α7 Nicotinic Acetylcholine Receptor Relieves Angiotensin II–Induced Senescence in Vascular Smooth Muscle Cells by Raising Nicotinamide Adenine Dinucleotide–Dependent SIRT1 Activity

Dong-Jie Li, Fang Huang, Min Ni, Hui Fu, Liang-Sheng Zhang, Fu-Ming Shen

Objective—α7 nicotinic acetylcholine receptor (α7nAChR) is a subtype of nAChR and has been reported to be involved in hypertension end-organ damage. In this study, we tested the role of α7nAChR in angiotensin II (Ang II)–induced senescence of vascular smooth muscle cells (VSMCs).

Approach and Results—Expression of α7nAChR was not induced by Ang II. Ang II induced remarkable senescent phenotypes in rodent and human VSMCs, including increased senescence-associated β-galactosidase activity, phosphorylation of H2A.X, phosphorylation of Chk1, reduced replication, and downregulation of proliferating cell nuclear antigen. Activation of α7nAChR with a selective agonist PNU-282987 blocked Ang II–induced senescence in cultured VSMCs. Moreover, PNU-282987 treatment attenuated the Ang II infusion–induced VSMC senescence in wild-type but not in α7nAChR−/− mice. PNU-282987 reduced the Ang II–enhanced reactive oxygen species, lipid peroxidation, and the expression of NADPH oxidase 1, NADPH oxidase 4, and p22phox in cultured VSMCs isolated from wild-type but not in α7nAChR−/− mice. Furthermore, PNU-282987 diminished Ang II–induced prosenescence signaling pathways, including p53, acetyl-p53, p21, and p16INK4a. Finally, although α7nAChR activation by PNU-282987 did not affect the Ang II–induced downregulation of sirtuin 1 (SIRT1), it significantly increased intracellular NAD+ levels, and thereby enhanced SIRT1 activity in an AMP-dependent protein kinase–independent manner. Depletion of SIRT1 by knockdown or SIRT1 inhibitor EX527 abrogated the antisenescence effect of α7nAChR against Ang II.

Conclusions—Our results demonstrate that activation of α7nAChR alleviates Ang II–induced VSMC senescence through promoting NAD+–SIRT1 pathway, suggesting that α7nAChR may be a potential therapeutic target for the treatment of Ang II–associated vascular aging disorders. (Arterioscler Thromb Vasc Biol. 2016;36:1566-1576. DOI: 10.1161/ATVBAHA.116.307157.)

Key Words: angiotensin II ■ hypertension ■ lipid peroxidation ■ mice ■ reactive oxygen species

Vascular aging is a physiological process that involves several deleterious changes in the cardiovascular system and, in particular, on the large arteries. Results from population studies have characterized the effects of aging on blood vessel structure and functions, including remodeling, intimal thickening, wall stiffness, reduced aortic pulse wave velocity, inflammation, oxidative stress, and endothelial dysfunction. These vascular aging phenotypes are associated with the increase of cardiovascular morbidity and mortality. Cell senescence is a state of essentially proliferative arrest and shows characteristic phenotypic changes in gene expression and morphology. Currently, cellular senescence has been suggested to be an important characteristic of aging and age-associated diseases. Vascular senescence may actively contribute toward the chronic inflammation associated with a proatherosclerotic state and other cardiovascular dysfunction.

In aging-related cardiovascular pathological changes, the renin–angiotensin system is a key factor. Angiotensin II (Ang II), the most potent component of the renin–angiotensin system, plays a central role in cardiovascular aging. Ang II concentration increases with aging in nonhuman primates. Moreover, Ang II mediates the age-dependent cardiomyopathy, mimics age-associated carotid arterial remodeling, promotes abdominal aortic aneurysms, and triggers age-related contractile dysfunction. In addition, inhibition of the Ang II type 1 receptor promotes longevity in mice. A wide range of studies aiming to understand the molecular mechanism of the effect of Ang II on aging indicated that Ang II accelerated cellular senescence of vascular smooth muscle cells (VSMCs) both in vitro and in vivo, suggesting a detrimental prosenescence role of Ang II in vascular aging. Thus, to explore Ang II–induced vascular senescence may provide...
a new approach to slow down Ang II–related cardiovascular aging and disorders.

Nicotinic acetylcholine receptors (nAChRs) are a class of ligand-gated ion channels that are widely distributed in central nervous system with specific functional and pharmacological properties. The α7 subtype of nAChR (α7nAChR), which was isolated and sequenced in 1990, is distinguished from other nAChRs by its rapid desensitization and low probability of channel opening. Recently, increasing evidence has extended the role of α7nAChR beyond that of a simple ion channel and has linked this receptor with the cholinergic anti-inflammatory pathway. Our previous report showed that α7nAChR was downregulated in aorta of spontaneously hypertensive rats and α7nAChR−/− mice. It was found that α7nAChR activation in human vascular smooth muscle cells (VSMCs) using α7nAChR agonist, PNU-282987, reduced the expression of proliferating cell nuclear antigen, a molecular marker of proliferation (Figure 1B in the online-only Data Supplement). Ang II treatment also lowered the expression of Ang II receptors (Ang II receptor, type 1 and Ang II receptor, type 2) in VSMCs (Figure IIC in the online-only Data Supplement). Ang II treatment also lowered the expression of proliferating cell nuclear antigen, a molecular marker of proliferation (Figure 1B), suggesting a pro senescent effect of Ang II. PNU-282987 treatment successfully blocked this effect. Ser139 phosphorylation of H2A.X, a nuclear histone that represents ≈10% of the total H2A histone proteins, is a response to DNA damage and represents a marker of replication senescence. Immunofluorescence staining of p-H2A.XSer139 in VSMCs showed that PNU-282987 significantly attenuated the increased fluorescence intensity of phospho-H2A.XSer139 induced by Ang II (Figure IC). Flow cytometer analysis confirmed this result (Figure ID). Edu incorporation assay showed that the Edu incorporation 3 days after Ang II incubation was largely reduced, which was partly reversed by PNU-282987 (Figure 1E). Phosphorylation of checkpoint kinase 1 (Chk1) at Ser317 site (p-Chk1Ser317), another marker of DNA damage, was measured by immunoblotting. Ang II incubation increased phospho-Chk1Ser317 level, which was reduced by PNU-282987 (Figure IF).

We also tested the effect of α7nAChR activation in human aortic smooth muscle cells. β-gal staining showed that the 3-day Ang II incubation–induced senescence was attenuated by PNU-282987 (Figure IIIA in the online-only Data Supplement). Edu incorporation assay and immunoblotting of p-Chk1Ser317 confirmed the inhibitory effects of PNU-282987 on Ang II–induced senescence (Figure IIIB and IIIC in the online-only Data Supplement). Immunoblotting also confirmed this result (Figure IC in the online-only Data Supplement).

Previously, we reported that α7nAChR was downregulated in aorta of rats with 2-kidney 1-clip for 4, 8, and 20 weeks. In this study, we confirmed the downregulation of α7nAChR protein in denuded aortae (without endothelium and perivascular fat) from rats with 2-kidney 1-clip for 4 and 8 weeks (Figure ID in the online-only Data Supplement). These results suggested that the hypertension itself or hypertension-related factors, such as high blood Ang II level, may be the cause of the α7nAChR downregulation in aortic media. To answer this question, we incubated rat VSMCs with different concentrations of Ang II (10–5–10–4 mol/L) for 3 days in vitro. However, we found that both α7nAChR mRNA (Figure 1E in the online-only Data Supplement) and protein (Figure IF in the online-only Data Supplement) were not altered by Ang II incubation. These results suggest that α7nAChR in vascular media was downregulated under hypertension conditions, whereas the increased blood Ang II level in hypertension may not be the direct cause of α7nAChR downregulation.

**Activation of α7nAChR Retards Ang II–Induced VSMC Senescence In Vitro**

To investigate the potential role of the α7nAChR downregulation in Ang II–induced vascular aging, we used PNU-282987, a selective chemical agonist of the α7nAChR. In primary rat VSMCs, activation of α7nAChR by PNU-282987 significantly alleviated the Ang II–induced VSMC senescence (P<0.05, Figure 1A). Pretreatment of VSMCs with methyllycaconitine, a high-affinity antagonist of α7nAChR, successfully abolished the protection of PNU-282987 on VSMC senescence (Figure IIA in the online-only Data Supplement). PNU-282987 or methyllycaconitine alone did not affect the SA-β-gal staining in VSMCs (Figure IIB in the online-only Data Supplement). Moreover, PNU-282987 alone did not affect the expression of Ang II receptors (Ang II receptor, type 1 and Ang II receptor, type 2) in VSMCs (Figure IIC in the online-only Data Supplement). Ang II treatment also lowered the expression of proliferating cell nuclear antigen, a molecular marker of proliferation (Figure 1B), suggesting a pro senescent effect of Ang II. PNU-282987 treatment successfully blocked this effect. Ser139 phosphorylation of H2A.X, a nuclear histone that represents ≈10% of the total H2A histone proteins, is a response to DNA damage and represents a marker of replication senescence. Immunofluorescence staining of p-H2A.XSer139 in VSMCs showed that PNU-282987 significantly attenuated the increased fluorescence intensity of phospho-H2A.XSer139 induced by Ang II (Figure IC). Flow cytometer analysis confirmed this result (Figure ID). Edu incorporation assay showed that the Edu incorporation 3 days after Ang II incubation was largely reduced, which was partly reversed by PNU-282987 (Figure 1E). Phosphorylation of checkpoint kinase 1 (Chk1) at Ser317 site (p-Chk1Ser317), another marker of DNA damage, was measured by immunoblotting. Ang II incubation increased phospho-Chk1Ser317 level, which was reduced by PNU-282987 (Figure IF).

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>α7nAChR</td>
<td>α7 subtype of nAChR</td>
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<tr>
<td>AMPK</td>
<td>AMP-dependent protein kinase</td>
</tr>
<tr>
<td>Ang II</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH oxidase</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>VSMCs</td>
<td>vascular smooth muscle cells</td>
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<td>WT</td>
<td>wild-type</td>
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online-only Data Supplement). These results together indicate that activation of α7nAChR is able to alleviate Ang II–induced VSMC senescence in vitro.

**Activation of α7nAChR Reduces Ang II–Induced VSMC Senescence In Vivo**

To assess the antisenescence effect of α7nAChR activation in vivo, we established a hypertensive mouse model using Ang II infusion for 14 days via ALZET osmotic pump in both WT and α7nAChR−/− mice and treated these mice with α7nAChR agonist PNU-282987. The systolic blood pressure was significantly enhanced after a 14-day infusion of Ang II in both WT and α7nAChR−/− mice (Figure 2A). PNU-282987 treatment did not reduce the Ang II–enhanced blood pressure (Figure 2A). We found that the Ang II–induced SA-β-gal staining in α7nAChR−/− aortae was more severe than that in WT aortae (Figure 2B). PNU-282987 remarkably attenuated the Ang II–induced SA-β-gal–positive
staining in WT but not in α7nAChR−/− aortae (Figure 2B). Immunohistochemistry staining showed that phospho-H2A.XSer139–positive staining (green arrow) was mainly located at the nuclei. Expression of nuclear phospho-H2A.X Ser139 was increased after Ang II infusion in WT aortae and, to a much greater degree, in α7nAChR−/− aortae (Figure 2C). PNU-282987 treatment reduced the Ang II–induced nuclear phospho-H2A.X Ser139 staining in WT but not in α7nAChR−/− aortae (Figure 2C). Immunoblotting analysis of phosphorylation of H2A.X at Ser139 site confirmed these results (Figure 2D).

Figure 2. Effect of α7 nicotinic acetylcholine receptor (α7nAChR) activation on angiotensin II (Ang II)–induced vascular smooth muscle cell senescence in aortae from wild-type (WT) and α7nAChR−/− mice. Mice were infused with saline (control) or Ang II (400 ng/kg per min) or Ang II (400 ng/kg per min)+PNU-282987 (PNU, 1 mg/kg per d) using Alzet osmotic minipumps for 14 d. **A**, Systolic blood pressure (SBP) at 14 d post drugs infusion. *P*<0.05; n=5. **B**, SA-β-gal staining assay on the aortae from WT and α7nAChR−/− mice receiving drugs infusion. *P*<0.05; **P**<0.01; n=5. **C**, Immunohistochemistry staining of p-H2A.XSer139 in WT and α7nAChR−/− aortae. *P*<0.05; n=5. Green arrows indicate the positive staining. **D**, Immunoblotting of p-H2A.XSer139 in WT and α7nAChR−/− aortae. *P*<0.05; n=5. **E**, Immunoblotting of p-Chk1Ser317 in WT and α7nAChR−/− aortae. *P*<0.05; n=5. NS indicates no significance; and p-H2A.X, phospho-H2A.X.
Activation of α7nACHR Suppresses Ang II–Induced Oxidant Stress in VSMCs

We compared the senescence in VSMCs isolated from α7nACHR−/− mice and aged-control WT mice. The senescence, reflected by the SA-β-gal staining, was similar between WT and α7nACHR−/− VSMCs (Figure 3A). Interestingly, PNU-282987 successfully decreased Ang II–induced senescence in WT VSMCs but not in α7nACHR−/− VSMCs (Figure 3A). We evaluated the oxidative stress in VSMCs treated by α7nACHR antagonist under Ang II stimuli. Dichloro-dihydro-fluorescein diacetate analysis was used to determine reactive oxygen species (ROS) level. Ang II treatment for 3 days increased dichloro-dihydro-fluorescein diacetate mean fluorescence density in VSMCs, suggesting that the intracellular ROS level in VSMCs was remarkably increased by Ang II (Figure 3B). Activation of α7nACHR by PNU-282987 partly lowered the increase of ROS level in WT but not in α7nACHR−/− VSMCs (Figure 3B). In addition, we measured H2O2 level using Amplex Red assays. PNU-282987 administration also attenuated H2O2 level in WT but not in α7nACHR−/− VSMCs (Figure 3C). The malondialdehyde (MDA) level, an index of lipid peroxidation, was elevated by Ang II treatment but was suppressed by PNU-282987 (Figure 3D). Total antioxidant level was decreased after Ang II treatment, which was partly prevented by PNU-282987 (Figure 3E). These effects of PNU-282987 on MDA and total antioxidant activity were observed in WT but not in α7nACHR−/− VSMCs (Figure 3D and 3E). These results demonstrate that activation of α7nACHR suppresses oxidant stress induced by Ang II in VSMCs.

Because activation of α7nACHR attenuated oxidative stress induced by Ang II, we further examined the influence of α7nACHR activation on NADPH oxidases (NOXs), a group of electron-transporting transmembrane enzymes whose function is to generate ROS.25 NOX1 and NOX4 are 2 major sources of superoxide in VSMCs in basal condition and Ang II stimulation,25,26 whereas p22phox is another critical component that is required for the formation of ROS on Ang II stimulation in VSMCs.37 We found that Ang II treatment significantly enhanced the protein expression of p22phox, NOX1, and NOX4 (Figure 3F). Activation of α7nACHR by PNU-282987 partly attenuated the Ang II–induced upregulation of p22phox, NOX1, and NOX4 in WT VSMCs but not in α7nACHR−/− VSMCs (Figure 3F).

Activation of α7nACHR Diminishes Ang II–Induced Activation of p53-p21 and p16INK4a Signaling Pathways in VSMCs

Activation of p53 is an important event for cellular senescence process.28 Ang II treatment increased p53 protein expression in VSMCs, which was blocked by PNU-282987 (Figure 4A). Activation of α7nACHR by PNU-282987 or blockade of α7nACHR by methyllycaconitine alone did not change the p53 level (Figure IV in the online-only Data Supplement). In addition, protein expression of p21, a main downstream factor of p53, was also increased by Ang II and partly reduced by PNU-282987 (Figure 4B). It is well established that p53 is a short-lived protein, and its activity is maintained at low levels in normal cells.29 Acetylation of p53 was found to be a key transcriptional mechanism to increase p53 stability and maintain p53 activity.29 Ang II treatment potently increased p53 acetylation, which was inhibited by PNU-282987 (Figure 4C). The p16INK4a pathway is another important regulator of senescence. It limits cell lifespan via a mechanism distinct from that used by p53.30 Ang II induced upregulation of p16INK4a in VSMCs, which was also diminished by PNU-282987 (Figure 4D).

Activation of α7nACHR Raises Intracellular NAD+ Level and Thereby Enhances Sirtuin 1 Activity in Ang II-Treated VSMCs

Sirtuin 1 (SIRT1), a nicotinamide adenine dinucleotide (NAD+)-dependent class III deacetylase,31 is considered to be a longevity protein connecting redox cascades such as NOX (including NOX1, NOX 4, and p22phox) with antiaging signaling pathways, such as p53 and p16INK4a.32,33 Decrease of SIRT1 activity is a characteristic of aging or senescence.31 Intriguingly, activation of α7nACHR by PNU-282987 administration partly rescued the decrease of SIRT1 activity in Ang II–treated cells (Figure 5A and 5B). However, pretreatment of α7nACHR antagonist methyllycaconitine almost totally blocked the rescue of SIRT1 activity by PNU-282987 (Figure 5A and 5B), confirming the essential role of α7nACHR in enhancing SIRT1.

SIRT1 activity is controlled by 2 factors: SIRT1 protein level and its cofactor NAD+.34 In line with previous results,35 Ang II treatment for 3 days in VSMCs resulted in a significant reduction of SIRT1 protein expression; however, activation of α7nACHR by PNU-282987 had no effect on SIRT1 protein level (Figure 5C). We further studied the effect of α7nACHR activation on NAD+ level. Activation of α7nACHR by PNU-282987 reversed the Ang II–induced decline of NAD+ levels (Figure 5D). Accordingly, PNU-282987 reversed the decline of NAD+/NADH ratio by Ang II (Figure 5E). These results suggest that activation of α7nACHR enhances SIRT1 activity through raising intracellular NAD+ level but not modulating SIRT1 protein expression in Ang II–treated VSMCs.

A recent study has shown that AMP-dependent protein kinase (AMPK) and SIRT1 can regulate each other, and AMPK can also increase intracellular NAD levels.36 So we also tested whether AMPK plays a role in the enhancement of the NAD+ pool by α7nACHR activation. Activation of α7nACHR failed to induce AMPK phosphorylation in VSMCs (Figure V in the online-only Data Supplement). Thus, the stimulation of NAD+–SIRT1 axis by α7nACHR activation is AMPK independent.

SIRT1 Activity Is Critical for the Antisenescence Effects by α7nACHR Activation

If the antisenescence effects of α7nACHR activation require SIRT1 activity, blockade of SIRT1 activity could abolish the
Figure 3. Effects of α7 nicotinic acetylcholine receptor (α7nAChR) activation on angiotensin II (Ang II)–induced free radical/oxidant stress in cultured vascular smooth muscle cells (VSMCs) isolated from wild-type (WT) and α7nAChR−/− mice. A, SA-β-gal staining showing the effect of α7nAChR activation on Ang II–induced senescence in WT and α7nAChR−/− VSMCs. *P<0.05 vs Ang II; n=5. B, Quantitative analysis of reactive oxygen species (ROS) using dichloro-dihydro-fluorescein diacetate assay in WT and α7nAChR−/− VSMCs. *P<0.05 vs control; #P<0.05 vs Ang II; n=4. C, H₂O₂ levels in WT and α7nAChR−/− VSMCs. *P<0.05 vs control; #P<0.05 vs Ang II; n=4. D and E, Effect of α7nAChR activation on intracellular malondialdehyde (MDA) and total antioxidant levels on Ang II treatment. *P<0.05 vs control; #P<0.05 vs Ang II; n=6. F, Representative images and quantitative analysis of p22phox, NADPH oxidase 1 (NOX1), and NOX4 protein levels. *P<0.05 vs control; #P<0.05 vs Ang II; n=4. Ang II, 10−7 mol/L; PNU, 5×10−6 mol/L. NS indicates no significance.
The α7nAChR was once thought to be a neural-specific receptor. However, the discovery of vagus nerve–dependent cholinergic anti-inflammatory pathway mediated by α7nAChR in peripheral organs has expanded our understanding on α7nAChR. In our previous studies, we have shown that α7nAChR is expressed in aorta and endothelial cells. The downregulation of α7nAChR was associated with increased inflammation in organs and contributed to pathogenesis of end-organ damage in hypertension. Notably, some previous data have suggested a close relationship between Ang II and α7nAChR. α7nAChR exerts neuroprotective effects via activation of the JAK2 cascade in brain, which can be blocked by Ang II. AR-R17779, another α7nAChR agonist, suppresses atherosclerosis and Ang II–induced aortic aneurysm formation in apolipoprotein–E–deficient mice. Another interesting issue is the endogenous ligand for α7nAChR in VSMCs. The well-accepted endogenous ligand for α7nAChR is acetylcholine. However, whether tunica media has cholinergic nerve terminals is not conclusive yet. Cholinergic nerve fibers are present in cerebral blood vessels. A few fibers were seen in close proximity to the vasa vasorum within the tunica media of the ascending aorta. Cholinergic nerve terminals were also visualized in nerve terminal appearing structures in the adventitia of small intramuscular arteries. We speculate that the acetylcholine released by cholinergic nerve terminals may diffuse into tunica media and functions on VSMCs. Indeed, we found that the vascular aging was significantly promoted by Ang II infusion for 2 weeks, which was more severe in α7nAChR−/− mice compared with WT mice. These results suggest that α7nAChR deficiency may result in an acceleration of vascular aging. Importantly, we also observed that the activation of α7nAChR by PNU-282987 relieved the Ang II–induced vascular aging in WT mice but not in α7nAChR−/− mice. These phenotypes were confirmed in cultured VSMCs isolated from WT and α7nAChR−/− mice. Taken together, our results add weight to the concept that α7nAChR activation may be a strategy against the detrimental effects of Ang II on blood vessels, such as prothrombotic phenotype and hypertension-associated abdominal aortic aneurysms, besides the well-known hypertension.

It is well established thatoxidant stress is one of the major factors in the process of aging. We found that pharmacological activation of α7nAChR by PNU-282987 remarkably...
suppressed the Ang II–induced oxidant stress in VSMCs, which is in line with our previous study in endothelial cells. PNU-282987 also reduced p53 acetylation and thereby inhibited p53-p21 signaling pathway via increasing NAD+ level. ROS acts as both an upstream signal that triggers p53 activation and a downstream factor that mediates p53-induced senescence. Inhibition of p53/p21, p16 INK4a, and ROS by activating α7nAChR in Ang II–treated VSMCs suggests that α7nAChR may affect a common path that lies upstream of p53/p21, p16INK4a, and ROS. Meanwhile, we found that Ang II activated p16INK4a pathways, which was partly prevented by the activation of α7nAChR. The proteins p16INK4a and p53 induce senescence via different mechanisms. Similar crosstalk between ROS and p16INK4a pathway was also observed. Thus, we consider that the inhibition on ROS production may be an important mechanism for the effects of α7nAChR. Indeed, the ROS scavengers, such as tempol or N-Acetyl cysteine, have been demonstrated to protect against Ang II–induced senescence in many previous studies. However, the inhibitory action on ROS production may be not the only mechanism underlying the effects of α7nAChR activation on senescence.

We next focused on the role of SIRT1 in the antisenescence effect of α7nAChR. SIRT1 is a member of the highly conserved gene family of sirtuins, which were originally identified in yeast as a major family of NAD+-dependent deacetylases. SIRT1 has been reported to deacetylate not only histones but also nonhistone substrates, such as p53, FoxO1, and FoxO3a. There is a growing body of evidence showing that SIRT1 is a longevity gene responsible for the life extension induced by calorie restriction. In addition, SIRT1 is a cardiovascular protector, protecting against aortic dissection during Ang II–induced hypertension. Importantly, the activity of SIRT1 requires the sustained intracellular NAD+ level. Without the cofactor NAD+, SIRT1 cannot function properly. NAD+ levels decline during the aging process, resulting in defects in nuclear/mitochondrial functions and age-associated pathologies. In our study, we for the first time found that Ang II treatment induced significant NAD+ decline in VSMCs. Morever, this effect was rescued by α7nAChR activation. Although α7nAChR activation was unable to upregulate SIRT1, it reversed the decline of NAD+ levels by Ang II and thereby increased SIRT1 activity.

![Figure 5](image-url)

**Figure 5.** Effects of α7 nicotinic acetylcholine receptor activation on sirtuin 1 (SIRT1) activity and intracellular NAD+ level on angiotensin II (Ang II) treatment in vascular smooth muscle cells (VSMCs). A and B, Effects of Ang II or Ang II+PNU on SIRT1 activity in VSMCs for 2 and 3 d under vehicle or methyllycaconitine (MLA) pretreatment conditions. *P*<0.05 vs control; #*P*<0.05 vs Ang II; n=6. C, SIRT1 protein expression was determined by immunoblotting. *P*<0.05 vs control; n=4. D and E, NAD+ concentration and NAD+/NADH ratio in VSMCs. *P*<0.05 vs control; #*P*<0.05 vs Ang II; n=6. Ang II, 10−7 mol/L; PNU, 5×10−6 mol/L. NS indicates no significance.
antisenescence effect mediated by α7nAChR. These findings underscore the importance of NAD+ pool and SIRT1 activity, as the molecular link between α7nAChR and Ang II in vascular aging pathogenesis. AMPK regulates energy expenditure by modulating intracellular NAD+ level and thereby SIRT1 activity. In our setting, activation of α7nAChR did not activate AMPK signaling pathway, suggesting that the promotion of NAD+–SIRT1 axis by activation of α7nAChR is AMPK independent. These results indicate that activation of α7nAChR may be a promising therapeutic target for Ang II–related vascular diseases. However, it is also important to bear in mind that activation of α7nAChR may accelerate angiogenesis and metastasis of lung cancer, involving JNK, PI3K, and PKA signal pathway. Therefore, it is important to pay careful attention to this potential detrimental role of α7nAChR in tumorigenesis when developing new chemical agents to target α7nAChR.

**Sources of Funding**

This work was supported by grants from the National Science Foundation of China (No. 81300081 to D.J. Li and No. 81370558 to F.M. Shen), Shanghai Natural Science Foundation (No. 13ZR1459300 to D.J. Li), and the Fundamental Research Funds for the Central Universities Multi-Subjects Crossing of Tongji University (No. 1501219097 to D.J. Li).

**Disclosures**

None.
References


Highlights

- Angiotensin II (Ang II) plays a detrimental role in vascular aging.
- α7 nicotinic acetylcholine receptor (α7nAChR) is a subtype of nAChR.
- Our previous study showed that α7nAChR was downregulated in hypertensive rat aortae.
- In this study, we demonstrate that pharmacological activation of α7nAChR protected rodent and human vascular smooth muscle cells from Ang II–induced senescent phenotypes, such as SA-β-gal activity and phosphorylation of histone H2A.Xα5935/Chk1α5375 in vivo and in vitro.
- Furthermore, α7nAChR activation by pharmacological agonist abolished the effects of Ang II on oxidative stress, lipid peroxidation, total antioxidant activity, and expression of NADPH oxidase 1, NADPH oxidase 4, p53, acetyl-p53, p21, and p16[AR].
- Finally, we described a novel mechanism that α7nAChR activation enhanced sirtuin 1 activity through elevating intracellular NAD+ level.
- These results may provide new insights into the molecular interaction between α7nAChR and Ang II in vascular smooth muscle cells and suggest that activating α7nAChR pharmacologically may be a useful intervention in the treatment of Ang II–related vascular aging.
α7 Nicotinic Acetylcholine Receptor Relieves Angiotensin II–Induced Senescence in Vascular Smooth Muscle Cells by Raising Nicotinamide Adenine Dinucleotide–Dependent SIRT1 Activity
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Arterioscler Thromb Vasc Biol, 2016;36:1566-1576; originally published online June 23, 2016;
doi: 10.1161/ATVBAHA.116.307157

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/36/8/1566

Data Supplement (unedited) at:
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Angiotensin II

VSMCs

Oxidative stress
NOX1/NOX4
ROS
H₂O₂
MDA

SIRT1 activity
NAD⁺

p53 acetylation
p21
p16INK4a

Senescence
SA-β-gal
p-H2A.XSer139
p-Chk1Thr317
PCNA

Crosstalk

Small molecules
Acetylcholine (endogenous)
PNU282987, ..., et al. (exogenous)

α7nAChR
Supplemental data

α7 nicotinic acetylcholine receptor relieves angiotensin II-induced senescence in vascular smooth muscle cells by raising NAD-dependent SIRT1 activity

Running title: α7AChR inhibits Ang II-induced VSMC senescence

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Effect of Ang II on $\alpha_7nAChR$ expression in VSMCs. (A-B) Immunofluorescence analysis showing the $\alpha_7nAChR$ is expressed in rat VSMCs and WT mice VSMCs. However, it is not expressed in $\alpha_7nAChR^{-/-}$ mice. $\alpha$-SMA is a specific marker of VSMCs (Green). DAPI is used to stain nuclei (Blue). $\alpha_7nAChR$ polyclonal antibody is used to stain $\alpha_7nAChR$ (Red). (C) Representative immunoblotting image of $\alpha_7nAChR$ expression in VSMCs and brain tissues from WT or $\alpha_7nAChR^{-/-}$ mice. $\alpha_7nAChR$ is abundantly expressed in brain tissue (positive control) of WT mice. (D) Protein expression of $\alpha_7nAChR$ in denuded aortae (without endothelium) of 2K1C rats for 4 and 8 weeks. $*P < 0.05$ versus control. N = 4. (E-F) Influence of Ang II ($10^{-8}$~$10^{-6}$ mol/L) on mRNA and protein levels of $\alpha_7nAChR$ in cultured rat VSMCs. N = 6. NS, no significance.
**Antagonistic effects of MLA on the anti-senescence action of α7nAChR activation in cultured rat VSMCs.** (A) MLA (1×10⁻⁵ mol/L) blocked the anti-senescence action of PNU-282987 (5×10⁻⁶ mol/L). *P < 0.05 versus control; #P < 0.05 versus AngII. &P < 0.05 versus Ang II + PNU. N = 4. (B) Incubation of PNU-282987 (PNU, 5×10⁻⁶ mol/L) or MLA (1×10⁻⁵ mol/L) alone for 3 days did not affect the senescence of VSMCs. Ang II (10⁻⁷ mol/L) was used as a positive control that induces VSMC senescence. NS, no significance. N=3. (C) Effect of PNU-282987 (PNU, 5×10⁻⁶ mol/L, 1 day or 3 days) on Ang II receptor type 1 (AT1R) and AT2R expression in VSMCs. NS, no significance. N = 4.
Effect of α7nAChR activation on Ang II-induced senescence in human aortic VSMCs (HAoSMCs). (A) SA-β-gal staining in HAoSMCs. Treatments were conducted as those in rat VSMCs. *P < 0.05 versus control; #P < 0.05 versus Ang II. N = 5. (B) Edu incorporation assay in HAoSMCs. *P < 0.05 versus control; #P < 0.05 versus Ang II. N = 5. (C) Effect of Ang II and PNU treatment on senescence marker phospho-Chk1Ser317 in HAoSMCs. p-Chk1Ser317, phospho-Chk1Ser317. *P < 0.05 versus control; #P < 0.05 versus Ang II. NS, no significance. N= 4. PNU, 5 × 10^-6 mol/L. Ang II, 10^-7 mol/L.
Incubation of PNU-282987 (PNU, $5 \times 10^{-6}$ mol/L) or MLA (1×10⁻⁵ mol/L) alone for 3 days did not affect the p53 protein level in cultured rat VSMCs. Ang II (10⁻⁷ mol/L) was used as a positive control that induces VSMC senescence. NS, no significance. N = 4.
Supplemental Figure V

Activation of α7nAChR failed to induce AMPK phosphorylation in cultured rat VSMCs. AICAR (1×10^{-4} mol/L), an AMPK activator, successfully induced AMPK phosphorylation at Thr172 site. In contrast, activation of α7nAChR by PNU-282987 (5×10^{-6} mol/L) did not induce AMPK phosphorylation. *P < 0.05 versus Ctrl. NS, no significance. N = 4.
Transfection of siRNA-scramble in primary rat VSMCs did not influence NAD$^+$ concentration (A), SIRT1 activity (B) and SA-β-gal activity (C). N = 6 per group. NS, no significance.
Effects of SIRT1 inhibitor EX527 on the anti-senescence action of α7AChR activation.

(A) EX527 treatment (50 μmol/L) blocked the attenuation of ROS by PNU-282987 (5×10^{-6} mol/L) in Ang II (10^{-7} mol/L)-treated rat VSMCs. (B) EX527 abolished the attenuation of MDA level by PNU-282987 in Ang II-treated rat VSMCs. (C) EX527 blocked the reduction of SA-β-gal staining by PNU-282987 in Ang II-treated rat VSMCs. Ang II was used to induce VSMC senescence. *P < 0.05 versus control; #P < 0.05 versus Ang II; &P< 0.05 versus Ang II + PNU. N = 4.
ATVB/2016/307157R2

Materials and Methods

Animals
Sprague-Dawley rats were purchased from Sino-British SIPPR/BK Lab Animal Ltd. (Shanghai, China). α7nAChR−/− mice (Stock No: 003232) were purchased from Jackson laboratory and described in our previous study.1 Animals were used in accordance with our institutional guidelines for animal care and the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health.

Reagents
Ang II (#A6778), AICAR (#A9978), PNU-282987 (#P6499), anti-PCNA (#SAB2701819), anti-SIRT1 (#S5322), GAPDH (#G8795) and anti-β-actin (#A1978) were obtained from Sigma Chemical Co. (St. Louis, MO). EX527 (#S1541) was purchased from Selleck Chemicals. Antibodies against α-smooth muscle actin (α-SMA, # AB5694), anti-α7nAChR (#AB10096), NADPH oxidase 1 (NOX1, #AB155831), NOX4 (#AB60940), p53 (#AB28), p21 (#AB109520) and p16INK4a (#AB189034) were purchased from Abcam (Cambridge, MA). Senescence-associated β-galactosidase (SA-β-gal) staining kit (#CBA-230) was from Cell BioLabs (San Diego, CA). Antibodies against p-Chk1 (#12302), t-Chk1 (#2360), p-AMPK (#4181), t-AMPK (#2532), acetyl-p53 (#2570), phospho-Histone H2AX Ser139 (for immunofluorescence, #5438) and PE-conjugated phospho-H2AX Ser139 (for flow cytometer, #5763) were purchased from Cell Signaling Technology Inc. (Beverly, MA). All secondary IRDye-conjugated antibodies were purchased from Li-Cor Biosciences (Lincoln, NE). Dichloro-dihydro-fluorescein diacetate (DCFH, #N7991), Alexa 488/555-conjugated secondary antibodies and DAPI fluorescent probe (#62248) were purchased from Invitrogen (Carslbad, CA). Enhanced chemiluminescence (#34095) and protease/phosphatase inhibitors (#88668) were purchased from Pierce (Rockford, IL). SIRT1 activity assay (#AK-555) was from Biomol (Plymouth Meeting, PA). Nicotinamide adenine dinucleotide (NAD+)/NADH assay (#600480) was from Cayman Chemical (Ann Arbor, MI). Commercial siRNA targeting SIRT1 (#SC-40987) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Reactive aldehydes assay kit (#STA-330) was purchased from Cell BioLabs (San Diego, CA). Click-iT EdU Microplate Assay (#C10214) was purchased from Invitrogen™ Thermo Fisher Scientific.

Human and rodent VSMCs culture
Human aortic smooth muscle cells (HAoSMCs) were purchased from Lifeline Cell Technology (Walkersville, MD). These cells were grown in Dulbecco’s modified Eagle’s medium-high glucose (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS) (Life Technology, Grand Island, NY), 50 U/ml penicillin and 50 µg/ml streptomycin in a humidified atmosphere of 5% CO2. The cells up to passage number 8 were used in the study. The rat and mouse VSMCs were isolated and cultured using a standard enzymatic digestion technique.2 Animals were sacrificed with an overdose of pentobarbital sodium (100 mg/kg) by intra-peritoneal injection before the aorta was excised. The vessels were cleaned of adipose and connective tissue and placed in an enzymatic solution (1 mg/ml collagenase II)
for 30 minutes. Then, the tissues were transferred to serum-free medium, cleaned for adventitia under dissection stereoscope, and placed in an enzymatic solution (1 mg/ml collagenase and 0.25 mg/ml elastase) for an additional 1 hour. After that, the partially digested tissue with enzyme solution was transferred into a 15 ml conical tube and gently triturated with a flame-polished glass pipette. The mixture was centrifuged at 1,000 rpm for 5 min and the supernatant was discarded. The dissociated cells were resuspended in 2-3 ml of culture medium (DMEM with 10% FBS) and incubated in 5% CO₂ plus 95% O₂. Experiments were performed using cells between passages 3 and 8.

**Ang II-infusion induced hypertension mouse model**

Ang II-infusion induced hypertensive mouse model was established as described previously.[3] Mice were infused with saline (control), Ang II (400 ng/kg/min)[3] and Ang II + PNU282987 (1 mg/kg/d)[4,5] using Alzet osmotic mini-pumps (#1002, DURECT Corp.) for 14 days. In mice receiving Ang II + PNU282987 infusion, the two agents were infused using two pumps respectively. Blood pressure was determined in these mice with a noninvasive, computerized tail-cuff system (ALC-NIBP, ALCBIO, Shanghai, CHINA). At the end of the experiment, mice were killed, and the aortae were dissected, and cut into three portions for SA-β-gal staining, immunohistochemistry staining and immunoblotting respectively.

**SA-β-gal staining**

SA-β-gal activity was determined as described using a commercial kit.[6] VSMCs were plated at low density (5 × 10⁴) in 12-well plates. Subconfluent VSMCs were cultured in DMEM containing 1% FBS overnight to induce a quiescent state before the following experiments with Ang II and PNU282987. PNU282987 was added into the medium for 4 hours and then Ang II was added. Moreover, the medium and stimulants/inhibitors were refreshed every day during 3 day incubation experiments. After incubation with Ang II and other compounds for 3 days, cells were washed twice with PBS and fixed with 0.2% glutaraldehyde in PBS for 10 min. Then, cells were incubated with β-gal staining solution (150 mM NaCl, 2 mM MgCl₂, 40 mM citric acid, 5 mM potassium ferrocyanide, 12 mM sodium phosphate, pH 6.0, containing 1 mg/mL X-gal) for 24 hours at 37°C. Cells were washed twice with PBS and kept in 70% glycerol. SA-β-gal images were got using light microscope (Olympus IX81 microscope) under blinded conditions. Photos of cells were manually randomized obtained (at least 5 visual fields), and the percentage of senescence staining was determined with Image J software (NIH, Bethesda, MD, USA). For SA-β-gal assay in vivo, the aortae were fixed with 0.2% glutaraldehyde in PBS for 20 min and then incubated with β-gal staining solution for 24 hours at 37°C. Aortae were washed twice with PBS. Under surgical microscope, the aortae were fixed using needles in plates. The images were then obtained using camera and analyzed using Image J software. The blue staining was considered to be positive staining.

**Real-time PCR**

Real-time PCR for determining α7nAChR mRNA was performed as described previously.[1] Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen) according to the instructions of the manufacturer. The RNA was treated with RNase-free DNase I to reduce the risk for genomic DNA contamination and 2 µg RNA was reverse transcribed to
cDNA using the M-MLV enzyme (Promega, Madison, WI). Real-time quantitative PCR was performed using the ABI7500 real-time PCR detection system (ABI System) and the SYBR Premix Ex Taq Mixture (Takara) with specific primers: rat α7nAChR, sense, 5'-GGTCGTATGGTGCCGTGGT-3'; anti-sense, 5'-TGCGGTTGGGAGAATATGCG-3'; rat GAPDH, sense, 5'-AGACCTCTATGCAACACAGCT-3'; anti-sense, 5'-GAGCCACCAATCCACACAGGT-3'. The PCR reactions were initiated with denaturation at 95°C for 10 s, followed by amplification with 40 cycles at 95°C for 10 s, and annealing at 60°C for 20 s (two-step method). Finally, melting curve analysis was performed from 60°C to 85°C. The rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control. All samples were performed in duplicate. The relative expression of the target genes was normalized to the level of GAPDH in the same cDNA.

Reactive oxygen species (ROS), lipid peroxidation and total antioxidant assays

ROS was measured using flow cytometer with DCFH as described previously. VSMCs were incubated with the DCFH fluorescent probe for 30 minutes. After being washed 3 times by ice cold PBS, the cells were subjected to flow cytometer. The fluorescence intensity was measured at an excitation wavelength of 488 nm. The mean fluorescence intensity was obtained. Lipid peroxidation was evaluated using malondialdehyde (MDA) content as described previously. Total antioxidant was measured using a commercial assay (OxiSelect™ Total Antioxidant Capacity Assay Kit) based on reduction of copper(II) to copper(I). The MDA and total antioxidant concentrations of each sample were detected according to the manufacturer’s instruction using microplate reader respectively. H₂O₂ level was determined using Amplex® Red Enzyme Assays (Life Technologies, Grand Island, NY, USA).

Immunoblotting

Immunoblotting analyses of cell-extracts were performed as described previously. Cells were lysed with RIPA buffer with protease and protein phosphatase inhibitors. Samples were separated in 12% SDS-PAGE, and transferred onto nitrocellulose membranes at 100V for 1-2 h. Nonspecific protein binding was blocked with 5% weight (w)/v nonfat milk and 0.1% v/v Tween-20 in Tris-buffered saline (TBS) followed by incubation with primary antibodies (PCNA, 1: 800; p53, 1: 1000; acetyl-p53, 1: 300; p21, 1: 1000; p16INK4a, 1: 1000; NOX1, 1:600; NOX4, 1: 1000; p-Chk1, 1:1000; Chk1, 1:1000; p-H2A.X, 1:1000; H2A.X, 1:2000; β-actin, 1: 5000; GAPDH, 1: 5000) diluted in TBS containing 2% w/v bovine serum albumin and 0.1% v/v Tween-20. Then the membranes were incubated with IRDye-labeled secondary antibody and detected by Odyssey system.

Immunofluorescence and immunohistochemistry

Immunofluorescence and immunohistochemistry were performed as described previously. For immunofluorescence, VSMCs were seeded at low density in confocal dishes approximately 24 h prior to imaging to ensure proper cell attachment. The cells were fixed with 4% paraformaldehyde and incubated with primary antibody overnight at 4°C and followed by Alexa-488 or Alexa-555 labeled secondary antibodies for 1 hour. DAPI was used to stain nuclei. Images were captured by a confocal laser scanning microscope (Olympus).
Fluoview FV1000, Tokyo, Japan). For immunohistochemistry, tissues were fixed in 4% paraformaldehyde and embedded in paraffin. The tissues were cut into 8-μm thick sections, which were blocked by 8% normal donkey serum (Jackson ImmunoResearch Labs, West Grove, PA, USA), and incubated in rabbit anti-p-H2A.X\textsuperscript{Ser139} (1:500) at 4°C for overnight. After being washed 3 times by PBST (0.1% Tween 20 in PBS [#70011-044, Gibco]), the sections were incubated with 0.3% H\textsubscript{2}O\textsubscript{2} in TBS for 15 min. Then, the sections were incubated with HRP-conjugated secondary antibody for 4 hours, washed for 3 times and incubated in chromogenic reagent. Finally, the sections were counterstained with hematoxylin. Images were obtained using a computerized microscope (Leica, Berlin, Germany).

For each antibody used in immunofluorescence and immunohistochemistry, we conducted validation before using it as follows: [1] performing immunoblotting analysis to demonstrate specific bands of the appropriate molecular weight, with minimal cross-reacting bands; [2] detect the antigens in paraffin-embedded tissues of known target expression levels to verify target specificity; [3] performing negative control in immunofluorescence and immunohistochemistry assays.

**Measurements of NAD\textsuperscript{+} and SIRT1 activity**

The assays for NAD\textsuperscript{+} level and SIRT1 activity were performed as described previously\textsuperscript{[10]}.

NAD\textsuperscript{+} levels were determined with a NAD\textsuperscript{+} quantification kit (BioVision). To evaluate the SIRT1 activity, extracts were obtained using a RIPA lysis buffer plus protease inhibitor mix. Samples were incubated for 10 minutes at 30°C to allow for NAD\textsuperscript{+} degradation, and incubated for 10 additional minutes with DTT 2 μM. SIRT1 in the tissues lysed by RIPA buffer was enriched by immunoprecipitation using anti-SIRT1 specific antibody (#07-131; Upstate Biotechnology) and were then subjected to a deacetylation assay using the SIRT1Fluorimetric Drug Discovery Kit (#AK-555, Biomol).

**siRNA transfection**

Knockdown by siRNA was performed with Lipofectamine® LTX Reagent (Invitrogen) according to the manufacturer’s instruction. Briefly, cells with confluence ~70% were washed by PBS twice and then added into prepared siRNA-SIRT1-lipid complexes. After incubation for 6 hours, the medium was removed. Three days later, the efficiency of knockdown of SIRT1 was checked by real-time PCR. The biochemical analyses were also performed on day 3 after transfection.

**Statistical analysis**

Data was expressed as mean ± SEM. All statistical calculations were performed using the SPSS software and GraphPad Prism 5 software. All data were tested with SPSS software to determine their distribution. For normal distribution data, unpaired t-tests or One-way ANOVA followed by Tukey correction was used. For non-normal distribution data, Mann-Whitney U test was used. Significance was set at $P < 0.05$. 


References


