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Original Article

SIRT1 inhibits angiotensin II-induced vascular smooth muscle cell hypertrophy

Li Li, Peng Gao, Huina Zhang, Houzao Chen, Wei Zheng, Xiang Lv, Tingting Xu, Yusheng Wei, Depei Liu*, and Chihchuan Liang†

National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100005, China
†Deceased.
*Correspondence address. Tel: +86-10-65296415; Fax: +86-10-65105093; E-mail: liudp@pumc.edu.cn

Angiotensin II (Ang II) stimulates vascular smooth muscle cell (VSMC) hypertrophy as a critical event in the development of vascular diseases such as atherosclerosis. Sirtuin (SIRT) 1, a nicotinamide adenine dinucleotide dependent deacetylase, has been demonstrated to exert protective effects in atherosclerosis by promoting endothelium-dependent vascular relaxation and reducing macrophage foam cell formation, but its role in VSMC hypertrophy remains unknown. In this study, we tried to investigate the effect of SIRT1 on Ang II-induced VSMC hypertrophy. Results showed that adenoviral-mediated over-expression of SIRT1 significantly inhibited Ang II-induced VSMC hypertrophy, while knockdown of SIRT1 by RNAi resulted in an increased [^{3}H]-leucine incorporation of VSMC. Accordingly, nicotinamide adenine dinucleotide phosphate oxidase 1 (Nox1) expression induced by Ang II was inhibited by SIRT1 in VSMCs. SIRT1 activator resveratrol decreased, whereas endogenous SIRT1 inhibitor nicotinamide increased Nox1 expression in A7r5 VSMCs. Furthermore, transcription factor GATA-6 was involved in the down-regulation of Nox1 expression by SIRT1. These results provide new insight into SIRT1's anti-atherogenic properties by suppressing Ang II-induced VSMC hypertrophy.

Keywords SIRT1; VSMC hypertrophy; Nox1; GATA-6; angiotensin II

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Introduction

Angiotensin (Ang) II, the main effector peptide of the renin–angiotensin system, not only modulates vasomotor tone and blood pressure, but also is implicated in the development of various vascular diseases characterized by the accumulation of vascular smooth muscle cells (VSMCs), such as atherosclerosis, hypertension, and restenosis after balloon angioplasty [1]. VSMC hypertrophy, a hallmark of vascular diseases, is induced by Ang II in the absence of other growth factors [2]. Accumulating evidence indicated that Ang II mediates its hypertrophy effects at least in part through reactive oxygen species (ROS), the production of which it stimulates [3–6]. In blood vessels, nicotinamide adenine dinucleotide (NAD) phosphate (NADPH) oxidases are the major sources of ROS.

NADPH oxidases are a family of enzyme complexes with each member or isoform being distinguished by the catalytic 'Nox' or 'Duox' subunit that it utilizes to generate superoxide and other downstream of ROS [7]. There are seven members of the NADPH oxidase family including Nox1- through 5- as well as Duox1- and 2-containing oxidases, in which three isoforms (Nox1, Nox4, and Nox5) were detected in VSMCs [3,8–10]. Among them, Nox1 was reported to be the mediator of Ang II-induced superoxide formation in VSMCs [3]. Moreover, Nox1 itself was reported to be involved in prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced hypertrophy of VSMCs in culture [11].

SIRT1, the closest homology to yeast Sir2 protein (silent information regulator 2) in the human sirtuins, functions as a NAD^+ dependent deacetylase, which has been implicated in the process of aging, metabolism, and tolerance to oxidative stress [12]. Emerging evidence suggested that SIRT1 functions as a metabolic sensor coupling energy and oxygen homeostasis to the growth and function of the vasculature [13]. Furthermore, SIRT1 has been demonstrated to be an anti-atherosclerosis factor through inhibiting oxidized low-density lipoprotein (oxLDL)-induced human umbilical vein endothelial cell apoptosis [14], promoting endothelium-dependent vascular relaxation [15], improving phagocytosis in peritoneal macrophages (pMΦs) [16] and reducing macrophage foam cell formation [17].

Ang II has been well established to stimulate protein synthesis and induce cellular hypertrophy in VSMCs. It may also participate in the induction of pathological states associated with atherosclerosis [18,19]. Despite recent

advances in our understanding of the protective effects of SIRT1 in atherogenesis, no information is currently available regarding the effect of SIRT1 on VSMC hypertrophy. The aims of this study are, therefore, to investigate the effect of SIRT1 on Ang II-induced VSMC hypertrophy and to elucidate the molecular mechanism that may be responsible for the putative effect of SIRT1.

Materials and methods

Materials and reagents

[^{3}H]-leucine was purchased from Moravek Biochemicals (Brea, USA). Synthetic human Ang II, resveratrol (RV), nicotinamide (NAM) and anti-β-actin antibody were purchased from Sigma-Aldrich (St. Louis, USA). Anti-mSirt1 (mouse Sirt1) antibody was purchased from Millipore (Billerica, USA). hSIRT1 (human SIRT1) rabbit polyclonal antibody and anti-c-myc antibody were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, USA). Antibodies against phospho-ERK1/2, ERK1/2 were purchased from Cell Signaling Technology (Beverly, USA).

Cell cultures

The A7r5 and A10 rat embryonic aortic VSMCs and 293A cell were obtained from the American Type Culture Collection (ATCC, Manassas, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories, Grand Island, USA) with 10% fetal bovine serum (FBS) (Gibco Laboratories) and antibiotics. Rat aortic VSMCs were isolated from the thoracic aorta of male Sprague–Dawley rats by enzymatic digestion, and then cultured in DMEM supplemented with 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. VSMCs were kept at 37°C in a humidified atmosphere with 5% CO_2 and were used until 80% confluent in the experiments at passages 3–5. The medium was changed before experiments.

Adenovirus generation and infection

The replication-defective adenoviral vectors expressing SIRT1 (Ad-SIRT1) or control green fluorescent protein (Ad-GFP), and adenovirus-mediated knockdown of SIRT1 (Ad-SIRT1 RNAi) or control vector (Ad-U6) were generated by using the Ad Easy Vector kit (Quantum Biotechnologies, Montreal, Canada) as described before [14,16]. VSMCs were infected for 12 or 24 h with the above adenovirus at a multiplicity of infection (MOI) of 100, washed, and incubated in the serum-free medium without virus for 24 or 48 h.

Western blot analysis

Cellular proteins were extracted with Radio Immuno Precipitation Assay (RIPA) buffer [25 mmol/l Tris–HCl (pH 7.6), 150 mmol/l NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)]. After complete homogenization on ice, samples were centrifuged at 4°C, 12000 g for 30 min to precipitate the cell debris. The supernatants were transferred into fresh tubes and the protein concentrations were determined by BCA method. Proteins were fractionated by 10% SDS–PAGE and electro-transferred onto a PVDF membrane (Millipore, Bedford, USA). The membranes were blocked with Tris-buffered saline containing 5% non-fat milk, probed with primary antibody and then HRP-conjugated secondary antibody at 4°C overnight. The blots were developed by using the ECL system (Pierce, Chicago, USA). Band intensities were quantified by densitometry. The results were normalized to β-actin as an internal control.

RT-PCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Two micrograms of total RNA was used to synthesize first-strand with cDNA M-MuLV reverse transcriptase (New England Biolabs, Beverly, USA) using random primers. RT-PCR was used to analyze Nox1, Nox4, and β-actin mRNA levels in cells. Primers used in PCR are listed as follows: human *SIRT1* sense 5′-CTTCAGGTCAAGGGATGGTAT-3′, antisense 5′-GCGTGTCTATGTTCTGGGTAT-3′; rat *Nox1* sense 5′-CACCTGCTCATTTTGCAACCACAC-3′, antisense 5′-CAACTCCTTTCATACT TATCCCACTC-3′; rat *Nox4* sense 5′-TTCGGGTGGCTTGTTGAAGTA-3′, antisense 5′-TGGTGACAGGTTTGTTGCTCC-3′; rat *β-actin* sense 5′-GAGAGGGA AATCGTGCGTGAC-3′, antisense 5′-TAGAGCCACCAATCCACACAGAG-3′; mouse *GATA-6* sense 5′-GCTGAGGGTGAGCCTGTGTG-3′, antisense 5′-CCTGAGGTGGTCGCTTGTGTA -3′.

[^{3}H]-leucine incorporation assay

VSMCs were plated onto 12-well plates at a density of 50,000 cells per well, and infected with adenovirus overnight, then serum starved for 48 h. After that, cells were incubated with [^{3}H]-leucine (2 μCi per well) in the presence or absence of 100 nmol/l Ang II for 24 h. At the end of the experiment, the cells were washed with cold PBS, scraped off the well, and then treated with 10% trichloroacetic acid at 4°C for 60 min. The precipitates were dissolved in NaOH (0.4 mol/l) and subsequently counted with a liquid scintillation counter.

Plasmids

The plasmid containing the coding sequence of human *SIRT1* (*pcDNA3.1-SIRT1*) was a kind gift from Prof. F. Ishikawa (Kyoto University, Kyoto, Japan). The plasmid containing coding sequence of full-length mouse *GATA-6* (*pCMVTag3b-GATA-6*) was kindly provided by Prof. Patricia A. Mericko (University of Pennsylvania, USA).

Co-immunoprecipitation assay

293A cells were cotransfected with the plasmids encoding myc-tagged GATA-6 and SIRT1. Then the whole-cell lysates were preincubated with 1.0 μg non-immune rabbit IgG and 20 μl of protein A-Sepharose beads (Millipore) at 4°C for 3 h and centrifuged. Cleared lysates (1 mg) were immunoprecipitated with the anti-hSIRT1 or anti-c-myc (for c-myc-GATA-6) antibodies at 4°C for 1 h and then incubated with protein A-Sepharose beads at 4°C overnight. The immunoprecipitated proteins were washed six times with lysis buffer and resuspended in the electrophoresis sample buffer. Samples of immunoprecipitated or total proteins (20–30 μg) were analyzed by western blot using anti-c-myc or anti-hSIRT1 primary antibodies.

Statistical analysis

Data are presented as mean ± SEM. Statistical analyses between groups were done by Student's *t*-test or one-way ANOVA followed by a Bonferroni *post hoc* test. A probability value of <0.05 was considered significant.

Results

SIRT1 inhibits Ang II-induced VSMC hypertrophy

First, we explored the role of SIRT1 in Ang II-induced VSMC hypertrophy. Ang II-induced VSMC hypertrophy was detected by [^{3}H]-leucine incorporation to determine the cell protein synthesis. Cells were stimulated with 100 nmol/l Ang II, a concentration previously shown to be effective in VSMCs [20–22]. As expected, Ang II strongly induced [^{3}H]-leucine incorporation. However, in Ad-SIRT1-infected cells, Ang II-mediated cellular hypertrophy was markedly reduced [**Fig. 1(A)**]. To further investigate the role of SIRT1 in VSMC hypertrophy, we used adenovirus-mediated RNAi of SIRT1, which had been proved efficiently in rat VSMCs. We found that knockdown of SIRT1 by RNAi in rat VSMCs resulted in an increased [^{3}H]-leucine incorporation of VSMCs under both basal and Ang II-induced conditions, which suggested that SIRT1 might act as a modulator of VSMC protein synthesis [**Fig. 1(B)**].

SIRT1 inhibits Nox1 gene expression induced by Ang II in VSMCs

Previous studies have shown that VSMC hypertrophy induced by Ang II requires ROS derived from NAD(P)H oxidases, specifically Nox1 [3,23,24]. We then detected whether SIRT1 regulates *Nox1* mRNA expression. *Nox1* mRNA was induced by Ang II, as expected [**Fig. 2(A, B)**]. Over-expression of SIRT1 significantly decreased *Nox1* mRNA induced by Ang II [**Fig. 2(C)**]. As Nox4 was expressed abundant and stood for NADPH oxidase activity largely under resting conditions in VSMCs [3,8], we checked whether SIRT1 regulated *Nox4* mRNA expression. Ang II decreased *Nox4* mRNA in a previous report [3], and over-expression of SIRT1 had no effect on Nox4 mRNA level [**Fig. 2(D)**]. Thus, down-regulation of *Nox1* but not *Nox4* mRNA by SIRT1 was involved in its inhibitory effect on Ang II-induced VSMC hypertrophy. Of note, SIRT1 activator resveratrol decreased *Nox1* mRNA level at a dose of 100 μM [**Fig. 2(E)**], whereas endogenous SIRT1 inhibitor NAM increased Nox1 expression in A7r5 VSMCs [**Fig. 2(F)**].

Transcription factor GATA-6 is involved in the down-regulation of Nox1 expression by SIRT1

Besides the activity regulation, the transcriptional regulation of Nox1 appears to be particularly important [25]. Transcription factor GATA-6 is implicated in the regulation

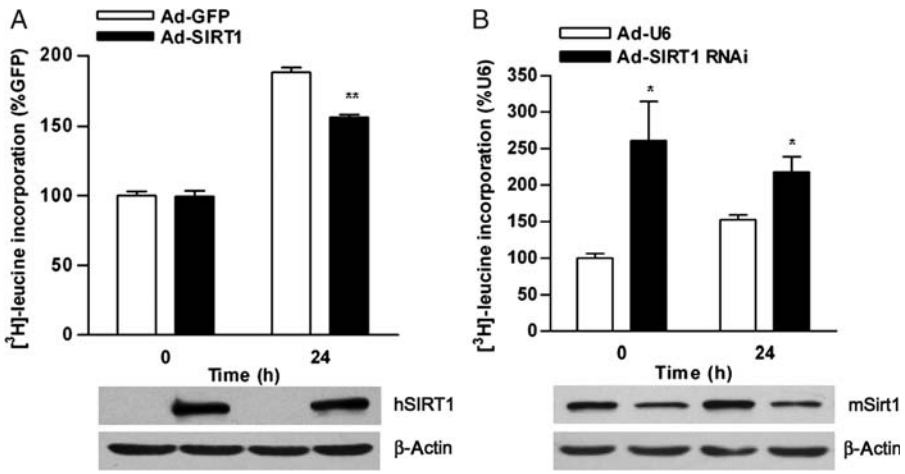

Figure 1 [^{3}H]-leucine incorporation in VSMCs (A, B) Effect of SIRT1 over-expression (A) or RNAi (B) on [^{3}H]-leucine incorporation of VSMCs. Rat aortic SMCs were adenoviral infected overnight, washed, following serum starvation for 48 h then incubated with [^{3}H]-leucine (2 μCi per well) in the presence or absence of 100 nmol/l Ang II for 24 h . Data are shown as mean ± SEM ($n = 3–5$). *$P < 0.05$, **$P < 0.01$ vs. Ad-GFP (A) or Ad-U6 (B) infected group under basal (oh) and Ang II-induced 24 h conditions, respectively. Representative western blots for hSIRT1 (120 kDa, antibody from Santa Cruz technology, Inc.), mSirt1 (110 kDa, antibody from Millipore) and β-actin (43 kDa) (lower panel).

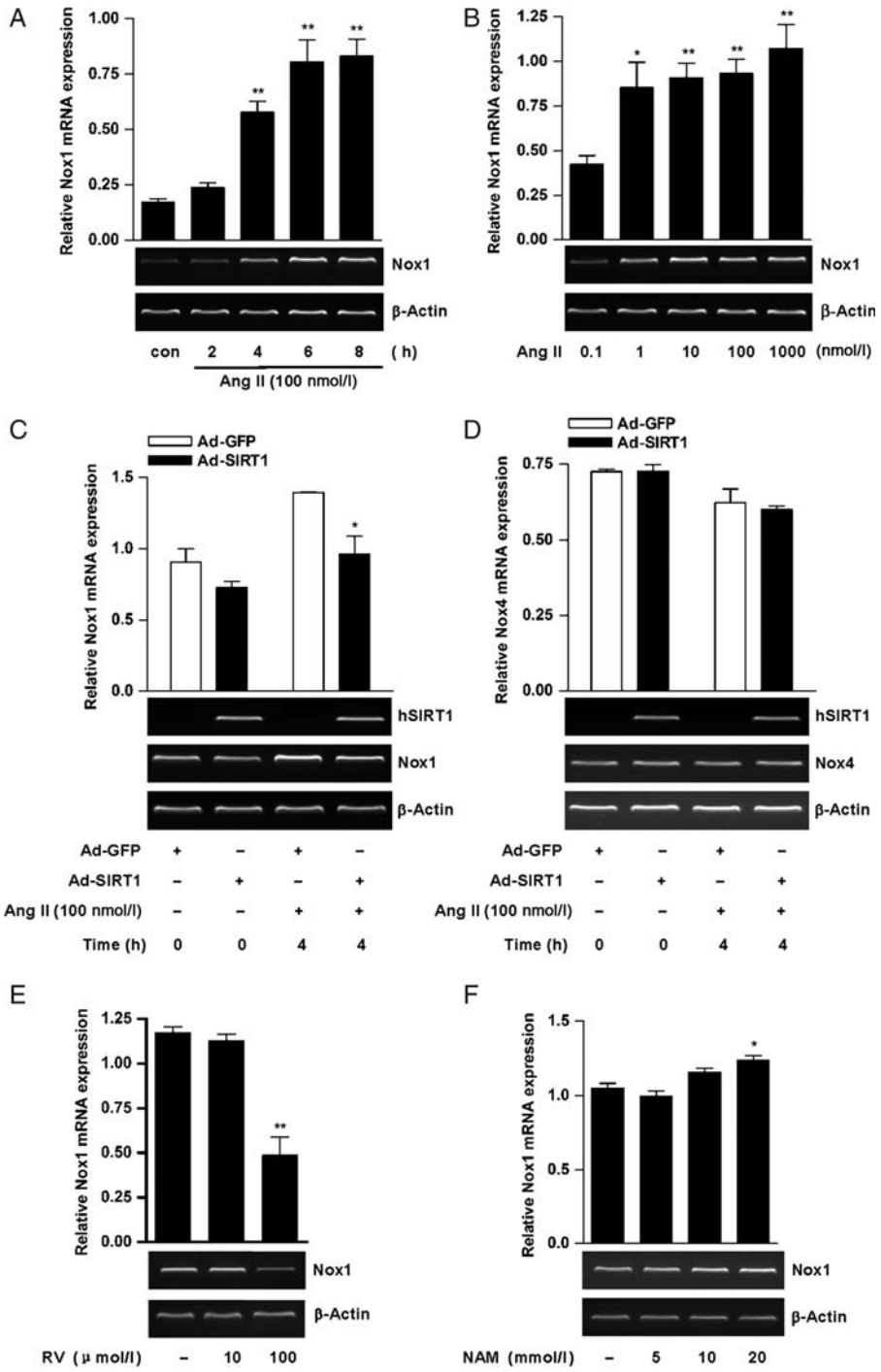

Figure 2 Nox1 (and Nox4) mRNA level(s) in VSMCs (A, B) Effect of Ang II on Nox1 mRNA level in VSMCs. Quiescent VSMCs were treated with Ang II at different time points (100 nmol/l) (A) and different concentrations (B). After that, RNA was isolated and applied to RT-PCR for Nox1.Values are expressed as mean ± SEM ($n = 3$). *$P < 0.05$, **$P < 0.01$ vs. control (A) or 0.1 nmol/l Ang II-treated group (B). (C, D) Effect of SIRT1 over-expression on Nox1 (C) and Nox4 (D) mRNA levels. Rat aortic SMCs were adenoviral infected for 24 h, washed, following serum starvation for 24 h then exposed to Ang II 100 nmol/l for 4 h. After that, RNA was isolated and applied to RT-PCR for Nox1, Nox4, or β-actin. Data are shown as mean ± SEM ($n = 3$). *$P < 0.05$ vs. Ang II-treated GFP control. (E, F) Effect of resveratrol/NAM on Nox1 mRNA level. Quiescent A7r5 VSMCs were treated with resveratrol (E) or NAM (F) in the indicated concentrations for 24 h, and then RNA was isolated and applied to RT-PCR. Data are shown as mean ± SEM ($n = 3$). *$P < 0.05$, **$P < 0.01$ vs. control.

of *Nox1* transcription by direct binding to the *Nox1* proximal promoter [26]. To elucidate the possible mechanism of Nox1 down-regulated by SIRT1, we co-transfected expression plasmids encoding GATA-6 and SIRT1 in A10 VSMCs and checked out the effect of SIRT1 on the transcriptional regulation of *Nox1* by GATA-6. As shown in **Fig. 3A**, *Nox1* mRNA expression was increased after GATA-6 plasmid transfection, but it was significantly

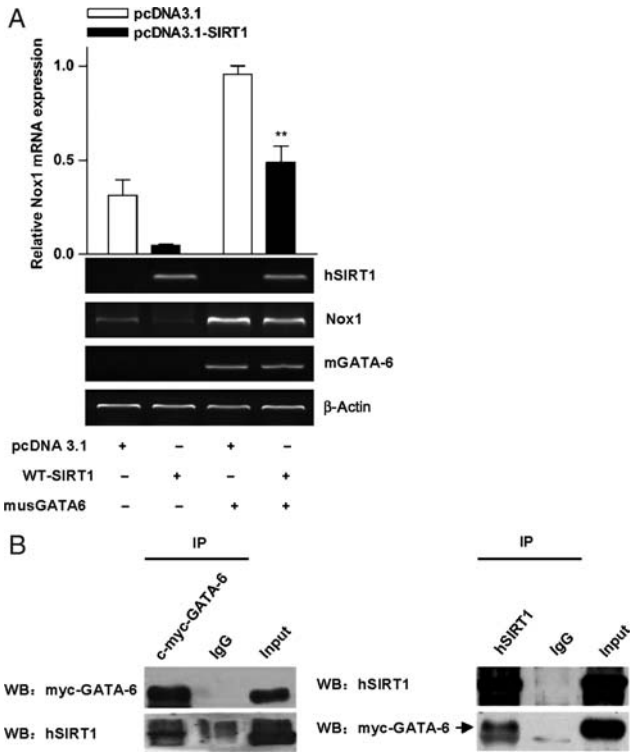

Figure 3 GATA-6 is involved in the down-regulation of Nox1 mRNA level by SIRT1 (A) Effect of cotransfecting of mus-GATA6 and human SIRT1 expression plasmids on Nox1 mRNA level. A10 VSMCs were transfected with plasmid(s) for 8 h, washed, following serum starvation for 40 h. Data are shown as mean ± SEM ($n = 3$). $^{**}P < 0.01$ vs. mus-GATA-6 and pcDNA3.1 expression plasmids cotransfecting group. (B) SIRT1 interacts with GATA-6. 293A cells were cotransfected with the plasmids encoding myc-tagged GATA-6 and SIRT1 for 36 h and were lysed for immunoprecipitation (IP) and western blotting (WB).

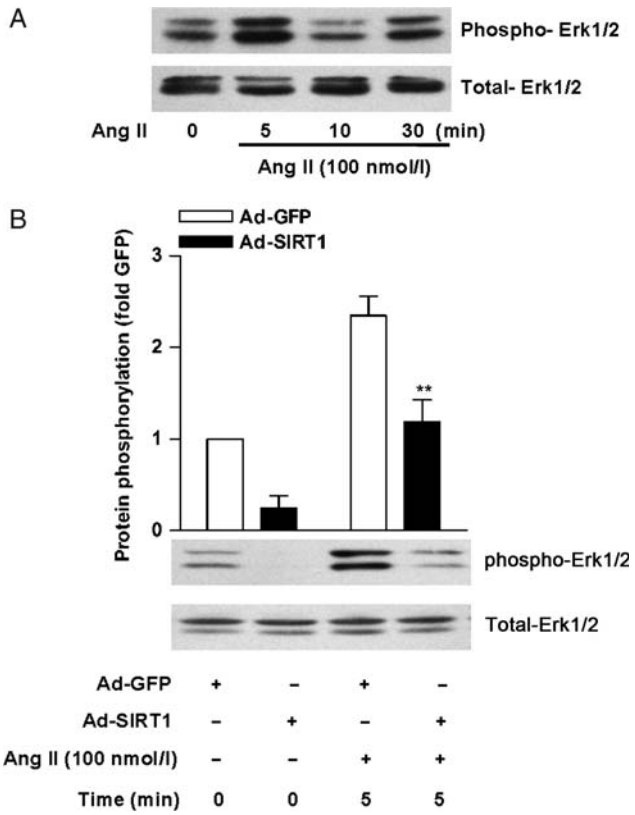

Figure 4 Effect of Ang II and SIRT1 over-expression on ERK1/2 phosphorylation Rat aortic SMCs were infected with Ad-GFP and Ad-SIRT1 adenoviruses for 24 h (B) or no infect (A), following serum starvation for 24 h then exposed to Ang II 100 nmol/l for the indicated time points. (A) Representative western blots of three experiments. (B) Effect of SIRT1 over-expression on ERK1/2 phosphorylation. Densitometric analysis of western blot (upper panel). Representative western blots for phospho-ERK1/2(42, 44 kDa) and ERK1/2 (42, 44 kDa) (lower panel). Data are shown as mean ± SEM ($n = 3$). $^{**}P < 0.01$, vs. Ang II-induced GFP control.

attenuated when co-transfecting with *SIRT1* plasmid, which suggested that SIRT1 could down-regulate *Nox1* mRNA expression through GATA-6. Furthermore, we performed immunoprecipitation experiments to investigate whether SIRT1 could interact with GATA-6 and found that SIRT1 co-immunoprecipitated with c-myc tagged GATA-6 as indicated, and vice versa [**Fig. 3(B)**], which may lead to the decreased Nox1 expression by SIRT1.

SIRT1 suppresses Ang II-induced ERK1/2 phosphorylation in VSMCs

Previous studies have shown that activation of GATA-6 by Ang II is mitogen-activated protein kinase-extracellular signal-regulated kinase (ERK)1/2 dependent [27]. To find out whether ERK/MAPKs signaling pathway was involved in the inhibitory effect of SIRT1 on Ang II-induced VSMC hypertrophy, we detected ERK1/2 phosphorylation by western blot analysis. As expected, Ang II significantly induced ERK1/2 phosphorylation, especially at 5 min after Ang II stimulation [**Fig. 4(A)**]. And phosphorylation of ERK1/2 was significantly inhibited by SIRT1 over-expression [**Fig. 4(B)**].

Discussion

Accumulating evidence has implied Nox1 to be a critical modulator of VSMC hypertrophy. In a rat VSMC derived cell line A7r5, depletion of *Nox1* mRNA by ribozymes significantly reduced the increased protein synthesis induced by $PGF_{2\alpha}$ [11]. Also, smooth muscle-specific Nox1 over-expression potentiated Ang II-induced vascular smooth muscle hypertrophy, and this potentiation of vascular hypertrophy was due to increased ROS formation [28]. While NOX1-deficient mice showed a marked reduction in Ang II-induced media hypertrophy [29]. In the present study, we found that over-expression of SIRT1 suppressed Ang II-induced VSMC hypertrophy at least in part via down-regulating *Nox1* mRNA expression. Alternatively, SIRT1-mediated inhibition of Ang II-induced VSMC hypertrophy might be through decreasing ROS originated from Nox1 induced by Ang II.

The activation state and protein expression of Nox/Duox enzymes, plus their regulatory subunits determine the

maximum capacity of the cell to generate ROS [25]. Rather, the transcriptional regulation of Nox1 appears to be particularly important in the modulation of its activity [3,10,30]. GATA-6, a member of the GATA family of zinc finger transcription factors, is the predominant one expressed in vascular SMCs and maintains the differentiated VSMC phenotype by inhibiting SMC proliferation [31,32]. GATA-6 acts to up-regulate Nox1 in colon epithelial cells by direct binding to a proximal site within the *Nox1* promoter [26]. Moreover, GATA-6 is stimulated by Ang II and regulates genes promoting synthetic functions in VSMCs [27,33], which suggests that GATA-6 activation plays a role in VSMC hypertrophy induced by Ang II. Thus, our findings that SIRT1 inhibited GATA-6-induced Nox1 expression provide the possible mechanism for SIRT1 suppressing Ang II-induced VSMC hypertrophy. In the future, it will be interesting to determine whether SIRT1 can deacetylate GATA-6 to mediate the inhibitory effects, for SIRT1 functions through deacetylating a variety of substrates, including histones, transcription factors and coregulators [12].

In VSMCs, p38MAPK, Akt and ERK1/2 signaling pathways activated by Ang II result in increased protein synthesis [1,34]. In the present study, we found that phosphorylation of ERK1/2 was significantly inhibited by SIRT1 over-expression in VSMCs, suggesting that suppressing this signaling pathway also contributes to SIRT1's anti-Ang II-induced VSMC hypertrophy effect. This result was in accordance with a previous study that SIRT1 activator resveratrol suppressed Ang II-induced AT1R expression and inhibited ERK1/2 phosphorylation in VSMCs, which contributed to the inhibition of Ang II-induced hypertension by resveratrol [35]. Nevertheless, we did not find the substantial change of AT1R protein expression by SIRT1 in Ang II-induced VSMCs (data not shown), which suggested that the down-regulation of Nox1 expression might be more important to mediate the suppressive effect of SIRT1 on Ang II-induced VSMC hypertrophy.

SIRT1 has been shown to control stress resistance by regulating the FOXO transcription factor FOXO3 and the tumor suppressor p53 [36–38]. It is also up-regulated in response to different stimuli such as oxidized oxLDL, hydrogen peroxide (H_2O_2) [14], and TNF-alpha in vascular cells [39]. In this study, we also found that hypertrophic agent Ang II treatment increased SIRT1 expression in VSMCs (data not shown), which seemed to be a compensative effect in protecting against Ang II-induced VSMC hypertrophy. Consistent with this notion, VSMCs infected with adenoviral SIRT1 attenuated Ang II-induced VSMC hypertrophy *in vitro*. We recently found that NF-κB, the mediator of the proinflammatory action of Ang II [40,41], mediated TNF-alpha-induced SIRT1 expression in VSMCs [39]. Hence, we guess that NF-κB may also mediate Ang II-induced SIRT1 expression in VSMCs, which needs to be verified in the future study.

In conclusion, our results showed that SIRT1 significantly inhibited Ang II-induced VSMC hypertrophy. And suppression of Nox1 mRNA at least in part contributed to the inhibitory effect of SIRT1 on Ang II-induced VSMC hypertrophy. Our work provides new insight into SIRT1's atheroprotective effects, and implicates SIRT1 as a potential target for the intervention of VSMC hypertrophy-associated vascular diseases.

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