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Chronic inhibition of nitric oxide synthase modulates calcium handling in rat heart.

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ABSTRACT

Systemic infusion of nitric oxide synthase (NOS) inhibitors increases peripheral vascular resistance due to inhibition of endothelial NOS leading to the activation of the arterial baroreceptor mechanisms and inhibition of central sympathetic outflow. In the current study, we explored that systemic NOS blockage activates protein kinase A (PKA)-mediated signaling pathway through maintained cGMP-dependent protein kinase (PKG) activation. Rats were treated with three different concentrations of N(ω)-nitro-L-arginine methyl ester (L-NAME) for 14 days. Systemic L-NAME treatment induced a dose-dependent increase in blood pressure, increased mRNA levels of atrial natriuretic peptide (ANP) and phosphorylation levels of p44/42 MAPK without any change in cardiac mass. The cardiac cGMP levels and PKG-mediated phosphorylation of vasodilator-stimulated phosphoprotein (VASP) (Ser239) did not alter in any group. At the highest dose of treatment (100mg/kg/day), PKA-mediated phosphorylations of VASP (Ser157) and troponin I (TnI) (Ser23/24) were enhanced significantly indicating the increase in PKA activation in response to chronic NOS blockage. Alterations in both phosphorylated phosholamban (Ser16/Thr17) and sarcoplasmic/endoplasmic Ca^{2+}-ATPase (SERCA2) levels can increase cytosolic Ca^{2+} load and impair Ca^{2+} handling. Our data suggest that the increased PKA activation in response to chronic NOS blockage appears to be responsible for cardiac abnormalities which occurs due to prolonged L-NAME treatment.

Keywords: nitric oxide, protein kinase A, cyclic GMP, atrial natriuretic peptide, phospholamban, SERCA, troponin I.

INTRODUCTION

Cyclic guanosine 3', 5'-monophosphate (cGMP) is one of the important intracellular second messengers in the cardiovascular system. cGMP is generated by guanylyl cyclase isoforms including soluble (sGC) and particulate (pGC) in response to nitric oxide (NO) or natriuretic peptides, respectively. cGMP activates different types of effector molecules such as cGMP-dependent protein kinase (PKG) and phosphodiesterases (PDEs) (Hammond and Balligand 2011). The cGMP/PKG signaling mediates vasorelaxation in vascular smooth muscle (Murad et al. 1985), inhibits development of cardiac hypertrophy and modulates cardiac contractility (Vila-Petroff et al. 1999; Takimoto et al. 2005).

It is well-known that increase in mean arterial blood pressure is physiologically controlled by enhanced baroreceptor activity thereby causing decreased sympathetic and increased vagal outflow to the heart which in turn results in bradycardia. Pharmacological inhibition of NO synthesis with N(ω)-nitro-L-arginine methyl ester (L-NAME) causes hypertension in a dose-dependent manner. L-NAME-induced hypertension results mainly from systemic vasoconstriction caused by inhibition of endothelium-dependent vasodilation. In addition, there is increasing evidence of a sympathetic activation induced by NOS blockage (Biancardi et al. 2007; Young et al. 2009). The contribution of increased central sympathetic drive in the maintenance of L-NAME-induced hypertension was demonstrated with chronic sympathectomy mediated by ganglionic blockage which attenuated the hypertension and total peripheral resistance in hypertensive groups (Cunha et al. 1993; Biancardi et al. 2007). These findings are also consistent with several studies which reported a progressive increase in sympathetic tone (Bergamaschi et al. 1999; Eshima et al. 2000; Thakali et al. 2006).

Sympathetic stimulation on the heart enhances heart rate and contractility by modulating Ca-cycling under physiological conditions (Kranias and Hajjar 2012). Therefore, the aim of this study was to determine whether chronic NOS blockage modulates calcium handling in rat heart. For this purpose, we examined possible changes in protein kinase A (PKA)- and PKG-mediated phosphorylations of several proteins which play role in calcium handling and myofilament response in L-NAME-induced hypertension model in addition to the alterations in blood pressure and heart rate.

MATERIALS AND METHODS

Animal care and procedures:

Eight-week-male Sprague-Dawley rats were purchased from Bilkent University Department of Molecular Biology and Genetics (Ankara, Turkey). All animal procedures were approved by Institutional Ethical Committee of Ankara University (Approval ID: 2014-7-43). Animals were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* (2011). The rats were housed in individual cages at 22±1ºC and 12-h light/darkness cycle. The rats were fed with standard rat chow (Purina, Turkey) and tap water *ad libitum.* The rats were divided into four groups randomly for chronic treatment protocols of NOS inhibitor L-NAME (Sigma-Aldrich Chemical, St. Louis, MO). The control group (C) were given tap water as drinking water and L-NAME was administered in the drinking water at 0,3 mg/mL (for 20mg/kg/day, L20), 0,75 mg/mL (for 50mg/kg/day, L50) and 1,5mg/mL (for 100mg/kg/day, L100) for 14 days remaining groups. Starting from the day 2, systolic blood pressure and heart rate measurements were performed in all groups at the same condition for 14 days. At the end of the 14-day-treatment period, rats were anesthetized by ether inhalation. The heart was quickly removed and placed in oxygenated Krebs solution (95%O_2 and 5%CO_2, pH 7.40) containing (in mmol/L): NaCl 118, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.50 and glucose 10. The pieces of left ventricle tissue were immediately frozen in liquid nitrogen and stored at -80°C for subsequent PCR, cGMP and western blot experiments.

Heart rate and systolic blood pressure measurements:

Heart rate and systolic blood pressure of rats in all groups were measured in triplicate using the tail cuff method (MAY 9610 Indirect Blood Pressure Recorder System, Commat Ltd., Turkey) prior to initiation of treatments (day 0) and during *in vivo* treatment (days 3, 7 and 14).

cGMP measurements:

cGMP level in left ventricular lysate was measured by ELISA using the cyclic cGMP Complete Kit (Enzo Life Sciences, NY, USA) as described previously. Briefly, left ventricular samples were homogenized in 0.1M HCl. The supernatants were used and the assays were performed according to the manufacturer's instructions. The absorbance at 405 nm were measured by SpectraMax 190 microplate reader (Molecular Devices, CA, USA). Each reaction was performed in duplicate. Results are expressed as an average of three independent experiments using left ventricular tissues of four-to-five rats.

Total RNA isolation, RT-PCR and real-time PCR experiments:

Left ventricular samples were powdered with liquid nitrogen and homogenized with an ultrasonic homogenizer (bandelin Electronics, Berlin, Germany) before RNA extraction. Total RNA was extracted with the TRIzol reagent (Sigma-Aldrich Chemical, St. Louis, MO) according to the manufacturer's protocol. The optical density values and amounts of RNA were determined spectrophotometrically using NanoDrop at wavelength 260nm and 280nm. In addition, 18S/28S bands were used to evaluate RNA integrity (data not shown). After DNase I treatment (Roche Diagnostics GmBH, Mannheim, Germany), 1ug of RNA from each sample was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Rovhe Diagnostics). Quantitative real-time PCR was performed using SYBR Green I Master (Roche Diagnostics) on Lightcycler 480 (Roche Applied Science, Indianapolis, IN) along with primers given in Table 1. All reactions were run in triplicates. Relative gene expression was normalized to TBP.

SDS-gel electrophoresis, immunoblotting and Western blot analysis:

Frozen left ventricular tissues were powdered with liquid nitrogen and homogenized in ice-cold radioimmunoprecipitation assay buffer (Sigma-Aldrich Chemical) including protease inhibitor cocktail (100X; Sigma-Aldrich Chemical) and sodium orthovanadate (1mM; Sigma-Aldrich Chemical). Homogenates were centrifuged at 1300g for 5 min at 4°C and the supernatants were centrifuged at 16000g for 30 min at 4°C and the 16000g-supernatants were used for immunoblotting. The protein concentrations of lysates were measured with the BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein from each heart lysate (20 µg/lane) were loaded onto 8% SDS-polyacrylamide gels for sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 10% tricine-SDS-polyacrylamide gels for phospho-phospholamban and phospholamban, separated in a minigel apparatus (Mini-PROTEAN-III, BioRad, Hercules, CA, USA) and transferred to polyvinylidene difluoride membranes (BioRad, Hercules, CA, USA). The membranes were blocked with 5% BSA (Equitech-Bio, Kerrville, TX, USA) in TBS-Tween and incubated overnight at 4ºC with SERCA2a antibody (1:5000), phospholamban (PLN) antibody (1:2000), phospho-phospholamban (Ser16/Thr17) (pPLN) antibody (1:2000), vasodilator-stimulated phosphoprotein (VASP) antibody (1:1000), phospho-vasodilator-stimulated phosphoprotein (Ser239) (pVASPSer239) antibody (1:1000), phospho-vasodilator-stimulated phosphoprotein (Ser157) (pVASPSer157) antibody (1:1000), phospho-troponin I (Ser23/24)

(pTnI) (1:1000), phospho-p44/42 MAPK (Thr202/Tyr204) antibody (1:1000), p44/42 MAPK antibody (1:1000) from Cell Signaling Technology (Beveryl, MA, USA), GAPDH (1:10000) from Millipore (Darmstadt, Germany) and troponin I (TnI) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antigens were detected by enhanced chemiluminescence method (ECL Western Blotting Substrate, Pierce, Rockford, IL, USA) with HRP-linked anti-rabbit IgG (1:30000) from Cell Signaling (Beveryl, MA, USA). Relative band densities were analyzed using Image J (http://rsbweb.nih.gov/ij/) and were normalized to GAPDH as loading controls.

Statistical analysis:

All results are expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using 1-way analysis of variance followed by the Newman-Keuls multiple comparison test. P value <0,05 was considered statistically significant.

RESULTS

Effects of chronic L-NAME treatment on blood pressure and heart rate:

As shown in Figure 1, chronic oral administration of L-NAME induced an increase in blood pressure and a reduction in heart rate of rats in a dose-dependent manner. 3 days after L-NAME treatment, the systolic blood pressure of all three groups were increased significantly compared to control (Figure 1A). Similarly, at the end of L-NAME treatment protocol, systolic blood pressures of rats rose ~17%, ~25% and ~31% compared to controls in L20, L50 and L100 groups respectively which demonstrated the dose-dependency of L-NAME treatment (Figure 1A). The heart rate of all L-NAME-treated groups were depressed significantly especially in the first 3-day of treatment which indicates a reflex mechanism against elevated peripheral resistance (Figure 1B). At the end of the treatment (14-day), significant negative chronotrophic effect still was observed in response to L-NAME treatment (Figure 1B).

Effects of chronic L-NAME treatment on cardiac mass and development of hypertrophy:

No significant difference was observed in body weight between four groups. Also chronic treatment of L-NAME did not cause any alteration in left ventricular mass evaluated as left ventricular weight-to-body weight ratio. Although downregulation of SERCA2a mRNA levels which is known as a marker of cardiac hypertrophy was not observed on our experimental conditions, the mRNA levels of ANP, another gene evaluated as a marker of cardiac hypertrophy were increased significantly in all L-NAME-treated groups (Figure 2A). Also, L-NAME treatment at the dose of 100mg/kg/day was increased phosphorylation levels of p44/42 MAPK in rat heart (Figure 2B).

Effects of chronic L-NAME treatment on cGMP levels and PKG-mediated phosphorylation of VASP:

The effect of NOS blockage on heart was evaluated with cardiac cGMP levels. However, as seen in Figure 3A, the cardiac cGMP levels did not change in response to any dose of L-NAME treatment. To evaluate the functionality of cGMP/PKG signaling pathway, Ser239 phosphorylation level of VASP protein was investigated. In response to chronic NOS blockage with L-NAME treatment, the phosphorylation levels of VASP at Ser239 in rat left ventricule was maintained which is consistent with preserved cardiac cGMP levels (Figure 3B).

Effects of chronic L-NAME treatment on PKA-mediated phosphorylations of VASP and TnI:

The Ser157 phosphorylation of VASP has been accepted as a marker for PKA activation (Sartoretto et al. 2009; Bibli et al. 2015). The phosphorylation levels of VASP at Ser157 increased significantly in L100 compared to L20 and L50 groups which demonstrates the increased activation of PKA in chronic NOS blockage (Figure 4A). Another target protein of PKA, phosphorylation of TnI at Ser23/24, was also investigated for assessment of PKA activation. Similar to the Ser157-phosphorylation of VASP, TnI phosphorylation at Ser23/24 was found to be increased in a dose-dependent manner with chronic NOS blockage (Figure 4B) suggesting a PKA-mediated activation in response to L-NAME treatment at 100mg/kg/day concentration.

Effects of chronic L-NAME treatment on Ca^{2+} handling:

The possible changes in phospholamban (PLN) and SERCA2a complex in response to chronic L-NAME treatment were investigated by alterations in PLN-to-SERCA2a ratio and phosphorylation status of PLN. The PLN-to-SERCA2a ratio was found to be increased significantly in L50 and L100 groups compared to control (Figure 5A). The phosphorylation of PLN which decrease the inhibitory effect of PLN on SERCA2a reduced significantly in L100 group compared to control (Figure 5B).

DISCUSSION

In the present study, we demonstrated that systemic L-NAME treatment (100mg/kg/day, 14 days) which induces a significant increase in blood pressure impaired through cardiac Ca^{2+} handling with maintenance of cGMP/PKG signaling pathway. L-NAME treatment with lower doses (20 and 50mg/kg/day) increased the blood pressure in a mild but significant manner without any alteration in the Ca^{2+} cycling of heart. Also L-NAME-induced transcriptional changes and remodeling which were demonstrated with increased ANP mRNA levels and phosphorylated p44/42 MAPK levels respectively were also observed in the current study. We have also shown that in the experimental conditions in which cardiac cGMP levels and PKG activation were maintained, phosphorylation levels of phospholamban and troponin I was found to be significantly altered. As evidenced with the highest dose of L-NAME treatment, efficient NOS blockage can modulate cardiac sympathetic drive and can lead to Ca^{2+} overload.

Pharmacological inhibition of NOS has been used extensively to establish both acute and chronic hypertension in many animal species (Baylis et al. 1992; Sander et al. 1995). According to the previous studies, L-NAME administration at the concentration of 60mg/kg/day for 4-6 weeks altered systemic and renal hemodynamics (Riberio et al. 1992) and at the concentration of 100-120 mg/kg/day for 8 weeks mediated hypertension-induced cardiac hypertrophy (Boe et al. 2013). In the current study, we evaluated the dose-dependent effects of systemic L-NAME treatment on specific phosphorylation targets of cardiac PKA and PKG at a single time-point. The shorter duration of the in vivo treatment protocol compared to other studies (Boe et al. 2013; Ozen et al. 2018) allowed us to assess the alterations in an earlier stage of the hypertensive model. The increase in systolic blood pressure after 2 weeks of L-NAME treatment was found to be quite similar to previous studies (Boe et al. 2013). It has been known that systemic infusion of NOS inhibitors increases blood pressure due to inhibition of eNOS, resulting in activation of the arterial baroreflex and subsequent inhibition of central sympathetic outflow (Young et al. 2009). In the current study, significant increases in the blood pressure were observed in all L-NAME-treated groups which demonstrated the vascular component of systemic NOS blockage in a dose-dependent manner. L-NAME-treated rats showed a profound but not persistent bradycardia which can be related to hypertension-induced baroreflex activation. As seen in the previous studies, present study showed that the effects of L-NAME on heart rate is recovered with more prolonged duration of L-NAME treatment (Scrogin et al.

1998). It is possible that the recovery of chronotropic alterations induced by L-NAME can be a result of increased cardiac sympathetic tone and/or attenuation of activated parasympathetic tone during in vivo treatment (Scrogin et al. 1998).

The heart responds to sympathetic stimulation as increased contractility, accelerated relaxation and [Ca^{2+}] decline under physiological conditions. The noradrenaline released from sympathetic nerve endings bind to the cardiac β-adrenoceptors thereby activating Gs-protein/adenylyl cyclase/cyclic AMP (cAMP)/PKA signaling pathway (Bers 2002). The PKA-mediated phosphorylations of phospholamban and troponin I increase the rate of Ca^{2+} reuptake by sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) to sarcoplasmic reticulum and dissociation of Ca^{2+} from myofilaments, respectively (Bers 2002). Regulation of Ca^{2+}-cycling and contractility by the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase/phospholamban (SERCA2a/PLN) complex is mediated by phosphorylation status of PLN. Dephosphorylated PLN is actually an inhibitor of cardiac SERCA2a and phosphorylation decreases the inhibition of PLN on SERCA2a (Kranias and Hajjar 2012). We investigated the alteration of cardiac sympathetic tone in response to L-NAME treatment in terms of signaling partners which are responsible for the cardiac excitation-contractin coupling and other targets of PKA such as VASP. The increased PLN-to-SERCA2a ratio in response to L50 and L100 groups indicate enhanced inhibitory effect of PLN on SERCA2a which can lead to impairment of Ca^{2+} handling. The reduced phosphorylation levels of PLN in L100 group suggested a decrease in the rate of cytosolic Ca^{2+} removal in the presence of NOS blockage at the highest dose used in the current study. The increased PLN-to-SERCA2a ratio and decreased phospho-PLN levels strongly demonstrated that impaired control of Ca^{2+} handling could lead to Ca^{2+} overload especially in L100 group. L-NAME-induced increase in phosphorylated TnI levels in L100 group provided evidence for increased PKA activation and subsequently enhancement of relaxation rate (Stelzer et al. 2007). Enhanced cardiac relaxation via increased TnI phosphorylation could be a compensation mechanism for increased cytosolic Ca^{2+} load and/or peripheral vascular resistance.

In addition to Ca^{2+}-handling proteins (e.g. phospholamban and SERCA2a) and myofilament protein (e.g. TnI), PKA can also phosphorylate members of actin-binding protein family such as VASP (Reinhard et al. 2001). VASP protein has been extensively used as a substrate for identifying of PKA and PKG activation (Walter et al. 1993; Sartoretto et al. 2009). Three phosphorylation sites of VASP (Ser157, Ser239,

Thr278) have been identified in human (Smolenski et al. 1998; Oelze et al. 2000). Ser157 is the preferred site for phosphorylation by PKA, whereas Ser239 is the site preferentially phosphorylated by PKG (Oelze et al. 2000). In the current study, the phosphorylation of VASP from two different sites were assessed for the evaluation of possible changes in PKA and PKG activation in response to L-NAME treatment. With enhanced ANP mRNA levels and maintained cGMP levels, the preserved phosphorylation levels of VASP at Ser239 indicated the compensatory role of natriuretic peptides on cGMP production via particular guanylate cyclase activation and the maintained activation of cGMP/PKG signaling cascade in the present experimental setting. On the other hand, the phosphorylation of VASP at Ser157 was enhanced in L-NAME treatment which provided evidence for increased PKA activity in the presence of NOS blockage which was also supported by increased TnI phosphorylation.

Maintanence of the cardiac cGMP levels and PKG-induced VASP phosphorylation levels at Ser239 in response to L-NAME treatment could be related with increased ANP mRNA levels which is a possible compensation mechanism depending on the duration of treatment protocol. The possible compensation effect of ANP on PKG activity was also supported with the results that short term ANP incubation mediated the phosphorylation of VASP at Ser239 in cardiac myocytes without affecting Ser157 phosphorylation (Sartoretto et al. 2009).

One of the potential limitations of the present study is the lack of changes in cardiac cAMP levels in response to L-NAME treatment. As validation of cardiac cGMP levels with VASP phosphorylation at Ser239, the phosphorylation status of TnI at Ser23/24 and VASP at Ser157 could be evaluated better with cAMP levels. Other limitations are the lack of cytosolic Ca^{2+} concentration measurements in cardiomyocytes and evaluation of cardiac contractility in the presence of NOS inhibition.

In summary, we demonstrated that the systemic NOS blockage enhanced PKA-mediated phosphorylations which provides evidence for augmented cardiac PKA activation in the presence of maintained PKG signaling. The increased PKA activation induced by L-NAME treatment disturbed the Ca^{2+} handling and could lead to Ca^{2+} overload in sustained systemic NOS inhibition. In addition to the involvement of NO in central sympathetic control, our findings provide important evidence that NO is also involved in the regulation of PKA activity without affecting cGMP/PKG signaling pathway in rat heart.

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FIGURE CAPTIONS

Figure 1. The effects of L-NAME treatment at three different concentrations (20, 50 and 100mg/kg/day) on **(A)** systolic blood pressure and **(B)** heart rate. Measurements were performed throughout the course of the study at given days. Values are means±SEM. n=4-6 for control (C), 20mg/kg/day (L20), 50mg/kg/day (L50) and 100mg/kg/day (L100) L-NAME. *, p<0,05 vs C; #, p<0,05 vs L20; ϕ, p<0,05 vs L50.

Figure 2. The effect of chronic L-NAME treatments on development of cardiac hypertrophy. **(A)** Cardiac ANP mRNA levels which were normalized to TBP and **(B)** Thr202/Tyr204 phosphorylation of p44/42 MAPK which were normalized to total p44/42 MAPK of left ventricular samples from L-NAME-treated rats were shown. Representative western blotting images are located below the bar graph. Values are means±SEM. n=4-6 for control (C), 20mg/kg/day (L20), 50mg/kg/day (L50) and 100mg/kg/day (L100) L-NAME. *, p<0,05 vs C.

Figure 3. The effect of chronic L-NAME treatments on cardiac cGMP/PKG signaling. **(A)** Cardiac cGMP levels and **(B)** Ser239 phosphorylation of VASP which were normalized to total VASP of left ventricular samples from L-NAME-treated rats were shown. Representative western blotting images are located below the bar graph. Values are means±SEM. n=4-6 for control (C), 20mg/kg/day (L20), 50mg/kg/day (L50) and 100mg/kg/day (L100) L-NAME. #, p<0,05 vs L20; ϕ, p<0,05 vs L50.

Figure 4. The effect of chronic L-NAME treatments on PKA-mediated phosphorylations. **(A)** Ser157 phosphorylation of VASP and **(B)** Ser23/24 phosphorylation of troponin I (TnI) of left ventricular samples from L-NAME-treated rats were normalized to total VASP and cardiac TnI, respectively. Representative western blotting images are located below each bar graph. Values are means±SEM. n=4-6 for control (C), 20mg/kg/day (L20), 50mg/kg/day (L50) and 100mg/kg/day (L100) L-NAME. #, p<0,05 vs L20; ϕ, p<0,05 vs L50.

Figure 5. The effect of chronic L-NAME treatments on cardiac Ca^{2+} handling. **(A)** Phospholamban (PLN)/SERCA2a, **(B)** phospho-PLN/PLN and (C) representative western blotting images are shown. Values are means±SEM. n=4-6 for control (C), 20mg/kg/day (L20), 50mg/kg/day (L50) and 100mg/kg/day (L100) L-NAME. #, p<0,05 vs L20.

TABLES

Table 1. Nucleotide sequences for the primers and size of PCR products of each primer set.

Gene	GenBank ID		Primers	Product size (bp)
SERCA2a	NM_001110823.2	F	CTCTGAGAGTTGACCAGTCGAT	176
		R	AGTATTGACTCCAGTCGCCA	
ANP	NM_012612	F	GGTAGGATTGACAGGATTGG	192
		R	AGATGAAGACAGGAAGCTGC	
TBP	NM_001004198	F	GCAATCAACATCTCAGCAGC	154
		R	TGGTGTGGCAGGAGTGATAG	

All the genes are from rat origin.

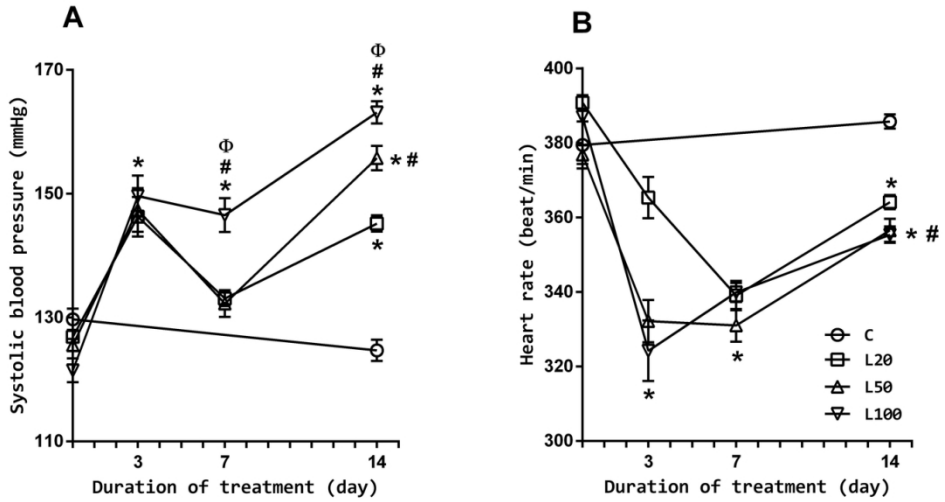

Figure 1. The effects of L-NAME treatment at three different concentrations (20, 50 and 100mg/kg/day) on (A) systolic blood pressure and (B) heart rate. Measurements were performed throughout the course of the study at given days. Values are means±SEM. n=4-6 for control (C), 20mg/kg/day (L20), 50mg/kg/day (L50) and 100mg/kg/day (L100) L-NAME. *, p<0,05 vs C; #, p<0,05 vs L20; ϕ, p<0,05 vs L50.

121x68mm (300 x 300 DPI)

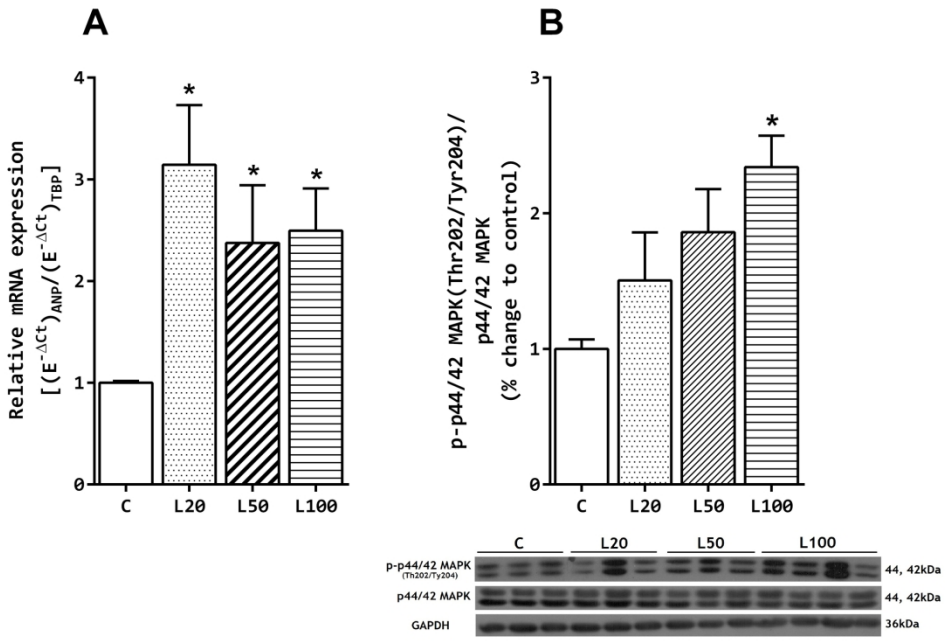

Figure 2. The effect of chronic L-NAME treatments on development of cardiac hypertrophy. (A) Cardiac ANP mRNA levels which were normalized to TBP and (B) Thr202/Tyr204 phosphorylation of p44/42 MAPK which were normalized to total p44/42 MAPK of left ventricular samples from L-NAME-treated rats were shown. Representative western blotting images are located below the bar graph. Values are means±SEM. n=4-6 for control (C), 20mg/kg/day (L20), 50mg/kg/day (L50) and 100mg/kg/day (L100) L-NAME. *, p<0,05 vs C.

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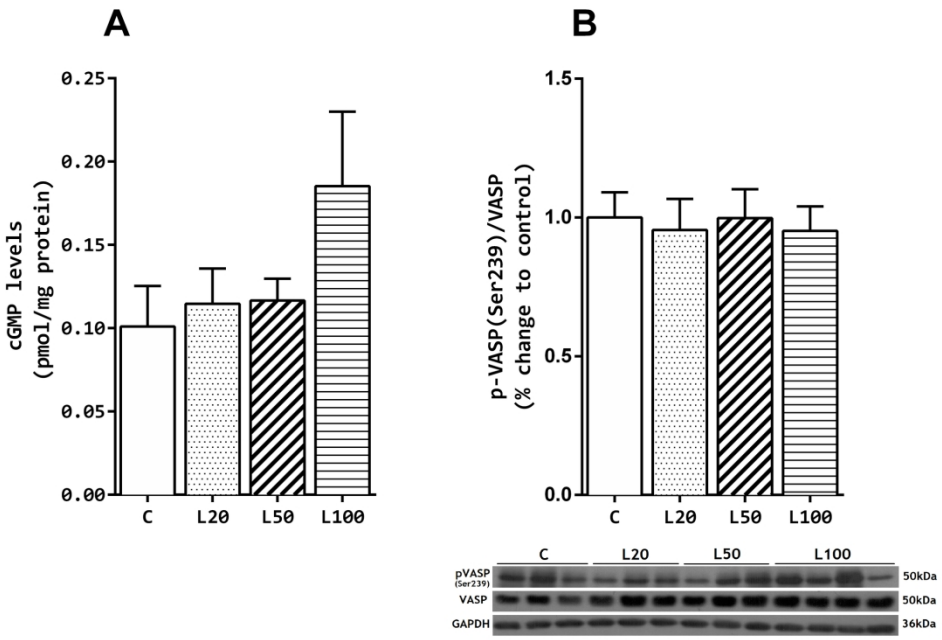

Figure 3. The effect of chronic L-NAME treatments on cardiac cGMP/PKG signaling. (A) Cardiac cGMP levels and (B) Ser239 phosphorylation of VASP which were normalized to total VASP of left ventricular samples from L-NAME-treated rats were shown. Representative western blotting images are located below the bar graph. Values are means±SEM. n=4-6 for control (C), 20mg/kg/day (L20), 50mg/kg/day (L50) and 100mg/kg/day (L100) L-NAME. #, p<0,05 vs L20; ϕ, p<0,05 vs L50.

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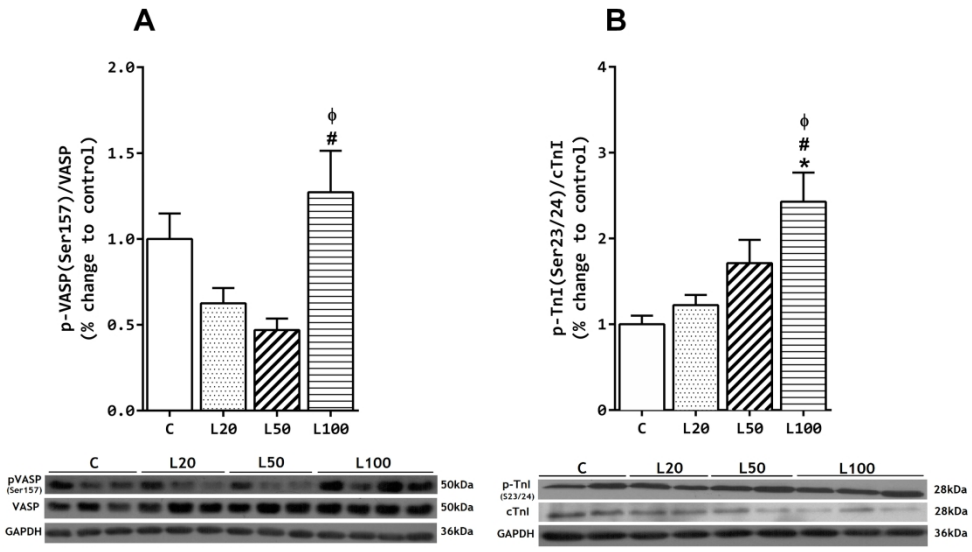

Figure 4. The effect of chronic L-NAME treatments on PKA-mediated phosphorylations. (A) Ser157 phosphorylation of VASP and (B) Ser23/24 phosphorylation of troponin I (TnI) of left ventricular samples from L-NAME-treated rats were normalized to total VASP and cardiac TnI, respectively. Representative western blotting images are located below each bar graph. Values are means±SEM. n=4-6 for control (C), 20mg/kg/day (L20), 50mg/kg/day (L50) and 100mg/kg/day (L100) L-NAME. #, $p<0,05$ vs L20; ϕ, $p<0,05$ vs L50.

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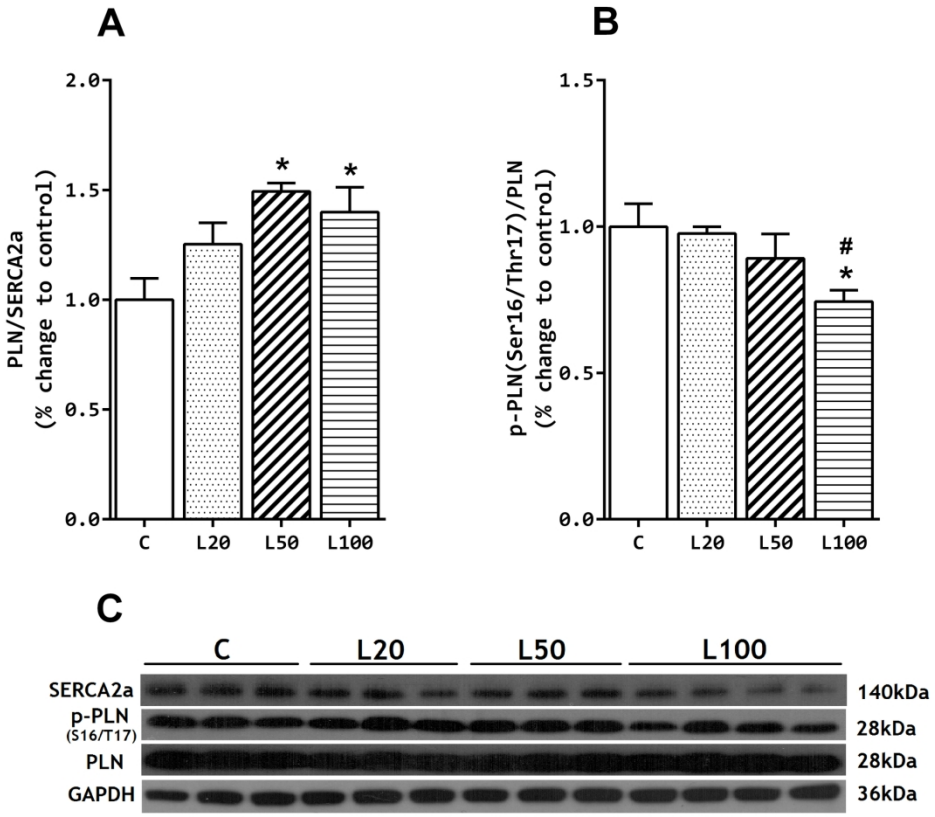

Figure 5. The effect of chronic L-NAME treatments on cardiac Ca2+ handling. (A) Phospholamban (PLN)/SERCA2a, (B) phospho-PLN/PLN and (C) representative western blotting images are shown. Values are means±SEM. n=4-6 for control (C), 20mg/kg/day (L20), 50mg/kg/day (L50) and 100mg/kg/day (L100) L-NAME. #, p<0,05 vs L20.

182x159mm (300 x 300 DPI)