ORIGINAL ARTICLE

Notch activation mediates angiotensin II-induced vascular remodeling by promoting the proliferation and migration of vascular smooth muscle cells

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Notch signaling is involved in an intercellular communication mechanism that is essential for coordinated cell fate determination and tissue morphogenesis. The biological effects of Notch signaling are context-dependent. We investigated the functional and hierarchical relationship between angiotensin (Ang) II receptor signaling and Notch signaling in vascular smooth muscle cells (VSMCs). A fluorogenic substrate assay revealed directly that the enzymatic activity of γ-secretase was enhanced after 10 min of Ang II stimulation in HEK293 cells expressing Ang II type 1 receptor. Notch cleavage by γ-secretase was consistently induced and peaked at 10 min after Ang II stimulation, and the Ang II-stimulated increase in Notch intracellular domain production was significantly suppressed by treatment with the γ-secretase inhibitor DAPT. Treatment with DAPT also significantly reduced the Ang II-stimulated proliferation and migration of human aortic VSMCs, as revealed by BrdU incorporation and the Boyden chamber assay, respectively. Systemic administration of the γ-secretase inhibitor dibenzazepine reduced Ang II-induced medial thickening and perivascular fibrosis in the aortas of wild-type mice. These findings suggest that the hierarchical Ang II receptor–Notch signaling pathway promotes the proliferation and migration of VSMCs, and thereby contributes to the progression of vascular remodeling.

INTRODUCTION

Both the renin–angiotensin system (RAS) and the Notch signaling pathway exhibit pleiotropic effects on the structure and function of multiple organ systems. RAS not only plays a homeostatic role in the regulation of blood pressure and the balance of sodium and water, but also has diverse pathological effects on the heart and vessels.[1] Angiotensin II (Ang II) is the primary bioactive molecule of RAS, and most of the pathophysiological actions of Ang II in the cardiovascular system are mediated primarily through the Ang II type 1 (AT_1) receptor.[2] The AT_1 receptor is a seven-transmembrane spanning G-protein-coupled receptor (GPCR), and activation of AT_1 receptor elicits multiple cellular responses via G-protein-dependent and -independent signaling pathways.[3,4] The principal targets of Ang II in the vasculature are vascular smooth muscle cells (VSMCs), and AT_1 receptor activation stimulates growth, proliferation and migration of VSMCs via the mitogen-activated protein kinase pathway.[4–6] AT_1 receptor activation also stimulates vascular fibrosis by enhancing synthesis and deposition of the extracellular matrix protein via transactivation of epidermal growth factor receptor and generation of reactive oxygen species.[4,7] In spite of the central role of AT_1 receptor signaling in multiple vascular pathobiological processes, the full scope of the specific signal transduction pathways leading to vascular remodeling remains to be fully elucidated.

The Notch signaling pathway plays a fundamental role in cell fate determination, differentiation, proliferation and apoptosis during diverse developmental and physiological processes.[8,9] Binding of Notch ligands to the surfaces of neighboring cells triggers sequential proteolytic cleavage of the transmembrane Notch receptor by ADAM-family metalloproteases and the γ-secretase complex to release the Notch intracellular domain (NICD) into the cytoplasm.[8–10] Once cleaved, NICD migrates into the nucleus, and forms a complex with the DNA-binding protein RBP-Jκ, the co-activator mastermind-like (MAML) and other co-activators to further activate the transcription of target genes.[8–10] It has been reported that Notch signaling controls

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proliferation and phenotypic switching of VSMCs after vascular injury, as well as the embryonic developmental program of the vasculature.[11,12]

We investigated the role of the functional and hierarchical relationship between AT$_1$ receptor signaling and Notch signaling in the pathogenesis of vascular remodeling. We found that Ang II stimulated the enzymatic activity of γ-secretase and thereby activated the Notch signaling pathway. The pharmacological inhibition of γ-secretase significantly reduced not only Ang II-stimulated proliferation and migration of VSMCs in vitro but also Ang II-induced aortic medial thickening and perivascular fibrosis in vivo. These results provide mechanistic insights into the hierarchical Ang II receptor–Notch signaling pathway that contributes to the progression of vascular remodeling.

METHODS

Cell cultures
HEK293 cells expressing the AT$_1$ receptor (HEK293-AT$_1$ cells)[13,14] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and were starved under a serum-free condition for 48 h before stimulation with 10^{-7} M Ang II (Sigma-Aldrich, St Louis, MO, USA). Human aortic smooth muscle cells (AoSMCs) were purchased from Lonza (Basel, Switzerland) and routinely cultured in smooth muscle medium-2 (SmGM-2, Lonza,) containing 5% fetal bovine serum with growth factors and antibiotics (SmGM-2 Bullet Kit, Lonza). AoSMCs were starved under a serum-free condition for 48 h before stimulation with 10^{-6} M Ang II. The γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT) was from Calbiochem (La Jolla, CA, USA). Actinomycin D and cycloheximide were from Sigma-Aldrich. The experiments were repeated at least three times in triplicate, and representative data are shown.

Western blot analysis
Nuclear extract was isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's instructions. Protein samples were fractionated with SDS-PAGE and transferred to PVDF membranes (GE Healthcare Biosciences, Piscataway, NJ, USA). The blotted membranes were incubated with primary antibody and then with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Immunoreactive signals were visualized using an ECL Plus Western Blotting Detection System (GE Healthcare Biosciences). The following antibodies were used: rabbit polyclonal anti-NOTCH 1 (Cleaved N Terminal) antibody (Rockland Immunochemicals, Gilbertsville, PA, USA), rabbit polyclonal anti-cleaved Notch1 (Val1744) antibody (Cell Signaling Technology, Beverly, MA, USA), goat polyclonal anti-DNA topoisomerase I (Topo I) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal anti-nucleoporin p62 (BD Transduction Laboratories, San Diego, CA, USA).

γ-Secretase-mediated peptide cleavage assay
The cells were homogenized in lysis buffer (25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 0.5 mM EGTA) plus protease inhibitors (Complete mini; Roche Applied Science, Indianapolis, IN, USA), and lysates were centrifuged at 800 g for 10 min to pellet unbroken cells and nuclei. The supernatant was discarded, and the pellets were resuspended in assay buffer (50 mM Tris-HCl, pH 6.8, 2 mM EDTA, 0.25% CHAPSO). The protein (15 μg) was incubated at 37 °C in assay buffer containing 8 μM fluorogenic γ-secretase substrate (Calbiochem). After incubation, the reaction mixtures were centrifuged at 16 100 g for 15 min and placed on ice. The supernatants were transferred to a 96-well plate, and fluorescence was measured using a microplate reader (FlexStation 3; Molecular Devices, Sunnyvale, CA, USA) with excitation wavelength 355 nm and emission wavelength 440 nm. The slope of the linear range was correlated with γ-secretase activity after subtraction of the nonspecific signal.

Luciferase assays
The luciferase reporter plasmids were transfected using FuGENE HD Transfection Reagent (Roche Applied Science) according to the manufacturer's instructions. pRL-SV40 (Promega, Madison, WI, USA) was co-transfected as an internal control. Luciferase activities were measured 24 h after Ang II stimulation using a dual-luciferase reporter assay system (Promega). The experiments were repeated at least three times in triplicate, and representative data are shown. The luciferase reporter plasmids were generous gifts from Dr T Honjo (Kyoto University), M Kengaku (RIKEN Brain Science Institute) (RBP-Jκ-Luc[15]) and Dr R Kageyama (Kyoto University) (Hes1-Luc and Hes5-Luc[16]).

Proliferation and migration assays
Cell proliferation was measured by bromodeoxyuridine (BrdU) incorporation during DNA synthesis of proliferating cells using a Cell Proliferation ELISA Kit (Roche Applied Science). After 48 h of incubation at 37 °C in serum-free medium, AoSMCs were labeled with BrdU with or without 10^{-6} M Ang II for 8 h. DNA synthesis was assayed by measuring the amount of BrdU incorporation according to the manufacturer's instructions. Cell migration was measured using Transwell 24-well plates (Corning Costar 3422; Corning Inc., Corning, NY, USA) with an 8-μm-pore polycarbonate membrane. The underside of the polycarbonate membrane was coated with 50 μg ml^{-1} collagen type I (Cellmatrix; Nitta Gelatin Inc., Osaka, Japan) overnight at 4 °C. AoSMCs (passage 7–10) were plated on the upper well at 5000 cells per well. After incubation in SmGM-2 with a SmGM-2 Bullet Kit for 48 h, the cells were washed in PBS twice and starved in Dulbecco's modified Eagle's medium supplemented with 0.2% fetal bovine serum for 24 h. The cells were pretreated with DAPT (2×10^{-5} M) or vehicle for 30 min, and placed in the lower chamber in the presence or absence of 10^{-6} M Ang II. After 6 h of incubation at 37 °C, the cells on the upper surface were washed, fixed and stained with hematoxylin and eosin. The number of cells that migrated to the lower (outer) surface of the filters was determined by counting under a microscope.

Ang II infusion and histological analysis
Twelve-week-old C57BL/6J male mice were treated with Ang II (0.9 mg kg^{-1} per day) or vehicle for 2 weeks using an osmotic mini-pump (ALZET model 2002; Durect Corp., Cupertino, CA, USA), as described previously.[17] The mice were randomly assigned to orally receive vehicle alone (0.4% methylcellulose) or the γ-secretase inhibitor dibenzazepine (1.0 mg kg^{-1} per day; Santa Cruz Biotechnology)[18] during Ang II infusion. The abdominal aortas were excised, fixed immediately in 10% neutralized formalin, and embedded in paraffin. Serial sections (thickness 5 μm) were stained with hematoxylin and eosin for morphological analysis, or with Masson's trichrome for detection of fibrosis. To evaluate aortic wall thickness and perivascular fibrosis, short-axis images were scanned at $\times 200$ magnification. The medial thickness, wall to lumen ratio (the ratio of medial thickness to internal diameter) and area of perivascular fibrosis (the ratio of the fibrotic area surrounding the vessel to the total vessel area) were calculated. All protocols were approved by the Institutional Animal Care and Use Committee of Chiba University.

Real time RT–PCR analysis
Total RNA was extracted using an RNeasy Kit (Qiagen, Valencia, CA, USA), and single-stranded cDNA was transcribed using a SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocols. We conducted quantitative real-time PCR analysis using Universal ProbeLibrary Assays (Roche Applied Science) according to the manufacturer's instructions. The amplification conditions were initial denaturation for 10 min at 95 °C followed by 45 cycles of 10 s at 95 °C and 25 s at 60 °C. Individual PCR products were analyzed by melting point analysis. The expression level of a gene was normalized relative to that of GAPDH using a comparative Ct method. The primer sequences and Universal Probe numbers were designed using ProbeFinder software (Roche Applied Science) as follows: NOTCH1, 5'-CGCACAAGGTGTCTTCCAG-3' and 5'-AGGATCAGTGGCGTCGTG-3', No. 85; NOTCH2, 5'-GGCAGACTGGTGACTTCACTT-3' and 5'-CTCTCACAG GTGCTCCCTTC-3', No. 8; NOTCH3, 5'-GCCAAGCGGCTAAAGGTA-3' and 5'-CACTGACGGCAATCCACA-3', No. 30; NOTCH4, 5'-CCTCTCTGCAA

CCTTCCACT-3′ and 5′-GCCTCCATTGTGGCAAAG-3′, No. 51; *JAG1*, 5′-GA
ATGGCAACAAAACTTGCAT-3′ and 5′-AGCCTTGTCGGCAAATAGC-3′, No.
42; *JAG2*, 5′-TGGGACTGGGACAACGATAC-3′ and 5′-ATGCGACACTCGC
TCGAT-3′, No. 17; *DLL1*, 5′-CTTCCCCTTCGGCTTCAC-3′ and 5′-GGGTT
TTCTGTTGCGAGGT-3′, No. 2; *DLL3*, 5′-CAACTGTGAGAAGAGGGTGGA-
3′ and 5′-CCAGGTCCAGGCAGAGTC-3′, No. 46; *DLL4*, 5′-CCCTGGCAATG
TACTTGTGAT-3′ and 5′-TGGTGGGTGCAGTAGTTGAG-3′, No. 23; *GAPDH*,
5′′-AGCCACATCGCTCAGACAC-3′ and 5′-GCCCAATACGACCAAATCC-3′,
No. 60.

Statistics

All data are presented as means ± s.e.m. Two-group comparisons were
performed using unpaired two-tailed Student's *t* test, and multiple-group
comparisons were performed by a one-way ANOVA followed by Fisher's PLSD
test for comparison of means. A *P* value < 0.05 was considered to be
statistically significant.

RESULTS

Ang II stimulation induces nuclear accumulation of NICD in HEK293 cells expressing the AT$_1$ receptor

To elucidate the functional and hierarchical relationship between AT$_1$
receptor signaling and Notch signaling, we examined the time-
dependent changes of nuclear NICD levels in HEK293-AT$_1$ cells after
stimulation with Ang II. HEK293 cells possess functional GPCR
signaling pathways without detectable expression of the AT$_1$ recep-
tor.[19] Western blot analysis using an antibody specific to NICD
revealed that nuclear NICD levels increased and peaked at 10 min

after Ang II stimulation (Figure 1a). These results suggest that
activation of the AT$_1$ receptor induces nuclear accumulation of NICD.

Ang II stimulation activates Notch signaling via the enhancement of γ-secretase activity

As the nuclear accumulation of NICD may have resulted from
enhanced proteolysis of Notch protein by γ-secretase,[10] we
examined the effect of Ang II stimulation on γ-secretase activity in
HEK293-AT$_1$ cells. We measured the enzymatic activity of γ-secretase
directly using a fluorogenic substrate assay[20] and found that
γ-secretase activity was significantly enhanced 10 min after
stimulation with Ang II, and that the enhancing effect was
suppressed by treatment with the γ-secretase inhibitor DAPT
(Figure 1b). Ang II-induced nuclear accumulation of NICD was
consistently reduced by treatment with DAPT (Figure 1c).

To elucidate the functional importance of the Ang II-induced
increase in nuclear NICD, we examined Notch-dependent activation
of luciferase-based reporters. Ang II strongly enhanced luciferase
activity driven by the Notch-dependent CBF1-responsive element
(*RBP-Jκ*-Luc), *Hes1* promoter (*Hes1*-Luc) and *Hes5* promoter (*Hes5*-
Luc) in HEK293-AT$_1$ cells (Figures 1d-f). The Ang II-induced
transactivation of Notch-dependent reporters was significantly inhib-
ited by treatment with DAPT (Figures 1d-f). These findings suggest
that Ang II stimulation activates Notch signaling via the enhancement
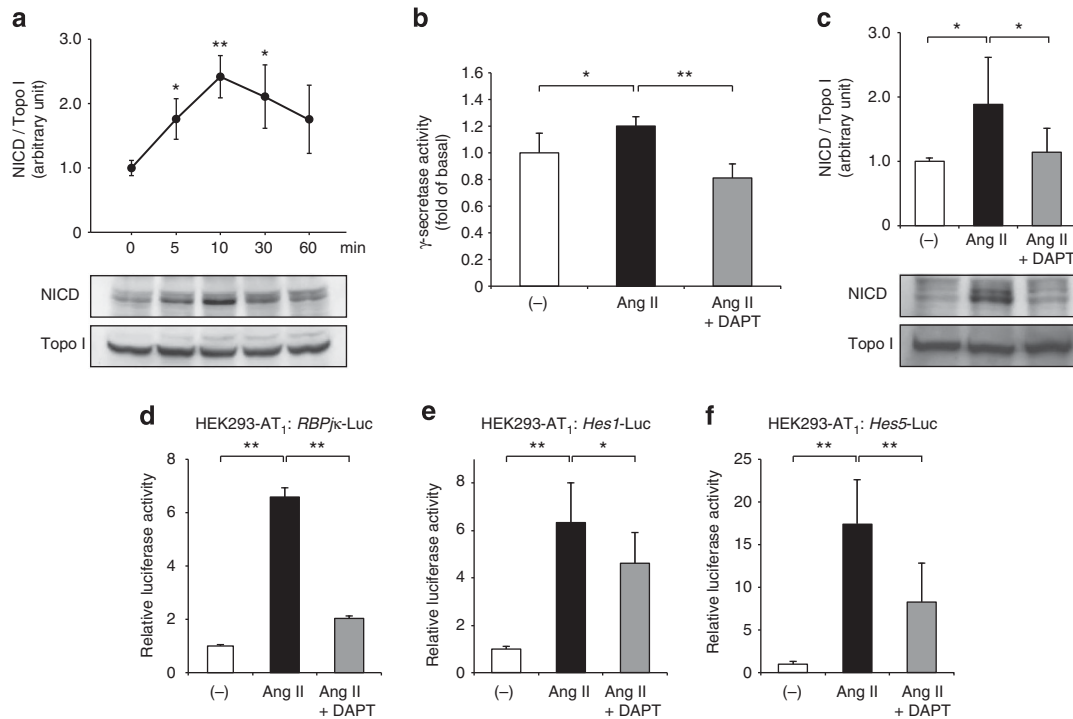of γ-secretase activity.

Figure 1 Ang II stimulates γ-secretase and activates Notch signaling in HEK293-AT$_1$ cells. (**a**) HEK293-AT$_1$ cells were stimulated with Ang II (10^{-7} M) for
the indicated periods, and NICD protein in the nuclear fraction was visualized by immunoblotting. Topo I was used as an internal control for the amount of
nuclear protein. The NICD/Topo I ratios were quantified by densitometry and plotted (upper panel). The data are presented as the mean ± s.e.m. *P<0.05,
P<0.01 *vs.* the value at 0 min. (b**) HEK293-AT$_1$ cells were pretreated with DAPT and stimulated with Ang II (10^{-7} M) for 10 min, and γ-secretase activity
was determined using a fluorogenic substrate assay. The data are presented as the mean ± s.e.m. *P<0.05, **P<0.01. (**c**) HEK293-AT$_1$ cells were
pretreated with or without DAPT (2×10^{-5} M) and stimulated with Ang II (10^{-7} M) for 10 min, and NICD and Topo I in the nuclear fraction were visualized
by immunoblotting. The NICD/Topo I ratios were quantified and presented as a bar graph (upper panel). The data are presented as the mean ± s.e.m.
*P<0.05. (**d–f**) HEK293-AT$_1$ cells were transfected with *RBPjk*-luciferase reporter (**d**), *Hes1*-luciferase reporter (**e**) or *Hes5*-luciferase reporter (**f**). The cells
were pretreated with or without DAPT (2×10^{-5} M) and stimulated with Ang II (10^{-7} M) for 24 h before the measurement of luciferase activities. The data
are presented as the mean ± s.e.m. *P<0.05, **P<0.01.

Ang II stimulation activates Notch signaling independently of de novo synthesis of Notch receptors and ligands

As most Notch ligands are also transmembrane proteins, the propagation of Notch signaling is essentially restricted to neighboring cells.[8–10] We considered the possibility that Notch signaling is enhanced as a consequence of the upregulation of Notch receptors and ligands by Ang II stimulation. However, in quantitative real-time RT–PCR analysis, the mRNA expression levels of Notch receptors (*NOTCH1-4*) and Notch ligands (*JAG1, JAG2, DLL1, DLL3* and *DLL4*) in HEK293-AT₁ cells were not significantly changed following Ang II stimulation (Figure 2a). Ang II stimulation increased nuclear NICD levels in HEK293-AT₁ cells even in the presence of the transcription inhibitor actinomycin D or the translation inhibitor cycloheximide (Figure 2b). These findings suggest that *de novo* synthesis of Notch receptors and ligands is not involved in the process of hierarchical activation of the AT₁ receptor–Notch signaling pathway.

Notch signaling mediates Ang II-stimulated proliferation and migration of human AoSMCs

We investigated the functional significance of Notch signaling in mediating the pathological actions of Ang II in VSMCs. In human AoSMCs, nuclear NICD levels increased and peaked at 30 min after Ang II stimulation (Figure 3a), indicating that Ang II stimulation activates Notch signaling in VSMCs as well as in HEK293-AT₁ cells. Ang II is an important growth factor for VSMCs, and Ang II stimulation significantly increased DNA synthesis in human AoSMCs as measured by incorporation of the nucleotide analog BrdU (Figure 3b). Ang II is also a potent chemoattractant for VSMCs,

and Ang II stimulation significantly enhanced the migration of human AoSMCs as measured by a Boyden chamber assay (Figure 3c). The enhanced DNA synthesis and migration of AoSMCs in response to Ang II stimulation were significantly suppressed by treatment with DAPT (Figures 3b and c). These findings suggest that Notch signaling mediates the Ang II-induced enhancement of VSMC proliferation and migration.

Notch signaling mediates Ang II-induced vascular remodeling in mice

Ang II contributes to structural remodeling of the aorta, which is characteristic of the vascular damage in hypertension and atherosclerosis. Analysis of hematoxylin and eosin staining of tissue sections revealed that the medial thickness of the abdominal aorta of wild-type mice was increased 1.6-fold after 2 weeks of Ang II infusion (Figures 4a and b). Masson's trichrome staining indicated that Ang II infusion induced perivascular fibrosis (Figure 4a). Systemic administration of the γ-secretase inhibitor dibenzazepine significantly reduced Ang II-induced medial thickening and perivascular fibrosis in the aorta of mice (Figures 4a and b). These findings suggest that Notch signaling mediates Ang II-induced vascular remodeling in mice.

DISCUSSION

The results of this study provide a more comprehensive insight into the role of AT₁ receptor signaling in the pathogenesis of vascular remodeling and help clarify the mechanism of action of this pathway. Upon activation by Ang II, the AT₁ receptor stimulates the Notch signaling pathway via the enhancement of γ-secretase enzymatic activity. Following such stimulation, Notch signaling promotes the
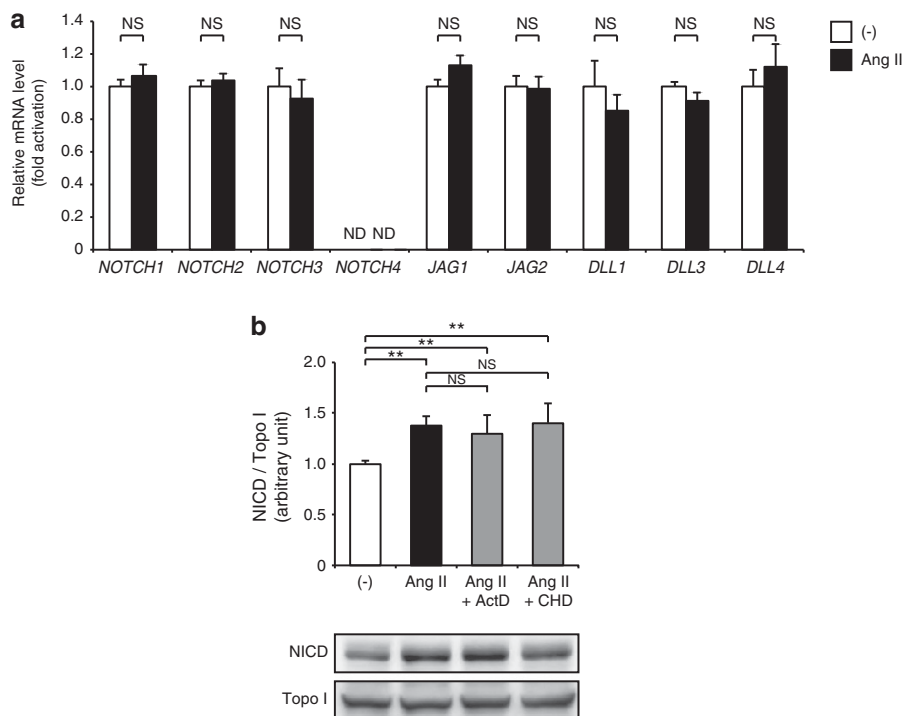

Figure 2 Ang II activates Notch signaling independently of the *de novo* synthesis of Notch receptors and ligands. (a) The mRNA expressions of *NOTCH1*, *NOTCH2*, *NOTCH3*, *NOTCH4*, *JAG1*, *JAG2*, *DLL1*, *DLL3* and *DLL4* in HEK293-AT₁ cells were determined 10 min after stimulation with Ang II (10^{-7} M) or vehicle. The experiments were repeated five times in duplicate. The data are presented as the mean ± s.e.m. ND, not determined; NS, not significant. (b) HEK293-AT₁ cells were pretreated with or without actinomycin D ($1 \mu g\,ml^{-1}$) or cycloheximide ($7.5 \mu g\,ml^{-1}$) and stimulated with Ang II (10^{-7} M) for 10 min. NICD and Topo I in the nuclear fraction recere visualized by immunoblotting. The NICD/Topo I ratios were quantified and presented as a bar graph (upper panel). The data are presented as the mean ± s.e.m. ActD, actinomycin D; CHD, cycloheximide; NS, not significant. **$P < 0.01$.

proliferation and migration of VSMCs, and contributes to the progression of vascular remodeling, which is characterized by aortic medial thickening and perivascular fibrosis. Our results provide the

Figure 3 Ang II stimulates the proliferation and migration of human aortic SMCs via activation of Notch signaling. (**a**) AoSMCs were stimulated with Ang II (10^{-7} M) for the indicated periods, and NICD protein in the nuclear fraction was visualized by immunoblotting. Nucleoporin was used as an internal control for the amount of nuclear protein. (**b**) Effect of inhibiting Notch signaling on Ang II-induced proliferation of AoSMCs. Proliferation of AoSMCs following stimulation with Ang II (10^{-7} M) in the presence or absence of DAPT (2×10^{-5} M) was monitored by BrdU incorporation. The data are presented as the mean ± s.e.m. *$P < 0.05$, **$P < 0.01$. (**c**) The effect of inhibiting Notch signaling on Ang II-induced migration of AoSMCs. The number of migrated AoSMCs in the presence or absence of DAPT (2×10^{-5} M) was counted after stimulation with Ang II (10^{-7} M). The data are presented as the mean ± s.e.m. **$P < 0.01$.

first experimental evidence for hierarchical and functional crosstalk between two major signaling pathways, AT$_1$ receptor signaling and Notch signaling.

It remains unclear how γ-secretase activity is enhanced as a consequence of AT$_1$ receptor activation. It has been reported that the amplitude and duration of Notch signal activation can be influenced at various points of the pathway, depending on the cellular context, by the regulation of endocytosis, trafficking of ligands and receptors, ectodomain shedding by ADAM metalloprotease, intramembrane proteolysis by γ-secretase and stochastic binding of Notch ligands to Notch receptors.[9] We found that *de novo* synthesis of Notch receptors and ligands was not involved in the activation of the AT$_1$ receptor–Notch signaling pathway (Figure 2), and that the amplitude of Notch signal activation by AT$_1$ receptor stimulation was unchanged when HEK293-AT$_1$ cells were cultured at various initial cell-seeding densities (Supplementary Figure S1). The direct measurement of γ-secretase activity revealed that γ-secretase enzymatic activity was enhanced following AT$_1$ receptor stimulation (Figure 1b). We therefore speculate that AT$_1$ receptor stimulation activates Notch signaling simply by enhancing the proteolytic activity of γ-secretase without any effect on the kinetics and dynamics of ligand–receptor binding. A previous study indicated that activation of the β$_2$-adrenergic receptor, a typical GPCR, stimulates γ-secretase activity and accelerates amyloid plaque formation.[21] Such enhancement involves the association of β$_2$-adrenergic receptor with presenilin-1, and requires the agonist-induced endocytosis of β$_2$-adrenergic receptor and the trafficking of γ-secretase to late endosomes and lysosomes.[21] It will be interesting to elucidate the precise molecular mechanism whereby activation of GPCRs leads to increased enzymatic activity of γ-secretase.
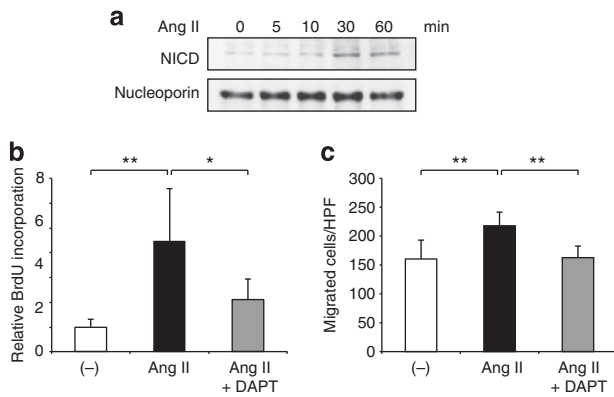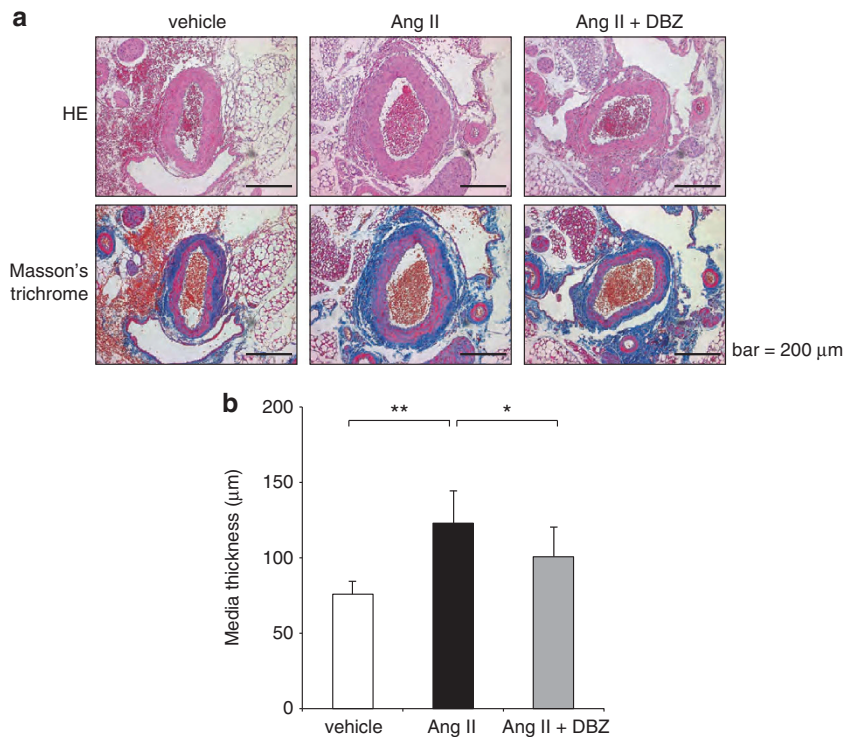

Figure 4 Ang II promotes vascular remodeling via activation of Notch signaling. (**a**) Representative hematoxylin and eosin (HE) staining (upper panels) and Masson's trichrome staining (lower panels) of the thoracic aortas of mice treated with vehicle, Ang II or Ang II/dibenzazepine (DBZ). Scale bars: 200 μm. (**b**) Medial thickness of the abdominal aortas of mice treated with vehicle, Ang II or Ang II/DBZ ($n = 4$ sections from five to six mice in each group). The data are presented as the mean ± s.e.m. *$P < 0.05$, **$P < 0.01$.

Notch signaling has been reported to control the cell fate, proliferation, differentiation and migration of postnatal VSMCs.[11,12,22] The established vascular target genes of the Notch signaling pathway include the *Hes* and *Hey* gene families.[8,12] Interestingly, Notch 3 receptor signaling promoted the proliferation of VSMCs by decreasing mRNA expression of the cell-cycle inhibitor p27^{Kip1}.[23] As p27^{Kip1} is expressed abundantly in quiescent VSMCs and Ang II promotes proliferation particularly when the expression levels of p27^{Kip1} are low,[24] it is reasonable to hypothesize that Notch signal activation mediates the Ang II-induced proliferation of VSMCs via reduction of p27^{Kip1} expression levels. Further studies are necessary to identify the Notch target genes responsible for mediating AT$_1$ receptor-induced proliferation and migration of VSMCs.

In spite of the abundant literature supporting the crucial role of Notch signaling in cardiovascular development,[25] the role of Notch signaling in the pathogenesis of cardiovascular remodeling under pathological conditions has not been extensively investigated. Studies using genetic disruption or activation of Notch signaling have shown that Notch signaling mediates cardiac repair and regeneration following myocardial infarction.[26] Notch signaling has also been found to promote atherosclerosis, metabolic disorders and pulmonary vascular remodeling in animal models.[27–29] A potential limitation of the present study is that γ-secretase inhibitors exert nonspecific effects on other type I transmembrane proteins (for example, amyloid precursor protein, ErbB4, CD44 and E-cadherin) that have been shown to be substrates for γ-secretase.[18] As a follow-up to our finding that the AT$_1$ receptor–Notch signaling pathway is involved in the pathogenesis of vascular remodeling, further studies using sophisticated genetic approaches will lead to a greater understanding of the functions and roles of the AT$_1$ receptor–Notch signaling pathway in a wide variety of cardiovascular and metabolic diseases.

In conclusion, Notch signaling is activated as a consequence of the enhanced enzymatic activity of γ-secretase by AT$_1$ receptor activation. This hierarchical pathway stimulates the proliferation and migration of VSMCs, and contributes to vascular remodeling in mice. Vascular remodeling is a crucial feature of end-organ damage associated with hypertension and atherosclerosis, which contributes to clinical morbidity and mortality. A growing body of evidence suggests that the use of AT$_1$ receptor blockers leads to a significant decrease in mortality and morbidity in patients with cardiovascular complications.[30,31] The AT$_1$ receptor activates multiple downstream signaling pathways, and some novel AT$_1$ receptor blockers function as biased ligands, which selectively activate only those pathways that lead to beneficial effects.[32,33] Elucidation of the molecular basis of the hierarchical AT$_1$ receptor–Notch signaling pathway will therefore be helpful in developing novel AT$_1$ receptor blockers with higher efficacy in preventing vascular remodeling.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Hypertension Research website (http://www.nature.com/hr)