Orphan Nuclear Receptor Nur77 Inhibits Angiotensin II–Induced Vascular Remodeling via Downregulation of β-Catenin

Mingli Cui,* Zhaohua Cai,* Shichun Chu, Zhe Sun, Xiaolei Wang, Liuhua Hu, Jing Yi, Linghong Shen, Ben He

Abstract—Angiotensin II (Ang II) is the predominant effector peptide of the renin–angiotensin system. Ang II contributes to vascular remodeling in many cardiovascular diseases (eg, hypertension, atherosclerosis, restenosis, and aneurysm). Orphan nuclear receptor Nur77 has a crucial role in the functional regulation of vascular cells. The objective of this study was to define the specific role of Nur77 in Ang II–induced vascular remodeling. Nur77 expression was initially found to be elevated in medial vascular smooth muscle cells (VSMCs) of thoracic aortas from mice continuously infused with Ang II for 2 weeks using a subcutaneous osmotic minipump. Cellular studies revealed that Nur77 expression was upregulated by Ang II via the MAPK/PKA-CREB signaling pathway. Ang II–induced proliferation, migration, and phenotypic switching were significantly enhanced in VSMCs isolated from Nur77−/− mice compared with wild-type VSMCs. Consistent with the role in VSMCs, we found that compared with wild-type mice, Nur77−/− mice had elevated aortic medial areas and luminal diameters, more severe elastin disruption and collagen deposition, increased VSMC proliferation and matrix metalloproteinase production, and decreased VSMC-specific genes SM-22α and α-actin expression, after 2 weeks of exogenous Ang II administration. The results of additional experiments suggested that Nur77 suppressed Ang II–induced β-catenin signaling pathway activation by promoting β-catenin degradation and inhibiting its transcriptional activity. Our findings indicated that Nur77 is a critical negative regulator of Ang II–induced VSMC proliferation, migration, and phenotypic switching via the downregulation of β-catenin activity. Nur77 may reduce Ang II–induced vascular remodeling involved in many cardiovascular diseases. (Hypertension. 2016;67:153-162. DOI: 10.1161/HYPERTENSIONAHA.115.06114.) • Online Data Supplement

Key Words: angiotensin II ■ beta catenin ■ cardiovascular diseases ■ hypertension ■ orphan nuclear receptor Nur77 ■ vascular remodeling

Vascular remodeling is a complicated pathophysiological process implicated in many cardiovascular diseases. Accumulating evidence supports the pivotal role of the activation of systemic or local renin–angiotensin system (RAS) in vascular remodeling during hypertension, atherosclerosis, vascular injury and restenosis, and aneurysm formation.1 Angiotensin II (Ang II), the predominant effector peptide of RAS, contributes to vascular remodeling by promoting vascular smooth muscle cell (VSMC) proliferation and migration, inflammatory processes and matrix deposition, and leading to medial thickening and vascular fibrosis.2 Diverse signaling pathways and molecules that include mitogen-activated protein (MAP) cascades, protein kinase C, and protein kinase A (PKA) are activated by Ang II during the remodeling process.3,4 Vascular remodeling associated with RAS hyperactivation has been clearly implicated in end organ damage and is associated with poor cardiovascular prognosis.5 A more detailed understanding of the molecular mechanisms underlying this pathophysiological process is of great importance.

The orphan nuclear receptor Nur77, also known as TR3, NGFI-B or NR4A1, is an immediate early gene that plays a crucial role in the functional regulation of cell differentiation, proliferation, apoptosis, and inflammation.5-7 Numerous studies indicate that Nur77 is involved in many cardiovascular diseases (eg, atherosclerosis, cardiac hypertrophy, and cardiac ischemia/reperfusion injury).8-10 Recently, Arkenbout et al11 reported that Nur77 acts as a modulator of neointima formation by inhibiting VSMC proliferation. Nur77 also inhibits flow-induced vascular remodeling in SMC-specific overexpression of Nur77 transgenic mice.12 Nur77 is upregulated
by Ang II in adrenocortical cells and cardiomyocytes and has critical roles in hypothalamic–pituitary–adrenal axis function and development of cardiac hypertrophy.\textsuperscript{9,11} We speculated that Nur77 may participate in Ang II–induced proliferative or dedifferentiation signals during the vascular remodeling associated with RAS hyperactivation.

The Wnt/β-catenin pathway is an important signaling pathway that regulates cardiac and vascular development and homeostasis.\textsuperscript{14,15} Many studies have revealed the critical pathophysiological roles of the Wnt/β-catenin pathway in vascular remodeling.\textsuperscript{16–19} Tsaousi et al\textsuperscript{18} found that Wnt/β-catenin signaling induces VSMC proliferation and is associated with intimal thickening in ligated arteries. Diverse physiological stimuli, including oxidized low-density lipoprotein and growth factors, activate β-catenin signaling in VSMCs.\textsuperscript{18,19} Activation of Wnt signaling results in the accumulation of cytosolic nonphosphorylated β-catenin (stable and active forms), and its subsequent translocation to the nucleus to induce the transcription of multiple target genes (eg, cyclin D1, c-myc, matrix metalloproteinases [MMPs]), which promote VSMC proliferation and migration. The interplay between Nur77 and the Wnt/β-catenin signaling pathway in cancer cells has been well reported in in vitro and in vivo settings.\textsuperscript{20,21} Nur77 significantly suppresses Wnt/β-catenin activity via distinct mechanisms.\textsuperscript{20,21} However, there is no direct information is available about the role of β-catenin in Ang II–induced vascular remodeling, and the mechanism by which β-catenin is regulated.

We developed a mouse model of vascular remodeling that used exogenous Ang II administration to examine the specific role of Nur77 in Ang II–induced vascular remodeling and to identify the crosstalk between the orphan nuclear receptor Nur77–mediated and Wnt/β-catenin signaling pathways. This study is the first to find that the Nur77 is abundantly expressed in mouse aortic SMCs and is significantly upregulated by Ang II in cultured VSMCs in vitro. The results of additional experiments indicated that Nur77 inhibited Ang II–induced VSMC proliferation, migration, and phenotypic switching via the downregulation of β-catenin activity. These findings implicate Nur77 as a critical negative regulator of the vascular remodeling associated with RAS hyperactivation.

Materials and Methods
Detailed description of Materials and Methods is available in the online-only Data Supplement.

Results
Nur77 Is Highly Expressed in VSMCs In Vivo and In Vitro in Response to Ang II
For evaluation of the potential role of Nur77 in Ang II–induced vascular remodeling, C57BL/6 mice were continuously infused with Ang II using an osmotic minipump. The thoracic aortas were isolated after 1 and 2 weeks. Nur77 staining was markedly increased in the thoracic aortas after vascular remodeling (Figure 1A and 1B). Dual immunofluorescence staining colocalized Nur77 predominantly to the medial VSMCs. The results of the quantitative real-time polymerase chain reaction analysis consistently indicated that there was a significant upregulation of Nur77 mRNA in the thoracic aorta sections of the Ang II–infused mice compared with the control mice (Figure 1C).

We next examined the expression of Nur77 in cultured VSMCs in response to Ang II stimulation in vitro. Nur77 mRNA was rapidly induced by Ang II (500 nmol/L) and reached a plateau at 1 hour (Figure 1D). The protein expression of Nur77 increased in a dose-dependent manner and peaked 3 hours after Ang II stimulation (Figure 1E and 1F).

Nur77 Expression Is Regulated by the MAPK/PKA-CREB Pathway
The transcription factor cAMP response element–binding protein (CREB) has previously been demonstrated to regulate Nur77 expression in various cell types.\textsuperscript{22,23} Moreover, there were 4 CREs located in the promoter of the Nur77 gene (Figure 2A). To explore the molecular mechanism by which Nur77 expression is regulated, we probed for activation of CREB signaling by Ang II. Ang II rapidly induced phosphorylation of CREB, which peaked at 5 minutes (Figure 2B). Inhibition of P38 MAPK by SB203580 and of ERK1/2 by PD98059 attenuated the Ang II–induced phosphorylation of CREB (Figure 2C). Inhibition of JNK did not affect the level of CREB phosphorylation. The PKA inhibitor H-89 also markedly attenuated phosphorylation of CREB (Figure 2D). These results indicated that CREB might be a downstream target of the P38/ERK1/2/PKA pathway after Ang II stimulation.

Furthermore, we performed loss-of-function studies using CREB-specific siRNA. The upregulation of Nur77 protein expression after Ang II stimulation was attenuated in VSMCs transfected with si-CREB (Figure 2E). Consistently, an ≈75% knockdown of CREB in cultured VSMCs markedly reduced the Ang II–induced upregulation of Nur77 mRNA expression (Figure 2F). Moreover, we found that AngII–induced Nur77 expression was markedly inhibited by ERK inhibitor PD98059, P38 inhibitor SB203580, and PKA inhibitor H-89 (Figure 2G). Therefore, these results revealed that Nur77 upregulation induced by Ang II is partially mediated via MAPK/PKA and the transcription factor CREB.

Nur77 Deletion Enhances Ang II–Induced VSMC Proliferation, Migration, and Phenotypic Switching
To establish the functional significance of Nur77, we examined the effects of Nur77 on Ang II–induced VSMC proliferation, migration, and phenotypic switching. Cell proliferation assays revealed that VSMCs isolated from Nur77\textsuperscript{−/−} mice (Nur77\textsuperscript{−} VSMCs) exhibited greater proliferation after 2 to 3 days in culture, with or without Ang II treatment, compared with wild-type (WT) cells (Figure 3A and 3B). Consistent with this result, the protein levels of the proliferative markers (PCNA and cyclin D1) were upregulated, whereas the protein level of P27, an inhibitor of cyclin-dependent kinase, was downregulated in Nur77\textsuperscript{−} VSMCs (Figure 3C).

We used in vitro scratch-wound and transwell migration assays to investigate whether Nur77 deletion caused abnormal VSMC migration. The in vitro scratch-wound assay
revealed that cell migration induced by Ang II was enhanced in Nur77−/− VSMCs (Figure 3D). In addition, the transwell migration assay also demonstrated that Ang II–stimulated cell migration increased by ≈3-fold in Nur77−/− VSMCs compared with WT cells (Figure 3E).

To investigate the effect of Nur77 on the phenotypic switching of VSMCs, we used Western blotting and quantitative real-time polymerase chain reaction assays to analyze the expression of VSMC-specific genes, including α-actin, SM-22α, and SM-MHC. Expression of these genes was significantly downregulated in Nur77−/− VSMCs, under both quiescent and Ang II–stimulated conditions (Figure 3F and 3G). Platelet-derived growth factor is another major driving factor of VSMC phenotypic switching. We also found that under platelet-derived growth factor stimulation, Nur77 deletion significantly decreased the expression of VSMC-specific contractile genes (Figure S1 in the online-only Data Supplement). These results revealed that Nur77 deletion facilitates phenotypic switching of VSMCs from a quiescent contractile phenotype to an active synthetic phenotype.

Taken together, these results indicated that Nur77 is a critical smooth muscle cell phenotypic modulator that inhibits proliferation, migration, and phenotypic switching of VSMCs.

**Nur77 Deletion Exacerbates Ang II–Induced Vascular Remodeling In Vivo**

Age- and sex-matched WT and Nur77−/− mice were continuously infused with Ang II (2.1 mg/kg per day) to further define the in vivo role of Nur77 in Ang II–induced vascular remodeling. The hematoxylin and eosin staining results indicated that 2 weeks after Ang II infusion, Nur77−/− mice showed markedly enhanced outward vascular remodeling compared with WT mice (Figure 4A). The quantitative morphometric analysis revealed marked increases in the medial areas (197.000±7091 versus 174.000±5927 μm²; P<0.05) and luminal diameters (880.7±24.13 versus 825.2±10.38 μm; P<0.05) of Ang II–induced remodeling thoracic aortas.
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from Nur77−/− mice compared with WT mice (Figure 4A). The Verhoeff-Van Gieson staining results revealed that there was more severe elastin disruption after Ang II infusion in Nur77−/− mice (Figure S2). Similarly, picrosirius red staining results indicated that collagen deposition was more pronounced in the aortas from Nur77−/− mice (Figure S2). These results indicated that Nur77 inhibits Ang II–induced vascular remodeling in vivo.

The body weight and blood pressure of each mouse was measured once per week after the initiation of systemic Ang II administration. There were no differences between the body weight and the increase in systolic blood pressure or mean blood pressure induced by Ang II in WT and Nur77−/− mice (Figure S3A–S3C). These results indicated that the exacerbation of Ang II–induced vascular remodeling that occurred in Nur77−/− mice was not because of alterations in blood pressure, but was because of the effects of the biological functions of the VSMCs.

To further test the effects of Nur77 on the biological functions of VSMCs in vivo, we analyzed the expression of some key cell markers of proliferation (PCNA), migration (MMP-8 and MMP-13), and VSMC phenotypic switching (SM-22α and α-actin) by immunofluorescence staining of aorta sections from WT and Nur77−/− mice. Immunofluorescence staining of PCNA, a proliferative cell nucleus marker, revealed an increase in percentage of PCNA-positive VSMCs in the aortas of Nur77−/− mice compared with WT mice (82.89±3.83% versus 39.22±2.60%; P<0.05; Figure 4B). Consistently,
immunofluorescence staining of MMP-8 and MMP-13 showed that the relative fluorescence intensity of MMP-8 and MMP-13 in the intima area were significantly increased in remodeling aortas from Nur77−/− mice (Figure 4C). In contrast, compared with WT mice, the expression of VSMC-specific genes, SM-22α and α-actin, was significantly decreased in the aortas from Nur77−/− mice (Figure 4D).

Ang II Activates the β-catenin Signaling Pathway, Which Is Attenuated by Nur77

Growing evidence indicates that β-catenin signaling is involved in the regulation of the proliferation and migration of VSMCs and vascular remodeling.16–19 Therefore, we first investigated whether β-catenin signaling was activated in Ang II–induced vascular remodeling. Ang II significantly
induced the upregulation of active β-catenin (reaching a plateau at 30 to 60 minutes; Figure 5A). Consistently, the results for the time course of subcellular localization of active β-catenin after Ang II stimulation showed an increase in the nuclear fraction, which indicated that active β-catenin translocated into the cell nuclei (Figure 5B). These results were also supported by the immunofluorescent microscopy results (Figure 5C).

To investigate the effects of Nur77 upregulation on the expression and activation of β-catenin, we directly overexpressed Nur77 in rat primary VSMCs and HEK 293T cells. Overexpression of green fluorescent protein (GFP)–Nur77 in rat primary VSMCs led to a decrease in the protein levels of endogenous β-catenin in a dose-dependent manner (Figure 5D). This effect also occurred in HEK 293T cells cotransfected with Myc-Nur77 and HA-β-catenin (Figure 5E). The interaction between Nur77 and β-catenin was revealed using communoprecipitated assay in exogenous settings in HEK 293T cells (Figure 5F). We also found that Nur77-induced β-catenin degradation was blocked by the proteasome inhibitor, MG-132 (Figure 5G). These results indicated that Nur77 may mediate β-catenin degradation via direct interaction. Overexpression of Nur77 also reversed the upregulation of β-catenin transcriptional activity induced by β-catenin overexpression (Figure 5H). Nur77 overexpression downregulated β-catenin, accompanied by decreased

Figure 4. Nur77 deletion exacerbates angiotensin II (Ang II)–induced vascular remodeling in vivo. A, Representative images of hematoxylin and eosin staining and quantification of medial area and luminal diameter results in wild-type (WT) and Nur77−/− mice (n=11–12, *P<0.05). B, Representative images of dual immunofluorescence staining of PCNA (green) and SM-α-actin (red), and quantification of percentage of PCNA-positive VSMCs in Ang II–induced remodeling aortas from WT and Nur77−/− mice (n=6, *P<0.05). C and D, Representative images of immunofluorescence staining of matrix metalloproteinase (MMP)-8, MMP-13, SM-22α, and α-actin, and quantification of their relative fluorescence intensity in Ang II–induced remodeling aortas from WT and Nur77−/− mice (n=6, *P<0.05).
target gene expression (eg, cyclin D1; Figure 5I). The in vivo expression of β-catenin was markedly increased in the Ang II–induced remodeling aortas from Nur77−/− mice compared with WT mice (β-catenin area: 26.60±2.50% versus 37.80±2.67%, P<0.05; β-catenin+ cells: 48.80±4.16% versus 67.80±2.78%, P<0.05; Figure 6). Taken together, these results suggest that Ang II induces β-catenin activation, which is attenuated by Nur77 via the promotion of β-catenin degradation and inhibition of its transcriptional activity.

**Discussion**

Growing evidence indicates that Nur77 has critical roles in the pathogenesis of a variety of cardiovascular diseases (eg, vascular remodeling, atherosclerosis, cardiac hypertrophy,
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In VSMCs, Nur77 can be induced by multiple stimuli, including cytokines, growth factors, oxidized low-density lipoprotein, and vascular injury. We found that Nur77 was highly expressed in medial VSMCs of thoracic aortas from Ang II–infused mice in vivo and in cultured VSMCs after Ang II stimulation in vitro. Several signaling pathways and transcription factors, including MAPK and nuclear factor-κB signaling pathways and MEF-2, AP-1, and CREB, have been reported to regulate the expression of Nur77 in various cell types. The results of our study indicated that Ang II–induced Nur77 expression was regulated by the P38/ERK1/2/PKA-CREB pathway in VSMCs. This finding is consistent with the results of several previous studies that indicate that Nur77 expression is regulated by the transcription factor, CREB.

The most important finding of this study was that Nur77 deletion exacerbates vascular outward remodeling induced by systemic Ang II administration. There have been previously several studies from us and others concerning the roles of Nur77 in functional regulation of VSMCs and in vascular remodeling. Bonta et al found that Nur77 inhibits flow-induced outward vascular remodeling in SMC-specific overexpression of Nur77 transgenic mice. One of our recent studies (unpublished data) revealed that Nur77 deletion promoted the development of vascular remodeling induced by low shear stress. Our current study was the first to use Nur77−/− mice to investigate the role of Nur77 in Ang II–induced vascular remodeling, which mimics the pathological process induced by hyperactivation of RAS. We found that Nur77 inhibited medial thickening and vascular fibrosis in Ang II–induced remodeling aortas, which was independent of alterations in blood pressure. Thus, our results have implicated Nur77 as a critical negative regulator of the vascular remodeling associated with RAS hyperactivation.

Phenotypic switching of VSMCs, from a differentiated to a dedifferentiated phenotype, has an important role in the pathogenesis of vascular remodeling. Dedifferentiated VSMCs exhibit increased cell proliferation and migration, accompanied by reduced expression of VSMC-specific genes (α-actin, SM-22α, and SM-MHC). This study revealed that Nur77 is a novel negative phenotypic modulator of VSMCs. VSMCs isolated from Nur77−/− mice exhibited increased proliferation and migration, accompanied by reduced expression of SM-specific contractile genes, under basal, Ang II–induced, and platelet-derived growth factor–induced conditions. This discovery was further confirmed in vivo in thoracic aorta sections after vascular remodeling induced by systemic Ang II administration. Early studies implicated the critical roles of Nur77 in VSMC proliferation. To the best of our knowledge, this study is the first to reveal the functional importance of Nur77 in VSMC phenotypic switching.

We focused on Wnt/β-catenin signaling to define the possible molecular mechanisms by which Nur77 exerts its effects on VSMCs. This signaling has critical roles in the regulation of VSMC proliferation and migration and in vascular remodeling. We found that the β-catenin signaling pathway was activated under Ang II stimulation. Overexpression of exogenous Nur77 in VSMCs or 293T cells downregulated the expression of β-catenin, accompanied by reduction in the transcriptional activity of β-catenin, and the expression of its target gene that controls VSMC proliferation. Additional experiments revealed that Nur77 can interact with β-catenin, promote its ubiquitination and proteasomal degradation, and subsequently attenuate the activation of β-catenin signaling. Most importantly, in vivo experiments revealed that there was increased β-catenin expression in Ang II–induced remodeling aortas from Nur77−/− mice compared with WT mice. These findings indicated that Nur77 inhibits the proliferation and migration of VSMCs and vascular remodeling, at least in

Figure 6. Effect of wild-type (WT) and Nur77-deficient genotype on β-catenin expression after systemic angiotensin II (Ang II) administration. Representative images of immunohistochemical staining of β-catenin, and quantification of β-catenin-positive area (% of intima-media area) and percentage of β-catenin–positive cells (n=6, *P<0.05).
part, via the downregulation of β-catenin activity. Nur77 may act as an important negative regulator of the Wnt/β-catenin signaling pathway during Ang II–induced vascular remodeling. This result was consistent with the results of previous studies with regard to the interplay between Nur77 and the Wnt/β-catenin signaling pathway in colon cancer cells and in osteoblasts.20,21,22 This signaling crosstalk may help to identify potential therapeutic targets for vascular remodeling associated with RAS hyperactivation during hypertension, atherosclerosis, vascular injury, restenosis, and aneurysm formation.

In summary, we found that Nur77 was upregulated in VSMCs in vitro and in vivo in response to Ang II during vascular remodeling. Most importantly, our results indicated that Nur77 deletion enhanced VSMC proliferation, migration, and exacerbated vascular remodeling, which suggests that Nur77 may act as an important negative regulator of the Wnt/β-catenin signaling pathway during Ang II–induced vascular remodeling. This result was consistent with the results of previous studies with regard to the interplay between Nur77 and the Wnt/β-catenin signaling pathway in colon cancer cells and in osteoblasts. This signaling crosstalk may help to identify potential therapeutic targets for vascular remodeling associated with RAS hyperactivation during hypertension, atherosclerosis, vascular injury, restenosis, and aneurysm formation.

In summary, we found that Nur77 was upregulated in VSMCs in vitro and in vivo in response to Ang II during vascular remodeling. Most importantly, our results indicated that Nur77 deletion enhanced VSMC proliferation, migration, and aneurysm formation. This work was supported by the Grant Numbers 81330006, 30971185, 81370399, and 81070239 from the National Natural Science Foundation.

Disclosures
None.

References
What is New?

- Nur77 was highly expressed in medial vascular smooth muscle cells of the mouse thoracic aorta in response to systemic angiotensin II infusion.
- Nur77 deletion enhances vascular smooth muscle cell proliferation, migration, and phenotypic switching and exacerbates angiotensin II–induced vascular remodeling.
- Nur77 suppresses angiotensin II–induced β-catenin signaling pathway activation by promoting β-catenin degradation and inhibiting its transcriptional activity.

What is Relevant?

- Vascular remodeling associated with renin–angiotensin system hyperactivation has been clearly implicated in end organ damage and is associated with poor cardiovascular prognosis.

Novelty and Significance

- Nur77 plays an important role in the pathological process of vascular remodeling associated with renin–angiotensin system hyperactivation.
- Nur77 might represent a novel therapeutic target for vascular remodeling.

Summary

We have identified Nur77 as a critical negative regulator of angiotensin II–induced vascular remodeling.
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Orphan Nuclear Receptor Nur77 Inhibits Angiotensin II-induced Vascular Remodeling via Downregulation of β-catenin

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Short title: Nur77 inhibits Ang II-induced vascular remodeling

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Supplemental Materials and Methods

Animal Experiments

All animal experiments were performed in accordance with the NIH guidelines for the Care and Use of Laboratory Animals. The study protocol was approved by the Committee on the Ethics of Animal Experiments of the Shanghai Jiao Tong University School of Medicine. Male wild type (WT) and Nur77−/− mice on the C57BL/6 background (12–16 weeks of age, Jackson Laboratory, Bar Harbor, Maine, USA) were used for the experiments. The mice were randomly assigned to one of two groups: Ang II-infusion group (Ang II group) or the sham control group (Con group).

Ang II-induced vascular remodeling was established as previously described. Briefly, WT and Nur77−/− mice were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal injection), followed by subcutaneous implantation of an Alzet osmotic minipump (Model 2002, ALZA Scientific Products, Mountain View, CA, USA) containing only saline, or Ang II (Sigma, A9525) dissolved in saline. Mice were then continuously infused with saline or Ang II (2.1 mg/kg/d) for 2 weeks. The body weight and blood pressure of each mouse was measured weekly using a digital bathroom scale and a non-invasive tail cuff sphygmomanometer (BP-98A, Softron, Shanghai, China), respectively.

Tissue Collection and Processing

Each mouse was perfused with ice cold isotonic saline at 100 mmHg until the perfusate cleared. The thoracic aorta was then isolated by blunt dissection. The thoracic aorta of each mouse was cut into two sections. One section was stored at -80°C for RNA extraction. The other section was fixed with 4% paraformaldehyde overnight, and was then embedded in paraffin or optimal cutting temperature (OCT) embedding material for morphometric analysis and immunofluorescence staining. Paraffin-embedded sections (8 μm thick) or OCT-embedded sections (5 μm thick) were cut every 200 μm over a 2-mm length of thoracic aortas from the proximal thoracic aortas specimens.

Histology and Immunohistochemistry

The OCT- and paraffin-embedded thoracic aorta sections were stained using hematoxylin and eosin (H&E), picrosirius red, or Verhoeff-Van Gieson (vvG) stains, according to standard protocols. All images were recorded using an Olympus digital camera (Tokyo, Japan), and were analyzed using ImagePro Plus software (Media Cybernetics, Rockville, MD, USA).

For immunohistochemical staining, OCT-embedded sections were preincubated with 5% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and then incubated with primary antibody against β-catenin (1:200, BD Biosciences, Franklin Lakes, NJ, USA). Anti-mouse immunoglobulin G (ready-to-use; K5007, DAKO, Agilent Technologies, Santa Clara, CA, USA) was used as secondary antibody. Immunohistochemical staining was visualized using an ABC kit (Invitrogen, Carlsbad, CA, USA) according to the
manufacturer’s instructions; 3,3′-diamino-benzidine was the enzyme substrate. The sections were counterstained with hematoxylin.

Immunofluorescence

Immunofluorescence staining was performed as previously described. Briefly, OCT-embedded sections were washed with PBS, fixed by 4% paraformaldehyde at 4°C for 20 min, and permeabilized with 0.2% Triton X-100 (Dow Chemical, Midland, MI, USA) for 8 min. After blocking with 5% FBS for 30 min, the sections were incubated with primary antibodies against Nur77 (1:100, Abcam, Cambridge, UK), α-actin (1:300, Millipore, Billerica, MA, USA), PCNA (1:100, Abgent, San Diego, CA, USA), β-catenin (1:100, Cell Signaling Technology [CST], Danvers, MA, USA), MMP-8 (1:100, Abcam), MMP-13 (1:100, Abcam), and SM-22α (1:100, CST), overnight. Secondary antibodies (1:300; donkey-anti-mouse, 555 nm, red fluorescence; donkey-anti-rabbit, 488 nm, green fluorescence; Invitrogen) were used to react with the primary antibodies for 1 h at 37°C, followed by nuclear DNA staining using 4, 6-diamino-2-phenylindole (blue fluorescence, DAPI) (Beyotime, Shanghai, China) for 8 min. Fluorescence signals were evaluated using confocal microscopy (LSM 710, Zeiss, Oberkochen, Germany).

Isolation and Culture of Aortic VSMCs

Primary VSMCs were isolated from the thoracic aortas of Sprague-Dawley rats and WT and Nur77−/− mice as previously described. Rat and mouse VSMCs were cultured in DMEM and DMEM/F-12 media (Hyclone, Logan, UT, USA), respectively, supplemented with 10% FBS at 37°C. The cells were serum-starved for 24 h before drug treatment. The VSMCs were used in the experiments between passages 3 and 8.

Western Blotting and Co-Immunoprecipitation Assays

Total proteins were prepared from cultured VSMCs, and western blotting was performed as briefly mentioned below. Proteins were quantified with a BCA assay (Pierce, Waltham, MA, USA), separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk dissolved in TBST at 37°C for 1h, and incubated with primary antibodies against Nur77 (1:1000, Abcam), cyclinD1 (1:1000, CST), PCNA (1:1000, Abgent), P27 (1:1000, CST), SM 22α (1:1000, CST), CREB (1:1000, CST), p-CREB (1:1000, CST), β-catenin (1:1000, CST), active β-catenin (1:1000, CST), flag (1:2000, Invitrogen), myc (1:2000, Invitrogen), GFP (1:2000, Invitrogen), and β-actin (1:5000, Abcam) at 4°C overnight. After incubation with horseradish peroxidase-conjugated secondary antibodies at 37°C for 1h, proteins were detected by enhanced chemiluminescence (Millipore) and quantified using Quantity One 4.4.0 software (Bio-Rad, Hercules, CA, USA).

For co-immunoprecipitation assay, cells were lysed in cold lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM EGTA [pH 8.0], 0.2 mM sodium ortho-vanadate, 1 mM PMSF, 0.5% protease inhibitor cocktail, 0.5% IGEPAL CA-630) on ice for 30 min. Following sonication, the lysates were centrifuged for 30 min at
12,000 rpm. The supernatants were added with flag-beads and incubated at 4°C overnight. The flag-beads were washed five time using lysis buffer. After the final wash, the proteins were eluted from the flag-beads in elution buffer and were then subjected to western blotting.

**Nuclear-Cytosolic Fractionation**

Nuclear and cytosolic protein extractions were performed as previously described. Primary VSMCs were washed with cold PBS and collected by centrifugation at 1000 rpm at 4°C for 5 min. The cell pellets were resuspended in 200 μl buffer A (10 mM Hepes-KOH [pH 7.4], 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM EGTA, containing protein inhibitor cocktail). After 15 min on ice, the cell lysates were passed through a syringe with a 23-gauge needle 28–32 times, until the plasma membrane was disrupted. The lysates were then centrifuged at 1000 g for 5 min. The supernatants (cytosolic fractions) were transferred to a new Eppendorf tube and centrifuged for 15 min at 12000 rpm to clear the debris. The pellets were washed with buffer A (1ml, twice), resuspended in 50 µl cold buffer B (10 mM Hepes-KOH [pH 7.4], 0.42 mM NaCl, 2.5% v/v glycerol, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM EGTA, 1mM DTT, containing protein inhibitor cocktail), and gently shaken at 4°C for 30 min. Nuclear protein extracts were obtained after centrifugation at 12000 rpm at 4°C for 10 min. The supernatants (nuclear fractions) were transferred to new Eppendorf tubes. Cytosolic and nuclear protein extracts were quantified using a BCA assay and were then subjected to western blotting.

**RNA Extraction and Quantitative Real-time PCR**

Total RNA was extracted from the cultured VSMCs or from thoracic aortas from Ang II-infused mice or control mice using Trizol reagent (Invitrogen), according to the manufacturer’s instructions. Total RNA (2 μg) was reverse-transcribed into first-strand cDNA, and RT-PCR amplification was performed using SYBR Green dye and the ABI Prism 7500 fast system (Applied Biosystems, Carlsbad, CA, USA). The primer sequences used for the detection of β-actin, rat Nur77, mouse Nur77, SM α-actin, SM-22α, SM-MHC, and CREB are presented in Supplemental Table S1. Relative mRNA expression was calculated using the comparative ΔΔCT method and the resulting values were normalized to β-actin expression. PCR was performed in triplicate for each experiment. The results presented represent three independent experiments.

**Cell Proliferation Assay (Cell Counting and CCK-8 assay)**

For cell counting, primary VSMCs isolated from the WT and Nur77−/− mice were seeded into 6-well plates at a density of 1×10⁵ cells/well. The VSMCs were starved in serum-free medium for 24 h to render cells quiescent. They were then stimulated with Ang II (500 mM) for 48 h, and then trypsinized and counted on a hemocytometer while viewing them through an inverted microscope.

For the CCK-8 assay, mouse VSMCs were seeded into 96-well plates at a density of
5×10³ cells/well. The cells were serum-starved and stimulated with Ang II as previously mentioned. Cell proliferation was then assessed using a Cell Counting Kit-8 (CCK-8, Yeasen, Shanghai, China), according to the manufacturer’s protocol.

Cell Migration Assay (In vitro Scratch-Wound Assay and Transwell Assay)

For the scratch-wound assay, primary VSMCs from the WT and Nur77−/− mice were seeded into 6-well plates at a density of 2×10⁵ cells/well. After reaching 80–90% confluency, the cells were serum-starved for 24 h, scraped by sterilized 10 μL pipette tips, washed with PBS to remove the cell debris, and stimulated using Ang II (500 mM) for an additional 20 or 36 h. Photomicrographs were taken using an Olympus inverted microscope. The numbers of cells that migrated into the wound area were quantified using Diskus software (Hilgers, Königswinter, Germany).

The migration assay was performed using a 24-well plate containing transwell inserts (Corning Inc., Corning, NY, USA) with 8 μm pore membrane filters. Starved VSMCs were seeded into the upper chamber at a density of 5×10⁴ cells/well and serum-free medium containing Ang II was added to the lower chamber; the plates were incubated for 6 h. The cells on the upper or lower surfaces of the transwell inserts were then fixed with ice cold methanol for 20 min, washed with PBS, and stained using crystal violet at room temperature for 1 h. Cells on the upper surface of the filter were scraped. The cells that migrated into the lower section were counted in five random fields (20× magnification) using an Olympus inverted microscope.

SiRNA Transfection

Small interfering RNA (siRNA) duplexes against rat CREB were designed and synthesized by Ribobio (Guangzhou, China). Primary VSMCs were seeded into a 6-well plate at a density of 1×10⁵ cells/well 1 day prior to transfection. Transfection of CREB siRNA (si-CREB) and control siRNA (si-CTL) (100 nM final concentration) was performed using Hiperfect Transfection Reagent (Qiagen, Venlo, Limburg, the Netherlands) according to the manufacturer’s protocol.

Recombinant Adeno-associated Viral (AAV) Production and VSMC Infection

AAV-Nur77 and AAV-control were produced using plasmid pAOV-CMV-Nur77-EGFP (GFP-Nur77) or pAOV-CMV-EGFP vector (GFP) (Obio Technology, Shanghai, China). Primary VSMCs were seeded in 6-well plates to approximately 75% confluence and infected with AAV-Nur77 and AAV-control at increasing MOI.

Plasmid Transfection and Dual-Luciferase Assay

The plasmids were transiently transfected into HEK-293T cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions.

The dual-luciferase assay was performed as previously described. Briefly, cells transfected with dual-luciferase reporters were incubated with 1× lysis buffer (luciferase assay kit, Promega, Madison, WI, USA) for 15 min and then harvested. Luciferase and
Renilla activities were measured using the dual-luciferase assay protocol. Each assay was repeated three times.

Statistical Analysis

Results were expressed as mean ± standard error of the mean (SEM) values. The Student’s t-test was used for comparison of two groups and the one-way ANOVA was used for multiple comparisons. A p-value<0.05 was considered to indicate a statistically significant result.

References

### Supplemental Table S1. Summary of the quantitative RT-PCR primer sets used.

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<th>Primer</th>
<th>Sequence (5’- 3’)</th>
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<tr>
<td>β-actin</td>
<td>Forward: 5’-GGCATCGTCACCAACTGGGAC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CGATTTCCCGCTCGGCGTG-3’</td>
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<tr>
<td>Rat Nur77</td>
<td>Forward: 5’-GCTCATTTCTGCTCAGGCCT-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CAGACGTCAGGCAGCTGCG-3’</td>
</tr>
<tr>
<td>Mouse Nur77</td>
<td>Forward: 5’-CTCGCCATCTACACCAACT-3’</td>
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<td></td>
<td>Reverse: 5’-AGCTTAGGCAACTGCTCTG-3’</td>
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<td>SM α-actin</td>
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<tr>
<td></td>
<td>Reverse: 5’-AGGATAGGGACACGACACA-3’</td>
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<td>SM-22α</td>
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<tr>
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</tr>
<tr>
<td>CREB</td>
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</tr>
<tr>
<td></td>
<td>Reverse: 5’-CAACAACCTTGTTGCTGGGACTA-3’</td>
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Supplemental Figures

**Figure S1.** Nur77 deletion facilitates VSMC phenotypic switching under PDGF stimulation. Primary VSMCs isolated from WT and Nur77<sup>−/−</sup> mice were serum-starved for 24 h and then stimulated with PDGF (20 ng/ml) for another 24 h. mRNA levels of VSMC-specific genes were assessed by real-time PCR (n=3, *p<0.05).
Figure S2. Collagen deposition and elastin disruption in Ang II-induced remodeling aortas from WT and Nur77\(^{-/-}\) mice. Representative images of Verhoeff-Van Gieson staining and picrosirius red staining, and quantification of the percentage of collagen area in WT and Nur77\(^{-/-}\) mice (*p<0.05, n=6). Arrows point to disrupted elastin structure.
**Figure S3.** Body weight and blood pressure in WT and Nur77<sup>−/−</sup> mice. (A) Quantification of body weight of WT and Nur77<sup>−/−</sup> mice. (B and C) Blood pressure of WT and Nur77<sup>−/−</sup> mice was measured using a non-invasive tail cuff sphygmomanometer before and 1 week and 2 weeks after Ang II infusion (2.1 mg/kg/d). Quantification of (B) systolic blood pressure (SBP) or (C) mean blood pressure (MBP) (n=12).
Figure S4. A working model depicting how Nur77 regulates Ang II-induced vascular remodeling via downregulation of β-catenin signaling.