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Research Article

β_2-Adrenoceptor and Expression of MuRF1 and Atrogin-1 Under Stress

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Abstract

Background: Stress is an important risk factor for cardiovascular diseases. During the stress reaction, catecholamines released by the sympathetic nervous system and the adrenal medulla couple to β-adrenoceptors (β-AR) in almost every organ. In the heart, β_1- (β_1-AR) and β_2-adrenoceptors (β_2-AR) are expressed in an 80:20 ratio, and control heart functioning (via the Gs protein-adenylyl-cyclase signaling pathway) as well as cardiomyocytes life cycle (via the Gi-PI3K-Akt pathway). Changes in β_1-AR/β_2-AR ratio were associated with poor cardiac performance and can be triggered by stress. The putative consequences of those stress-induced changes on cardiac atrophy/pathological hypertrophy were not explored yet. This work aims to investigate the role played by β_2-AR in the expression of components of the PI3K-Akt signaling pathway in the heart of rats submitted to foot shock stress.

Material and Method: Adult male rats were distributed in four groups: control, stress, ICI118,551 treated, and stress + ICI118,551 treated. Rats were submitted to one daily session of foot-shock stress (30 min duration, 1 mA, 1 s duration at random intervals between 5 and 25 s) during 3 days. ICI was administered i.p., 2 days before and during the stress period. The expression of proteins was analyzed by Western blot in the heart of rats sacrificed immediately after the last stress session.

Results: Foot-shock stress induced a reduction in the expression of MuRF1 whereas atrogin-1 and C2 proteasome expressions were unaltered. In the heart of stressed rats treated with the β_2-AR antagonist (ICI118,551) there was down regulation of pAkt and atrogin-1 whereas MuRF1 and C2 proteasome were up regulated.

Conclusion: The β_2-AR plays a role in the control of the signaling pathways leading to the expression of MuRF1, atrogin-1 and C2 proteasome under stress, probably protecting the heart against atrophy and pathological hypertrophy.

Keywords: *β_2 adrenoceptor; Atrogin-1; MuRF1; Cardiac Remodeling; Stress*

Abbreviations

AC: Adenylyl Cyclase; Akt: Serine/Threonine Kinase; β-AR: β Adrenoceptor; CO: Control; FoxO: Fork Head Transcriptional Factor; Gi: Inhibitory G Protein; Gs: Stimulatory G Protein; ICI: ICI118,551; MAPK: Mitogen-Activated Protein Kinase; mTOR: Mammalian Target of Rapamycin; PI3K: Phosphoinositide 3-Kinase; PKA: Protein Kinase A; ST: Stress; UPS: Ubiquitin Proteasome System

Introduction

Stress is the organism reaction to any challenge. The main effectors of the stress reaction are the hypothalamus-pituitary-adrenal cortex axis and the sympathetic nervous system-adrenal medulla that release, respectively, glucocorticoids and catecholamines. The heart is a target of those stress mediators. Acute stress is usually harmless and aims to guarantee survival. However, chronic stress may impose an excessive workload to the heart that may lead to its enlargement in an endeavor to manage the increased workload.

That process is known as hypertrophy and, when associated to cardiac damage, it is referred as pathological hypertrophy and represent an independent risk factor for heart failure [1]. Since the cardiomyocytes do not replicate, hypertrophy depends on the addition of sarcomeres in series, causing cell elongation. Parallel to the increase in myocytes size there is an increase in the number of fibroblasts, causing fibrosis, increased myocardial stiffness and loss of cardiomyocytes by apoptosis [2].

The forkhead transcriptional factor 1 (FoxO1) is one of the regulators of cardiomyocytes apoptosis while FoxO3 controls the cardiomyocytes size [3]. FoxOs activity is controlled by Akt [4] and stimulates atrogin-1 and MuRF1 expression [5-8]. In a cardiac context, atrogin-1 and MuRF1 were reported to inhibit hypertrophy [9-11] and to regulate cardiac myosin-binding protein C which is related to familial hypertrophic cardiomyopathy [12]. Being upstream to FoxO, Akt activation is accomplished by many mediators including PI3K [13], which, in the heart, can be activated by β_2-AR-Gi protein [14-17].

β_2-AR coexists with β_1-AR in cardiomyocytes in a 20:80 ratio [18-20]. Altered expression of β_1-AR and β_2-AR has been associated with heart failure [21,22], aging [23], diabetes mellitus [24], and stress [25,26]. In the model of foot shock stress [27,28] cardiac β_1-AR down regulation is accompanied by β_2-AR up regulation. The consequences are sub-sensitivity of the chronotropic and inotropic responses of the isolated atria to β_1-AR selective agonists and super-sensitivity to non-selective and β_2-AR selective agonists [28]. Additionally, cAMP production by atrial membranes in response to isoproterenol when β_2-ARs were antagonized by ICI118,551 is attenuated [28], and the expression of several proteins involved in the Gs-AC-PKA and Gi-PI3K-Akt signaling pathways is altered [29]. The expression of some proteins remained altered 5 days after the end of the stress period even though the β_2-AR expression had returned to normal values 24 h after the end of the stress period [30]. Based on those evidences, we hypothesized that stress may induce alterations in the expression of proteins belonging to the Gi-PI3K-Akt signaling pathway and that the β_2-ARs play a role in this process.

Aim of the Study

The aim of this work was to investigate the role played by β_2-AR in the expression of components of the PI3K-Akt signaling pathway, including atrogin-1 and MuRF1, in the heart of rats submitted to foot shock stress.

Materials and Methods

Animals

Male Wistar rats (*Rattus norvegicus*; 250 - 300g, 12-week-old) were purchased from the Center for the Development of Experimental Models (CEDEME), of the Federal University of São Paulo (São Paulo, SP, Brazil). They were housed in standard cages in a temperature-controlled room (22ºC) on a 12/12h light/dark cycle with the lights on at 7:00 a.m. Laboratory chow for rodents and tap water were available *ad libitum*. All procedures were in accordance with the ethical standards of the institution and experimental protocols were approved by the Institutional Committee for Ethics in Animal Experimentation (CEUA 743716).

Experimental groups and study design

Two weeks after arrival to the animals' room, rats were distributed in four experimental groups as follows: control (CO, no stress); ICI (no stress treated with ICI118,551), stress (ST, three foot-shock sessions), and ICIST (ICI118,551 treatment + three foot-shock sessions). Those rats in the ST and ICIST groups were submitted to foot shock sessions as described below. Rats in the CO and ICI groups were placed in the foot shock cage as well, but did not receive foot-shocks. The rats body weight was determined before the first and the last stress session. Rats were euthanized by decapitation immediately after the third foot shock stress session or the third period in the foot shock cage without receiving foot shocks. The heart was dissected, and the cardiac chambers were stored at -80°C until assayed by the proteins of interest.

Stress protocol

A Plexiglas chamber (26 cm long x 21 cm wide x 26 cm high) provided with a grid floor consisting of stainless-steel rods (0.3 cm in diameter and spaced 1.0 cm apart) was used to apply the foot shocks. During the 30 min sessions, which occurred once a day for three consecutive days, between 8:00 a.m. and 11:00 a.m., the shocks were delivered from a constant current source controlled by a microprocessor-based scrambler produced by the Center for Biomedical Engineering at the State University of Campinas (UNICAMP, Campinas, Sao Paulo, Brazil). Each rat received 120 foot-shocks (intensity: 1.0 mA; duration: 1.0s) at random intervals of 5 - 25s. The rats in the control group were placed in a similar Plexiglas chamber during 30 min, once a day for three consecutive days, between 8:00 a.m. and 11:00 a.m. However, they did not receive foot shocks. This stress protocol and experimental design were previously used by our research group to investigate the effects of stress on the chronotropic and inotropic responses to catecholamines in isolated atria of rats [31]. The plasmatic level of corticosterone after each stress session was higher than control. However, they were similar to control 24h later [32]. The anxiety levels of stressed rats were similar to those of control rats as measured in the elevated plus-maze, they did not reduce food or water intake, neither the body weight [33].

Rats' treatment with ICI118,551

One group of rats (ICI) received during 5 days, 500 µg/Kg/day, i.p. of ICI118,551((±)-1- [2,3- (Dihidro-7-metil-1H-inden-4-il) oxi]-3-[1-metilletil) amino]-2-butanol), a competitive antagonist of β_2-AR [34]. Another group of rats was treated with ICI118,551 and submitted to foot shock stress as above described, in the 3rd, 4th and 5th days of treatment (ICIST).

Western blot analysis

The left ventricles were homogenized using a T-18 Ultra-Turrax homogenizer (Ika Works Inc., Wilmington, NC, USA) containing 1.0 mL of assay buffer (4°C) of the following composition: 1% Triton X-100 (BioRad, Hercules, CA, USA), 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 µg/ml Aprotinin (Amresco, Solon, Ohio), 1 mmol/L PMSF, 0.25 mmol/L sodium orthovanadate and 0.1% cocktail of protease inhibitors. The samples were centrifuged for 20 min at 11,000g and the supernatant was collected and assayed for total protein concentration using the Bradford method (Bio Rad Laboratories Inc, Hercules, CA, USA). Samples were stored at −80°C until assay. One hundred micrograms of total protein were separated by Bolt Bis-Tris Plus Gel (ThermoFisher Scientific, MA, USA) and transferred to nitrocellulose membranes using iBlot 2 Dry Bloting System (ThermoFisher Scientific, MA, USA). Membranes were blocked with 4% BSA in TBS buffer containing 0.1% Tween 20, for 1 hour. The following primary antibodies (all from Abcam, Cambridge, MA, USA) were incubated at 4°C overnight: β_1-AR rabbit polyclonal (ab3442); β_2-AR rabbit polyclonal (ab36956); Gi rabbit monoclonal (ab140125); Gs rabbit polyclonal (ab83735); phospho Akt rabbit monoclonal (ab81283); mTOR rabbit polyclonal (ab2732); atrogin-1 rabbit monoclonal (ab168372); MuRF1 rabbit monoclonal (ab172479); and proteasome C2 rabbit monoclonal (ab109530). GAPDH rabbit polyclonal antibody (sc25778; Santa Cruz Biotechnology, Dallas, USA) was used as endogenous control of protein expression. The membranes were

subsequently rinsed three times (5 min each) in buffer solution and then incubated with the respective HRP-conjugated secondary antibody 1:2000 dilution (Sigma-Aldrich, St. Louis, MO, USA) for 1h, then rinsed in buffer. Using an enhanced chemiluminescence kit (Super Signal®, West Pico Chemiluminescent Substrate, ThermoFisher Scientific, Rockford, IL, USA), the blots were developed on ChemiDoc XRS+ System (BioRad, Hercules, CA, USA). Densitometric analyses were done using Image *J* Launcher software.

RT-PCR

One fragment of no more than 100 mg of the left ventricle was homogenized in 1 mL Trizol (Invitrogen®, Carlsbad, CA, EUA) using a T-18 Ultra-Turrax homogenizer (Ika Works Inc., Wilmington, NC, EUA) in an ice bath. Then, chloroform (200 µl) was added, vortex for 15s. After 3 minutes at room temperature, it was centrifuged at 12,000g, 15 minutes, 4ºC. The water phase was transferred to a clean tube. Then, 500 µl of isopropanol was added and incubated during 10 minutes at room temperature. After that, the tube was centrifuged at 7,500g during 10 minutes at 4ºC for mRNA precipitation. The pellet was washed with 1 ml ethanol at 100%, suspended in 30 µl of water treated with 0.1% dietilpyrocarbonate (DEPC UltraPure, Invitrogen®, Carlsbad, CA, EUA), and stored at -80ºC. The sample mRNA concentration and degree of purity were determined in Nanodrop 2000 c (ThermoScientific®, Canada) under 260/280 nm. The mRNA was treated with DNAse (Deoxyribonuclease Amp Grade I, Invitrogen®, Carlsbad, CA, EUA), according to the manufacturer indication. The final volume was 10 µL. After mRNA purification, complementary DNA (cDNA) was obtained, using the High-Capacity cDNA Reverser Transcription kit (Applied Biosystems®, Carlsbad, CA, USA), according to the manufacturer's indication, in a final volume of 20 µL. The mRNA expression was analyzed using previously designed primers as shown in the table 1. Glyceraldehyde triphosphate dehydrogenase (GAPDH) was used as endogenous control. Real time-PCR was done using SYBRGreen PCR Master Mix (Applied Biosystems®, Carlsbad, CA, USA) in a Step One Plus Real Time PCR System (Applied Biosystems®, Carlsbad, CA, USA). The results were normalized in relation to the values obtained for the endogenous gene and were expressed as $2^{-\Delta\Delta Ct}$.

Statistical analysis

The results were expressed as means ± s.e.m. Student's unpaired *t*-test or Analysis of Variance followed by the Tukey test were used to compare groups. Differences were considered significant at $p \leq 0.05$. Statistical analyses were done using Prism v.6 (GraphPad Software Inc., San Diego, CA).

Results

The stress protocol used here have been previously reported to significantly increase corticosterone plasma level [28]. None of the treatments caused death, reduction of body weight (Table 1) or any paw lesion. Stressed rat's anxiety levels were similar to those seen in the control group [33] and no alteration was seen in the cardiac mass or structure as observed in the optical microscope (data not shown).

The mRNA and protein expression of β_1-AR and β_2-AR in the left ventricle of control rats, stressed rats, and rats treated with ICI are shown in the figure 1. The stress protocol induced down-regulation of β_1-AR at protein level. On the contrary, β_2-AR protein expression was enhanced in the left ventricle of stressed rats. The β_2-AR blockade with ICI118,551 in the absence of stress did not modify the expression of β_2-AR or β_1-AR (Figure 1).

The expressions of both Gs and Gi proteins (Figure 2A and 2B, respectively) were not significantly altered by foot shock stress neither by the β_2-AR blockade.

	Control	Stress
Before	275 ± 4	271 ± 6
After	286 ± 4	280 ± 5

Table 1: *Body weight (g) of rats before and after foot-shock stress.*

Rats in the stress group were submitted to one daily session of foot shock stress during 3 days. The foot shock session was 30 minutes duration and pulses were of 1 mA, 1s, at random intervals between 5 and 25s. There was not difference between groups ($p > 0.05$; one way ANOVA).

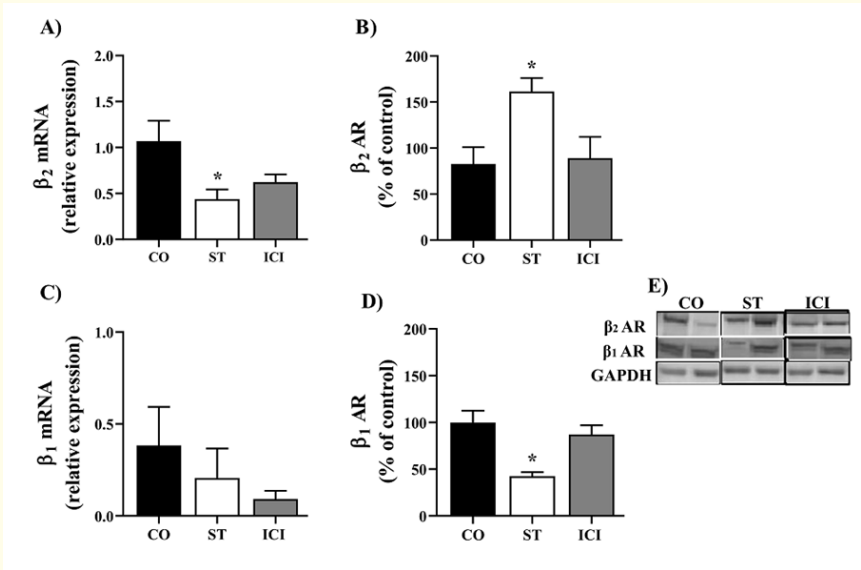

Figure 1: *Gene and protein expression of β2-adrenoceptor (β2-AR, panels A and B) and β1-adrenoceptor (β1-AR, panels C and D) in the left ventricle of control (CO), stressed (ST) rats, and rats treated with ICI118,551 (ICI). RT-PCR: n = 4/group, Western blot: n = 8/group. *p ≤ 0.05 compared to control, Student's t test. The representative Western blots images are in panel E.*

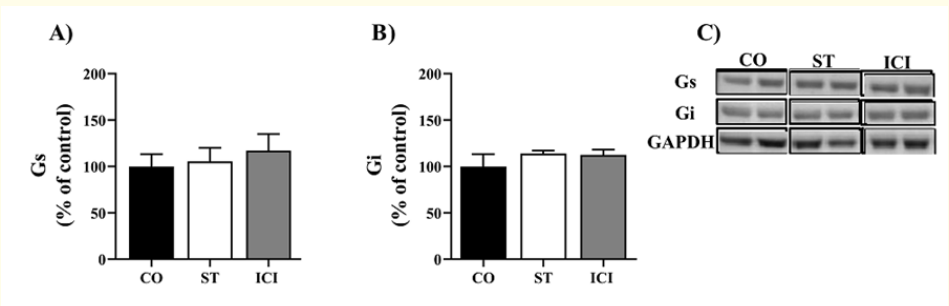

Figure 2: *Protein expression of stimulatory (Gs, panel A) and inhibitory (Gi, panel B) G proteins in the left ventricle of control (CO), stressed (ST) rats, and rats treated with ICI118551 (ICI) n = 6/group. There were no significant differences between groups, $p > 0.05$, Student's t test. The representative Western blots images are in panel C.*

The phosphorylation of Akt was not changed by foot shock stress or by the ICI- treatment (Figure 3A). Accordingly, the expressions of mTOR, a downstream substrate of Akt, and of atrogin-1 were unaltered, too (Figure 3B and 3C, respectively). Also unaltered was the expression of C2 proteasome (Figure 3E), a component of the ubiquitin-proteasome system (UPS). However, the expression of MuRF1 was reduced in cardiac tissue of rats submitted to stress as compared to control non-stressed rats and to ICI-treated rats (Figure 3D)

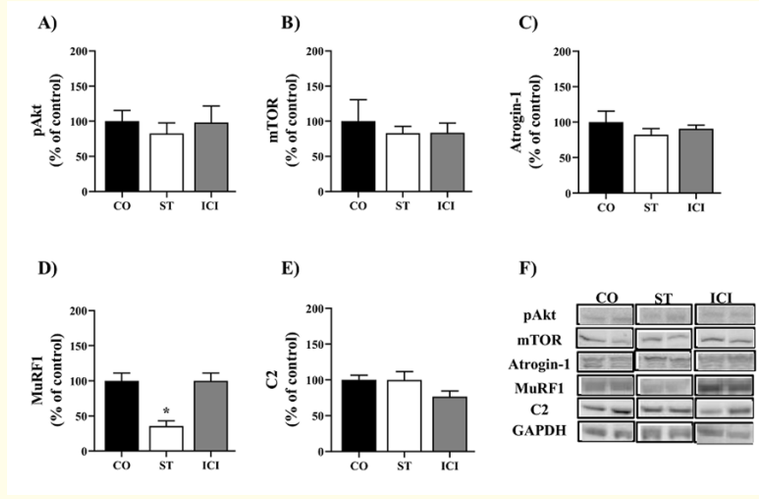

Figure 3: *Protein expression of phosphorylated Akt (panel A), mTOR (panel B), atrogin-1 (panel C), MuRF1 (panel D), and C2 proteasome (panel E) in the left ventricle of control (CO), stressed (ST) rats, and rats treated with ICI118551 (ICI) n = 6/group. There were no significant differences between groups, p > 0.05, Student's t test. The representative Western blots images are in panel F.*

In the heart of rats treated with ICI118,551 and submitted to stress, Akt phosphorylation was reduced (Figure 4A) and no change was observed in mTOR expression (Figure 4B). Whereas the expression of atrogin-1 was lower than control (Figure 4C), the expressions of MuRF1 (Figure 4D) and C2 proteasome (Figure 4E) were higher than control.

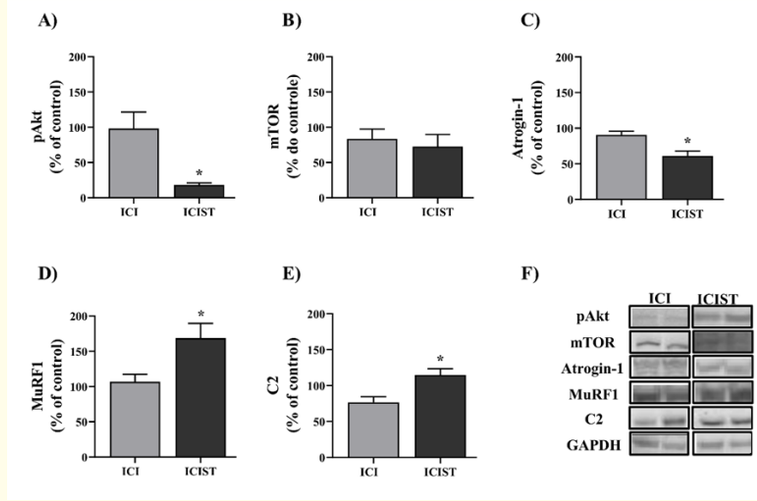

Figure 4: *Protein expression of phosphorylated Akt (panel A), mTOR (panel B), atrogin-1 (panel C), MuRF1 (panel D), and C2 proteasome (panel E) in the left ventricle of rats treated with ICI118551 (ICI) and rats treated with ICO118,551 and submitted to stress. n = 6/group. There were no significant differences between groups, p > 0.05, Student's t test. The representative Western blots images are in panel F.*

Discussion

The present data show that, in the heart of foot shock stressed rats, the expression of atrogin-1 and C2 proteasome was unaltered but the expression of MuRF1 was decreased. Both atrogin-1 (which expression was unaltered) and MuRF1 (that increased) are part of a family of biochemically related molecules involved in protein degradation during the process of skeletal muscle atrophy and myocardial remodeling. MuRF1 not only activates anti-hypertrophic pathways within the myocardium; it also mediates cardiac atrophy *in vivo*. In fact, it was demonstrated, in the model of dexamethasone-induced cardiac atrophy, that MuRF1 knockout mice were more resistant than wild-type mice to hypertrophy regression [35]. Both atrogin-1 and MuRF1 participate in the ubiquitination of proteins to be degraded by the UPS of which C2 proteasome is a component, too [7]. UPS shares with autophagy-lysosome system the responsibility for proteins homeostasis in cardiomyocytes [36]. Disturbances in the UPS are thought to be involved in the development of heart failure [37]. Moreover, MuRF1 and atrogin-1 are upregulated in human heart failure leading to increased protein ubiquitination and degradation, thereby leading to a molecular state that favors heart muscle loss and left ventricular dysfunction [43].

The cellular targets ubiquitinated by MuRF1 are myofibrillar proteins like troponin I, titin, nebulin, myosin light chain 2, as well as metabolic enzymes involved in energy production [39,40]. Troponin I significantly decrease in heart failure, when MuRF1/atrogin-1 are up-regulated [38]. A negative correlation between troponin I degradation and maximal force generation has been previously reported [41]. TNF-α seems to induce MuRF1 and atrogin-1 in the myocardium and is involved in cardiac remodeling [42].

In the model of foot shock stress, atrogin-1 expression was unaltered whereas the expression of MuRF1 was lower than control. These data suggest that the heart is protected from the effect of stress on atrophy/pathological hypertrophy. However, when the rats were submitted to stress and β_2-AR was pharmacologically blocked by ICI118,551 there was an increase in the expression of MuRF1 and a decrease of atrogin-1. Although intriguing, the regulation of these two E3 ligases in opposite directions is possible because they are regulated differently. TNF-α activation and the expression of atrogin-1 occurs through p38 MAPK, whereas the activation of MuRF-1 is mediated by p42/44 MAPK [38].

The expression of MuRF1 and atrogin-1 is also stimulated by FoxO1/3a [5,6], which are considered the key downstream targets of Akt [13]. Once phosphorylated, FoxO leave the cell nucleus interrupting the fork head transcriptional program [4]. The phosphorylated Akt was unaltered in the heart of stressed rats, where β_2-AR was up regulated. Nevertheless, it was lower than control when stress was applied after β_2-AR blockade (by ICI118,551). Akt activation is accomplished by PI3K and many other post-translational mediators [13] such as insulin, platelet derived growth factor, vascular endothelial growth factor, epidermal growth factor, basic fibroblast growth factor [43] and some phosphatase inhibitors [44]. Numerous phosphorylation sites on Akt have been identified and some of them have been related to Akt function [45]. Phosphorylation at S473, that we evaluated, is considered, together with T308, as rate-limiting and obligatory for maximal Akt activation downstream of PI3K [13]. Despite that, in the presence of an up regulated β_2-AR, Akt phosphorylation was not altered, in the heart of rats submitted to stress under β_2-AR blockade, Akt activation was reduced. A similar pAkt reduction was reported to occur in the human failing heart. Decrease of Akt activity leads to activation of FoxO3a and to the induction of atrogin-1. The higher expression of atrogin-1 leads to increased protein ubiquitination and degradation [46,47].

Whether this signaling cascade can be activated by β_2-AR is still controversy. Acute overall β-AR stimulation and specific β_2-AR overstimulation were reported to induce an increase in Akt phosphorylation in mouse hearts [17]. Unlike that, in skeletal muscle, norepinephrine did not induce any changes in Akt phosphorylation [6]. High levels of atrogin-1 were reported to suppress cardiomyocyte hypertrophy induced by adrenergic stimulation *in vitro* and by pressure overload *in vivo* [10].

Stress under β_2-AR blockade resulted in reduction on Akt phosphorylation, increase in MuRF-1 and C2 proteasome, and reduction of atrogin-1 expression, which might disorganize the mechanism that maintain the proteostasis in the cardiomyocytes. Therefore, the data

presented here suggest that β_2-AR plays a role in the control of the signaling pathways leading to the expression of MuRF1, atrogin-1 and C2 proteasome under stress, probably protecting the heart against atrophy and pathological hypertrophy. Of course, the duration of the stress protocol and the experimental design used here were too short to allow the detection of a putative hypertrophy. However, we have recently reported that, five days after the end of the stress period, MuRF1 was still down regulated and atrogin-1 expression had returned to the control value [30]. We also reported that foot shock stress induces changes in the cardiac profile of gene expression [29].

Limitation of the Study

A limitation of this work is that we did not evaluate the total poly-ubiquitinated protein or the consequences of the described changes on the heart function. Additional investigation of the mechanisms involved in the effects of stress on cardiac muscle atrophy and putative left ventricular dysfunction might be the key to develop novel therapeutic strategies to halt the link between stress and cardiovascular diseases. The importance of clarify such mechanisms is obvious when one take into account that stress is prevalent and cardiovascular diseases are the leading cause of death all over the world.

Conclusion

It is concluded that β_2-AR plays a role in the control of the signaling pathways leading to the expression of MuRF1, atrogin-1 and C2 proteasome under stress, probably protecting the heart against atrophy and pathological hypertrophy.

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Conflict of Interest

No financial interest or any conflict of interest exists.

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