

Chronic Stress Improves NO- and Ca²⁺ Flux-Dependent Vascular Function: A Pharmacological Study

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Abstract

Background: Stress is associated with cardiovascular diseases.

Objective: This study aimed at assessing whether chronic stress induces vascular alterations, and whether these modulations are nitric oxide (NO) and Ca²⁺ dependent.

Methods: Wistar rats, 30 days of age, were separated into 2 groups: control (C) and Stress (St). Chronic stress consisted of immobilization for 1 hour/day, 5 days/week, 15 weeks. Systolic blood pressure was assessed. Vascular studies on aortic rings were performed. Concentration-effect curves were built for noradrenaline, in the presence of L-NAME or prazosin, acetylcholine, sodium nitroprusside and KCl. In addition, Ca²⁺ flux was also evaluated.

Results: Chronic stress induced hypertension, decreased the vascular response to KCl and to noradrenaline, and increased the vascular response to acetylcholine. L-NAME blunted the difference observed in noradrenaline curves. Furthermore, contractile response to Ca²⁺ was decreased in the aorta of stressed rats.

Conclusion: Our data suggest that the vascular response to chronic stress is an adaptation to its deleterious effects, such as hypertension. In addition, this adaptation is NO- and Ca²⁺-dependent. These data help to clarify the contribution of stress to cardiovascular abnormalities. However, further studies are necessary to better elucidate the mechanisms involved in the cardiovascular dysfunction associated with stressors. (*Arq Bras Cardiol.* 2015; 104(3):226-233)

Keywords: Stress, Physiological / physiopatology; Hypertension; Nitric Oxide / physiology; Rats; Vasoconstrictor Agents / pharmacology.

Introduction

Stress is known as a complex and multidimensional condition¹. The responses to stressor agents depend on the intensity, frequency, duration, and type of stressor agent. Hypothalamus-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) are the major responsible systems that modulate the organism to stressor agents². When stimulated, HPA and SNS release glucocorticoid hormone, such as cortisol, and biogenic amines, such as adrenaline and noradrenaline, respectively³.

Stress triggers different dysfunctions and pathologies including asthma, allergy, depression, anxiety, ulcer, metabolism dysfunction and cardiovascular diseases, such as stroke, hypertension and infarction^{2,4-8}.

The literature and previous data from our laboratory have shown that stress induces cardiac alterations, such as

fibrosis, systolic and diastolic left ventricle (LV) dysfunction and Ca²⁺ transit alteration^{6,9-13}. Moreover, cardiovascular changes are not restricted only to the heart, some evidence implicates that different types of acute stress induce modulated vascular response to different agonists, such as increased acetylcholine response and decreased contractile effect of noradrenaline, which are nitric oxide (NO) and endothelium-dependent^{7,14-16}. However, more studies are necessary to elucidate the mechanisms involved in modulated stress-induced responses.

Given that information, the aim of the present study was to assess whether chronic stress induces vascular alterations, and whether these modulations are NO- and Ca²⁺-dependent, in addition, the real involvement of α 1-adrenoreceptor also was analyzed. Our hypothesis was that chronic stress promotes adaptive vascular NO- and Ca²⁺-dependent responses and desensitization of α 1-adrenoreceptor. To understand the involvement of mediators and of α 1-adrenoreceptor, pharmacological tools were used.

Methods

Animals

Thirty-day-old male *Wistar* rats (70-100 g) obtained from the Animal Center of Botucatu Medical School (Botucatu, São Paulo, Brazil) were housed in individual cages. The environment

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Manuscript received March 07, 2014; revised manuscript August 29, 2014; accepted September 09, 2014.

DOI: 10.5935/abc.20140207

was controlled in terms of light (12-hour light/dark cycle starting at 6 AM), clean-air, room temperature ($23 \pm 3^\circ\text{C}$), and relative humidity ($60\% \pm 5\%$). After 7 days of acclimatization, the rats were distributed into two groups: control (C, $n = 8$) and chronic stress (St, $n = 8$). All experiments and procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*, published by the National Research Council (1996), and were approved by the Ethics Committee of the Instituto de Biociências UNESP-Botucatu (protocol nº 95/08-CEEA).

Chronic stress

The stress characteristics taken into consideration were quality, frequency, type, physical or emotional, as well as the animal species being studied¹⁷. Immobilization stress is a model of emotional stress and one of the most used in research¹⁸. After 37 days of age, the St group animals were immobilized individually in metal capsules at room temperature (25°C) for 1 hour per day, five days a week, for 15 weeks.

During the stress session, the C group animals remained in cage at room temperature (25°C), receiving neither food nor water. Then, the St group animals were returned to their cages. Forty-eight hours after the last stress session, the animals were subjected to experimental protocols.

Cardiac and adrenal hypertrophy test

The animals were sacrificed, and hypertrophy of adrenals glands and LV was assessed. The glands and LV were removed, dissected and weighed. Cardiac hypertrophy was assessed by using the LV/tibia (mm) ratio and, on echocardiography, the LV (g)/final body weight (FBW) ratio, according to our previous study^{7,8,13}.

Systolic blood pressure (SBP)

Systolic blood pressure was assessed at the end of stress exposure by use of the non-invasive tail-cuff method with a Narco BioSystems® Electro-Sphygmomanometer (International Biomedical, Austin, TX, USA). The mean of two SBP readings was recorded for each animal.

Corticosterone level

Animals were submitted to a 12-15-hour fasting, anesthetized with sodium pentobarbital (50 mg/kg i.p.), and sacrificed by use of decapitation. Blood samples were collected in heparinized tubes, centrifuged at 3000 X g for 15 minutes at 4°C , and the serum was separated and stored at -80°C for further analysis. Corticosterone level was measured by using a specific radioimmunoassay kit (Coat-A-Count Rat Corticosterone – Diagnostic Products Corporation).

Vascular function

After 15 weeks of stress exposure, the rats were decapitated. The descending thoracic aorta was excised and trimmed free of adhering fat and connective tissue. Two transverse rings of the same artery, measuring 4 mm in length each, were cut and mounted at the optimal

length for isometric tension recording in organ chambers. One ring served as control, while the endothelium was mechanically removed from the others by gently rubbing the luminal surface. The preparations were mounted in organ baths containing 7 mL of Krebs-Henseleit solution, whose composition in mM was as follows: NaCl 113.0; KCl 4.7; CaCl_2 2.5; KH_2PO_4 1.2; MgSO_4 1.1; NaHCO_3 25.0; glucose 11.0; ascorbic acid 0.11. The bathing fluid, kept at $37.0 \pm 0.5^\circ\text{C}$, was saturated with a gas mixture of 95% O_2 and 5% CO_2 , and the pH was 7.4. The preparations were allowed to equilibrate for at least 1 h under a resting tension of 1.5 g, which is ideal for inducing maximum contraction. Tension was recorded by use of a myograph (Ugo Basile).

Cumulative concentration-effect (CCE) curves were constructed from the tissue response to potassium chloride (KCl) and to noradrenaline. Cumulative concentration-effect curves to noradrenaline were constructed in the absence and presence of L-NAME (3×10^{-4} M, inhibitor of NO synthase - NOS) or prazosin (10^{-8} M, α_1 -adrenoreceptor antagonist) (Sigma Chemical Co., St Louis, Missouri, USA).

In another set of experiments, CCE curves were constructed for acetylcholine, in intact aortic rings (+E), and for sodium nitroprusside (SNP), in endothelium denuded aortic rings (-E) (Sigma Chemical Co., St Louis, Missouri, USA).

Contribution of intracellular and extracellular Ca^{2+} in the decreased response of endothelium-free aortic rings to noradrenaline

Adapted from Tirapelli et al¹⁹, we investigated the contribution of intracellular Ca^{2+} release on the decreased vascular function to noradrenaline, contractile response to this agonist was obtained in Krebs' solution without Ca^{2+} . The rings were exposed to this solution for 1 minute, then stimulated with 10^{-7} and 10^{-6} M noradrenaline, and then the tension was assessed. Furthermore, the role of extracellular Ca^{2+} mobilization was investigated by using CaCl_2 -induced contraction in the presence of noradrenaline. In Ca^{2+} -free solution containing EDTA (1 mM), endothelium-free aortic rings were contracted with noradrenaline (10^{-4} M) to deplete the intracellular Ca^{2+} stores and then rinsed in Krebs solution without Ca^{2+} and EDTA and containing noradrenaline (0.1 mM). The process was repeated several times until the extinction of noradrenaline-induced contraction, when we considered that Ca^{2+} was completely depleted.

Statistical analysis

Data are reported as means \pm standard error of the mean (SEM). The cardiac mass and adrenal hypertrophy, corticosterone levels and final SBP of the groups were compared by using *t*-test and *post hoc* Tukey-test with the GraphPad Prism 6.04 software. Individual concentration-effect curves were fitted into a curve by use of non-linear regression analysis. The negative logarithm of EC50 values (pD2) and the maximal response were compared by use of Student *t* test or ANOVA, when appropriate. The significance level of 5% was adopted.

Results

Chronic stress did not increase cardiac mass as follows: LV (g)/tibia (mm) values in the C and St groups were 0.15 ± 0.02 vs 0.16 ± 0.03 , respectively; and the results of the echocardiography test [LV (g)/FBW (g)] in the C and St groups were 1.45 ± 0.16 vs 1.52 ± 0.11 , respectively. However, chronic stress increased the wet adrenal weight (C = 0.57 ± 0.08 vs St = 0.76 ± 0.05). In addition, animals exposed to chronic stress developed high blood pressure [C = 118.3 ± 12.3 vs St = $148.8 \pm 9.43^*$ (mmHg)] and had increased corticosterone levels in plasma [C = 48.3 ± 10.2 vs St = $97.2 \pm 16.3^*$ (ng/mL)] * $p < 0.05$.

Chronic stress promoted decreased maximal response to KCl in aortic rings with or without endothelium [+E (C: 2.65 ± 0.48 vs St: $2.06 \pm 0.26^*$); -E (C: 2.62 ± 0.64 vs St: $2.18 \pm 0.62^*$)]. Moreover, no pD_2 difference was observed in rings with and without endothelium [(C: 3.47 ± 0.10 vs St: 3.36 ± 0.09) and (C: 11.51 ± 2.80 vs St: 11.17 ± 2.81)] (Figure 1) * $p < 0.05$.

Similarly to the KCl response, aortic rings with or without endothelium from stressed rats also had decreased maximal response to noradrenaline. Pre-incubation with L-NAME blunted these changes. No pD_2 difference was observed in the experimental groups without L-NAME pre-incubation. L-NAME pre-incubation increased the sensitivity to noradrenaline in both rings, endothelium-intact and denuded (Figure 2 and Table 1).

Prazosin, α_1 competitive antagonist, was used to assess the real α_1 adrenoreceptor involvement. It shifted the noradrenaline response to the right in endothelium-free aortic rings; however, there was no pD_2 difference between the C and St groups (C = 7.82 ± 0.08 ; St = 7.81 ± 0.09 ; C/Prazosin = $6.02 \pm 0.05^*$; St/Prazosin = $6.14 \pm 0.06^*$) * $p < 0.05$ (Figure 3).

Chronic stress enhanced the maximal response to acetylcholine in aortic rings with endothelium, as well as its sensitivity (Figure 4.A and Table 2). Moreover, endothelium-denuded aortic rings from stressed rats showed shift to the left for NO donor, SNP, and no difference was observed in the maximal response parameter (Figure 4.B and Table 2).

Stressed rats had decreased contractile response to noradrenaline in Krebs solution without Ca^{2+} . Furthermore, CCE curves for $CaCl_2$, in presence of noradrenaline, in endothelium-free aortic rings from chronically stressed rats also had decreased maximal response. No difference was observed in pD_2 (Figure 5 and Table 3).

Discussion

In the present study, chronic stress increased adrenal wet weight and plasma corticosterone levels, which suggest increased HPA axis activity, and corroborate the literature that shows these same effects in different stress models^{17,20,21}. Stress can lead to hypertension through the production of several mediators or hyperactivation of some systems, including renin-angiotensin-aldosterone, and vasoactive amines, such as adrenaline, that are associated with blood pressure regulation²²⁻²⁴. Our stress model led to high blood pressure that might be associated with adrenaline release by adrenal glands, because we found increased adrenal mass, which indicates SNS hyperactivation, corroborating findings from literature^{13,22}.

Stress, in acute or chronic models, improves vascular function to different agonists^{7,14,16,25}. Our results corroborate these data, in which increased responses to noradrenaline and acetylcholine were observed. In addition, we can suggest that these responses are NO-dependent for two reasons: i) the previous incubation with NOS inhibitor abolished the decreased maximal response to noradrenaline in aortic rings from stressed rats compared with that of the control group; ii) we found that acetylcholine-induced relaxation was higher in aortic rings from the St group than from the C group. Acetylcholine, an endothelium-dependent agonist, is able to release NO when it binds to a muscarinic receptor located in endothelial cells, leading to vascular relaxation²⁶.

Another interesting finding from our study shows that vascular smooth muscle from stressed rats is more sensitive to NO than that from the non-stressed group, because the NO donor, SNP, induced shift to the left in endothelium-free aortic rings. We did not assess pathways associated with NO

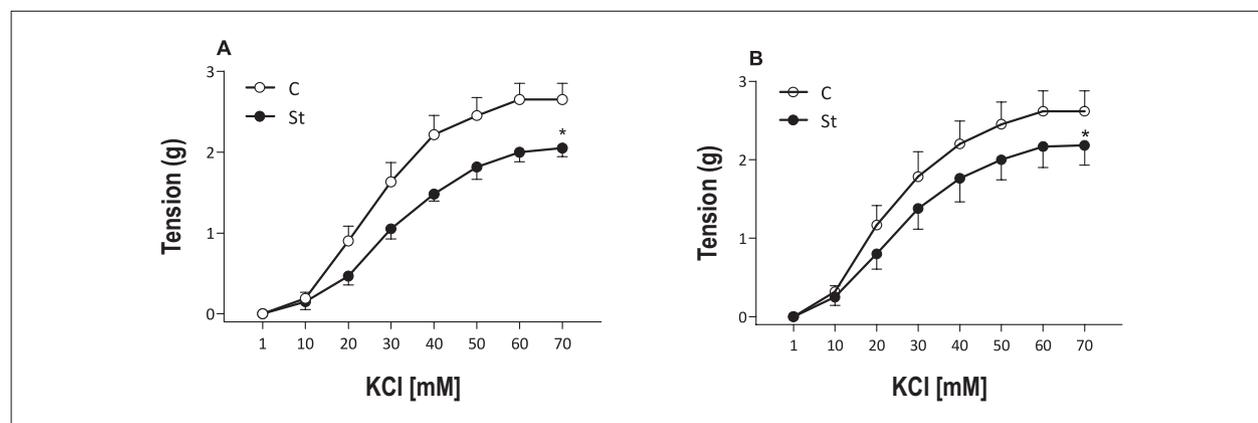


Figure 1 – Concentration-effect curves for KCl obtained with two rings, one with (A) and the other without (B) endothelium, of the same thoracic aorta from control (empty symbol) and stressed (solid symbol) rats. Data are reported as means \pm SEM ($n = 6$) * $p < 0.05$.

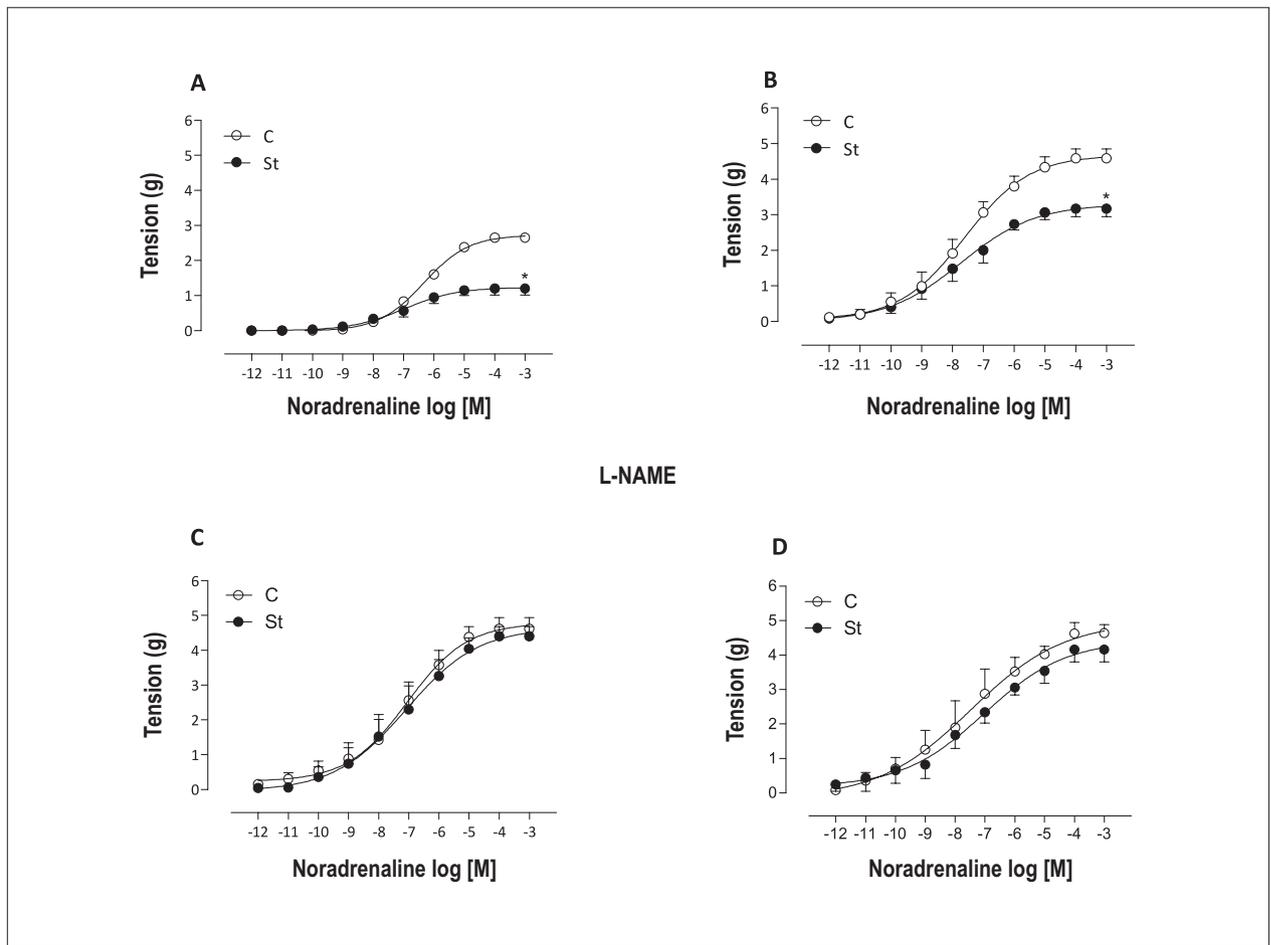


Figure 2 – Concentration-effect curves for noradrenaline obtained in intact endothelium (E+) (A and C) and in denuded endothelium (E-) aortic rings (B and D), in presence (C and D) or absence (A and B) of L-NAME (3×10^{-4} M), from control (empty symbol) and stressed (solid symbol) rats. Data are reported as means \pm SEM (n = 5-7) *p < 0.05.

Table 1 – Vascular reactivity to noradrenaline in presence or absence of L-NAME

Groups	Agonist	Parameters			
		Maximal response		pD ₂	
		+E	-E	+E	-E
C	Nor	2.65 \pm 0.21	4.58 \pm 0.64 [#]	6.33 \pm 0.07	7.60 \pm 0.23 [#]
	Nor/L-NAME	4.62 \pm 0.70 [§]	4.40 \pm 0.63 [#]	7.05 \pm 0.29 [§]	7.47 \pm 0.52
St	Nor	1.20 \pm 0.40*	3.87 \pm 0.58 ^{#*}	6.49 \pm 0.32	7.27 \pm 0.21 [#]
	Nor/L-NAME	4.64 \pm 0.55 [§]	4.16 \pm 0.78 ^{§*}	7.08 \pm 0.38 [§]	7.03 \pm 0.44

Effects of chronic stress on maximal response and pD₂ (negative logarithm of the EC₅₀) for noradrenaline (Nor) in aortic rings from Wistar rats, in L-NAME presence or absence (3×10^{-4} M). Concentration-effect curves (CCE) were constructed in intact endothelium (E+) and denuded endothelium (E-) aortic rings. Results are shown as means \pm SEM of 5-7 rats in each experimental group. *p < 0.05 C vs St; [#]p < 0.05 C vs St; [§]p < 0.05 L-NAME vs Nor; [#]p < 0.05 -E vs +E; C: control group; St: stress group.

production, such as AKT (protein kinase B), which is able to phosphorylate NO synthase²⁷, but we can confirm that both NO releasing and sensitivity to NO are involved in the modulated vascular response to chronic stress.

We assessed whether α 1-adrenoreceptor participates in the vascular function of stressed rats by using a competitive α 1-adrenoreceptor antagonist. We concluded that α 1-adrenoreceptor activity does not change in the aorta of stressed

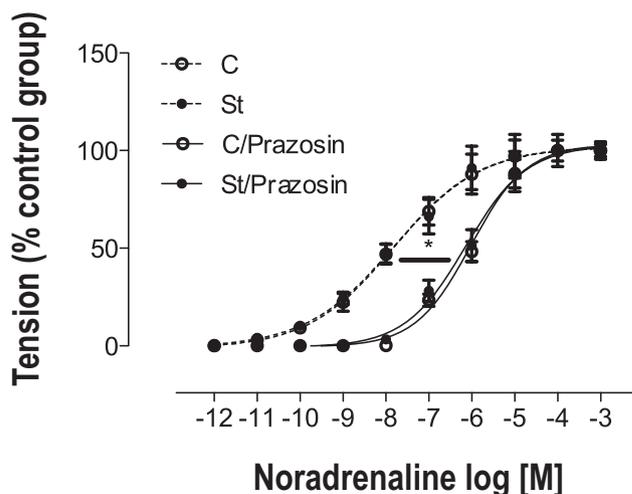


Figure 3 – Concentration-effect curves for noradrenaline in denuded endothelium (E-) aortic rings, in presence (solid line) or absence (dotted line) of prazosin (10^{-8} M) from control (empty symbol) and stressed (solid symbol) rats. Data are reported as means \pm SEM ($n = 6$) * $p < 0.05$.

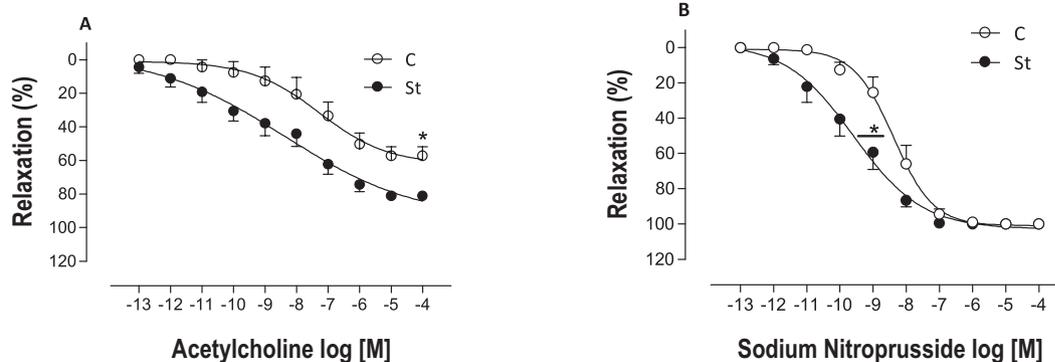


Figure 4 – Concentration-effect curves for acetylcholine (A) obtained in intact endothelium (E+) aortic rings and sodium nitroprusside (B) obtained in denuded endothelium (E-) aortic rings from control (empty symbol) and stressed (solid symbol) rats. Data are reported as means \pm SEM ($n = 5-7$) * $p < 0.05$.

rats, and the decreased maximal response observed in the experimental group might be linked to downstream events to α 1-adrenoreceptor, such as NO release and sensitivity²⁸, or NO release might be associated with α 2-adrenoreceptor activation by noradrenaline²⁹.

Similarly to noradrenaline response, KCl, a contractile agonist not receptor-dependent, also had decreased maximal response in aorta rings of the St group. These data, together with prazosin and noradrenaline, strengthen that some intracellular mediator is involved in stress vascular response, NO being a strong candidate, since the KCl-induced vascular contraction does not depend on a specific receptor, but on action potential.

Ca²⁺ plays a crucial role in vascular homeostasis, modulating vascular function and structure, the intracellular Ca²⁺ and uptake being essential for perfect operation³⁰. We examined whether intracellular Ca²⁺ and uptake are involved in the attenuated vascular contraction to noradrenaline in stressed rats. Attenuated vascular response to single concentrations of noradrenaline was observed in this study. So, we could suggest that intracellular Ca²⁺ release, which occurs in the endoplasmic reticulum (ER) after interaction of inositol trisphosphate (IP₃) with its receptor located in the ER membrane^{31,32}, or the low Ca²⁺ concentration in the ER could participate in this modulation. Moreover, Ca²⁺ uptake by some channel, such as L-type calcium channels^{32,33}, might have low activity to uptake Ca²⁺ in the vasculature from the St group, because the CCE curve for

Table 2 – Vascular reactivity to acetylcholine

Groups	Agonist	Parameters	
		Maximal response	pD ₂
+E			
C	ACh (%)	62.4 ± 8.63	7.27 ± 0.47
St		95.3 ± 16.2*	8.37 ± 0.63*
-E			
C	SNP (%)	101 ± 5.4	8.38 ± 0.10
St		103 ± 5.7	9.58 ± 0.22*

Effects of chronic stress on maximal response and pD₂ (negative logarithm of the EC₅₀) for acetylcholine (ACh) in intact endothelium (E+) aortic rings and sodium nitroprusside (SNP) in denuded endothelium (E-) aortic rings from Wistar rats. Concentration-effect curves (CCE) were constructed. Results are shown as means ± SEM of 5-7 rats in each experimental group. *p < 0.05 C vs St; C: control group; St: stress group.

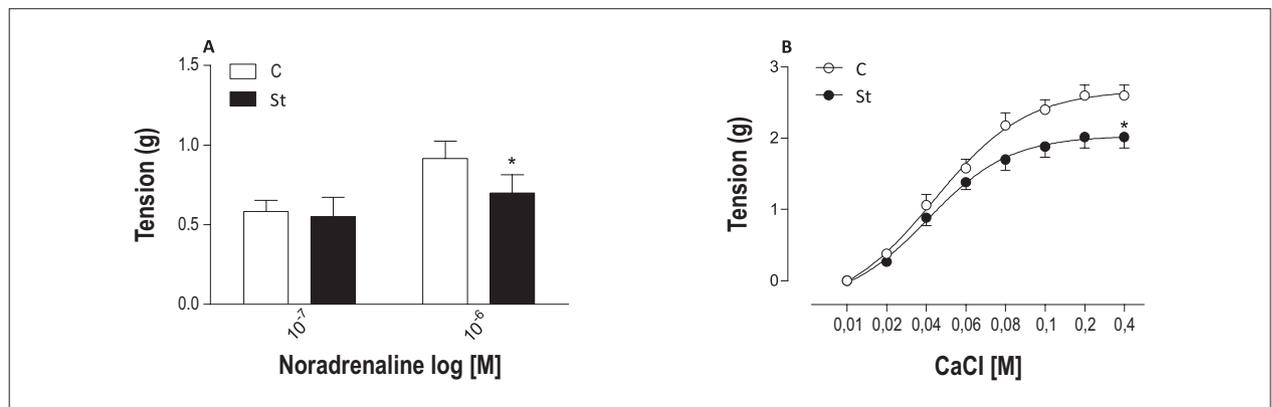


Figure 5 – Contractile response for noradrenaline in denuded endothelium (E-) aortic rings from control (empty bar) and stressed (solid bar) rats in Krebs solution without Ca²⁺ (A). Concentration-effect curves for CaCl₂ in aortic rings without endothelium from control (empty symbol) and stressed (solid symbol) rats. Data are reported as means ± SEM (n = 5-7) *p < 0.05.

Table 3 – Vascular reactivity to CaCl₂

Groups	Agonist	Parameters	
		Maximal response	pD ₂
-E			
C	CaCl ₂	2.76 ± 0.13	8.81 ± 0.34
St		2.03 ± 0.10*	8.97 ± 0.37

Effects of chronic stress on maximal response and pD₂ (negative logarithm of the EC₅₀) for CaCl₂ in aortic rings from Wistar rats. Concentration-effect curves (CCE) were constructed in denuded endothelium (E-) aortic rings. Results are shown as means ± SEM of 5-7 rats in each experimental group. *p < 0.05 C vs St; C: control group; St: stress group.

Ca²⁺, in a Ca²⁺-free medium in presence of noradrenaline, was attenuated in endothelium-free aortic rings of stressed rats.

Conclusion

Our study advances the understanding and identifies new mediators involved in the attenuated vascular response to noradrenaline in stressed rats. Nitric oxide and Ca²⁺ fluxes are the possible mediators. We believe these mediators are positively

activated to counterbalance the deleterious cardiovascular effects caused by stressful conditions. However, more studies are necessary to better elucidate the adaptive response to chronic stress.

Author contributions

Conception and design of the research, Statistical analysis and Obtaining financing: Bruder-Nascimento T, Cicogna AC, Cordellini S; Acquisition of data: Bruder-Nascimento T,

Campos DHS; Analysis and interpretation of the data and Critical revision of the manuscript for intellectual content: Bruder-Nascimento T, Campos DHS, Cicogna AC, Cordellini S; Writing of the manuscript: Bruder-Nascimento T.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Sources of Funding

This study was funded by Fapesp add the process number 2009/03771-2.

Study Association

This article is part of the thesis of master submitted by Thiago Bruder do Nascimento, from Universidade do Estado de São Paulo.

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