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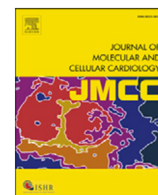

Original article

Myofilament Ca^{2+} desensitization mediates positive lusitropic effect of neuronal nitric oxide synthase in left ventricular myocytes from murine hypertensive heart[☆]

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Neuronal nitric oxide synthase (NOS1 or nNOS) exerts negative inotropic and positive lusitropic effects through Ca^{2+} handling processes in cardiac myocytes from healthy hearts. However, underlying mechanisms of NOS1 in diseased hearts remain unclear. The present study aims to investigate this question in angiotensin II (Ang II)-induced hypertensive rat hearts (HP). Our results showed that the systolic function of left ventricle (LV) was reduced and diastolic function was unaltered (echocardiographic assessment) in HP compared to those in shams. In isolated LV myocytes, contraction was unchanged but peak $[Ca^{2+}]_i$ transient was increased in HP. Concomitantly, relaxation and time constant of $[Ca^{2+}]_i$ decay (tau) were faster and the phosphorylated fraction of phospholamban (PLN-Ser^{16}/PLN) was greater. NOS1 protein expression and activity were increased in LV myocyte homogenates from HP. Surprisingly, inhibition of NOS1 did not affect contraction but reduced peak $[Ca^{2+}]_i$ transient; prevented faster relaxation without affecting the tau of $[Ca^{2+}]_i$ transient or PLN-Ser^{16}/PLN in HP, suggesting myofilament Ca^{2+} desensitization by NOS1. Indeed, relaxation phase of the sarcomere length–$[Ca^{2+}]_i$ relationship of LV myocytes shifted to the right and increased $[Ca^{2+}]_i$ for 50% of sarcomere shortening (EC_{50}) in HP. Phosphorylations of cardiac myosin binding protein-C (cMyBP-C^{282} and cMyBP-C^{273}) were increased and cardiac troponin I (cTn$I^{23/24}$) was reduced in HP. Importantly, NOS1 or PKG inhibition reduced cMyBP-C^{273} and cTn$I^{23/24}$ and reversed myofilament Ca^{2+} sensitivity. These results reveal that NOS1 is up-regulated in LV myocytes from HP and exerts positive lusitropic effect by modulating myofilament Ca^{2+} sensitivity through phosphorylation of key regulators in sarcomere.

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1. Introduction

Systemic hypertension is one of the major risk factors for developing cardiac hypertrophy and heart failure due to mechanical and neurohormonal (*e.g.* increased renin–angiotensin system) disturbance of intracellular Ca^{2+} handling and myofilament responses to Ca^{2+} in the myocardium [1,2]. Nitric oxide (NO) has been implicated to be one of the major influences of cardiac contractile function in both healthy and diseased hearts [3,4]. Recently, neuronal nitric oxide synthase (nNOS or NOS1) protein expression and activity are shown to be increased in hypertrophic or failing LV myocardium both in animal models and in humans [5,6], indicating a significant role NOS1 plays in regulating cardiac function. Indeed, NOS1 has been demonstrated to be instrumental in preventing the deterioration of systolic and diastolic dysfunction in myocardial infarction-induced or ischemic HP [7,8]. However, mechanisms mediating the effect of NOS1 on myocyte contractility in diseased heart remain unidentified.

Using NOS1 gene knockout mice model, our previous work and that of others have shown that NOS1 in the sarcoplasmic reticulum (SR) attenuates cardiac contraction by reducing peak L-type Ca^{2+} currents (I_{Ca-L}) [9,10] or by modulating SR Ca^{2+} loading following *s*-nitrosylation of ryanodine receptors and increasing Ca^{2+} leak [11,12]. Furthermore, NOS1 facilitates basal cardiac relaxation predominantly by increasing phospholamban Ser^{16} phosphorylation

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(PLN-Ser[16]) and SERCA activity, which subsequently improves Ca^{2+} reuptake into SR [13,14]. It is plausible to suggest that NOS1 up-regulation in hypertrophic or failing myocardium may strengthen its negative inotropic and positive lusitropic effects by regulating intracellular Ca^{2+} transients ($[Ca^{2+}]_i$) secondary to reduced I_{Ca-L} density and increased PLN-Ser[16]. However, despite the fact that NOS1 is up-regulated and is translocated from SR to the caveolae (where I_{Ca-L} resides) [5], I_{Ca-L} density is genuinely unaffected [15,16] in hypertrophic or failing myocardium. Furthermore, PLN-Ser[16] is significantly reduced and SERCA activity is impaired [17], suggesting that NOS1 may regulate myocardial contractility *via* a mechanism that is different from I_{Ca-L} or PLN-Ser[16] in diseased myocardium.

In the present study, we aim to examine the mechanisms of NOS1 in regulating contraction and relaxation in LV myocytes from angiotensin II (Ang II)-induced systematic hypertensive rats. Our results reveal that NOS1 is up-regulated in LV myocytes from hypertensive heart and modulates myofilament Ca^{2+} sensitivity by increasing PKG-dependent phosphorylations of cMyBP-C-Ser[273] and cTnI[23/24]. This novel mechanism is important in understanding NOS1 regulation of myocardial contractile function in hypertension.

2. Methods & materials

Detailed information of the Methods and materials can be found in *Online Supplementary Data*.

2.1. Animals

Sprague–Dawley rats (12 weeks old, male) were subjected to Ang II infusion subcutaneously using osmotic minipump for 4 weeks, and were paired with a sham-operated group. Blood pressure was measured using Non-Invasive Blood Pressure System, tail-cuff method (CODA).

2.2. Functional assessment of the rat hearts by echocardiography

Transthoracic echocardiogram was performed in Ang II (4 weeks) and in shams with a portable echocardiographic system (Vivid Q Portable Ultrasound Machine; GE Healthcare, Pittsburgh, PA, USA) equipped with a 10-MHz transducer (10S-RS; GE Healthcare, Pittsburgh, PA, USA).

2.3. Immunoblotting

Immunoblots were performed in rat LV myocyte lysates using specific antibodies against NOS1 (Santa Cruz) and NOS3 (BD Transduction Laboratories), total PLN (Affinity Bioreagents) and its Ser[16] phosphorylated fraction (PLN-Ser[16], Upstate), total and phosphorylated fraction of cardiac troponin I: cTnI (Santa Cruz) and cTnI[23/24] (Cell Signaling), total and phospho-specific cMyBP-C antibodies [18].

3. Results

3.1. Systolic and diastolic blood pressures and LV contractile functions in HP rats

Both systolic and diastolic blood pressures were increased 1 week after Ang II infusion (125 ng/min/kg) and continuously increased up to the period studied (Fig. 1A). At 4 weeks, systolic blood pressure was 127.8 ± 2.9 mm Hg in shams *vs.* 156.6 ± 4.9 mm Hg with Ang II ($P < 0.001$, n = 26); diastolic blood pressure was 92.8 ± 2.8 mm Hg in shams *vs.* 113.4 ± 4.7 mm Hg with Ang II ($P < 0.001$, n = 26). Heart rate was significantly lower in hypertensive rats (HP, heart rate, beats/min: 457.1 ± 5.8 in shams and 426.7 ± 10.6 in HP, $P = 0.02$, n = 26 and n = 26). Autopsy weight of LV myocardium and dimensions of LV myocytes were measured to assess whether myocardial hypertrophy was induced by systemic hypertension in these rats. As shown in Table 1, heart weight, heart/body weight ratio or average width of LV myocytes was not increased in HP (heart weight in g, 1.98 ± 0.07 in shams *vs.* 2.10 ± 0.08 in HP, $P = 0.5$, n = 16 and 18; heart/body weight ratio × 100%, 0.53 ± 0.02% in shams *vs.* 0.57 ±0.02% in HP, $P = 0.2$, n = 16 and n = 18; LV myocyte width, μm, 26.1 ± 0.35 in shams *vs.* 26.6 ± 0.35 in HP, $P = 0.3$, n = 197 and n = 210), the slack length of sarcomere was decreased ($P = 0.005$, n = 197 and n = 210).

Fig. 1B is the representative echocardiograms of shams and HP and Table 2 shows average results. None of the geometrical parameters of LV were affected by Ang II treatment at the time points studied (Table 2). However, LVEF and LVFS were significantly lower in Ang II group (LVEF was 61.83 ± 3.66% in Ang II rats *vs.* 72.5 ± 2.08% in shams, $P = 0.03$, n = 6; LVFS was 29.83 ± 2.5 *vs.* 37.17 ± 1.82%, $P = 0.04$, n = 6, Fig. 1C), indicating contractile dysfunction in this group. However, despite the fact that blood pressure is significantly increased in Ang II rats, diastolic parameters including E velocity, A velocity, E/A ratio, E′ velocity and E/E′ ratio were not different between the two groups (Table 2). These results demonstrate that Ang II infusion induced hypertension in rats; at 4 weeks, systolic function was impaired whereas diastolic function was maintained.

3.2. Protein expression and activity of NOS1 in LV myocytes from HP rats

We detected whether NOS1 is up-regulated in LV myocyte homogenates from HP rats. As shown in Figs. 2A & B, NOS1 protein expression was significantly increased ($P = 0.03$ compared to that in shams, n = 9 and 9). Conversely, NOS3 (eNOS) protein expression was significantly reduced ($P = 0.004$, n = 8 and 8, Figs. 2A & B).

To determine whether increased NOS1 protein expression in LV myocyte homogenate is associated with its greater activity, we compared NOS1 production of NO (nitrite content) between the groups. As shown in Fig. 2C, NO production was significantly greater in HP compared to shams ($P = 0.02$, n = 18 and 18). Pre-treatment of LV myocytes with selective and potent NOS1 inhibitors, S-methyl-L-thiocitrulline (SMTC, 100 nM, 30 min) or vinyl-l-N-5-(1-imino-3-butenyl)-l-ornithine (L-VNIO, 100 μM, 30 min) reduced NO production in both groups (in shams, Ctr *vs.* SMTC, $P < 0.001$; Ctr *vs.* L-VNIO, $P = 0.04$; n = 18 for each group; in HP, Ctr *vs.* SMTC, $P < 0.001$ & Ctr *vs.* L-VNIO, $P = 0.002$, n = 20 and n = 18, 18 & 14, respectively, Fig. 2C). In the presence of NOS1 inhibitors, NO level was no longer different between the two groups ($P = 0.2$, between SMTCs and $P = 0.97$ between L-VNIOs). These results suggest that NOS1 is the isoform that is up-regulated and contributes to the production and the maintenance of NO in cardiac myocytes from HP rats.

3.3. NOS1 regulation of LV myocyte contraction and relaxation

Next, we tested whether up-regulation of NOS1 in LV myocytes from HP is associated with its greater effects on myocyte contractile function. Figs. 3A & B showed that LV myocyte relaxation was significantly faster in HP compared to that in shams (time to 50% relaxation, TR_{50}, $P < 0.001$, n = 183 and 237) but myocyte contraction was not different between the groups (amplitude of sarcomere shortening, $P = 0.7$, n = 183 and 237, Figs. 3A & C). Inhibition of NOS1 (SMTC, 100 nM) did not affect myocyte contraction (amplitude of sarcomere shortening, $P = 0.6$ between control and SMTC in shams; $P = 0.5$ between control and SMTC in HP, n = 183 *vs.* 67 and n = 237 *vs.* 85, Figs. 3A & C). L-type Ca^{2+} current (I_{Ca-L}) density and its response to SMTC were similar between the groups (peak I_{Ca-L} density at 0 mV was −6.70 ± 0.52 pA/pF in shams and was −6.87 ± 0.43 pA/pF in HP, $P = 0.8$, n = 16 and n = 30; 9.07 ± 0.64 pA/pF with SMTC in shams and was −8.39 ± 0.42 pA/pF with SMTC in HP, n = 10 and 23, $P = 0.36$).

In contrast, SMTC significantly prolonged LV myocyte relaxation both in shams (TR_{50}, $P = 0.03$, n = 183 and 67, Fig. 3B) and in HP (TR_{50}, $P < 0.001$, n = 237 and 85, Fig. 3B) and abolished the difference in relaxation between two groups ($P = 0.8$). Similar to SMTC, L-VNIO

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