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Neuronal NO synthase mediates plenylephrine induced cardiomyocyte hypertrophy through facilitation of NFAT-dependent transcriptional activity

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ABSTRACT

Neuronal nitric oxide synthase (NOS1) has been consistently shown to be the predominant isoform of NOS and/or NOS-derived NO that may be involved in the myocardial remodeling including cardiac hypertrophy. However, the direct functional contribution of NOS1 in this process remains to be elucidated. Therefore, in the present study, we attempted to use silent RNA and adenovirus mediated silencing or overexpression to investigate the role of NOS1 and the associated molecular signaling mechanisms during OKphenylephrine (PE)-induced cardiac hypertrophy growth in neonatal rat ventricular cardiomyocytes (NRVMs). We found that the expression of NOS1 was enhanced in PE-induced hypertrophic cardiomyocytes. Moreover, LVNIO treatment, a selective NOS1 inhibitor, significantly decreased PE-induced NRVMs hypertrophy and [^{3}H]-leucine incorporation. We demonstrated that NOS1 gene silencing attenuated both the increased size and the transcriptional activity of the hypertrophic marker atrial natriuretic factor (ANF) induced by PE stimulation. Further investigation suggested that deficiency of NOS1-induced diminished NRVMS hypertrophy resulted in decreased calcineurin protein expression and activity (assessed by measuring the transcriptional activity of NFAT) and, an increased activity of the anti-hypertrophic pathway, GSK-3β (estimated by its augmented phosphorylated level). In contrast, exposing the NOS1 overexpressed NRVMs to PE-treatment further increased the hypertrophic growth, ANF transcriptional activity and calcineurin activity. Together, the results of the present study suggest that NOS1 is directly involved in controlling the development of cardiomyocyte hypertrophy.

1. Introduction

Cardiovascular disease continues to be the leading cause of morbidity and deaths in industrialized countries, and left ventricular hypertrophy is an independent risk factor for cardiovascular mortality and morbidity. At first, cardiac hypertrophy is a critical adaptive response to preserve cardiac function in response to various stresses. However, continued cardiac stress caused pathological cardiac hypertrophy leading to heart failure [1,2].

Although the physiological importance of nitric oxide (NO)-dependent signaling in heart hypertrophy has been long discussed, the precise role of cardiomyocyte nitric oxide synthase isoforms (NOS) in the development of cardiac hypertrophy is still controversial [3,4]. However, it appears that the main source of NO that modulates cardiomyocyte growth is produced by the neuronal nitric oxide synthase (NOS1). NOS1 expression and activity were consistently increased in the hypertrophic or failing rodent myocardium and in failing human hearts [5–7]. NOS1 gene deletion was associated with more severe cardiac remodeling following myocardial infarction [8,9]. In contrast, we reported that mice with cardiomyocyte specific overexpression of NOS1 exhibited a greater cardiac hypertrophic response in a model of pressure overload-induced cardiac hypertrophy, as compared to wild-type mice [10].

Although these previous studies have demonstrated that NOS1 was associated with cardiac hypertrophic growth, the direct functional contribution of NOS1 in this process remains to be elucidated. Hence, in the present study we attempted to use silent RNA and adenovirus

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mediated silencing or overexpression to investigate the role of NOS1 during phenylephrine (PE)-induced cardiac hypertrophy growth in neonatal rat ventricular cardiomyocytes (NRVMs). We demonstrate that NOS1 gene suppression clearly blunts NRVMs hypertrophy and the corresponding foetal gene program in response to PE. This inhibition of NRVMs growth was, at least, mediated through inhibition of the calcineurin signaling pathway. In contrast, exposing the NOS1 overexpressed NRVMs to PE-treatment markedly increased the hypertrophic growth and marker gene expression. Together, the results of the present study suggest that NOS1 is directly involved in controlling the development of cardiomyocyte hypertrophy.

2. Materials and methods

The present study was conducted in accordance with institutional guidelines and those formulated by the European Community for experimental animal use (L358-86/609/EEC). We used Wistar rat pups on post-natal day 1 to isolate neonatal rat ventricular myocytes (NRVMs).

2.1. NRVMs culture, transfections and manipulation

Monolayer cultures of neonatal rat cardiac cells were prepared as previously described. Briefly, hearts were removed and ventricular cells were dispersed by digestion with collagenase A (0.45 mg/ml) (Boehringer Mannheim) and pancreatin (0.05 mg/ml) (GIBCO) in Ads buffer (116 mM NaCl, 20 mM HEPES, 1 mM NaH2PO4, 5.4 mM KCl, 5.5 mM glucose, 0.8 mM MgSO4, pH 7.35). Cardiomyocytes were purified on a discontinuous Percoll gradient (1.059/1.085) collected by centrifugation at $3000 \times g$ and resuspended (0.3×10^6 cells/ml) in Dulbecco's modified Eagle medium (DMEM, 1.8 mM Ca^{2+}), 17% Medium 199 (GIBCO), 10% horse serum, 5% newborn calf serum, 1% penicillin and 1% streptomycin.

In order to manipulate NOS1 activity or expression, we used the selective NOS1 inhibitor Vinyl-*l*-N-5-(1-imino-3-butenyl)-L-ornithine (LVNIO, Alexis Biochemical, 1.10^{-6} mol/L), silent RNA (siRNA) against mouse NOS1 or adenovirus coding for the human NOS1. siRNA duplex for NOS1 (si-NOS1) and a siRNA containing the same nucleotides in a scrambled order (si-Scramb) were designed and provided from Quiagen France SAS. The sense and antisense oligonucleotides of NOS1 siRNA were, respectively, r(CAA UAC UAC UCA UCC AUU A)dTdT and r(UAA UGG AUG AGU AGU AUU G)dGdT. All the siRNAs were transfected using Lipofectamine® Reagent (Thermo Fisher) according to the manufacturer's protocol. The human NOS1 (*h*NOS1, α isoform) coding sequence was a kind gift of Dr. Bernd Mayer (Department of Pharmacology and Toxicology, Karl Franzens Universität Graz, Austria). Adenovirus coding for NOS1 (Ad.NOS1) was constructed and amplified by Dr. Graziella Griffith. For all experiments using adenoviruses, control cells were infected with a control empty adenovirus and so called “Ad.Empty”.

After 24 h, NRVMs were starved and hypertrophy was induced by phenylephrine stimulation (PE, Sigma aldrich, 10^{-4} mol/L) for 48 h. For experiments employing adenoviruses, the day after seeding, cells were infected overnight with adenoviruses. The day after, excess of adenoviruses is removed and cells were replaced in SFM and stimulated with PE. Viruses were used at a multiplicity of infection (MOI) of 100. Identical procedure was used when cells were transfected with siRNAs.

2.2. Incorporation of amino acids and cell size measurement

To quantify the incorporation of amino acids, NRVMs were incubated with [^{3}H]-leucine for the last 24 h with (1.0 μCi/ml, Perkin Elmer, USA). After three washes with PBS 1 ×, cells were treated for 2 h at 4 °C with trichloroacetic acid (10% w/v) followed by solubilization in 0.1 N NaOH (0.2 M) for 2 h under agitation at 37 °C. The radioactivity was determined by scintillation counting and expressed as percentage of control cells.

To perform cell size measurements, cells were fixed in 4% PFA and then stained with anti-alpha actinin antibody (Sigma Aldrich) and Hoescht staining for nucleus. Pictures were recorded using a fluorescent microscope (Zeiss Imager Z1, France). A minimun of 10 fields per condition was recorded at × 20. Cardiomyocytes areas were determined using Image J Software.

2.3. Hypertrophic program determination using reporter assay

To determine the role of NOS1 on the hypertrophic program, cells were cotransfected with Lipofectamine 2000 (Invitrogen Life Technologies, France) in the presence of 1 μg of ANF-Luciferase plasmid construct (generously provided by Dr K. Knowlton). The day after transfection, cells were stimulated with PE for 48 h and assayed for Luciferase activity. Results are expressed as percentage activation of control.

2.4. Superoxide measurement using Lucigenin-enhanced chemiluminescence

To determine if NOS1-derived superoxide anions production was involved in the hypertrophic response following PE stimulation, NOS1-derived superoxide production was measured in cardiomyocytes homogenates using Lucigenin-enhanced chemiluminescence. Briefly, cells homogenates were placed into a 96-well microplate luminometer together with dark-adapted lucigenin (5 μmol/L), and NADPH (300 μmol/L) was added before recording chemiluminescence. NOS1-derived superoxide was assessed by pre-incubating cells homogenates with the selective NOS1 inhibitor LVNIO (1.10^{-6} mol/L). Superoxide production was expressed as arbitrary light units over 20 min. Results are expressed as percentage activation of control.

2.5. Immunoblots

NRVMs were homogenized and subjected to Western Blot analysis. After migration on SDS-PAGE gels, proteins were transferred onto nitrocellulose membranes (Amersham, Chalfont St. Giles, UK). Afterwards, the membranes were incubated overnight at 4 °C with the following primary antibodies in blocking solution: anti-NOS1 (Affinity Bioreagent), anti-NOS3 (Santa Cruz Biotechnology Inc.), anti-calcineurin (Becton Dickinson), anti-GSK-3β and anti-Phospho-GSK-3β (Cell signaling). After several washes, membranes were incubated with a secondary antibody coupled to horseraddish peroxidase (1:5000; Amersham, Chalfont St. Giles, UK). Protein detection was performed using ECL + on a Fuji system and quantified by Image Gauge. Membranes were also blotted with an anti GAPDH antibody (Chemicon) to normalize.

2.6. Calcineurin NFAT activity

Calcineurin activity was quantified using the measurement of the NFAT-Luciferase reporter activity. The luciferase reporter plasmid driven by four NFAT consensus binding sites (NFAT-Luc) was obtained from Stratagene. NRVMs were cotransfected with Lipofectamine 2000 in the presence of 1 μg of the plasmid construct according to the manufacturers'instructions. The day after transfection, cells were stimulated by PE for 48 h and were assayed for Luciferase activity. Results were expressed as percentage activation of control.

2.7. Statistical analysis

Data are expressed as mean ± sem. Statistical analysis was performed with Prism. ANOVA followed by a Bonferroni-Dunn test was performed. $P < 0.05$ after Bonferroni test is considered significant.

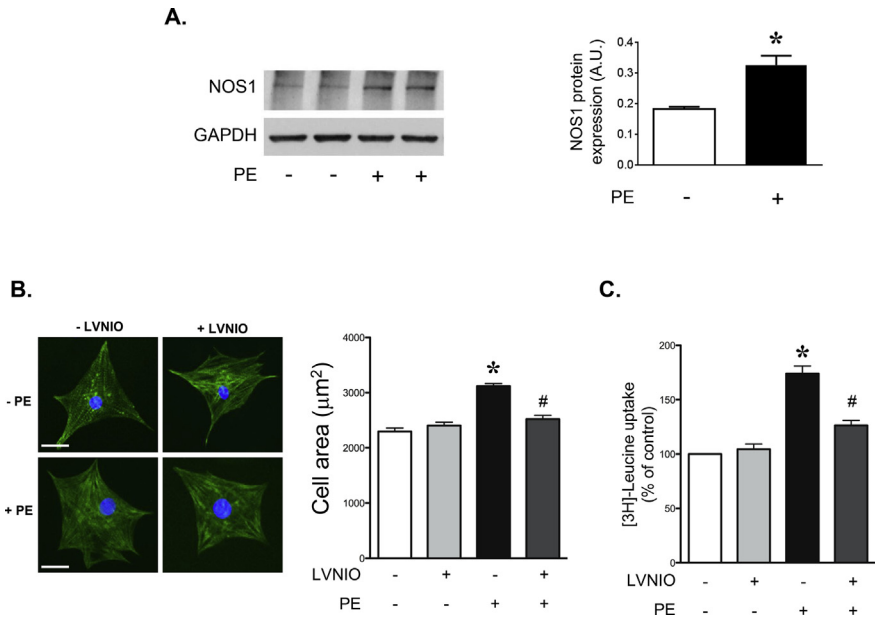

Fig. 1. Selective neuronal nitric oxide synthase inhibition blocks cardiomyocyte hypertrophy in vitro. **A,** Representative immunoblot showing NOS1 expression in response to PE stimulation. Values are expressed as mean ± SEM of triplicates from three independent experiments. **B,** Representative immunofluorescence of alpha-actinin (green) and nuclear staining (blue) and corresponding quantification of the cardiomyocyte cell surface area for each treatment condition. Scale bar = 50 µm. **C,** Tritiated leucine incorporation as a function of LVNIO (10^{-6} Mol/L) and/or phenylephrine treatments. Results are expressed as percentage activation of control. $^{*}P < 0.05$ versus non treated cells, $^{\#}P < 0.01$ versus PE. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. NOS1 is upregulated and promotes the induction of cardiomyocyte hypertrophy induced by PE

Because we have previously demonstrated that increased expression and/or activity of NOS1 expression were associated to the progression of cardiac hypertrophy, we tested whether PE stimulation may regulate NOS1 protein expression and activity and whether blocking NOS1 activity would influence the development of cardiomyocyte hypertophy. As shown in Fig. 1A, PE treatment significantly increased NOS1 protein expression, as compared to non-stimulated cardiomyocytes ($P < 0.05$ versus basal). As expected, PE-induced cardiomyocyte hypertrophy was demonstrated by a 36% increase in cell size (Fig. 1B) and a 74% induction in [^{3}H]-leucine incorporation (Fig. 1C). In line with our hypothesis, LVNIO treatment, the selective NOS1 inhibitor, significantly decreased PE-induced NRVMs hypertrophy and [^{3}H]-leucine incorporation ($P < 0.01$ versus PE). Note that LVNIO treatment in absence of PE had no effect.

To determine if NOS1-derived superoxide anions production was involved in the hypertrophic response following PE stimulation, NOS1-derived superoxide production was measured in cardiomyocytes homogenates using Lucigenin-enhanced chemiluminescence. As expected, PE induced a significant increase in superoxide production (+114% versus non-stimulated cells). However, LVNIO pre-incubation had no effect on superoxide production, suggesting that NOS1-derived superoxide was not involved in the hypertrophic response mediated by PE (Supplemental Fig. 1A).

3.2. NOS1 is involved in the induction of cardiomyocyte hypertrophy induced by PE

To further investigate the possible effects of NOS1 on cardiomyocyte hypertrophy, we used complementary strategies. To explore the role of native NOS1 in the hypertrophic effect of PE, we used a specific silent RNA targeting NOS1 (si-NOS1). As expected, NRVMs transfected with si-NOS1 showed a decreased level of NOS1 compared with silent RNA sequence control (si-Scramb, Fig. 2A). To mimic the results previously obtained in vivo and those obtained in vitro after PE stimulation, we constructed an adenovirus encoding the human NOS1 protein (Ad.NOS1). As expected, NRVMs infected with Ad.NOS1 showed an increased level of NOS1 compared with a control empty adenovirus

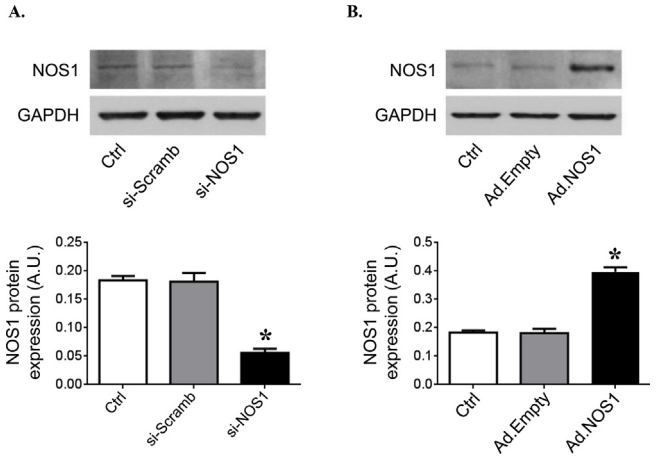

Fig. 2. Modulation of NOS1 expression by specific siRNA or adenovirus is efficient into neonatal rat cardiomyocytes. Representative immunoblots and quantification for NOS1 and GAPDH of NRVMs treated with si-NOS1, Ad.NOS1 and their respective controls. Values are expressed as mean ± SEM from six independent experiments. $^{*}P < 0.05$ versus non treated cells.

(Ad.Empty, Fig. 2B). Both the transfection and infection efficiencies were maintained for at least 72 h.

Then we investigated cardiomyocytes transfected with the si-NOS1 or Ad.NOS1 followed by PE treatment. As shown in Fig. 3A and B, It can be observed that silencing of NOS1 significantly attenuated the increase in cell surface and in [^{3}H]-leucine incorporation induced by PE stimulation. Similar findings were obtained on another marker of cardiomyocyte hypertrophy, ANF expression. Indeed, silencing NOS1 expression significantly inhibited PE-induced ANF-Luciferase gene transcriptional activity (Fig. 3C). Consistent with this finding, upregulation of NOS1 in NRVMs with the Ad.NOS1 further exacerbated the effect of PE on cell surface area compared with NRVMs infected with control adenovirus Ad.Empty (Fig. 3A). The effect of Ad.NOS1 on cell growth was also confirmed by protein synthesis measurement (Fig. 3B). Finally, the ANF-Luciferase gene transcriptional activity was also further increased in NRVMs infected with Ad.NOS1 compared to cells treated with PE only. In absence of PE stimulation either silencing or overexpressing NOS1 has no effect on cardiomyocyte hypertrophy. In the same way, the si-Scramb and Ad.Empty had no effects on NRVMS

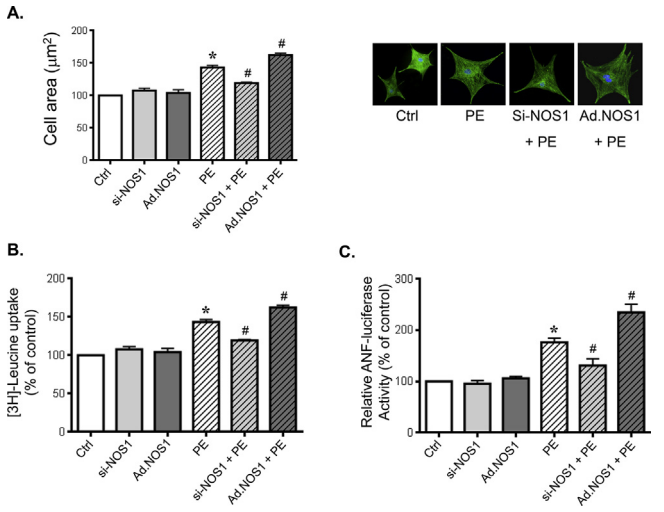

Fig. 3. NOS1 is involved in PE-induced NRVMs hypertrophy. **A,** Representative immunofluorescence images showing α-actinin (green) and DAPI (blue) -stained neonatal rat cardiomyocytes and bar graph depicting average cell surface area for each treatment condition. Scale bar, 50 μm. **B,** Tritiated leucine incorporation as a function of PE treatment and regulation of NOS1 protein expression using si-NOS1 or Ad.NOS1. **C,** NRVMS cotransfected with ANF-Luc and si-NOS1 or Ad.NOS1 were treated or not with PE for 48 h and were assayed for luciferase activity. Results are expressed as percentage activation of control. Values are expressed as mean ± SEM of triplicates from three independent experiments. $^{*}P < 0.05$ versus non treated cells, $^{\#}P < 0.05$ versus PE. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

hypertrophy with or without PE stimulation (data not shown). We finally examined if the effects on cardiomyocytes hypertrophy induced by NOS1 could be partially mediated through NOS3 protein expression modulation. As shown in Supplemental Fig. 1B, silencing or overexpression NOS1 didn't induce any change in NOS3 protein expression after PE stimulation. Since the selective inhibitor LVNIO abolished PE-induced cardiomyocyte hypertrophy, NOS3 or potential NOS3-derived NO production seemed to be negligible in the development of NRVMs hypertrophy.

Taken together, these date support the hypothesis that NOS1 participate in PE-induced cellular hypertrophy.

3.3. NOS1 mediates PE-induced cardiomyocyte hypertrophy in a NFAT-dependent signaling pathway

Several calcium-dependent signal transduction pathways have been implicated in cardiac hypertrophy. The calcium/calmodulin-dependent protein phosphatase calcineurin is an especially potent inducer of myocardial hypertrophy in vivo and in vitro [11]. Calcineurin dephosphorylates a large number of cellular targets that could influence cardiac growth, including the nuclear factor of activated T cell (NFAT) proteins [12].

Since we have previously demonstrated that NOS1 cardiomyocyte specific overexpression in mice was associated with an increase in calcium content, we further characterize the NOS1 hypertrophic signaling pathway and investigate the effect of NOS1 protein modulation on calcineurin expression and activity (using the NFAT-luciferase reporter assay to monitor transcriptional activity of NFAT). Fig. 4A and B shows that calcineurin protein expression and activity were both significantly increased in NRVMs treated with PE, as compared to non-treated cells. In line with the above results on cells growth, these increases induced by PE were prevented by the selective si-NOS1. Interestingly, overexpressing NOS1 using Ad.NOS1 further increased calcineurin protein expression and activity in response to PE (Fig. 4B).

In contrary, glycogen synthase kinase-3β (GSK-3β) is a ubiquitous serine/threonine protein kinase that phosphorylates a series of sites of NFAT proteins, inhibiting its transcriptional activity. GSK-3β is highly active in unstimulated cells and becomes inactivated in response to hypertrophic stimulation. Conversely, dephosphorylation results in activation of the kinase [13]. To gain further insight on the mechanism of retarded cardiomyocyte hypertrophy induced by NOS1 gene silencing, we thus determined if GSK-3β activation (determined by the ratio phosphorylated form to non-phosphorylated form) is affected following PE stimulation. As shown in Fig. 4C, PE induced a significant increase in phospho/total ratio indicating that gsk3β is inactive and cannot repress hypertrophy. Upon PE stimulation, silencing NOS1 leads to a decrease of the phospho/total ratio indicating that GSK-3β is active and can limit hypertrophy development.

All of these data demonstrate that NOS1 acts as a prohypertrophic factor by involving calcineurin pathway and limiting action of the GSK-3β one.

4. Discussion

The results of the present study reveal for the first time a key and direct contribution of NOS1 and NOS1-derived NO in PE-induced neonatal cardiomyocyte hypertrophy. Indeed, not only PE stimulation is associated with an increased expression of NOS1 but also a specific inhibitor of NOS1 prevents PE-induced NRVMs growth. In addition, silencing NOS1 expression blocked the hypertrophic effect of PE. Finally, overexpression of NOS1, using an adenovirus, further exacerbated NRVMs growth following PE stimulation.

Although we did not observe any effect of either silencing or overexpressing NOS1 on cardiomyocyte hypertrophy in the absence of PE stimulation, the present results confirmed our in vivo data where we did not observe any impact of NOS1 overexpression on adult cardiomyocyte hypertrophy without stressing the heart (following increased pressure-overload) [10]. In fact, NOS1 binds calmodulin and NOS1 activity is regulated by Ca^{2+} and calmodulin. In NOS1, calmodulin binding is brought about by an increase in intracellular Ca^{2+}. Thus, to unmask any effect of NOS1 expression modulation, we need to promote its activation by increasing cardiomyocyte calcium level [14]. Although we did not quantify Ca^{2+} transients in response to PE, this hypertrophic stimulus has been shown to increase intracellular Ca^{2+} influx in NRVMs via Gq protein activation. The activated Gq protein activates phospholipase C and in turn cleaves phosphatidyl inositol 4,5-biphosphate into the second messengers inositol 1,4,5-triphosphate and diacylglycerol leading to the increase of calcium in the cardiomyocyte [15]. After PE stimulation, calcium-calmodulin complex levels increased resulting in increased NOS1 activity. In non-stimulated cardiomyocytes, calcium level are unchanged which may explain that silencing or overexpressing NOS1 have no impact on cardiomyocytes hypertrophy. In the same way, ATP was shown to regulate cardiomyocyte NO production through modulation of calcium influx [16].

Experimental evidence has indicated that constitutive NO production may play an important part in regulating cardiac growth in response to stress [17,18]. However, selective gene deletion of the second NOS isoform present in the cardiomyocyte, NOS3, has not produced consistent results on cardiac remodeling, and NOS3 expression and activity have been found to be significantly suppressed or even absent in the myocytes of the hypertrophic heart [5,6,19–21]. In contrast, the increase of NOS1 expression following PE stimulation is consistent with previous in vivo experimental evidence, demonstrating that NOS1 expression and activity were increased in the hypertrophic or failing rodent myocardium and in failing human hearts [5,6,10]. Similarly, NOS1 expression and activity are significantly increased in the myocardium or isolated cardiomyocytes of hypertensive rats and in isolated LV myocytes following angiotensin II treatment, confirming the upregulation of cardiac NOS1 and/or activity with cardiomyocyte hypertrophy [22–25].

Emerging but conflicting data suggest that NOS1 may have

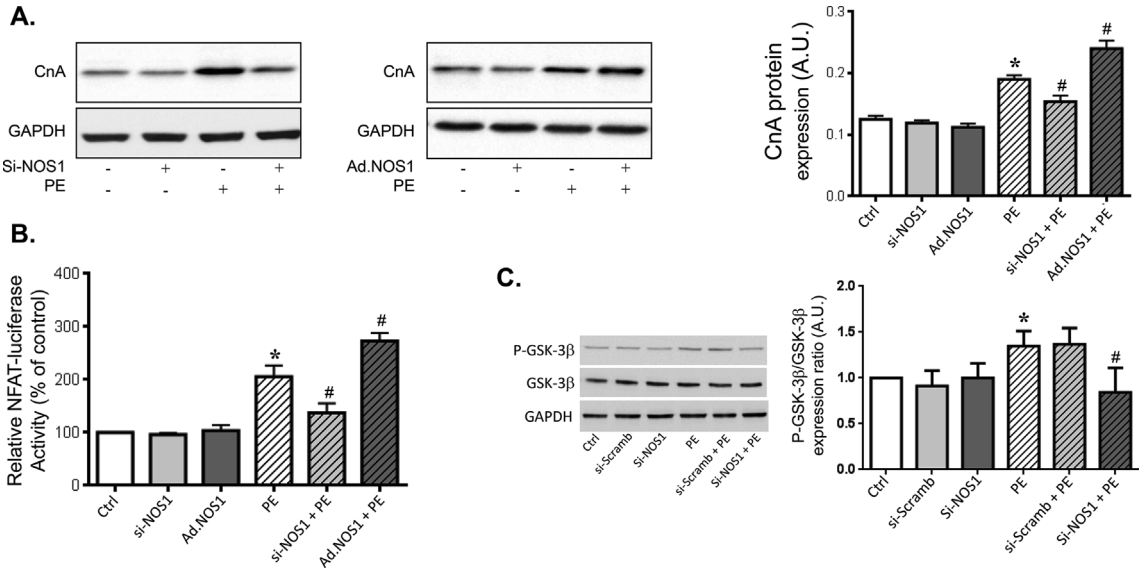

Fig. 4. NOS1 effect on cardiomyocyte hypertrophy is mediated by the NFAT signaling pathway. **A,** Representative immunoblots for calcineurin and its corresponding quantification normalized to GAPDH. **B**, NRVMS cotransfected with NFAT-Luc and si-NOS1 or Ad.NOS1 were treated or not with PE for 48 h and were assayed for luciferase activity. Results are expressed as percentage activation of control. **C**, Representative phospho-GSK-3β and total GSK-3β immunoblots and corresponding level of activation determined by the ratio phospho to total GSK-3β normalized to GAPDH. Values are expressed as mean ± SEM of triplicates from three independent experiments. $^{*}P < 0.05$ versus the non-treated cells, $^{\#}P < 0.05$ versus PE.

important functions in the pathophysiology of adverse cardiac hypertrophy. Here, we provide the first in vitro evidence that both NOS1 and NOS1-derived NO act as positive regulators of cardiomyocyte hypertrophy. These results are in sharp contrast with previous in vitro studies, which demonstrated that non-selective inhibition of NOS isoforms potentiated cardiomyocyte hypertrophy [16,26,27]. However, these studies never used selective inhibitor of the different NOS isoforms neither manipulated NOS isoforms expression using specific silent RNA or adenovirus. In contrast, our results, demonstrating that NOS1 is a pro-hypertrophic factor in vitro, extend our data obtained in vivo previously. Although we didn't use the same experimental approach to overexpress NOS1, using a transgenic mouse with conditional cardiomyocyte specific NOS1 overexpression, we provided evidence that NOS1 overexpressing hearts undergo further hypertrophy retaining concentric left ventricular remodeling and full contractile function, whereas WT mice showed pronounced chamber dilation and impaired contractility in response to chronic pressure overload. In this study, we further demonstrated that isolated cardiomyocytes from NOS1 overexpressing mice had greater shortening, intracellular Ca^{2+} transients and sarcoplasmic reticulum Ca^{2+} load. The current results in NOS1 transgenic mice were consistent with the idea that NOS1 may prevent adverse remodeling and cardiac deterioration by preserving calcium cycling and contractile function even though the extent of hypertrophy is increased in response to pressure overload [10]. Consistent with an adaptive and protective role for myocardial NOS1 overexpression, studies found that NOS1 in the left ventricular myocardium of chronically infarcted mice appears to attenuate adverse left ventricular remodeling and functional deterioration [9]. More recently, Rosello-Lleti and collaborators demonstrated an increased NOS1 protein expression in ventricular tissue obtained from explanted human ischemic cardiomyopathy hearts. They also found a significant relationship between NOS1 protein levels and systolic ventricular function, indicating that an increased NOS1 level was associated with a better ventricular performing. Of note, the authors found no increase in NOS1-derived NO production in their experiments and suggested that the increased levels of NOS1 could be a mechanism to compensate the loss of its activity. The authors concluded that NOS1 may be important in the physiopathology of cardiac dysfunction in ischemic heart disease with a preservative role in maintaining myocardial homeostasis [28]. Thus, increasing NOS1 protein expression within the cardiomyocyte may be a potential therapeutic target for heart disease. We now have to test our hypothesis in vivo using this adenovius-induced cardiomyocyte NOS1 protein expression (using AAV9 serotype) in different experimental models of heart failure induced by an increased pressure-overload or myocardial infarction.

In the same way, NOS1 and NOS1-derived NO were shown to be key triggers of skeletal muscle hypertrophy. Using a genetic model of Duchenne muscular dystrophy (MDX mouse) engineered to lack NOS1, the authors demonstrated that NOS1 depletion from MDX mice prevented compensatory skeletal muscle hypertrophy [29]. In another model of overload-induced skeletal muscle hypertrophy, based on compensatory adaptation of the plantaris muscle after ablation of the tendons of the functionally synergistic muscles, NOS1 was found to modulate overload-induced fiber hypertrophy. Ito and collaborators [30] demonstrated that, 7 days after ablation, NOS1-null mice showed a significant diminished increase in muscle weight, compared to in wild-type mice. Overload also increased the fiber size and total protein contents in the wild-type plantaris, whereas no such substantial increases were found in the NOS1-null plantaris. In this study, the authors also validated that the observed effects were mediated through an increase in NOS1-derived NO, using the specific inhibitor 7-nitroindazole. The latter results are similar to those we have obtained in our study demonstrating that LVNIO prevented PE-induced cardiomyocyte hypertrophy.

Cardiomyocyte growth is finely controlled by Ca^{2+}-mediated signaling [31]; however, it was still unknown whether the action of NOS1 involved Ca^{2+}-mediated pathways to induce positive regulation during hypertrophy. A major new paradigm in NO biology has emerged in the last few years to suggest that NOS1-derived NO plays an important role in the physiological regulation of myocyte calcium fluxes [27]. Although we did not quantify Ca^{2+} transients in response to PE, this drug has been shown to increase intracellular Ca^{2+} influx in NRVMs. In addition, we have previously demonstrated that cardiomyocytes overexpressing NOS1 higher Ca^{2+} transients than control myocytes. Accordingly, we found that silencing NOS1 expression decreased both the expression and activity (measuring the NFAT transcriptional activity) of the Ca^{2+}-dependent prohypertrophic protein calcineurin. The opposite results were obtained when NOS1 was overexpressed. On the other

hand, transcriptional activity of NFAT is also controlled by GSK-3β. As previously described, PE-induced cardiomyocyte hypertrophy induced an increase in phosphorylated GSK-3 β, the inactive form. In contrary, silencing NOS1 leads to a decrease of the phospho/total GSK-3β ratio indicating that GSK-3 β was more active and can limit the development of myocyte hypertrophy. Our results are in agreement with data from Drenning and colleagues [32] who demonstrated that NO could facilitate NFAT-dependent transcription in mouse myotubes through regulation of both pathways described in our study. More recently, NOS1 was shown to induce neuronal differentiation through inactivation of the GSK-3 β pathway [33].

In conclusion, we have provided in vitro evidence that NOS1 and NOS1-derived NO promote cardiac cells hypertrophy through a signaling pathway that includes calcineurin and GSK-3 β. These data improve our understanding of the regulation of myocyte hypertrophy by NOS1.

Disclosures

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2019.100620.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrep.2019.100620.

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