPKs (extracellular signal-regulated kinases, ERK1/2; c-Jun-N-terminal kinases, JNK1/2, and p38).

Material and Methods

Animal model

CAMα$_{1B}$-AR transgenic male adult mice (10 to 12 weeks old; Jackson Laboratory, USA) were maintained and used (7). They harbored a genetic profile in which the mutant α$_{1B}$ gene was incorporated by non-homologous insertion through the α-myosin heavy chain (α-MHC) promoter coupled to the coding sequence of CAMα$_{1B}$-AR. Non-transgenic C57BL/6J mice (WT; Jackson Laboratory) served as controls. All animal procedures were approved by the Ethics Committee of Universidade Federal de São Paulo, in accordance with the Principles of Laboratory Animal Care published by the National Institutes of Health (NIH Publication #86-23, revised 1996, USA).

Tissue collection and membrane preparations

Mice were weighed and their hearts excised, dissected, washed internally with a 320 mM sucrose, 1 mM EDTA and 5 mM imidazole-HCl buffer, pH 7.2, blotted on filter paper and weighed. Tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until use. Subcellular preparations were obtained as described (25). Organs from both transgenic and WT mice (approximately five hearts per preparation) were minced and homogenized in a 320 mM sucrose, 5 mM imidazole-HCl buffer plus 0.1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, USA) using an Ultraturrax disperser (9500 rpm for 10 s plus two steps of 13,500 rpm for 10 s with a 20-s interval between steps), followed by filtration through gauze and ultracentrifugation at 100,000 g_{av} for 45 min. A crude preparation was chosen because it is more suitable for quantification and comparison of molecular entities in muscles under conditions of plasticity, yielding a high and reproducible protein recovery (26). The pellet was resuspended in the same buffer without PMSF and stored at -80°C. Protein concentration was determined by the Lowry protein assay, using bovine serum albumin as standard.

[^{3}H]-prazosin binding assay

Saturation experiments were carried out at 25°C in 1 mL of a buffered solution, pH 7.4, containing 1 mM EDTA, 50 mM Tris-HCl, 0.5 nM [^{3}H]-prazosin (specific activity 80 Ci/mmol; New England Nuclear, USA) and increasing concentrations of unlabeled prazosin (0.1-20 nM), in parallel for both preparations (CAMα$_{1B}$-AR and WT mice). This protocol, classically referred to as competition experiment, allows the use of lower amounts of radioligand and preparation (25). The reaction was started by adding 200 µg protein and was stopped after 45 min by adding 4 mL ice-cold 5 mM Tris-HCl buffer, pH 7.4, twice and then subjected to vacuum filtration through a glass fiber filter (Filtrak GMF 3, Germany). Filters were then washed twice with 8 mL of the same buffer, dried and added to 10 mL of the scintillation solution (toluene containing 0.1 g/L POPOP and 4.0 g/L PPO). Radioactivity was then measured with a Tri-Carb liquid scintillation analyzer (Packard Instrument Co., USA). Nonspecific binding was determined in the presence of 1 µM unlabeled prazosin.

Na$^+$/K$^+$-ATPase activity

The ATPase activity assay was performed at 37°C for 1 h and started by adding 20-40 µg protein to a medium

consisting of 20 mM maleate-Tris buffer, pH 7.4, 87.6 mM NaCl, 3 mM MgCl$_2$, 1.2 mM ATPNa$_2$, 10 mM NaN$_3$, and 1 mM EGTA in the presence of 3 mM KCl (total activity), or 1 mM ouabain (ouabain-resistant activity; Sigma-Aldrich). ATPase activity was determined by measuring the release of inorganic phosphate (Pi) from ATP. The Na$^+$/K$^+$-ATPase activity was considered as the difference between total and ouabain-resistant ATPase activities (27).

Ca^{2+}-ATPase activity

ATPase activity was assessed in a medium containing 5 mM Na$_2$ATP, 0.3 mM EGTA, 4 mM MgCl$_2$, 10 mM NaN$_3$, 5 µM A$_{23187}$ (a Ca^{2+} ionophore), traces of [γ^{32}P]-ATP and 50 mM HEPES-Tris buffer, pH 7.4, by measuring the radioactivity derived from the released [^{32}P]-Pi. Ca^{2+}-ATPase activity was calculated by subtracting the basal ATPase activity (measured in the absence of Ca^{2+}) from the total ATPase activity (measured in the presence of 10 µM free Ca^{2+}, with or without 3 µM thapsigargin; Sigma-Aldrich). The thapsigargin-resistant Ca^{2+}-ATPase activity (assumed to be due to PMCA pumps) was subtracted from the total Ca^{2+}-ATPase activity to obtain the thapsigargin-sensitive Ca^{2+}-ATPase activity (assumed to be due to SERCA pumps) (28).

Immunoblotting assays

Proteins were separated by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Blots were incubated for 1 h in 5% non-fat dry milk dissolved in Tris-buffered saline solution containing 0.1% Tween 20. Subsequently, membranes were incubated for 1 h at room temperature with one of the following primary antibodies: monoclonal mouse IgG anti-Na$^+$/K$^+$-ATPase α$_1$ (McK1) and α$_2$ (McB2) isoforms (Dr. K. Sweadner, Harvard Medical School, Boston, MA, USA), anti-PMCA (clone 5F10, Sigma Chemical, USA), anti-SERCA2 (Calbiochem, EMD Chemicals, USA) or polyclonal rabbit anti-total or phospho-MAPKs antibodies (ERK1/2, p38 or SAPK/JNK; Cell Signaling Technology, USA) and for 1 h with horseradish peroxidase-conjugated anti-mouse (ATPases) or anti-rabbit (MAPKs) antibody (Promega, USA). Proteins were detected using an ECL detection kit (GE Healthcare Life Sciences, USA) and blot images were captured with a Bio-Rad GS-700 Imaging Densitometer and analyzed using the Quantity One Imaging software (Bio-Rad Laboratories, USA) (25,28). Loading control was performed by densitometric examination of Ponceau red staining as well as total MAPK blotting.

Data analysis

Values from equilibrium binding experiments (dissociation constant, K_d, and maximal number of binding sites, B_{max}) were calculated assuming the existence of one class of specific binding sites in the range of concentrations used, by means of computerized non-linear regression analysis of the untransformed data (Prism 4.0, GraphPad Software Inc., USA), as reported earlier (28). Statistical analysis for comparing transgenic to wild-type values was performed using the unpaired Student t-test (P < 0.05).

Results

Phenotype analysis of CAMα$_{1B}$-AR mice

Increases in heart/body weight ratios are classically associated with the occurrence of myocardial hypertrophy (7,25). A small but significant increase of this ratio (22%) was observed in transgenic mice (WT = 3.95 ± 0.05 and CAMα$_{1B}$-AR = 4.85 ± 0.08 mg/g, N = 30; P < 0.01), which resulted exclusively from an increase in cardiac mass (WT = 116.9 ± 2.7 and CAMα$_{1B}$-AR = 148.0 ± 3.3 g, N = 30; P < 0.01), as the body weight did not change (WT = 29.5 ± 0.4 and CAMα$_{1B}$-AR = 30.5 ± 0.3 g, N = 30). This mild hypertrophy is consistent with a previous report (7).

The quantification of α$_{1B}$-AR tissue density is not possible due to the lack of a selective ligand for this subtype of α$_1$-AR (2,3,7). Therefore, the global estimation of such receptors through binding assays with [^{3}H]-prazosin or [^{3}H]-HEAT has become a consensual method (7). Data obtained in [^{3}H]-prazosin binding assays showed an increase in total α$_1$-AR binding (B_{max}) of approximately 70% (WT = 123.7 ± 31.3 and CAMα$_{1B}$-AR = 214.6 ± 32.0 fmol/mg, N = 6; P < 0.01), indicating a significant increase of α$_1$-AR density in transgenic hearts.

Ca^{2+}-ATPase activity and protein expression

Maximal cardiac Ca^{2+}-ATPase activity was similar for WT and CAMα$_1$-AR mice (Table 1). On the other hand, the relative contribution of SERCA and PMCA to the total Ca^{2+}-ATPase activity was statistically different: while in our preparation the contribution of the two Ca^{2+}-ATPases was not different in WT mice, the thapsigargin-resistant PMCA contribution was significantly higher than the thapsigargin-sensitive SERCA contribution in transgenic mice (Table 1).

Table 1. Cardiac P-type ATPase activities of wild-type and myocardial-targeted constitutively active mutant α$_{1B}$-AR (CAMα$_{1B}$-AR) mice.

	Enzyme activity (µmol Pi·mg protein^{-1}·h^{-1})	
	Wild-type	CAMα$_{1B}$-AR
Total Ca^{2+}-ATPase	6.98 ± 2.92	5.13 ± 1.53
Thapsigargin-sensitive Ca^{2+}-ATPase	2.88 ± 0.50	1.07 ± 0.73
Thapsigargin-resistant Ca^{2+}-ATPase	4.10 ± 0.71	4.06 ± 0.48*
Total Na$^+$/K$^+$-ATPase	1.17 ± 0.17	1.30 ± 0.16

Data are reported as means ± SEM (N = 6 preparations of 5 hearts each). *P < 0.05 compared to CAMα$_{1B}$-AR thapsigargin-sensitive Ca^{2+}-ATPase (unpaired Student t-test).
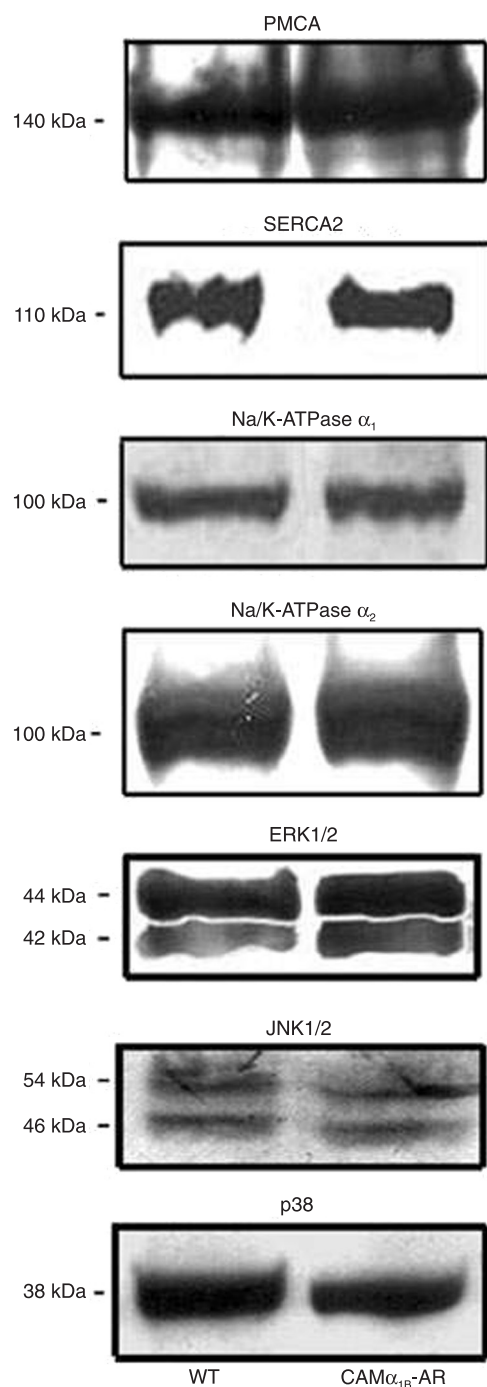
Figure 1. Protein expression of cardiac plasmalemmal (PMCA) and sarco/endoplasmic reticulum type 2 (SERCA2) Ca^{2+}-ATPases, Na$^+$/K$^+$-ATPase α$_1$ and α$_2$ subunit isoforms and phosphorylated ERK1/2, JNK1/2 and p38. Representative blots of heart preparations from wild-type (WT) and myocardial-targeted constitutively active mutant α$_{1B}$-AR (CAMα$_{1B}$-AR) mice.

Western blot assays performed to determine the expression of the SERCA2 isoform in this model showed bands with similar relative absorbance in both groups (WT = 100 ± 9 and CAMα$_{1B}$-AR = 108 ± 5%, N = 3; Figure 1), in accordance with functional biochemical data. On the other hand, the amount of PMCA protein was significantly increased in transgenic heart preparations, i.e., about 30% compared to WT (WT = 100 ± 7 and CAMα$_{1B}$-AR = 132 ± 4%, N = 6; P < 0.05; Figure 1).

Na$^+$/K$^+$-ATPase activity and protein expression

Maximal Na$^+$/K$^+$-ATPase activity of heart preparations did not differ between groups (Table 1). Similarly, there was no significant difference between groups in the expression pattern of the two major cardiac α subunit isoforms of Na$^+$/K$^+$-ATPase (α$_1$: WT = 100 ± 11 and CAMα$_{1B}$-AR = 113 ± 12%, N = 6; and α$_2$: WT = 100 ± 8 and CAMα$_{1B}$-AR = 115 ± 7%, N = 6; Figure 1).

Stress-signaling MAPK protein expression

In order to determine whether MAPK pathways are activated in the present model of mild heart hypertrophy, we performed immunoblot assays to evaluate the expression of the three main MAPKs (ERK1/2, JNK1/2 and p38) in their activated (phosphorylated) form. All three MAPKs could be detected in every heart preparation. An equivalent expression pattern was found for both total (data not shown) and phosphorylated forms of ERK1/2 (WT = 100 ± 11 and CAMα$_{1B}$-AR = 91 ± 11%, N = 6), JNK1/2 (WT = 100 ± 13 and CAMα$_{1B}$-AR = 88 ± 9%, N = 6) and p38 (WT = 100 ± 10 and CAMα$_{1B}$-AR = 106 ± 6%, N = 6; Figure 1).

Discussion

Due to their lower density in mammalian hearts compared to the β-AR and the lack of drugs with suitable selectivity, the role of α$_1$-AR subtypes in cardiac function is still poorly understood. The hypertrophic effect of α$_1$-AR agonists on isolated rat cardiomyocytes has been known for a long time (4) and has been claimed to be mediated by the α$_{1A}$-AR subtype (29). Nevertheless, results from transgenic mouse models indicated that α$_{1B}$-AR, but not α$_{1A}$-AR, is probably involved in the adaptive growth phenomenon in the heart (2,3,6). In fact, the cardiac phenotype of the CAMα$_{1B}$-AR mice used in this study indicated a mild hypertrophy, in agreement with data reported by Milano et al. (7). Furthermore, binding data revealed a 1.7-fold increase of α$_1$-AR density, similar to the 2- to 3-fold increase detected by others (7,16). Interestingly, different reports described the absence of heart hypertrophy with the same model, probably because of epigenetic characteristics like diet and housing conditions, suggesting that myocardial α$_{1B}$-AR activation *per se* would be a permissive more than a causative factor (15,16). Considering our results (discussed below), we can presume that chronic cardiac α$_{1B}$-AR self-stimulation alone,

at least at this age, is a weak inducer of pathological changes in the heart, as also deduced elsewhere (6,9).

The present study revealed for the first time that the activity and expression of important cardiac P-type ATPases and MAPKs were predominantly unaltered in CAMα_{1B}-AR mice. SERCA2 and Na$^+$/K$^+$-ATPase α_2 isoform down-regulation has been observed in conditions of cardiac overload, (19,20,25), with no change of the housekeeping Na$^+$/K$^+$-ATPase α_1 isoform (17,25). Interestingly, cardiac-specific overexpression of WT α_{1B}-AR (70- to 150-fold increase) did not affect SERCA2 mRNA content (9,18), as it was reported at the initial hypertrophic stage (30). Our results also agree with previous reports showing that chronic α_1-AR activation had no effect on Na$^+$/K$^+$-ATPase activity *in vivo* (31) or protein expression *in vitro* (32,33). On the other hand, PMCA expression and PMCA-to-SERCA relative contribution to total Ca^{2+}-ATPase activity increased in CAMα_{1B}-AR mice, suggesting that alterations of sarcolemmal calcium transport might be a maladaptive mechanism during the development of cardiac hypertrophy (34). Interestingly, PMCA has been associated with myocardial growth (35) and, via neuronal nitric oxide synthase, exacerbates the adrenergic-induced hypertrophy (36), suggesting the active contribution of this entity to progression of the disease.

MAPK activation plays a role in the regulation of the hypertrophic response *in vivo*, although it depends on the pathological stage, experimental model and type of stimulus (22). In comparable experimental models, activated MAPKs were only detected in very young but not in adult mice (3 and 12 weeks old, respectively) (37-39). ERK1/2 and/or JNK1/2 stimulation has been reported in cardiac-selective WT α_{1B}-AR (11) and systemic CAMα_{1B}-AR (13) overexpression. Nonetheless, a direct comparison with the present model should be considered with caution. Benoit et al. (11) showed a small but significant ERK1/2 activation, but not of the immediate upstream kinases MEK1/2, and their mice expressed huge amounts of α_{1B}-AR (a 150-fold increase), which can lead to promiscuous receptor (trans) activation. Chalothorn et al. (13) detected ERK1/2 and JNK1/2 activation, but their animals exhibited autonomic failure and hypotension, which may evoke compensatory hypertensive mechanisms. This can significantly affect heart α_{1B}-AR-independent signaling. Finally, persistent α_{1B}-AR activation *in vivo* desensitizes the MAPK response *in vitro* (40).

The present results demonstrate that long-lasting stimulation of α_{1B}-AR in murine heart provokes mild hypertrophy without a major influence on the expression of many proteins involved in cardiac homeostasis - transport ATPases (Na$^+$/K$^+$-ATPase and SERCA) and MAPKs (ERK1/2, JNK1/2 and p38) - but an up-regulation of PMCA, which may indicate an initial adaptation related to an emergent hypertrophic process. Considering that constitutive α_{1B}-AR activation persisted during the mouse lifetime, our findings might suggest that α_{1B}-AR probably has a minor pathophysiological function in the mouse heart.

Acknowledgments

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