Vascular ADAM17 as a Novel Therapeutic Target in Mediating Cardiovascular Hypertrophy and Perivascular Fibrosis Induced by Angiotensin II

Takehiko Takayanagi,* Steven J. Forrester,* Tatsuo Kawai, Takashi Obama, Toshiyuki Tsuji, Katherine J. Elliott, Elisa Nuti, Armando Rossello, Hang Fai Kwok, Rosario Scalia, Victor Rizzo, Satoru Eguchi

See Editorial Commentary, pp 849–850

Abstract—Angiotensin II (AngII) has been strongly implicated in hypertension and its complications. Evidence suggests the mechanisms by which AngII elevates blood pressure and enhances cardiovascular remodeling and damage may be distinct. However, the signal transduction cascade by which AngII specifically initiates cardiovascular remodeling, such as hypertrophy and fibrosis, remains insufficiently understood. In vascular smooth muscle cells, a metalloproteinase ADAM17 mediates epidermal growth factor receptor transactivation, which may be responsible for cardiovascular remodeling but not hypertension induced by AngII. Thus, the objective of this study was to test the hypothesis that activation of vascular ADAM17 is indispensable for vascular remodeling but not for hypertension induced by AngII. Vascular ADAM17–deficient mice and control mice were infused with AngII for 2 weeks. Control mice infused with AngII showed cardiac hypertrophy, vascular medial hypertrophy, and perivascular fibrosis. These phenotypes were prevented in vascular ADAM17–deficient mice independent of blood pressure alteration. AngII infusion enhanced ADAM17 expression, epidermal growth factor receptor activation, and endoplasmic reticulum stress in the vasculature, which were diminished in ADAM17–deficient mice. Treatment with a human cross-reactive ADAM17 inhibitory antibody also prevented cardiovascular remodeling and endoplasmic reticulum stress but not hypertension in C57Bl/6 mice infused with AngII. In vitro data further supported these findings. In conclusion, vascular ADAM17 mediates AngII-induced cardiovascular remodeling via epidermal growth factor receptor activation independent of blood pressure regulation. ADAM17 seems to be a unique therapeutic target for the prevention of hypertensive complications. (Hypertension. 2016;68:949-955. DOI: 10.1161/HYPERTENSIONAHA.116.07620.) ● Online Data Supplement

Key Words: end-organ damage ■ fibrosis ■ hypertension ■ renin angiotensin system ■ signal transduction

The prevalence and morbidity of hypertension is growing steadily.1 End-organ damage is the most important clinical consequence of hypertension causing cardiac failure, renal failure, and stroke. Despite great achievements in blood pressure therapy, optimally treated hypertensive patients still have a 50% greater risk than untreated normotensive subjects,2 suggesting the urgent need of add-on therapy to specifically target hypertensive end-organ damage. Vascular remodeling has been strongly implicated in hypertensive end-organ damage and associated with poor cardiovascular outcomes. The remodeling predisposes to end-organ damage, and pharmacological intervention in vascular remodeling should have special clinical efficacy for prevention of hypertensive organ damage.3 The renin angiotensin system has been strongly implicated in hypertension and its complications. Importantly, it has been suggested that the mechanisms by which angiotensin II (AngII) elevates blood pressure and enhances cardiovascular remodeling and end-organ damage may be distinct.4 Although many downstream signaling cascades and target genes/proteins of AngII have been identified, the proximal key event primarily responsible for vascular remodeling, such as vascular hypertrophy and fibrosis, independent of blood pressure, remains largely unclear.5,6 AngII mediates vascular smooth muscle cell (VSMC) contraction via G_{q}-mediated intracellular Ca^{2+} elevation and G_{12/13}-mediated Rho kinase activation.7 ADAM (a disintegrin
ADAM17 gene transfer prevents neointimal hyperplasia. Others have shown that ADAM17 expression is enhanced in atherosclerosis and in the left ventricle on AngII infusion. However, whether vascular ADAM17 manipulation has therapeutic potential against hypertensive complications remains completely unknown. Therefore, using mice lacking VSMC ADAM17 or treated with a human cross-reactive ADAM17 antibody as well as with in vitro fibrosis assessment, we have tested our hypothesis that vascular ADAM17 is indispensable for cardiovascular remodeling but not for hypertension induced by AngII, thus, highlighting a unique therapeutic target in hypertension.

Methods

Animal Studies and the Tissue Analysis
Animal procedures were performed in accordance with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and Temple University IACUC guidelines. Eight- to 10-week-old male ADAM17αCre +/− mice and control αCre +/− mice were infused with AngII (Bachem, 1 μg/kg/min) for 2 weeks via osmotic mini-pump. Eight- to 10-week-old male C57BL6 mice (Jackson) were infused with AngII (Bachem, 1 μg/kg/min) and treated with human cross-reactive ADAM17 inhibitory antibody A9B8 or control human IgG2 (Athens Research & Technology) which was solubilized in PBS, 10 mg/kg/d intraperitoneal injection, at day 1 and day 7. Blood pressure and heart rate were evaluated in the conscious state by telemetry (Data Sciences International equipped with ADInstrument 6 software) via carotid catheter (PA-C10 transmitter). Cardiac function was measured using VisualSonics Velvo 2100 (M-mode). Plasma B-type natriuretic peptide and blood urea nitrogen concentrations were determined by the EIA kits (RayBiotech Inc and Stanbio Laboratories, respectively). Quantitative real-time polymerase chain reaction was performed with SYBR Green qPCR Master Mix (Fermentas) as described previously.

Cell Culture and Experiments
VSMCs were prepared from thoracic aortas of male Sprague–Dawley rats by the explant method as described previously. VSMCs were subcultured in DMEM containing 10% fetal bovine serum, penicillin, and streptomycin. Cells from passage 3 to 10 at 80% to 90% confluency were used for experiments.

Immunoblotting was performed as previously described. Quiescent VSMCs grown on 6-well plates were stimulated with 100 nmol/L AngII (Sigma) for specified durations. The reaction was terminated by the replacement of medium with 100 μL of 1×SDS lysis buffer. Forty microliter of the cell lysates were subjected to SDS-PAGE gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane. The membranes were then exposed to primary antibodies overnight at 4°C. After incubation with the
peroxidase-linked secondary antibody for 1 hour at room temperature, immunoreactive proteins were visualized using a chemiluminescence reaction kit.

To evaluate proinflammatory response, serum-starved VSMCs were stimulated with 100 mmol/L AngII for 48 hours, and extracellular cellular collagen content was quantified by Sirius Red collagen quantification kit (Chondrex) according to the manufacturer’s protocol. Recombinant adenoviral vector encoding rat ADAM17 siRNA was created, and specificity and efficiency has been reported.13 VSMCs were infected with 100 m.o.i. adenovirus before the AngII stimulation as reported previously.13 ADAM17-selective inhibitor compound no 21 (JG26)8 was generated as reported. VSMCs were pretreated with 1 μmol/L JG26 or vehicle (0.1% DMSO in final) for 30 minutes before the AngII stimulation. 4-Phenylbutyrate was obtained from Scandinavian Formulas and solubilized in DMEM. VSMCs were pretreated with 10 mmol/L 4-phenylbutyrate for 30 minutes. Erlotinib (OSI Pharmaceuticals) was obtained from Genentech. VSMCs were pretreated with 1 μmol/L erlotinib for 30 minutes.

Antibodies
Antibodies against Tyr<sup>1068</sup>-phosphorylated EGFR for immunohistochemistry (2234) and Ser<sup>1033</sup>-phosphorylated eIF2α were purchased from Cell Signaling. Antibody against Tyr<sup>1068</sup>-phosphorylated EGFR for immunoblotting (4478G) was purchased from Invitrogen. Antibody against KDEL for detection of an endoplasmic reticulum (ER) stress marker GRP78 (ADI-SPA-827) was purchased from Enzo Life Sciences. Antibodies against ADAM17 for immunoblotting (sc-13973), EGFR (sc-03), and an ER stress marker, CHOP-10/GADD153 (sc-575), were purchased from Santa Cruz Biotechnology. Antibodies against ADAM17 for immunohistochemistry (ab59163) were purchased from Abcam. Antibodies against Cre recombinase (MAB3120) and GAPDH (MAB374) were purchased from Millipore.

Statistical Analysis
Data are presented as mean±SEM or SD where appropriate. Differences between the multiple groups were analyzed by 1-way or 2-way analysis of variance, followed by the Tukey’s post hoc test. Statistical significance was set at P<0.05.

Results
Prevention of AngII-Induced Cardiovascular Remodeling in Mice Lacking VSMC ADAM17
VSMC ADAM17–deficient (ADAM17<sup>fl/fl</sup> sm22αCre<sup>cre</sup>) mice were generated,16 and vascular selective Cre expression was confirmed (Figure S1A in the online-only Data Supplement). VSMC ADAM17–deficient mice and littermate control ADAM17<sup>fl/fl</sup> sm22αCre<sup>cre</sup> mice were infused with 1 μg/kg/min AngII for 2 weeks. In control littermate mice, 2 weeks of AngII infusion caused vascular hypertrophy in aorta and coronary arteries that was markedly prevented in VSMC ADAM17–deficient mice. Perivascular fibrosis induced by AngII infusion was also prevented in VSMC ADAM17–deficient mice (Figure 1A; Figure S1B). In control mice, AngII infusion for 2 weeks induced cardiac hypertrophy assessed by heart weight to body weight ratio and echocardiogram (Figure 1B; Table S1A). Serum B–type natriuretic peptide and blood urea nitrogen concentrations were also elevated in these mice (Figure S1C). These cardiac and renal alterations by AngII infusion were attenuated in VSMC ADAM17–deficient mice. In contrast, hypertension was induced in both groups infused with AngII at 2 weeks (Figure 1C; Table S1B). AngII-induced vascular remodeling in control mice was associated with vascular-dominant EGFR activation and ER stress assessed by immunohistochemistry. These AngII responses were attenuated in VSMC ADAM17–deficient mice. ADAM17 expression was barely detectable in heart or kidney but was significantly induced on AngII infusion in the vasculature. No such induction was observed in VSMC ADAM17–deficient mice (Figure 2; Figure S2). Quantitative polymerase chain reaction analysis of aortic mRNA confirmed ADAM17 induction by AngII as well as vascular ADAM17 silencing. ADAM17 mRNA was also increased in whole heart with AngII infusion in control mice but not in VSMC ADAM17–deficient mice (Figure S3). In addition, although statistically insignificant, cardiac ADAM17 mRNA expression tends to be less in saline-infused VSMC ADAM17–deficient mice compared with saline-infused control mice.

Human Cross-Reactive ADAM17 Antibody Attenuates Cardiovascular Remodeling Induced by AngII
To ascertain that ADAM17 represents a novel therapeutic target contributing to target organ remodeling, AngII-infused C57Bl6 mice were treated with a human cross-reactive ADAM17 inhibitory antibody A9B8.18 C57Bl6/6 mice were
infused with 1 μg/kg/min AngII for 2 weeks with treatment of ADAM17 antibody or control IgG (10 mg/kg IP on day 1 and 7). A9B8 prevented AngII-induced cardiovascular hypertrophy and perivascular fibrosis but not hypertension or its development. These responses were associated with suppression of ER stress (Figure 3; Figure S4 and Table S2A and S2B).

ADAM17 Antibody, a Small Molecule ADAM17 Inhibitor or Adenovirus Encoding ADAM17 siRNA, Attenuates a Fibrotic Response Induced by AngII In Vitro

To test for the role of ADAM17 in AngII-induced vascular fibrosis, extracellular collagen content was evaluated in VSMCs stimulated with AngII. Adenovirus encoding ADAM17 siRNA11 attenuated the AngII-induced enhancement in collagen content (Figure 4A and 4B). Treatment of VSMCs with ADAM17 antibody A9B8, a highly selective ADAM17 inhibitor JG26/compound 21,22 an EGFR kinase inhibitor erlotinib or a chemical ER chaperone 4-phenylbutyrate also attenuated AngII-induced enhancement in collagen content (Figure 4C; Figure S5A–S5C). As shown with the ADAM17 siRNA,11 A9B8 as well as JG26 inhibited AngII-induced EGFR transactivation assessed with an auto-phosphorylation site antibody (Figure 4D; Figure S5D).

Discussion

The major finding of the present study is that AngII-induced cardiovascular hypertrophy and perivascular fibrosis but not hypertension were attenuated in mice lacking VSMC ADAM17 expression, as well as in mice treated with an ADAM17 antibody. The suppression of AngII-induced...
deficient mice.25 In our 2-week AngII infusion model, interstitial fibrosis is attenuated in proximal tubule EGFR-genic mice develop cardiac fibrosis.24 AngII-induced renal hypertension.17,26

Inhibition of the fibrotic response in VSMCs with ER stress has been implicated in cardiovascular diseases,33 whereas limited information is available for its role in hypertension.14 Our recent study suggests a potential prevention of hypertensive organ damage but not hypertension by 4-phenylbutyrate, which seems to involve VSMC ADAM17 as well as EGFR.35 Inhibition of the fibrotic response in VSMCs with ER stress inhibition further suggests the presence of ER stress–responsible downstream signal transduction leading to vascular fibrosis, which likely involves transcriptional upregulation of several distinct genes.17,20

Perspectives
The vascular ADAM17/EGFR signal transduction axis seems to be essential for cardiovascular remodeling associated with ER stress but not for hypertension in mice with AngII infusion. The signal seems to include a feed-forward mechanism involving vascular ADAM17 induction, which enhances EGFR ligand production and subsequent EGFR activation and vascular remodeling. Although the intervention with ADAM17 antibody in mice seems promising, additional research in large animals and humans will be necessary to seek for an add-on treatment against hypertensive complications.

Acknowledgment
We thank Dr Gillian Murphy for A9B8 preparation.

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Disclosures

None.

References


Novelty and Significance

What Is New?
• Analysis of blood pressure and vascular pathology in the heart, kidney, and aorta with vascular smooth muscle cell ADAM17 deletion or ADAM17 inhibition by an antibody established a role for this metalloproteinase in Angiotensin II–induced pathological vascular remodeling independent of hypertension in mice.
• The concept of the feed-forward induction of vascular ADAM17 to amplify the epidermal growth factor receptor pathway and subsequent endoplasmic reticulum stress.

What Is Relevant?
• Vascular-restricted ADAM17 signal transduction highlights the importance of vascular pathology for subsequent tissue dysfunction in hypertension.
• Results indicating prevention of vascular remodeling but not hypertension by human cross-reactive ADAM17 antibody provide a foundation to seek a potential add-on therapy to current pressure-lowering treatments for hypertension.

Summary
In vascular smooth muscle ADAM17–deficient mice or mice treated with ADAM17 inhibitory antibody, vascular hypertrophy and perivascular fibrosis but not hypertension were prevented. Angiotensin II infusion showed vascular ADAM17 induction, epidermal growth factor receptor activation, and ER stress, which were attenuated in these mice. Cultured vascular smooth muscle cells were used to confirm the involvement of the ADAM17/epidermal growth factor receptor signaling axis in the induction of vascular fibrosis.
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* These authors contributed equally to this work.

**Short title:** Vascular ADAM17 mediates organ damage by AngII

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### Supplementary Tables S1

#### S1A. M-mode echocardiography at 2 weeks after AngII infusion

<table>
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<tr>
<th>Parameters</th>
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<th>A17ffsmCre/-</th>
<th>A17ffsmCre+/-</th>
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<tr>
<td></td>
<td>saline</td>
<td>AngII</td>
<td>saline</td>
<td>AngII</td>
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<tr>
<td>IVSd (mm)</td>
<td>0.620±0.050</td>
<td>0.781±0.097*</td>
<td>0.590±0.049</td>
<td>0.581±0.040†</td>
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<td>LVIDd (mm)</td>
<td>3.72±0.15</td>
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<td>LVPWd (mm)</td>
<td>0.750±0.017</td>
<td>0.963±0.049*</td>
<td>0.638±0.060</td>
<td>0.755±0.070†</td>
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<td>LVIDs (mm)</td>
<td>2.95±0.20</td>
<td>2.09±0.15*</td>
<td>2.88±0.11</td>
<td>2.70±0.21†</td>
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<td>FS (%)</td>
<td>29.4±4.1</td>
<td>29.5±1.4</td>
<td>33.7±1.9</td>
<td>34.4±5.9</td>
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</tbody>
</table>

Mean±SD (n=8), *p<0.001 compared with saline* or AngII† infusion.

IVSd: interventricular septum thickness in diastole; LVIDd: LV internal diameter in diastole; LVPWd: LV posterior wall thickness in diastole; LVIDs: LV internal diameter in systole; FS: fractional shortening.

#### S1B. Effects of VSMC ADAM17 deletion on characteristics of mice infused with AngII

<table>
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<th>Parameters</th>
<th>A17ffCre/- saline</th>
<th>A17ffCre/- AngII</th>
<th>A17ffCre+- saline</th>
<th>A17ffCre+- AngII</th>
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<td>BW (g)</td>
<td>25.4±4.2</td>
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<td>24.5±2.4</td>
<td>21.8±3.7</td>
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<td>SBP/DBP (mmHg)</td>
<td>121±5/88±13</td>
<td>185±19*/142±19*</td>
<td>117±11/96±10</td>
<td>182±26*/131±18*</td>
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<tr>
<td>HR (beats/min)</td>
<td>496±159</td>
<td>570±100</td>
<td>463±146</td>
<td>585±58</td>
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</table>

Mean±SD (n=8), *p<0.001 compared with saline infusion. BW: body weight; SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate. No significance was detected among the parameters between Cre+/- and Cre/- animals regardless of the treatment.
### Supplementary Table S2

#### S2A. M-mode echocardiography at 2 weeks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>saline</th>
<th>AngII+IgG2</th>
<th>AngII+A9B8</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVSd (mm)</td>
<td>0.679±0.020</td>
<td>0.902±0.029*</td>
<td>0.774±0.025†</td>
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<td>LVIDd (mm)</td>
<td>3.84±0.07</td>
<td>3.58±0.10*</td>
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<td>LVPWd (mm)</td>
<td>0.707±0.020</td>
<td>0.847±0.020*</td>
<td>0.798±0.036†</td>
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<td>LVIDs (mm)</td>
<td>3.11±0.07</td>
<td>2.634±0.08*</td>
<td>2.67±0.15</td>
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<tr>
<td>FS (%)</td>
<td>28.7±0.7</td>
<td>30.2±0.7</td>
<td>29.7±1.0</td>
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Mean±SD (n=8), p<0.001 compared with saline* or AngII† infusion. IVSd: interventricular septum thickness in diastole; LVIDd: LV internal diameter in diastole; LVPWd: LV posterior wall thickness in diastole; LVIDs: LV internal diameter in systole; FS: fractional shortening.

#### S2B. Effects of A9B8 on characteristics of mice infused with AngII

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<tbody>
<tr>
<td>BW (g)</td>
<td>23.3±2.2</td>
<td>25.0±2.0</td>
<td>24.6±2.1</td>
</tr>
<tr>
<td>SBP/DBP (mmHg)</td>
<td>119±15/84±12</td>
<td>172±9*/137±17*</td>
<td>170±7*/141±6*</td>
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<td>HR (beats/min)</td>
<td>634±85</td>
<td>586±87</td>
<td>583±90</td>
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Mean±SD (n=6), *p<0.001 compared with saline infusion. BW: body weight; SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate.
Supplementary Figure S1. A. Expression of Cre in VSMC ADAM17 deficient mice. Cre expression was analyzed by immunohistochemistry with anti-Cre antibody. B. VSMC ADAM17 deficient mice and control mice were infused with AngII or saline as in Figure 1. Aortas were stained with Masson’s trichrome. Representative images are shown. Data are mean±SEM (n=6). C. Plasma BNP concentration. Plasma BUN concentration. Mean±SEM (n=6). *p<0.05 compared with saline control. †p<0.05 compared with AngII control.
Supplementary Figure S2. a. Suppression of vascular ER stress in VSMC ADAM17 deficient mice. VSMC ADAM17 deficient mice and control mice were infused with AngII or saline as in Fig 1. Tissues were immuno-stained with the antibodies indicated. Representative images are presented (n=4). Data are mean±SEM (n=4). *p<0.05 compared with saline control. †p<0.05 compared with AngII control.
Supplementary Figure S3. The aorta (A) and heart (B) samples were evaluated for ADAM17 mRNA expression by qPCR. Mean±SEM (n=6). *p<0.05 compared with saline control. †p<0.05 compared with AngII control.
Supplementary Figure S4. Effects of ADAM17 inhibitory antibody, A9B8, on hypertension development induced by AngII. **A**: C57Bl/6 mice were infused with AngII from Day 0 with or without treatment of A9B8 on Day 1. Arterial pressure was evaluated by telemetry on Day 1 and Day 2 (Mean±SEM, n=3). Significant blood pressure elevation in response to AngII infusion was observed at Day 2 compared with Day 1 regardless of the antibody treatment. *p<0.05 compared with corresponding Day1 values.
Supplementary Figure S5. A: Rat aortic VSMCs pretreated with ADAM17 inhibitor JG26 (1 μmol/L) or vehicle (DMSO final concentration 0.1%) for 30 min were stimulated with 100 nmol/L AngII for 48 hours and extracellular collagen accumulation was quantified. Mean±SD (n=4). B: VSMCs pretreated with EGFR inhibitor erlotinib (Erlo) or vehicle (DMSO final concentration 0.1%) for 30 min were stimulated with 100 nmol/L AngII for 48 hours and extracellular collagen accumulation was quantified. Mean±SD (n=4). C: VSMCs pretreated with or without PBA (10 mmol/L in DMEM) were stimulated with 100 nmol/L AngII for 48 h and extracellular collagen accumulation was quantified. Mean±SD (n=4). D: VSMCs pretreated with JG26 (1 μmol/L) or vehicle (DMSO final concentration 0.1%) for 30 min were stimulated with 100 nmol/L AngII for 2 min and immunoblottings were performed with antibodies as indicated. Mean±SD (n=4). *p<0.05 compared with vehicle control. †p<0.05 compared with AngII control.