Clinical & Experimental Cardiology

OPEN ACCESS Freely available online

Research Article

Persistent Effects of Subchronic Stress on Components of Ubiquitin-Proteasome System in the Heart

Marco A Cordeiro, Luana S Rodrigues, Daniela Ortolani, Ana Elisa TS de Carvalho, Regina C Spadari*

Department of Biosciences, Institute of Health and Society, Campus Baixada Santista, Federal University of São Paulo (UNIFESP), Santos, São Paulo, Brazil

ABSTRACT

We have previously described that rats submitted to foot shock stress present cardiac β_2-ARs up-regulation and alterations in components of the β_2-AR-Gi signaling pathway related to atrophy. The aim of this work was to investigate the time course of those stress induced alterations in cardiac components of the β_2-AR-Gi signaling pathway related to atrophy. Male rats were submitted to foot shock stress once a day for three consecutive days. Protein expressions were analyzed in the ventricle of rats euthanized immediately, 24 hours or 5 days after the last stress exposure. β_2-ARs were upregulated in the heart of rats sacrificed immediately after stress. However, PI3K, pAkt, and MuRF1 expressions that were lower than control immediately after stress remained lower than control 5 days after stress exposure; atrogin-1 expression was unaltered. It is suggested that MuRF1 and atrogin-1 expressions are independently regulated from each other and that atrophy might be blunted in the heart of stressed rats. Moreover, some intracellular alterations induced by stress are long lasting and remain even after the end of the stress exposure period.

Keywords: β2 adrenoceptor; Stress; atrogin-1; MuRF1; PI3K-Akt signaling; Cardiac remodeling; Atrophy

INTRODUCTION

The sympathetic nervous system influences cardiac physiology through the action of catecholamines on β-adrenoceptors (β-ARs). β-ARs stimulation promotes intracellular activation through G proteins signaling. β_1AR and β_2AR couple to stimulatory G protein (Gs), which stimulates the synthesis of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) signaling pathway [1-4]. Alternatively, β_2ARs couple to inhibitory G protein (Gi), which inhibits adenylyl cyclase activity thus attenuating the synthesis of cAMP [5]. The β_2AR-Gi signaling pathway has also been linked to cardioprotection for activating cell survival signaling controlled by phosphatidylinositol 3-kinase (PI3K) Akt [5,6]. In skeletal muscle cells, PI3K-Akt activation reduces the rate of overall proteolysis [7,8]. Accordingly, attenuation of muscle atrophy through inhibitory effects on the ubiquitin-proteasome system (UPS) has been reported in rats treated with clenbuterol, a selective β2-AR agonist [9].

UPS is the main mechanism of protein degradation in eukaryotic cells [10]. This system plays a role in the quality control of short-term proteins that regulate several basic cellular processes, such as the cell cycle, membrane receptors and ionic channels, and those of the inflammatory signaling pathways [11]. Firstly, identified misfolded proteins are ubiquitinated by a sequence of enzymes known as E-ligases (E1-, E2-, E3-ligase). Polyubiquitinated proteins are then recognized and transferred to a complex of specific enzymes, as atrogin-1 and MuRF1, to be metabolized by the 26S proteasome complex [12]. It has been suggested that atrogin-1 and MuRF1 activity stimulated by FoxO may cause cardiac muscle atrophy [13].

In the model of foot shock stress, cardiac β_2ARs are up-regulated [14,15] and the isolated atria are super-sensitive to the chronotropic and inotropic effects of non-selective, and to β_2AR selective, agonists [15]. If stress exposure causes alterations in components of the β_2AR-Gi signaling pathway related to atrophy is not clear yet. Moreover, it is unknown if the putative alterations induced by stress are limited to the stress exposure period or if those stress effects are long lasting.

The aim of this work was to investigate the time course of the effect of foot shock stress in components of the β_2AR-Gi signaling

Correspondence to: Spadari RC, Department of Biosciences, Institute of Health and Society, Campus Baixada Santista, Federal University of São Paulo (UNIFESP), Santos, São Paulo, Brazil, Tel: +55 (13) 32290194; E-mail: regina.spadari@unifesp.br

Received: September 10, 2020; **Accepted:** September 25, 2020; **Published:** October 02, 2020

Citation: Cordeiro MA, Rodrigues LS, Ortolani D, de Carvalho AET, Spadari RC (2020) Persistent Effects of Subchronic Stress on Components of Ubiquitin-Proteasome System in the Heart. J Clin Exp Cardiolog.11:676. DOI: 35248/2155-9880.20. 11.676.

pathway related to atrophy in the heart.

MATERIALS AND METHODS

Animals

Male Wistar rats (Rattus norvergicus; 300-350 g, 12 weeks old) were housed in standard cages in a temperature-controlled room (22°C) on a 12/12 h 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 Ethic Committee on Animal Use of the Federal University of São Paulo (CEUA/UNIFESP), CEUA 6793060318, in accordance with National Council for Control of Animal Experimentation (CONCEA, Brazil).

Stress protocol

A Plexiglas chamber (26 cm long × 21 cm wide × 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 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: 1.0 s) at random intervals of 5-25 s. The rats in the control group were placed in a similar Plexiglas chamber during 30 min, once a day for three 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, as described in [15-19]. None of the treatments caused death, reduction of body weight or any paw lesion.

Corticosterone concentration

The plasmatic concentration of corticosterone were determined by enzyme immunoassay (ELISA) using a commercial kit (Enzo Life Science Inc®, Ann Arbor, EUA) according to manufacturer's guidelines.

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 inhibitors protease. The samples were centrifuged for 20 min at 11,000 g 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 Blotting 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 (from Abcam, Cambridge, MA, USA) were incubated at 4°C overnight: β_2AR rabbit polyclonal (ab36956); Gi rabbit monoclonal (ab140125); Gs rabbit polyclonal (ab83735); phospho Akt rabbit monoclonal (ab81283); total Akt rabbit polyclonal (ab8805); PI3K rabbit monoclonal (ab151549); atrogin-1 rabbit monoclonal (ab168372); and MuRF1 rabbit monoclonal (ab172479). GAPDH rabbit polyclonal (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 1 h, 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.

Experimental groups and study design

Rats were distributed in four experimental groups as follows: Control (CO, no stress); submitted to stress and euthanized immediately after the last foot shock session (ST), submitted to stress and euthanized 24 hours after the last foot shock session (ST24H), and submitted to stress and euthanized 5 days after the last foot shock session (ST5D). The rats were euthanized by decapitation, their blood was collected and centrifuged, and plasma was collected and stored at 80°C. The left ventricle was also isolated and stored at 80°C.

STATISTICAL ANALYSIS

The results were expressed as means ± s.e.m. One way Analysis of Variance (ANOVA) followed by the Tukey test was used to compare groups. Differences were considered significant at $p \leq 0.05$. Statistical analyses were done using Prism v.8 (GraphPad Software Inc., San Diego, CA).

RESULTS

The corticosterone plasma level was higher than control in rats euthanized immediately after the last stress session. However, 24 hours and 5 days after the last stress session, corticosterone plasma level had returned to basal levels (Figure 1).

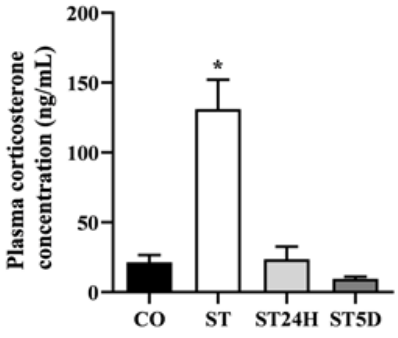

Figure 1: Plasmatic concentration of corticosterone in control non-stressed rats (CO) and in rats submitted to foot shock stress euthanized immediately (ST), 24 hours (ST24H) or 5 days (ST5D) after the last stress exposure. n=6 /group. *p ≤ 0.05 com- pared to control, one-way ANOVA plus Tukey's test.

Figure 2A shows that the protein expression of β_2AR in left ventricle of stressed rats was up-regulated immediately after the last stress session. However, 24 hours after that β_2AR expression returned to basal levels and remained so until 5 days afterwards. On the other hand, Gs and Gi proteins showed a progressive

increase that was significant for Gs only 5 days after the last stress session (Figure 2B).

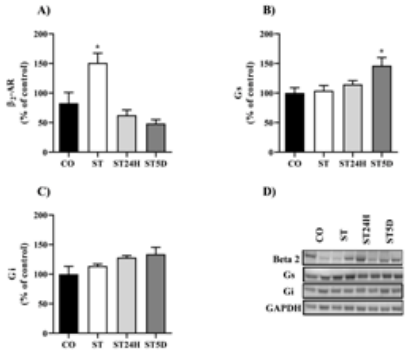

Figure 2: Representative Western blots (D) and protein expression of β_2-adrenoceptor (A), stimulatory G protein (Gs, B) and inhibitory G protein (Gi, C) in left ventricle of control non-stressed rats (CO) and in rats submitted to foot shock stress euthanized im- mediately (ST), 24 hours (ST24H) or 5 days (ST5D) after the last stress exposure. n=6/group. *p ≤ 0.05 compared to control, one-way ANOVA plus Tukey's test.

The expression of PI3K catalytic subunit and Akt phosphorylation (pAkt) exhibited similar time course, i.e., their expressions progressively decreased until 5 days after the stress (Figure 3). MuRF1 expression was lower in left ventricle of rats euthanized immediately, 24 hours or 5 days after the last stress session as compared to control whereas the expression of atrogin-1 was not altered (Figure 4).

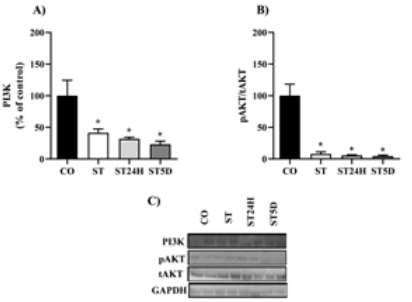

Figure 3: Representative Western blots (C) and protein expression of PI3K (A) and ratio of phosphorylated Akt (pAkt) and total AKT (tAkt) (B) in left ventricle of control non- stressed rats (CO) and in rats submitted to foot shock stress euthanized immediately (ST), 24 hours (ST24H) or 5 days (ST5D) after the last stress exposure. n=6 /group.*p ≤ 0.05 compared to control, one-way ANOVA plus Tukey's test.

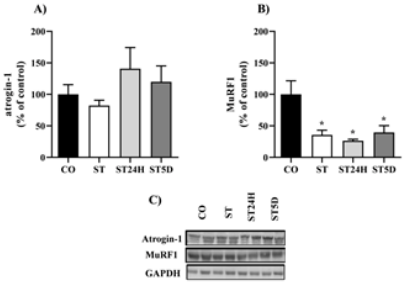

Figure 4: Representative Western blots (D) and protein expression of atrogin-1 (A) and MuRF1 (B) in left ventricle of control non-stressed rats (CO) and in rats submitted to foot shock stress euthanized immediately (ST), 24 hours (ST24H) or 5 days (ST5D) after the last stress exposure. n=6 /group. *p ≤ 0.05 compared to control, one-way ANOVA plus Tukey's test.

DISCUSSION

The present results have shown that the up regulation of β_2ARs previously reported in the heart of rats submitted to foot shock stress [15-21] is no longer present 24 hours or 5 days after the end of the last stress session. They also show that the expression of Gs protein follows an opposite time course, being similar to control in the heart of rats euthanized immediately after the last stress session and progressively increasing to be significantly higher than control only 5 days after the stress. Gi protein, although showing a similar profile, did not differ from control in any group. Different from G proteins, PI3K expression decreased immediately after the last stress session and remained lower than control 5 days after that. Similar time course was observed for pAkt.

Additionally, the present data show that in the heart of rats submitted to this stress protocol, the expression of MuRF1, a protein related to atrophy, was reduced. However, the expression of other enzyme mediating proteolysis (atrogin-1) was unaltered. It has been proposed that the expression of atrogenes is modulated by the signaling pathway starting with the activation of Gi-protein by β_2AR [13]. This signaling pathway seems to play a protective role against the cardiotoxic effects of excessive β_1-AR stimulation. Indeed, the expression of the central target of this signaling pathway, pAkt, was lower in the heart of stressed rats. Its upstream activator, PI3K, was reduced as well. Moreover, the expression of those proteins remained reduced even after the β_2-AR expression had returned to normal values.

Although both atrogin-1 and MuRF1 are recognized as targets for FoxOs [13], different from MuRF1, the expression of atrogin-1 was not altered by stress. Therefore, these data suggest that the expression of each atrogene can be specifically targeted. They also suggest that the alteration in Akt phosphorylation was not necessarily associated with changes in all the atrogenes expression. Indeed, independent transcription of those proteins has been described before [9,20]. Additionally, other transcription factors are recognized as modulators of those atrogenes expression, such as NF-κB, C/EBP β, KLF15, Smad 3, and the glucocorticoid receptor [20]. NF-κB expression is regulated by TNF-α, reactive oxygen species (ROS), and by endothelial (eNOS) and inducible (iNOS) nitric oxide synthases. The concentration of hydrogen peroxide was lower than control 5 days after stress as well as the expression of eNOS and iNOS (unpublished data).

CONCLUSION

It is not possible, at this time, to establish a causal relation between β2-AR upregulation and the lower expression of components of the PI3K-Akt signaling pathway. Nevertheless, it is plausible to suggest that the regulation of MuRF1 and atrogin-1 is independent from each other and that atrophy might be blunted in the heart of stressed rats. Moreover, data presented here suggest that some of the alterations in the expression and activity of components of the PI3K-Akt signaling pathway induced by stress remain even after the end of the stress exposure period.

ACKNOWLEDGEMENT

This work was supported by the "Fundação de Amparo à Pesquisa do Estado de São Paulo" (2016/20777-8, 2016/20784-4), "Coordenadoria de Aperfeiçoamento de Pessoal do Ensino Superior" (CAPES) and "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (424114/2016-0).

DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

No conflicts of interest, financial, or otherwise are declared by the authors.

REFERENCES

1. Emorine LJ, Marullo S, Delavier-Klutchko C, Kaveri CSV, Durieu-Trautmann O, A D Strosberg. Structure of the gene for human beta 2-adrenergic receptor: Expression and promoter characterization. Proc Nat Acad Sci. 1987; 20: 6995-6999.
2. Brodde OE. Pathophysiology of the beta-adrenoceptor system in chronic heart failure: Consequences for treatment with agonists, partial agonists or antagonists? Eur Heart J. 1991; 12: 54-62.
3. Brodde OE, Michel MC. Adrenergic and muscarinic receptors in the human heart. Pharmacol Rev. 1999; 4: 651-690.
4. Lefkowitz RJ. Seven transmembrane receptors: Something old, something new. Acta Physiol. 2007; 1: 9-19.
5. Zhu W, Petrashevskaya N, Ren S, Zhao A, Chakir K, Gao E, et al. Gi-biased β2AR signaling links GRK2 upregulation to heart failure. Circ Res. 2012; 2: 265-74.
6. Spadari RC, Cavadas C, de Carvalho AETS, Ortolani D, de Moura AL, Vassalo PF. Role of beta-adrenergic receptors and sirtuin signaling in the heart during aging, heart failure, and adaptation to stress. Cell Mol Neurobiol. 2018; 1: 109-120.
7. Kim YS, Sainz RD. β-adrenergic agonists and hypertrophy of skeletal muscles. Life Sci. 1991; 50: 397-407.
8. Lynch GS, Ryall JG. Role of β-adrenoceptor signaling in skeletal muscle: Implications for muscle wasting and disease. Physiol Rev. 2008; 88: 729-767.
9. Silveira WA, Gonçalves FA, Graça AL, Andrade-Lopes AL, Bergantin LB, Zanon NM, et al. Activating cAMP/PKA signaling in skeletal muscle suppresses the ubiquitin proteasome-dependent proteolysis: Implications for sympathetic regulation. J Appl Physiol. 2014; 117: 11-19.
10. Voges D, Zwickl P, Baumeister W. The 26S proteasome: A molecular machine designed for controlled proteolysis. An Rev Biochem. 1999; 68: 1015-1068.
11. Seo D, Hare JM. The transforming growth factor-beta/Smad3 pathway: coming of age as a key participant in cardiac remodeling. Circul. 2007; 116: 2096-2098.
12. Hicke L, Schubert HL, Hill, CP. Ubiquitin-binding domains. Nat Rev Molecul Cell Biol. 2005; 6: 610-621.
13. Zhang W, Yano N, Deng M, Mao Q, Shaw SK, Tseng YT. Adrenergic receptor-PI3K signaling crosstalk in mouse heart: Elucidation of immediate downstream signaling cascades. Plos ONE. 2011; 10: e26581, 2011.
14. Spadari RC, Bassani RA, De Moraes S. Supersensitivity to isoprenaline and epinephrine in right atria isolated from rats submitted to a single swimming session. Gen Pharmacol. 1988; 1: 129-35.
15. Moura AL, Hyslop S, Grassi-Kassisse DM, Spadari RC. Functional β2-adrenoceptors in rat left atria: Effect of foot-shock stress. Can J Physiol Pharmacol. 2017; 9: 999-1038.
16. Bassani RA, De Moraes S. Effects of footshock stress on the sensitivity of the isolated rat pacemaker to catecholamines. Braz J Med and Biol Res. 1987; 3-4: 467-470.
17. Santos IN, Spadari-Bratfisch RC. Chronotropic response to (+/-)-CGP12177 in right atria of stressed rats. Can J Physiol Pharmacol. 2001; 5: 393-399.
18. Santos IN, Spadari-Bratfisch RC. Stress and cardiac beta adrenoceptors. Stress. 2006; 9 2: 69-84.
19. Ortolani D, Oyama LM, Ferrari EM, Melo LL, Spadari-Bratfisch RC. Effects of comfort food on food intake, anxiety-like behavior and the stress response in rats. Phys Beh. 2011; 103: 487-492.
20. Bodine SC, Baehr LM. Skeletal muscle atrophy and the E3 ubiquitin ligases MuRF1 and MAFbx/atrogin-1. Am J Physiol: Endocrinol Metab. 2014; 307: E469–E484.
21. Conrado MR, Cordeiro MA, Medeiros A, Ortolani D, Araki DR, Carvalho AETS, et al. (2020) Cardiac β2-adrenergic receptors modulate stress-induced alteration in the expression of ubiquitin-proteasome system components. Submitted