BASIC RESEARCH

Sildenafil preserves diastolic relaxation after reduction by L-NAME and increases phosphodiesterase-5 in the intercalated discs of cardiac myocytes and arterioles

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OBJECTIVES: We investigated the influence of sildenafil on cardiac contractility and diastolic relaxation and examined the distribution of phosphodiesterase-5 in the hearts of hypertensive rats that were treated with by NG-nitro-L-arginine methyl ester (L-NAME).

METHODS: Male Wistar rats were treated with L-NAME and/or sildenafil for eight weeks. The Langendorff method was used to examine the effects of sildenafil on cardiac contractility and diastolic relaxation. The presence and location of phosphodiesterase-5 and phosphodiesterase-3 were assessed by immunohistochemistry, and cGMP plasma levels were measured by ELISA.

RESULTS: In isolated hearts, sildenafil prevented the reduction of diastolic relaxation (dP/dt) that was induced by L-NAME. In addition, phosphodiesterase-5 immunoreactivity was localized in the intercalated discs between the myocardial cells. The staining intensity was reduced by L-NAME, and sildenafil treatment abolished this reduction. Consistent with these results, the plasma levels of cGMP were decreased in the L-NAME-treated rats but not in rats that were treated with L-NAME + sildenafil.

CONCLUSION: The sildenafil-induced attenuation of the deleterious hemodynamic and cardiac morphological effects of L-NAME in cardiac myocytes is mediated (at least in part) by the inhibition of phosphodiesterase-5.

KEYWORDS: Hypertension; Phosphodiesterase-5 Inhibitor; Immunohistochemistry.

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INTRODUCTION

The inhibition of phosphodiesterase type 5 (PDE5) by selective inhibitors such as sildenafil enhances intracellular levels of cGMP, which can be beneficial in restoring physiological function in situations in which nitric OXIDE (NO) formation is reduced. Although the cyclic GMP–selective PDE5 has been thought to play a minor role in cardiac myocytes, recent studies using selective inhibitors have suggested that PDE5 can modulate chronic cardiac stress responses.[1-3] In addition, recent studies have demonstrated PDE5 expression and activity in cardiac myocytes and its targeted inhibition by sildenafil; moreover, a role for this PDE in cardiomyocyte hypertrophy modulation has been reported.[4-5]

The chronic inhibition of NO biosynthesis by the oral administration of the nonselective NO synthase (NOS) inhibitor NG-nitro-L-arginine methyl ester (L-NAME) is a well-established hypertension model[6-8] that is associated with reduced cardiac output, cardiac hypertrophy, extensive areas of fibrosis and myocardial necrosis, changes in myocardial contractility, and cardiomyocyte and vascular smooth muscle remodeling.[8-13] In a previous study, we demonstrated that sildenafil confers cardiovascular protection by inhibiting PDE5, thereby increasing the bioavailability of cGMP.[14]

Recently, PDE5 distribution in the heart was reported to be compartmentalized in the Z bands of myocardial tissue[15-16], and sildenafil has been reported to affect cardiac performance and vascular function in L-NAME–treated rats.[14] These latter findings seem to be related to sildenafil's vasodilatory effects that both reduce afterload and improve cardiac output. However, it is unknown whether sildenafil affects cardiac contractility and diastolic relaxation. Therefore, the aim of this study was to examine the effect of sildenafil on cardiac contractility and diastolic relaxation in isolated hearts and to confirm the association between the cardiovascular effects of sildenafil and the presence and distribution of PDE5 in the hearts of hypertensive L-NAME–treated rats.

METHODS

The animal experiments described here were approved by the Institutional Committee for Ethics in Animal Experimentation (CEEA/IB/Unicamp) and were performed in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and under the ethical guidelines that have been established by the Brazilian College for Animal Experimentation (Cobea).

Experimental design

Male Wistar rats (specific-pathogen free) weighing 180 ± 20 g were obtained from the Central Animal House Services (Cemib, a facility that is engaged in the production and reproduction of laboratory animals and is affiliated with the International Council for Laboratory Animal Science - ICLAS) at Unicamp and were divided into the following groups, each containing 10-15 rats: control (water alone); L-NAME (20 mg/rat/day);[17] sildenafil (45 mg/kg/day);[18] and L-NAME + sildenafil (20 mg/rat/day and 45 mg/kg/day, respectively). Each group was treated for eight weeks. Before starting the treatment, the mean volume of liquid that was ingested by the five rats in each cage was determined by measuring the volume of water that the rats drank and dividing this volume by five. This calculated volume was then used to determine the amount of each drug to directly dilute in the drinking water to deliver the desired dose per rat or kg of body weight per day. The L-NAME and sildenafil citrate were dissolved in the drinking water at concentrations of 1.1 mM and 1 mM to provide daily intakes of approximately 74 µmol/rat/day and 67 µmol/rat/day, respectively. In the L-NAME + sildenafil group, the two drugs were diluted in the same drinking bottle. The average daily intake of both water and food did not differ significantly between the L-NAME-treated and untreated rats. The control rats received the same volume of tap water alone. In addition, we monitored the water consumption by the animals in each group daily to verify that the correct dose was administered.

Non-Invasive (tail-cuff) blood pressure and body weight measurements

The systolic arterial blood pressure (SBP) of each rat was measured twice a week for eight weeks using the tail-cuff method (Codas system), and the mean of these two measurements was considered as the value for that week. The rats were also weighed twice a week after obtaining the blood pressure measurements, and the mean weekly weight gain was calculated as described for the blood pressure.

Isolated heart preparation (Langendorff)

The method for isolating the beating heart was originally described by Langendorff.[19] At the end of study, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). After opening the chest, the heart was isolated and perfused in a Langendorff apparatus (Isolated Heart Perfusion Apparatus, Harvard Apparatus, Hollister, MA, USA.) under a constant pressure of 70 mmHg. A collapsed latex balloon was placed in the left ventricular cavity via an incision in the left atrium, and the initial intraballoon pressure was adjusted to 4–6 mmHg. The left ventricular pressure was monitored via a pressure transducer (YS100, Transonic Systems Inc., NY, USA). Both pressure parameters (the left ventricular development pressure, or LVDP, dP/dt+, dP/dt-, the maximum rate of rise or fall in LVDP) and heart rate (HR) were continually recorded; the signals were acquired, amplified, and analyzed using an analog-to-digital interface (Dataq Instruments, Akron, OH, USA). The hearts were perfused with a Krebs-Henseleit solution.

PDE immunohistochemistry

PDE5 and PDE3 were measured by immunohistochemistry performed in slices of the left ventricle or vessels that were stained with IgG anti-PDE5 or anti-PDE3 antibodies (Zymed, Laboratories, South San Francisco, CA). In brief, the slices were deparaffinized with Citrosolv (Fisher Scientific, Fair Lawn, NJ). Before tissue rehydration, the endogenous peroxidase activity was blocked with hydrogen peroxide and methanol (1 : 9) for 20 minutes. Following rehydration, the samples were rinsed with phosphate-buffered saline (PBS). Fetal bovine serum (10% FBS in PBS) was used to block the nonspecific sites for 60 minutes at room temperature, followed by incubation in 2.5% fat-free dry milk (Molico, Nestlé, Brazil) for 30 minutes. The primary antibody—either rabbit anti–PDE5 or goat anti-PDE3—was diluted to 1 : 250 in 2% BSA in PBS and applied to the sections for 16-18 hours at 4℃. Subsequently, the samples were washed and incubated with the biotinylated secondary antibody (Zymed Laboratories, South San Francisco, CA) for 60 minutes at room temperature, followed by incubation with streptavidin-peroxidase complex (1 : 1000) for 60 minutes at room temperature. Finally, a chromogen solution comprised of 3,3′-diaminobenzidine (DAB) (6 mg), hydrogen peroxide (150 µl) and PBS (10 ml) was applied for two minutes in the dark at room temperature.

The samples were coded and then assessed by two independent blinded observers using an optical microscope (Q500YW, LEICA, UK) equipped with a 40x objective and coupled to an image analyzer (Quantimet Q500YW, LEICA, UK).

cGMP concentrations

The plasma cGMP concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Cayman Chemical Co., Ann Arbor, MI). The plasma samples were initially precipitated with trichloroacetic acid, extracted with water-saturated ethyl ether, evaporated to dryness, and then reconstituted in an assay buffer. The standards and samples were acetylated to allow the detection of nucleotides in the picomolar range.[20]

Statistical analysis

The results are expressed as the mean $\pm$ SEM. An analysis of variance (ANOVA) for repeated measurements model was used to assess the differences in body weight and tail-cuff pressure. A two-way ANOVA was used to compare the heart weight, left ventricular weight, left ventricular weight index, mean arterial pressure, cardiac output, and total peripheral vascular resistance. When the

ANOVA results were deemed significant, the Bonferroni test was used to examine the differences among the groups. In all cases, a two-sided p-value of <0.05 was considered to be significant.

RESULTS

Systolic blood pressure

The systolic blood pressure (SBP) increased to a similar extent in the L-NAME and L-NAME + sildenafil groups until the sixth week; in addition, the SBP was higher in both groups than in the control and sildenafil groups from two weeks onwards. After six weeks, the rats in the L-NAME group had higher blood pressures than did the other three groups ($p<0.05$), while blood pressure in the L-NAME + sildenafil group essentially returned to normal (Figure 1).

Isolated heart (Langendorff)

The systolic contractility of the isolated hearts was measured by the first temporal derivative of the LVDP positive development (dP/dt+, in mmHg/s), and the isovolumetric relaxation was measured by the first temporal derivative of the LVDP negative pressure (dP/dt-, in mmHg/s). We analyzed all the hearts using the same diastolic pressure range (4–6 mmHg).

A significant decrease in dP/dt+ was found in the L-NAME group (4028$\pm$77 mmHg/s) relative to the control and sildenafil groups (4514$\pm$102 and 4466$\pm$116 mmHg/s, respectively; $p<0.05$). Sildenafil prevented this dP/dt+ reduction in the L-NAME–treated rats (4165$\pm$82 mmHg/s; $p<0.05$). The L-NAME group showed relatively decreased dP/dt- (3090$\pm$95 mmHg/s) when compared to the control and sildenafil groups (3930$\pm$96 and 4079$\pm$113 mm Hg/s, respectively; $p<0.05$); however, sildenafil prevented this impaired cardiac relaxation (the dP/dt- in the L-NAME + sildenafil group was 3768$\pm$121 mmHg/s; $p<0.01$) (Table 1).

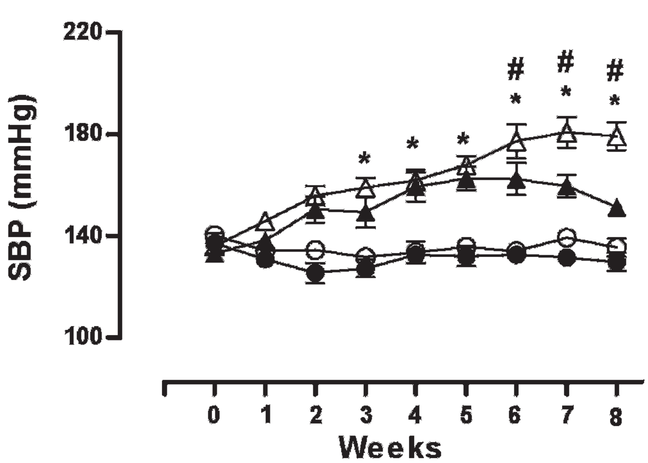

Figure 1 - The changes in systolic blood pressure (SBP, in mmHg) during the eight weeks of the study. Control (squares, n = 15); L-NAME (diamonds, n = 15); sildenafil (circles, n = 15) and L-NAME + sildenafil (triangles, n = 15). The data points and error bars represent the mean $\pm$ SEM. *$p<0.05$ for L-NAME and L-NAME + sildenafil groups *vs.* control group. #$p<0.05$ for L-NAME group *vs.* L-NAME + sildenafil group.

Table 1 - Isolated heart (Langendorff technique). Development of left ventricular systolic (LVSP, mmHg) and diastolic (LVDP, mmHg) pressures, first temporal derivative of LVDP positive development (dP/dt+, mmHg/s), first temporal derivative of the LVDP negative pressure (dP/dt-, mmHg/s) and heart rate (HR, bpm).

	CONTROL (n = 12)	SILDENAFIL (n = 10)	L-NAME (n = 10)	L-NAME+ SILDENAFIL (n = 12)
LVSP (mmHg)	138$\pm$16	132$\pm$17	127$\pm$12	129$\pm$18
LVDP (mmHg)	4.5$\pm$0.9	5.3$\pm$0.5	4.6$\pm$0.9	4.6$\pm$0.8
dP/dt+ (mmHg/s)	4514$\pm$102	4466$\pm$116	4028$\pm$77*	4165$\pm$82**
dP/dt- (mmHg/s)	3930$\pm$96	4079$\pm$113	3090$\pm$95*	3768$\pm$121**
HR (bpm)	275$\pm$25	291$\pm$19	270$\pm$28	297$\pm$18

*$p<0.05$ *vs.* CONTROL and sildenafil.
**$p<0.01$ *vs.* L-NAME.

PDE immunohistochemistry

PDE5. The PDE5 immunohistochemistry revealed positive staining in the tunicae media and intima but not in the adventitia of both arteries and veins. The L-NAME treatment markedly reduced the media staining in the 10-50-µm diameter arterioles when compared to the control group. However, this reduction was abolished in the L-NAME + sildenafil group. Conversely, the rats that were treated only with sildenafil showed an increase in staining when compared to the other groups (Figure 2A). Larger arteries (larger than 100 µm in diameter) did not show any relevant alterations in PDE5 staining and localization in the four groups.

Staining was also observed in the intercalated discs of the myocardial cells, and this was reduced by the L-NAME treatment. Sildenafil administered concomitantly with the L-NAME reversed the L-NAME effect to the control levels. In addition, sildenafil alone increased PDE5 intensity when compared to the control group (Figure 2B).

PDE3. The PDE3 immunohistochemistry revealed positive staining in the adventitia of both arteries and veins that was intensified in the group that received only sildenafil when compared to the control, L-NAME, and L-NAME + sildenafil groups. The endothelium also showed positive (albeit less intense) staining for PDE3. The cardiac myocytes had light positive staining for PDE3 in all four experimental groups (data not shown). Interestingly, areas of lesions showed an intense staining that was not affected by the sildenafil treatment (data not shown).

Plasma cGMP concentrations

After eight weeks, the plasma cGMP levels (in pmol/ml) were decreased in the L-NAME group and increased in the sildenafil group ($p<0.05$ *vs.* the control group). The cGMP levels were restored to the control values in the L-NAME + sildenafil group (Figure 3).

DISCUSSION

Using the isolated heart preparation, the decrease in dP/dt- that was observed in the L-NAME group was prevented in the L-NAME + sildenafil group. This result may be interpreted as an effect of sildenafil on the L-NAME–induced diastolic relaxation because the cardiac

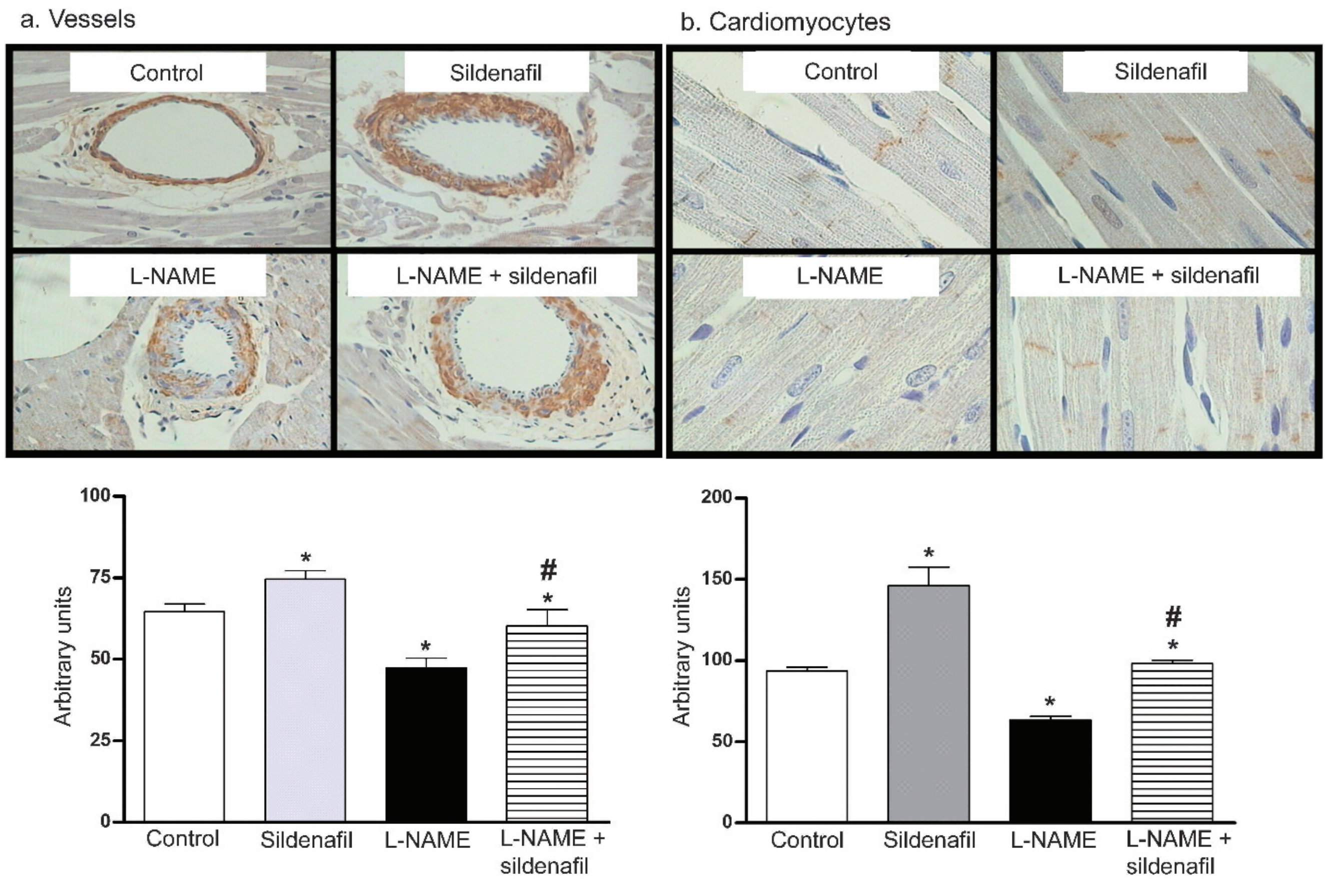

Figure 2 - A) The PDE5 immunohistochemistry in the cardiac arteries after 8 weeks of treatment. Representative photomicrographs and a summary graph of the quantitative analysis of the staining intensity in the vascular smooth muscle cells of small arteries are shown. B) The PDE5 immunohistochemistry in the cardiac tissue after 8 weeks of treatment. Representative photomicrographs and a summary graph of the quantitative analysis of staining intensity in the intercalary discs between myocytes. The data are expressed in arbitrary units; n = 5/group; **p*<0.05 *vs.* control; #*p*<0.05 *vs.* L-NAME.

load was pre-controlled and there were no significant differences in heart rates among the various groups. In cardiac myocytes, cAMP mediates catecholamine signaling, while cGMP mediates the effects of nitric oxide and atrial natriuretic peptide. cGMP activates PKG, which is then capable of countering the cAMP-PKA contractile stimulation.[21] Moreover, the duration and magnitude of these actions are determined by cGMP generation and by its hydrolysis, which is catalyzed by PDE5 that is compartmentalized within the cell, thereby facilitating the regulation of specific targeted proteins.[22]

While sildenafil had no effect on blood pressure in the normotensive (control) group, the compound produced a small but significant attenuation of the increase in blood pressure that was seen in the rats that were treated with L-NAME alone. Moreover, we have previously showed that enalapril and amlodipine decrease blood pressure in L-NAME–treated rats but do not prevent cardiac lesions.[17,36] Therefore, we suggest that this hemodynamic effect may have been at least partially mediated by an inhibition of PDE5 in arterial resistance vessels[23-24] and that it was associated with a slight reduction in the total peripheral vascular resistance and with enhanced cardiac output.[14] An alternative explanation could be sildenafil's effect of preventing the impaired diastolic relaxation (dP/dt-) that was diminished by L-NAME.

Inhibitors of nitric oxide synthesis may cause an afterload increment in hypertensive animals that can subsequently induce cardiac hypertrophy, fibrosis and/or ischemia.[25]

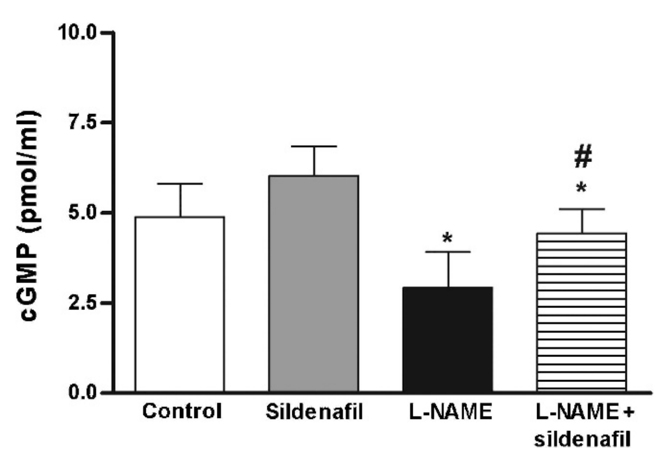

Figure 3 - The plasma cGMP levels after eight weeks of treatment in the control (open column, n = 15), L-NAME (black column, n = 15), sildenafil (grey column, n = 15) and L-NAME + sildenafil (horizontally striped column, n = 15) groups. The bars represent the mean ± SEM. **p*<0.05 *vs.* control group; #*p*<0.05 *vs.* L-NAME group.

These three changes, either alone or in combination, predispose the heart to impaired left ventricular relaxation. In addition, decreased left ventricular diastolic distensibility may arise from a dysfunction of the dynamics of ventricular relaxation.[26] Thus, the impaired cardiac output could be due to these alterations in ventricular diastolic function through a reduction in the isovolumic relaxation time (IVRT).

In support of this idea, another important finding in the present study is that sildenafil prevented the L-NAME–induced reduction in PDE5 staining in the myocardium and in the arterioles, while it did not alter the PDE3 staining. Consistent with previous studies,[15-16] we found that PDE5 is localized at the intercalated discs. Importantly, low concentrations of cGMP in unstimulated hearts can augment contractile function, and this effect is likely mediated by cross-talk with cAMP-dependent signaling.[27] However, PDE5 inhibition targets cGMP–PKG activity in a region that is strategically linked to adrenergic regulation. In this region, it depresses myofilament calcium sensitivity by increasing troponin I phosphorylation, thereby accounting for the positive lusitropic[28-29] and negative inotropic effects.[30] Thus, our immunohistochemical findings are consistent with previous findings in isolated hearts in which sildenafil prevented the L-NAME–induced impairment in diastolic relaxation (dP/dt-).

In contrast to our findings, however, a previous study has reported a shift in the intracellular localization of PDE5 from its normal Z band localization to a more diffuse cytosolic distribution following a chronic NOS inhibition by L-NAME that eliminated sildenafil's effectiveness even when exogenous NO was provided.[16] In the present study, we found no shift in PDE5 localization. These contradictory results may be explained by differences in the experimental protocols; the aforementioned study administered L-NAME for one or two weeks following the sildenafil treatment, while we studied the effect of co-treatment with L-NAME and sildenafil for eight weeks and found that sildenafil prevented the L-NAME–induced effects.

Another finding from our study is that sildenafil increased circulating cGMP and abolished the decrease that was caused by L-NAME. Importantly, although cGMP is considered to reflect natriuretic peptides in patients with cardiac dysfunction and to be an indicator of NO synthase activity in healthy subjects,[31] Castro et al has reported that PDE-5 regulation appears to be compartmentalized in cardiac myocytes, where it interacts with NO but not with natriuretic peptide-stimulated cGMP.[32] In addition, chronic L-NAME–induced NOS inhibition decreases cGMP,[8,33-35] suggesting that the natriuretic peptide pathway in this model is unable to rescue the impaired cGMP formation caused by NOS inhibition. Therefore, the changes in the plasma cGMP levels that we found in the present study are likely the result of NO pathway modulation.

CONCLUSIONS

In conclusion, our results suggest that the sildenafil-mediated attenuation of L-NAME–induced deleterious hemodynamic and morphological alterations is at least partially modulated by PDE5 inhibition in cardiac myocytes, as supported by our immunohistochemical and isolated heart findings. Finally, sildenafil's effects on cardiac relaxation should be studied further as a new therapeutic approach for treating hypertensive patients with diastolic dysfunction.

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