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ORIGINAL RESEARCH

Angiotensin II Type 1 Receptor Blocker Prevents Abdominal Aortic Aneurysm Progression in Osteoprotegerin-Deficient Mice via Upregulation of Angiotensin (1–7)

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BACKGROUND: Angiotensin II type 1 receptor blockers (ARBs) have been shown to limit the growth of abdominal aortic aneurysm (AAA), but their efficacy is controversial. This study aimed to investigate the molecular mechanism underlying the protective effect of ARBs against AAA progression.

METHODS AND RESULTS: Olmesartan, an ARB, was administered to wild-type and *osteoprotegerin*-knockout (*Opg*-KO) mice starting 2 weeks before direct application of $CaCl_2$ to aortas to induce AAA. The protective effect of olmesartan against AAA in wild-type and *Opg*-KO mice was compared at 6 weeks after AAA induction. Olmesartan prevented AAA progression in *Opg*-KO mice, including excessive aortic dilatation and collapse of tunica media, but not in wild-type mice. Deficiency of the *Opg* gene is known to cause excessive activation of the tumor necrosis factor–related apoptosis-inducing ligand–induced c-Jun N-terminal kinase/matrix metalloproteinase 9 pathway, resulting in prolonged AAA progression. Olmesartan attenuated the upregulation of phosphorylated c-Jun N-terminal kinase and matrix metalloproteinase 9 expression in the aortic wall of *Opg*-KO mice. In cultured vascular smooth muscle cells, tumor necrosis factor–related apoptosis-inducing ligand–induced c-Jun N-terminal kinase phosphorylation and matrix metalloproteinase 9 expression were inhibited by angiotensin (1–7), the circulating levels of which are increased by ARBs. Furthermore, administering an angiotensin (1–7) antagonist to *Opg*-KO mice diminished the protective effect of olmesartan against AAA progression.

CONCLUSIONS: Olmesartan prevented AAA progression in *Opg*-KO mice by upregulating angiotensin (1–7), suggesting that angiotensin (1–7) may be a key factor that mediates the protective effect of ARBs.

Key Words: abdominal aortic aneurysm ■ angiotensin II type 1 receptor blocker ■ osteoprotegerin ■ angiotensin (1–7)

Abdominal aortic aneurysm (AAA) is a common aortic disease associated with chronic inflammation and degenerative changes in the aortic wall. Smoking, hypertension, family history, male sex, and aging are major risk factors for AAA formation.[1] A lethal complication of AAA is aortic rupture, resulting in a mortality rate as high as 20% to 30%, even with surgical intervention.[2,3] Elective AAA repair is the only available treatment option for preventing aortic rupture, and pharmacologic therapies to limit AAA growth have not yet been established.[4] The lack of therapies for AAA has been a major burden on patients with AAA who are not indicated for surgery.

Angiotensin II is a peptide involved in AAA formation.[5,6] Several observational studies, including a nationwide cohort study, have demonstrated that angiotensin II type 1 receptor blockers (ARBs) can reduce the rate of death from AAA and the speed of

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CLINICAL PERSPECTIVE

What Is New?

- An angiotensin II type 1 receptor blocker, olmesartan, attenuates progression of $CaCl_2$-induced abdominal aortic aneurysm in the *osteoprotegerin*-deficient mice.
- Angiotensin (1–7) downregulates tumor necrosis factor–related apoptosis-inducing ligand–induced c-Jun N-terminal kinase phosphorylation and matrix metalloproteinase 9 expression in vascular smooth muscle cells.
- Administration of an angiotensin (1–7) antagonist diminishes the protective effect of olmesartan against the abdominal aortic aneurysm progression in our model.

What Are the Clinical Implications?

- Our findings support the potential of angiotensin II type 1 receptor blockers as therapeutic drugs to suppress abdominal aortic aneurysm growth.
- This study suggests that the potential role of angiotensin (1–7) should be considered in therapeutic effects of angiotensin II type 1 receptor blockers against abdominal aortic aneurysm.

Nonstandard Abbreviations and Acronyms

AAA	abdominal aortic aneurysm
Jnk	c-Jun N-terminal kinase
Mmp9	matrix metalloproteinase 9
Opg-KO	*osteoprotegerin* knockout
Trail	tumor necrosis factor–related apoptosis-inducing ligand
VSMC	vascular smooth muscle cell

AAA progression.[7–9] However, other clinical studies, including a recent randomized controlled trial, found that ARBs had no effect on AAA progression.[10,11] Thus, the efficacy of ARBs in treating human AAA is controversial. In mouse and rat AAA models induced by elastase infusion into an aorta or systemic angiotensin II infusion, ARBs reportedly inhibited AAA formation independently of their hypotensive effects.[12–14] However, the detailed molecular mechanism underlying how ARBs protect against AAA development is unclear. Clarification of the mechanism may help explain the discrepancy observed in the efficacy of ARBs against AAA in clinical settings.

The $CaCl_2$-induced AAA model is used to study AAA pathophysiology associated with inflammation. We previously reported that osteoprotegerin, a soluble receptor belonging to the tumor necrosis factor receptor superfamily,[15] has a protective role in this model. *Opg*-knockout (*Opg*-KO) mice subjected to $CaCl_2$-induced AAA exhibit serious AAA, including excessive aortic dilatation and collapse of aortic elastic fibers.[16] AAA development in *Opg*-KO mice is accompanied by prolonged and increased phosphorylation of c-Jun N-terminal kinase (Jnk) and matrix metalloproteinase 9 (Mmp9) expression,[16] which are responsible for aortic tissue destruction and AAA formation.[17–19] We also found that tumor necrosis factor–related apoptosis-inducing ligand (Trail), a cytokine belonging to the tumor necrosis factor superfamily, which is inhibited by osteoprotegerin,[15] increases the severity of AAA in *Opg*-KO mice. Trail induces Mmp9 and its own expression in vascular smooth muscle cells (VSMCs) via the Jnk pathway; this process is inhibited by osteoprotegerin.[16] Thus, *Opg* deficiency results in hyperactivation of the Trail-induced Jnk-Mmp9 pathway and AAA progression. The severe AAA progression model, associated with Trail-Jnk-Mmp9 pathway, may be useful for assessing the attenuation of AAA progression via the pleiotropic effects of ARBs beyond angiotensin II blockade.

Herein, we show that olmesartan, an ARB, prevents AAA progression in *Opg*-KO mice. In the $CaCl_2$-induced AAA model, administration of olmesartan in *Opg*-KO mice attenuated the excessive enlargement of AAA, the collapse of aortic tissue, and the increase in Jnk phosphorylation and Mmp9 expression in the aortic wall. Angiotensin (1–7), the serum levels of which are increased by ARBs, reduced Jnk phosphorylation and Mmp9 expression induced by Trail in cultured VSMCs. An antagonist of angiotensin (1–7) limited the protective effect of olmesartan against AAA progression in *Opg*-KO mice. Our findings suggest that ARBs mitigate activation of the Trail-induced Jnk-Mmp9 pathway to prevent AAA progression in *Opg*-KO mice, and the protective effect of ARBs may occur via the upregulation of angiotensin (1–7).

METHODS

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Murine AAA Model

Male wild-type and *Opg*-KO mice of the C57BL/6J strain (CLEA Japan, Inc) were used for experiments. At 7 weeks of age, AAAs were induced by the $CaCl_2$ method, as previously reported.[16] After mice were anesthetized by intraperitoneal administration of a mixture of anesthetic agents (0.3 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol), a

small piece of cotton soaked with 50 μL of 0.5 M $CaCl_2$ was placed on the abdominal aortic wall for 15 minutes. Mice were administered olmesartan (provided by Daiichi Sankyo Co, Ltd) at 20 mg/kg body weight per day by oral gavage starting 2 weeks before AAA induction to the date of euthanasia. The angiotensin (1–7) antagonist, [D-Ala 7]-angiotensin-(1–7) (A779; Tokyo Chemical Industry Co, Ltd), was administered using a micro-osmotic pump (ALZET model 2006; DURECT CORPORATION) from AAA induction to the date of euthanasia at a rate of 400 ng/kg per minute. The control group received vehicle (saline). Blood pressure was measured by using the tail-cuff method (BP-98A; Softron Ltd) in conscious mice. At 6 weeks after AAA induction, mice were euthanized with CO_2 gas, and serum and aortas were collected. Blood was sampled from the right ventricle, and serum was stored at −80 °C. Aortic tissue was fixed by perfusion and immersion in 4% paraformaldehyde. At 1 week after AAA induction, left ventricles and kidneys were harvested, submerged in RNAlater (Invitrogen, Thermo Fisher Scientific Inc), and stored at 4 °C. The experimental protocol was approved by the Committee of Animal Experimentation at Hiroshima University (A08-32).

Morphological and Histological Examination

The external diameter of the aorta was measured at the most enlarged portion under a microscope. To examine histological changes in the aorta, 6-μm-thick sections from paraffin-embedded aortic tissues were stained with hematoxylin and eosin or Elastica van Gieson using standard protocols. The width of the aortic medial layer, which was defined as the mean thickness between internal elastic lamina and external elastic lamina, was measured using Photoshop (Adobe Inc) and Image-J (National Institutes of Health).

Fluorescence Immunohistochemistry

Sections were preincubated in target retrieval solution (modified sodium citrate buffer, pH 6.1; Dako, Agilent Technologies Inc) at 90 °C for 45 minutes, then blocked with 1% BSA in 0.1% Tween-PBS and incubated with 2 primary antibodies. Antibodies for angiotensin-converting enzyme 2 (ACE2) (rabbit polyclonal, 1:200; Proteintech Group, Inc), Mmp9 (goat polyclonal, 1:40; R&D Systems, Inc), Trail (rabbit polyclonal, 1:100; Abcam plc), and phosphorylated stress-activated protein kinase (SAPK)/JNK (rabbit monoclonal, 1:100; Cell Signaling Technology Inc) were used. Subsequently, sections were incubated with appropriate secondary antibodies, including Alexa Fluor 488– or Alexa Fluor 594–conjugated anti-mouse, anti-rat, anti-rabbit, or anti-goat antibodies (donkey polyclonal, 1:500; Life Technologies, Thermo Fisher Scientific Inc), and counterstained with 4′-6-diamidino-2-phenylindole. Signals were detected with a DMI4000 fluorescence microscope (Leica Microsystems). The signal area in the tunica media was automatically detected on the basis of color information and calculated. Autofluorescence of elastic lamellae was observed in the staining control without primary antibodies in some sections (Figure S1). This region of elastic lamellae was not counted for the signal detection. The percentage expression areas of phosphorylated Jnk (pJnk), Mmp9, and Trail were calculated by dividing the signal area by the entire area of the tunica media. All measurements were performed using Photoshop (Adobe Inc) and Image-J (National Institutes of Health).

Cell Culture

VSMCs were harvested from the abdominal aorta of 5-week-old wild-type male mice, as previously described,[16,20] and cultured in DMEM with 10% fetal bovine serum. VSMCs at passages 4 to 8 were used for experiments. Before experiments, VSMCs were placed under serum starvation conditions for 24 hours. After incubation with A779 (Tokyo Chemical Industry Co, Ltd) for 1 hour, angiotensin (1–7) (PEPTIDE INSTITUTE, Inc) was added to VSMCs 20 minutes before recombinant mouse Trail (Sigma-Aldrich) stimulation. VSMCs were harvested at appropriate time points after Trail stimulation.

Western Blotting

Radioimmunoprecipitation assay lysis buffer was used to obtain cell lysates from VSMCs 20 minutes after Trail stimulation. Cell lysates were homogenized with sonication and centrifuged to remove debris, and the supernatant was used for Western blotting. Protein concentrations were measured using the DC protein assay (Bio-Rad Laboratories, Inc). Samples were mixed with sample buffer containing sodium dodecyl sulfate and dithiothreitol and heated at 95 °C for 5 minutes. Samples were then separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Membranes were blocked with 5% BSA-PBS, incubated with a primary antibody (phosphorylated SAPK/JNK, rabbit monoclonal, 1:1000, Cell Signaling Technology Inc; JNK, mouse monoclonal, 1:1000, Santa Cruz Biotechnology, Inc), and incubated with an appropriate horseradish peroxidase–linked secondary antibody. Signals were visualized using the Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc).

Real-Time Polymerase Chain Reaction

Total RNA was extracted using TRIzol (Invitrogen, Thermo Fisher Scientific Inc). The ReverTra Ace qPCR RT Kit (TOYOBO Co, Ltd) was used for reverse

transcription. Real-time polymerase chain reaction (PCR) was conducted with the Luna Universal qPCR Master Mix (New England BioLabs, Inc). PCR, measurement of PCR products, and analysis of results were performed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc). Amplification conditions were as previously described.[16] Sequences of the PCR primers are shown in Table S1. Glyceraldehyde-3-phosphate dehydrogenase (G3pdh) was used as an internal control.

Statistical Analysis

All statistical analyses were performed using Ekuseru-Toukei 2012 software (Social Survey Research Information Co, Ltd), and quantitative values are presented as mean±SD. Kruskal-Wallis test with Steel-Dwass or Steel post hoc analysis was used for multiple comparisons. Two-tailed unpaired Student *t* test was used for 2-group comparisons when the sample size was not suitable for nonparametric methods. $P<0.05$ was considered statistically significant.

RESULTS

Olmesartan Prevents the Progression of AAA Resulting From Osteoprotegerin Deficiency

We first confirmed whether olmesartan can prevent AAA progression. Olmesartan was orally administered to both wild-type and *Opg*-KO mice starting 2 weeks before AAA induction by $CaCl_2$ application to the aorta (Figure 1A). At 6 weeks after AAA induction, the external diameter of the aorta was significantly increased in both wild-type mice and *Opg*-KO mice, with *Opg*-KO mice showing a markedly increased diameter compared with wild-type mice (Figures 1B and 1C). This result is consistent with our previous studies.[16,21] However, administration of olmesartan to *Opg*-KO mice prevented aortas from excessively dilating at 6 weeks after AAA induction but had no impact on aortic dilatation in wild-type mice (Figures 1B and 1C). Aortic enlargement of both wild-type and *Opg*-KO mice at 1 week after AAA induction was unchanged by the administration of olmesartan (Figure S2A and S2B). These results suggest that olmesartan prevents AAA progression in *Opg*-KO mice but not in wild-type mice.

We next examined the degenerative changes in the aortic wall to assess the protective effect of olmesartan against AAA progression in *Opg*-KO mice. Histological analyses with Elastica van Gieson staining showed that, in wild-type mice, medial elastic lamellae were flattened with some small cracks at 6 weeks after AAA induction (Figure 2A). Meanwhile, consistent with our previous studies,[16,21] severe destruction of the aortic wall was apparent in *Opg*-KO mice compared with wild-type mice, including degradation of all elastic lamellae and a thickening of the medial layer with fibrosis (Figure 2A and 2B). This finding is suggestive of tunica media collapse. However, administration of olmesartan in *Opg*-KO mice attenuated the severe destruction of the tunica media to a comparable level seen in wild-type mice (Figure 2A). The thickening of the tunica media in *Opg*-KO mice was also significantly suppressed by the administration of olmesartan (Figure 2B). These results indicate that olmesartan can attenuate the severe degeneration of aortic tunica media caused by $CaCl_2$ application in *Opg*-KO mice.

Olmesartan Attenuates Jnk Phosphorylation and Mmp9 Expression in AAA of *Opg*-KO Mice

As discussed above, olmesartan prevents AAA progression in *Opg*-KO mice. Previously, we reported that the Jnk-Mmp9 pathway, which is activated by Trail, is likely involved in the aggravation of AAA in *Opg*-KO mice.[16,21] On this basis, we predicted that the protective effect of olmesartan against AAA progression in *Opg*-KO mice may involve inhibition of the Trail-induced Jnk-Mmp9 pathway. To test this, we performed immunohistochemical analysis to assess the levels of pJnk and Mmp9 in AAA. At 6 weeks after AAA induction, colocalization of pJnk and Mmp9 in the aortic tunica media was markedly increased in *Opg*-KO mice compared with wild-type mice (Figure 3A through 3C), consistent with our previous report.[16,21] However, administration of olmesartan reduced the colocalization in *Opg*-KO mice (Figure 3A through 3C). These results suggest that the protective effect of olmesartan against AAA progression in *Opg*-KO mice is associated with downregulation of the Jnk-Mmp9 pathway.

Angiotensin (1–7) Inhibits Trail-Induced Jnk Phosphorylation and Induction of Mmp9 mRNA Expression in VSMCs

In *Opg*-KO mice, olmesartan prevents AAA progression associated with the Trail-induced Jnk-Mmp9 pathway. We speculated that the protective effect of olmesartan against AAA progression is not directly caused by blocking angiotensin II but, rather, is mediated by an intrinsic factor. Angiotensin (1–7) levels reportedly increase in blood after administration of ARBs, including olmesartan.[22–25] In mice, the increase of angiotensin (1–7) by ARBs is accompanied by an increase in heart tissue of ACE2,[22,25] an enzyme that converts angiotensin II to angiotensin (1–7).[26] Also, in patients with cardiovascular disease, the ACE2 expression in heart tissue has been reported to be upregulated by ARBs.[27] Angiotensin (1–7) reduces Jnk phosphorylation, which

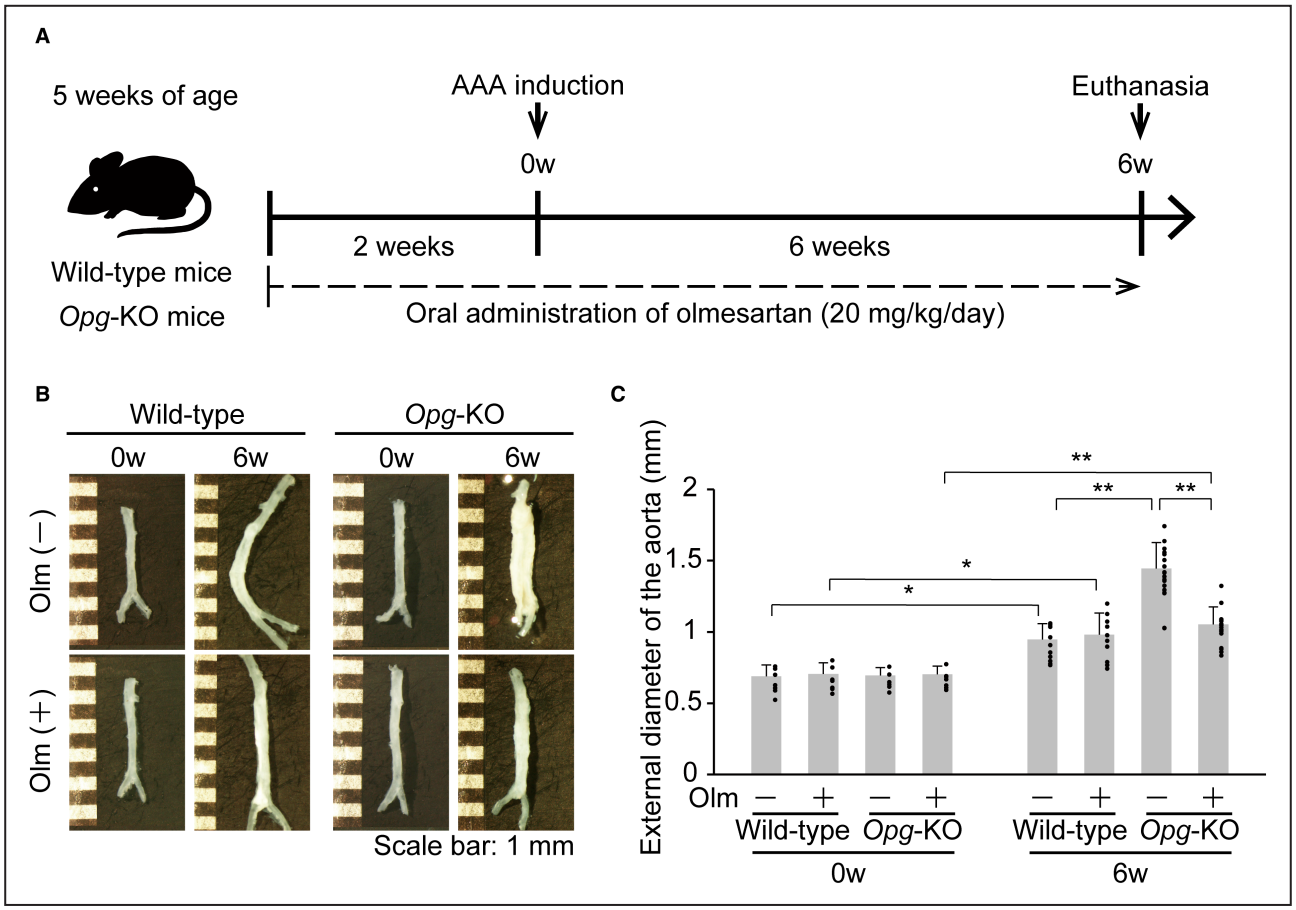

Figure 1. Olm protects against AAA progression in *Opg*-KO mice.
A, Experimental design. AAA was induced in wild-type and *Opg*-KO mice following the administration of Olm for 2 w. Mice were euthanized by 6 w after AAA induction, and Olm was administered to mice until euthanasia. **B**, Representative harvested aortas at 0 and 6 w after AAA induction in wild-type and *Opg*-KO mice, with or without Olm administration. **C**, External aortic diameter measurements at 0 and 6 w. Data are shown as mean±SD. Zero weeks: n=7. Six weeks: wild type, n=10 to 11; *Opg*-KO, n=18 to 19. **P*<0.05, ***P*<0.01. Kruskal-Wallis test with Steel-Dwass post hoc analysis was used for multiple comparisons between groups. AAA indicates abdominal aortic aneurysm; Olm, olmesartan; *Opg*-KO, *osteoprotegerin*-knockout; and w, weeks.

is upregulated by lipopolysaccharide or palmitate in endothelial cells.[28,29] Therefore, we hypothesized that angiotensin (1–7), which is increased by olmesartan via upregulation of ACE2, would decrease Mmp9 expression and Trail-induced Jnk phosphorylation.

To test this possibility, we evaluated the effect of olmesartan on expression of ACE2. Consistent with previous studies,[22,25] olmesartan increased *Ace2* mRNA expression in the heart of *Opg*-KO and wild-type mice, whereas no clear change in the ACE2 expression by olmesartan in AAA was observed (Figure S3A and S3B). We then examined the effect of angiotensin (1–7) on Trail-induced Jnk phosphorylation and downstream Mmp9 expression in cultured VSMCs. Pretreatment with angiotensin (1–7) effectively decreased Trail-induced Jnk phosphorylation (Figure 4A; full-size blots are included in Figure S4). Angiotensin (1–7) also attenuated the upregulation of *Mmp9* mRNA expression by Trail (Figure 4B). Moreover, A779, an antagonist of angiotensin (1–7) that can inhibit its binding to the Mas receptor, diminished these inhibitory effects on Trail-induced Jnk-Mmp9 pathway (Figure 4A and 4B). These results suggest that angiotensin (1–7), which may be increased via the increase in ACE2 expression by ARBs, may attenuate the Trail-induced Jnk-Mmp9 pathway via Mas receptor to prevent AAA progression in *Opg*-KO mice.

Angiotensin (1–7) May Mediate the Protective Effect of Olmesartan Against AAA Progression

Our in vitro experiments suggest that angiotensin (1–7) can attenuate the Trail-induced Jnk-Mmp9 pathway. Thus, we examined whether angiotensin (1–7) is important for the protective effect of olmesartan in the AAA model. To this end, we conducted experiments using A779, an antagonist of angiotensin (1–7)

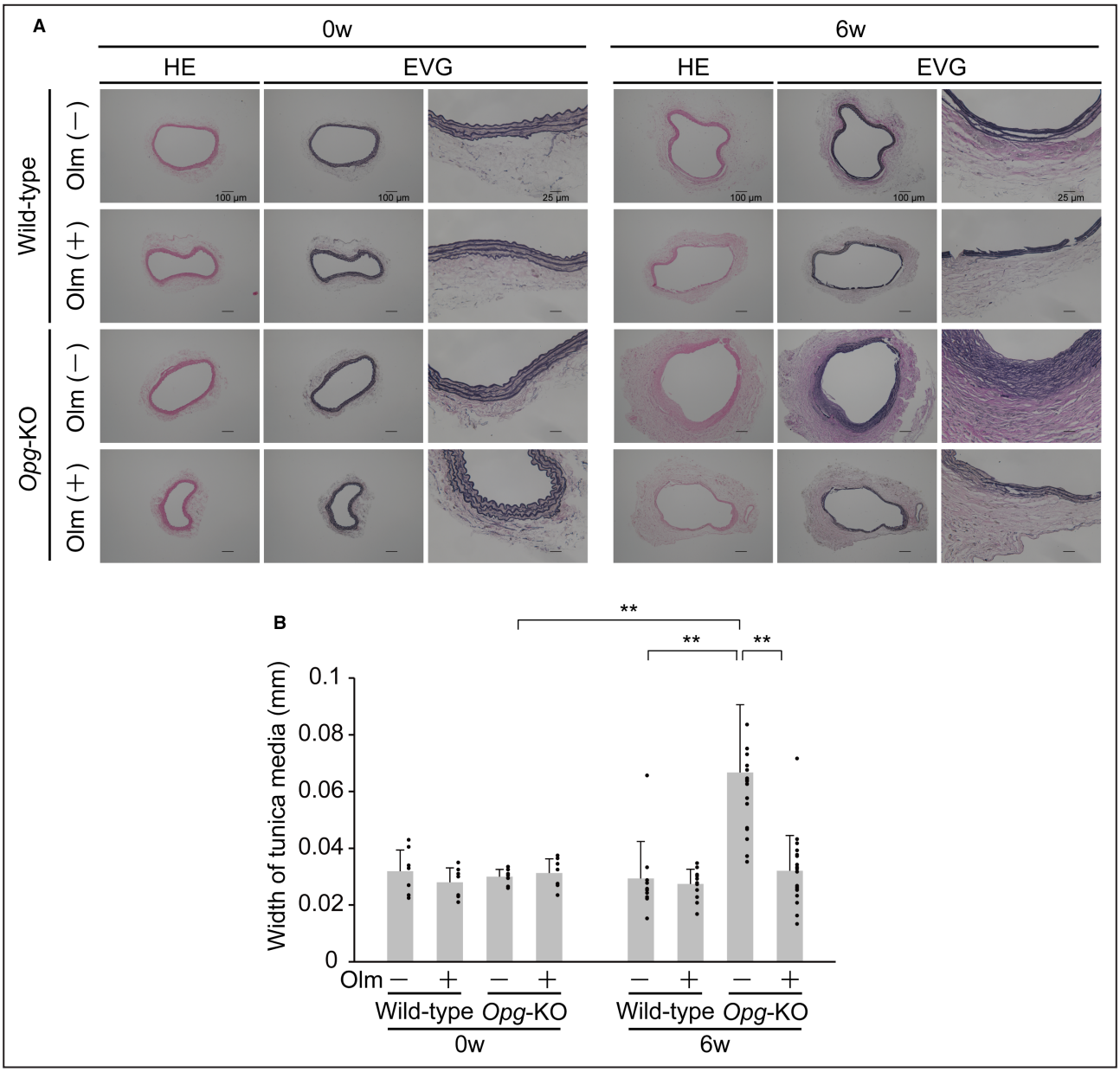

Figure 2. Administration of Olm to *Opg*-KO mice attenuates severe degenerative changes of the tunica media in $CaCl_2$-induced abdominal aortic aneurysm.

A, Representative images of HE- and EVG-stained aortic sections. **B**, Measurements of the width of the tunica media at 0 and 6 w. Data are shown as mean±SD. Zero weeks: n=7. Six weeks: wild type, n=10 to 11; *Opg*-KO, n=18 to 19. ***P*<0.01. Kruskal-Wallis test with Steel-Dwass post hoc analysis was used for multiple comparisons between groups. EVG indicates Elastica van Gieson; HE, hematoxylin and eosin; Olm, olmesartan; *Opg*-KO, *osteoprotegerin*-knockout; and w, weeks.

that inhibits angiotensin (1–7) binding to the Mas receptor (Figure 5A). The administration of olmesartan to *Opg*-KO mice tended to slightly lower blood pressure, but this trend was not altered by the administration of A779 (Table S2). The continuous administration of A779 with a micro-osmotic pump reversed the protective effect of olmesartan against severe aortic dilatation in *Opg*-KO mice at 6 weeks after AAA induction (Figure 5B and 5C). The administration of A779 also diminished the attenuation of tissue collapse and thickening in the tunica media by olmesartan in *Opg*-KO mice (Figure 5D and 5E). Immunohistochemical analysis revealed that the significant decrease in colocalization of pJnk and Mmp9 by olmesartan disappeared in the presence of the A779 administration (Figure 6A through 6C).

In a previous study, we reported that Trail increases the expression of Trail itself via the Jnk pathway.[21] The area expressing Trail in the tunica media was reduced by olmesartan, whereas A779 administration increased the expression of Trail (Figure 7A and 7B). This indicates

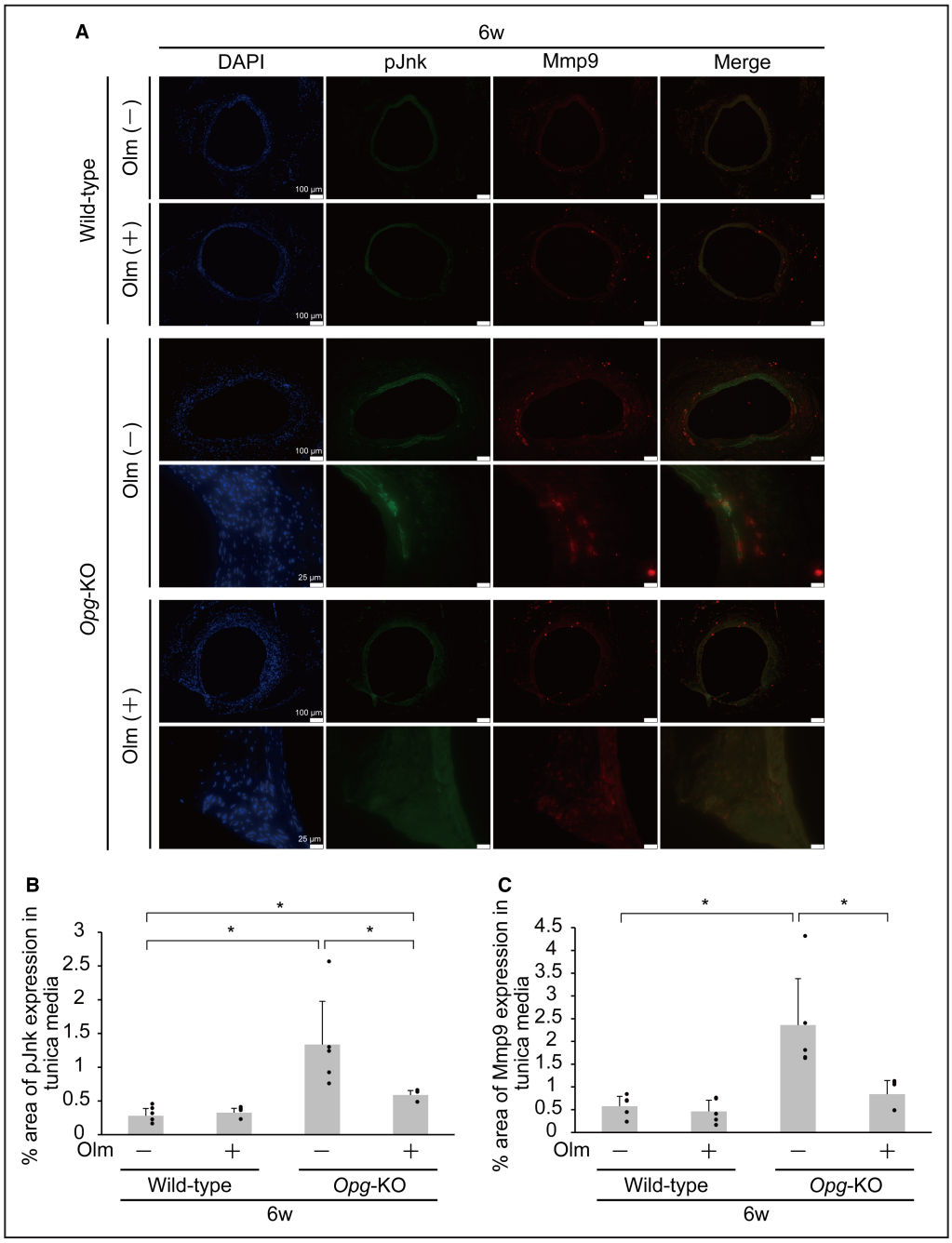

Figure 3. Olm inhibits the increase in colocalization of pJnk and Mmp9 in *Opg*-KO mice.
A, Representative images of double-immunofluorescent staining for pJnk (green) and Mmp9 (red) at 6 w after AAA induction in the aortas of wild-type and *Opg*-KO mice, with or without Olm administration. Nuclei are stained with DAPI (blue). **B** and **C**, Percentages of stained areas for pJnk (**B**) and Mmp9 (**C**) in the tunica media at 6 w after AAA induction. Data are shown as mean±SD. n=5. **P*<0.05. Kruskal-Wallis test with Steel-Dwass post hoc analysis was used for multiple comparisons between groups. AAA indicates abdominal aortic aneurysm; DAPI, 4′,6-diamidino-2-phenylindole; Mmp9, matrix metalloproteinase 9; Olm, olmesartan; *Opg*-KO, *osteoprotegerin*-knockout; pJnk; and w, weeks.

that the increase of angiotensin (1–7) by olmesartan might comprehensively mitigate the Trail-related vicious cycle associated with AAA progression in *Opg*-KO mice. These results also support our hypothesis that angiotensin (1–7) plays an important role in the protective effect of olmesartan against AAA progression in *Opg*-KO mice via downregulation of the Trail-induced Jnk-Mmp9 pathway.

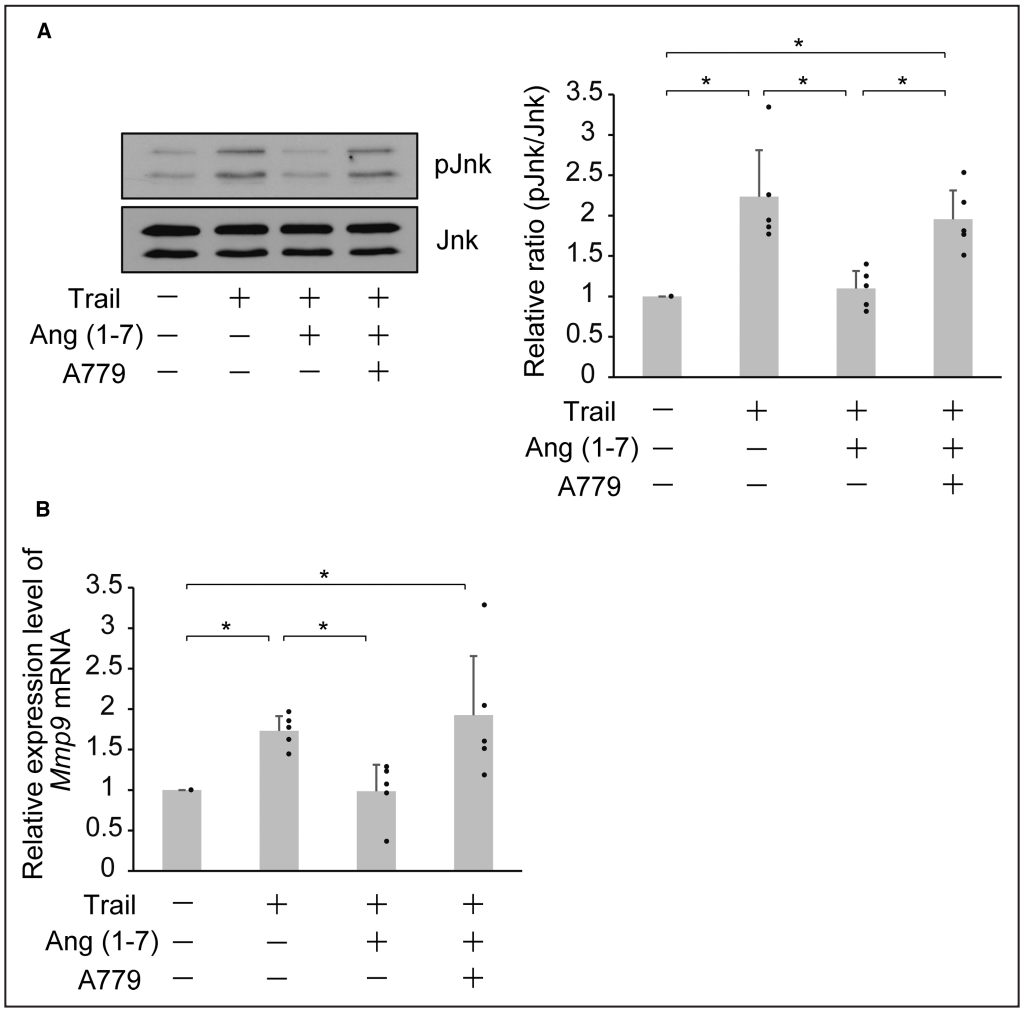

Figure 4. Ang (1–7) attenuates Trail-induced phosphorylation of Jnk and *Mmp9* mRNA expression. **A**, Western blot analysis of pJnk and Jnk in cultured wild-type VSMCs treated with Trail (50 ng/mL) for 20 minutes with or without preincubation with Ang (1–7) (100 nM) for 20 minutes and A779 (1 μM) for 60 minutes. **B**, Relative expression levels of *Mmp9* mRNA quantified with quantitative polymerase chain reaction in cultured VSMCs treated with Trail (50 ng/mL) for 6 hours with or without preincubation with Ang (1–7) (100 nM) for 20 minutes and A779 (1 μM) for 60 minutes. Each data point represents a biological replicate from different passaged cells. Data are shown as mean±SD (n=5). **P*<0.05. Kruskal-Wallis test with Steel-Dwass post hoc analysis was used for multiple comparisons between groups. Ang indicates angiotensin; Jnk, c-Jun N-terminal kinase; pJnk, phosphorylated Jnk; Trail, tumor necrosis factor–related apoptosis-inducing ligand; and VSMCs, vascular smooth muscle cells.

DISCUSSION

In the present study, we demonstrated that olmesartan prevents AAA progression in *Opg*-KO mice and that this protective effect was diminished by an angiotensin (1–7) antagonist. These results suggest that, in the treatment of AAA, an increase in angiotensin (1–7) levels by ARB treatment can attenuate AAA progression. Thus, the pleiotropic effects of ARBs, beyond the blockade of angiotensin II type 1 receptor, may be important for the protective effect of ARBs against AAA.

Angiotensin (1–7) is a heptapeptide converted from angiotensin II by ACE2 or from angiotensin I by endopeptidases, such as neprilysin.[26,30] ARBs reportedly increase the blood levels of angiotensin (1–7) together with the upregulation of ACE2 expression.[22,25] According to some studies, the increase of angiotensin (1–7) by administration of ARBs or ACE inhibitors has therapeutic effects on severe hypertension and end-organ damage,[31] insulin resistance,[32] and venous thrombosis[33] in animal models. Several animal studies have also shown that angiotensin (1–7) attenuates the formation of aneurysms associated with angiotensin II.[34–36] However, it was unclear whether angiotensin (1–7) can affect AAA models that are not associated with exogeneous angiotensin II. It is also unknown whether the therapeutic effects of ARBs are mediated by angiotensin (1–7). In the present study, olmesartan

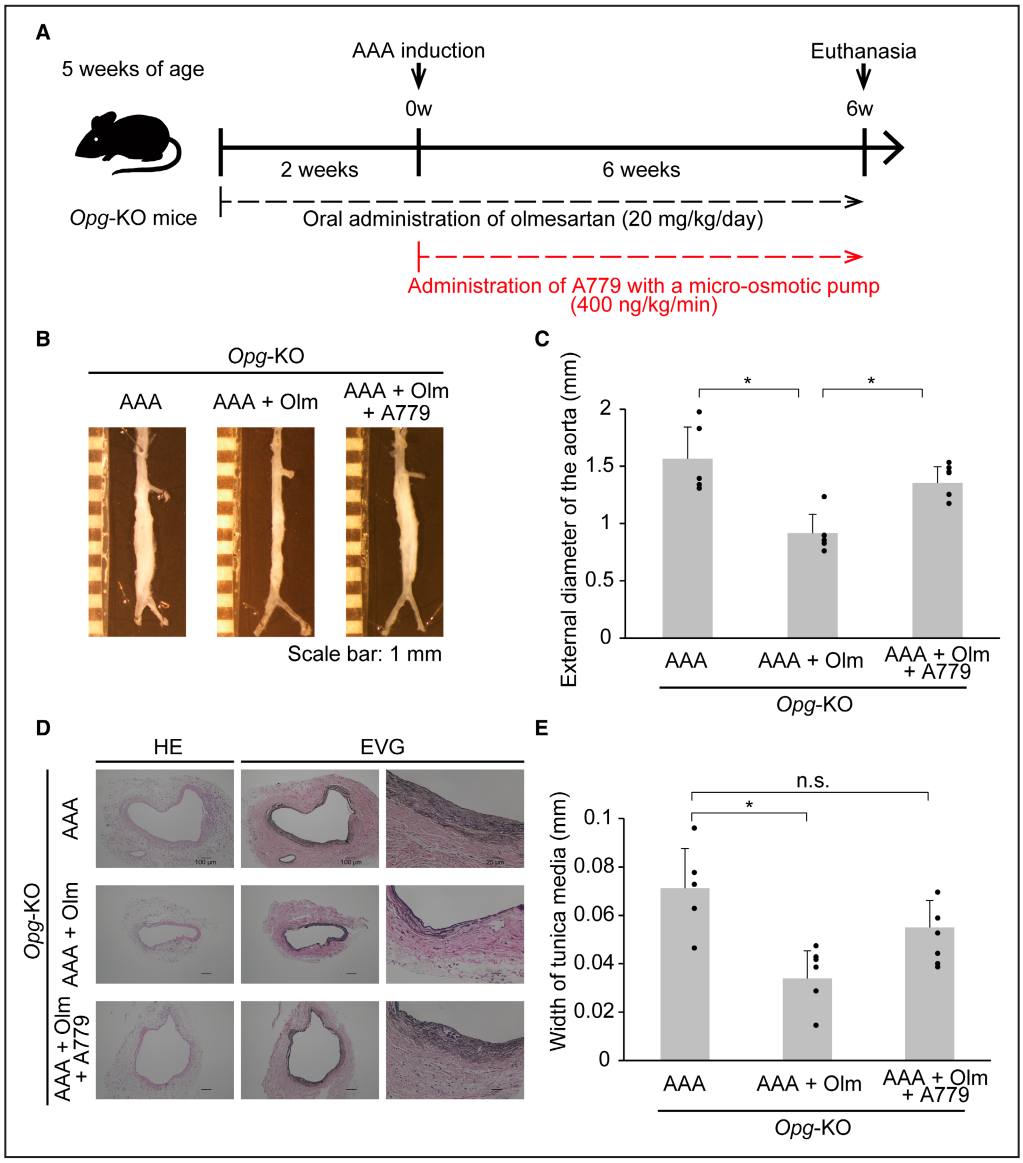

Figure 5. Blockade of angiotensin (1–7) signaling with A779 reduces the protective effect of Olm against AAA progression in *Opg*-KO mice.
A, Experimental design. AAA was induced in *Opg*-KO mice following the administration of Olm for 2 w. Mice were euthanized at 6 w after AAA induction, and Olm was administered to mice until euthanasia. A779 was administered with a micro-osmotic pump from AAA induction until euthanasia. **B**, Representative aortas of *Opg*-KO mice at 6 w after AAA induction. AAA is the control group. AAA+Olm is the group administered Olm. AAA+Olm+A779 is the group administered Olm and A779. **C**, External aortic diameter measurements at 6 w. **D**, Representative images of HE- and EVG-stained aortic sections at 6 w. **E**, Measurements of tunica media width at 6 w. Data are shown as mean±SD (n=5–6). AAA indicates abdominal aortic aneurysm; EVG, Elastica van Gieson; HE, hematoxylin and eosin; N.s., not significant; Olm, olmesartan; *Opg*-KO, *osteoprotegerin*-knockout; and w, weeks. **P*<0.05. Kruskal-Wallis test with Steel-Dwass post hoc analysis was used for multiple comparisons between groups.

prevented AAA progression in *Opg*-KO mice, and this protective effect was accompanied by an increase in *Ace2* mRNA expression. AAA progression in *Opg*-KO mice is associated with excessive activation of the Trail-induced Jnk-Mmp9 pathway.[16,21] Olmesartan effectively decreased the expression of Trail, pJnk, and Mmp9 in AAA of *Opg*-KO mice (Figures 3, 6, and 7). Moreover, in vitro experiments demonstrated that angiotensin (1–7) can attenuate Trail-induced Jnk phosphorylation and *Mmp9* mRNA levels (Figure 4). Angiotensin (1–7) reportedly inhibits Jnk phosphorylation, which is induced by lipopolysaccharide or palmitate.[28,29] We speculate that this inhibitory effect is similar to the mechanism seen with Trail-induced

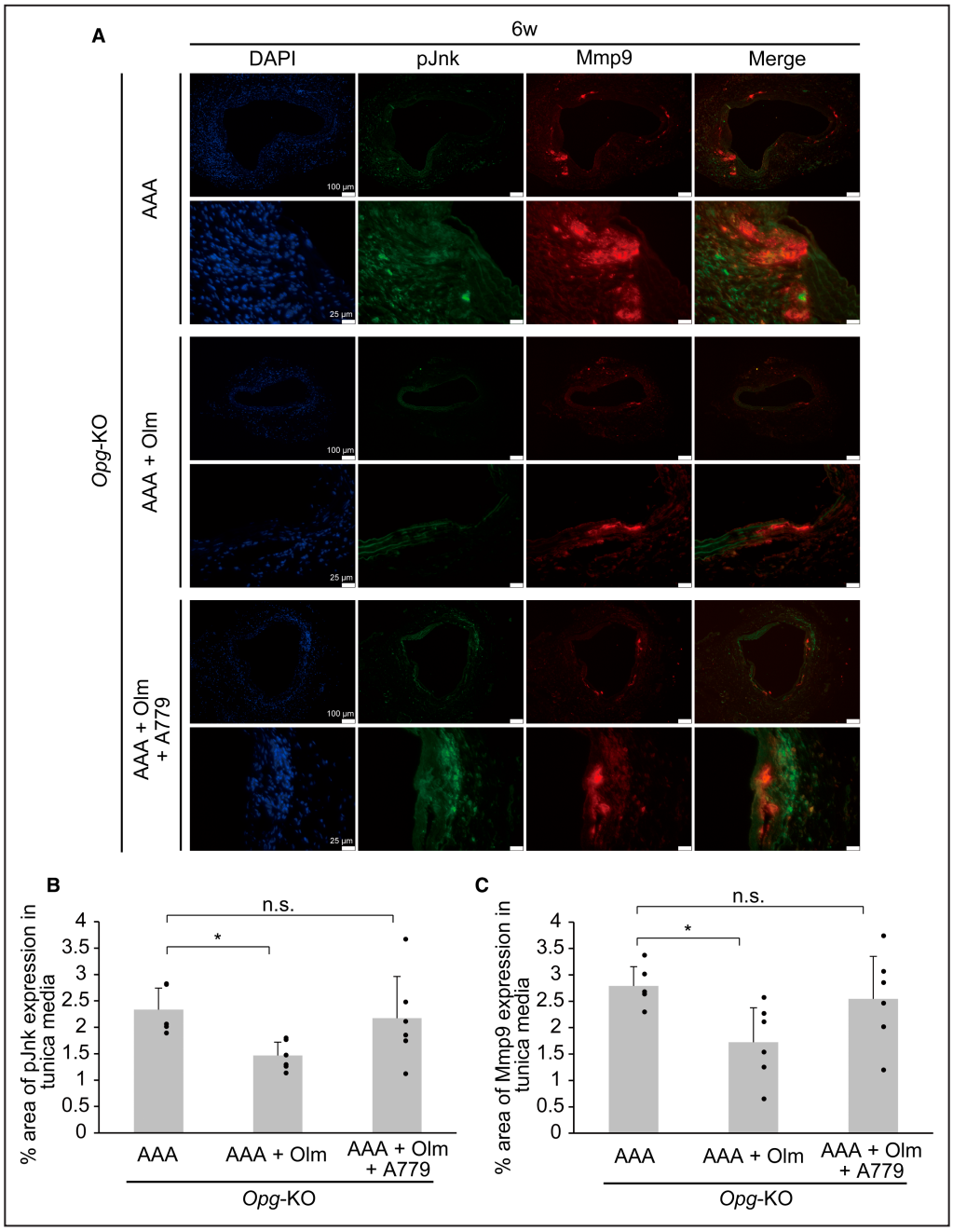

Figure 6. Blockade of angiotensin (1–7) signaling with A779 diminishes the attenuation of colocalized expression of pJnk and Mmp9 in *Opg*-KO mice by Olm.
A, Representative images of double-immunofluorescent staining for pJnk (green) and Mmp9 (red) at 6 w after AAA induction in the aortas of *Opg*-KO mice. AAA+Olm is the group administered Olm. AAA+Olm+A779 is the group administered Olm and A779. Nuclei are stained with DAPI (blue). **B** and **C**, Percentages of stained areas for pJnk and Mmp9 in the tunica media at 6 w after AAA induction. Data are shown as mean±SD (n=5–6). AAA indicates abdominal aortic aneurysm; DAPI, 4′,6-diamidino-2-phenylindole; Mmp9, matrix metalloproteinase 9; N.s., not significant; Olm, olmesartan; *Opg*-KO, *osteoprotegerin*-knockout; pJnk, phosphorylated c-Jun N-terminal kinase; and w, weeks. **P*<0.05. Kruskal-Wallis test with Steel-Dwass post hoc analysis was used for multiple comparisons between groups.

Jnk phosphorylation. Furthermore, blockade of angiotensin (1–7) by A779 reduced the protective effect of olmesartan against AAA progression in *Opg*-KO mice, including excessive aortic enlargement, collapse of the tunica media, and upregulation of Trail, pJnk, and Mmp9 expression (Figures 5 through 7). These results collectively suggest that olmesartan upregulates the angiotensin (1–7) signaling to prevent AAA progression

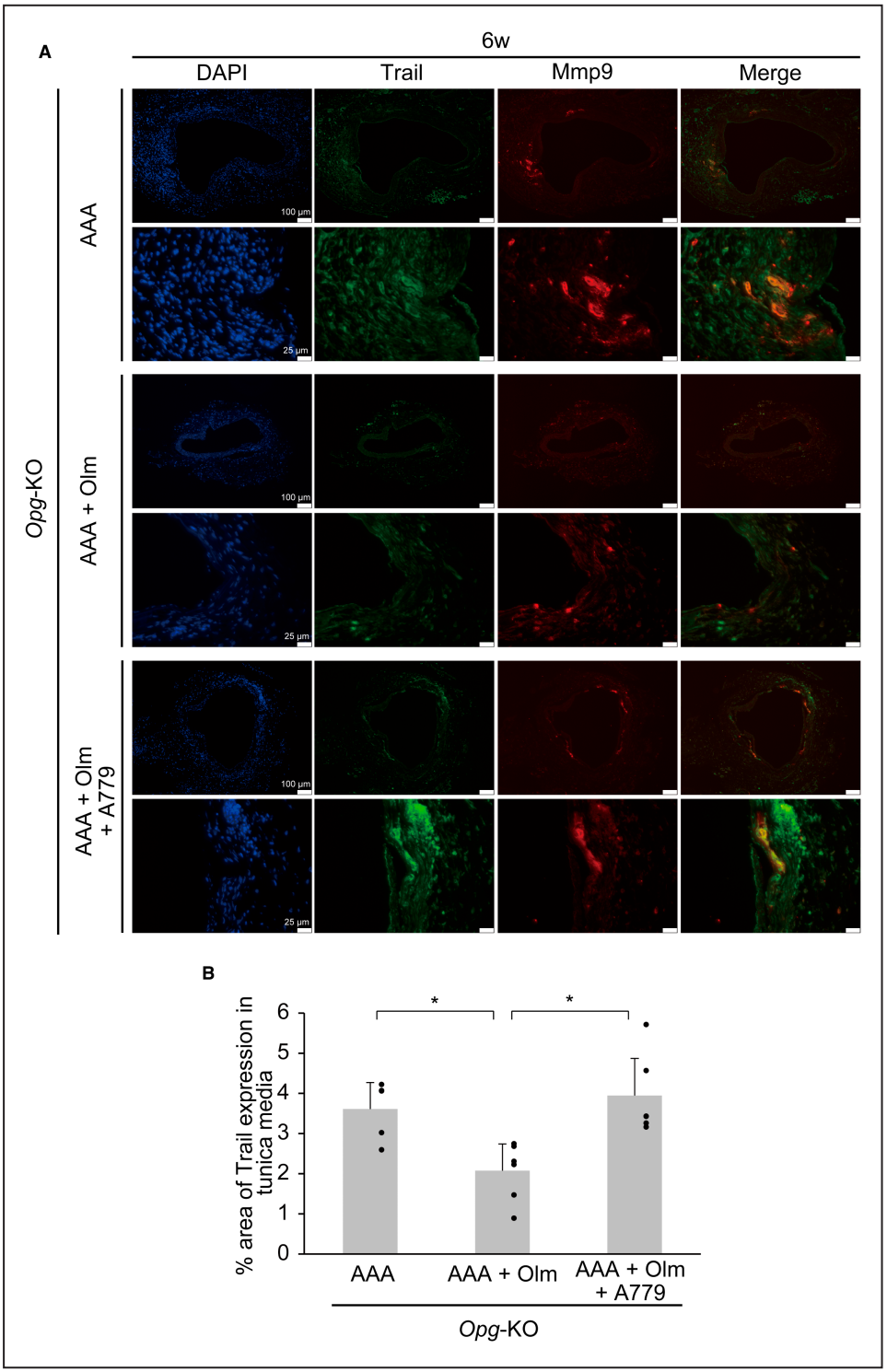

Figure 7. A779 reverses the downregulation of Trail expression by Olm in *Opg*-KO mice.
A, Representative images of double-immunofluorescent staining for Trail (green) and matrix metalloproteinase 9 (Mmp9) (red) at 6w after AAA induction in the aortas of *Opg*-KO mice. AAA+Olm is the group administered Olm. AAA+Olm+A779 is the group administered Olm and A779. Nuclei are stained with DAPI (blue). **B**, Percentages of stained areas for Trail in the tunica media at 6w after AAA induction. Data are shown as mean±SD (n=5–6). *P<0.05. Kruskal-Wallis test with Steel-Dwass post hoc analysis was used for multiple comparisons between groups. AAA indicates abdominal aortic aneurysm; DAPI, 4′,6-diamidino-2-phenylindole; Mmp9, matrix metalloproteinase 9; Olm, olmesartan; Opg-KO, osteoprotegerin-knockout; pJnk, phosphorylated c-Jun N-terminal kinase; Trail, tumor necrosis factor–related apoptosis-inducing ligand; and w, weeks.

induced by a combination of $CaCl_2$ application and osteoprotegerin deficiency. To the best of our knowledge, the present study provides the first evidence that intrinsic angiotensin (1–7) may be a key factor underlying the protective effect of ARBs against AAA progression.

Olmesartan prevented AAA progression but may not inhibit onset in our mouse AAA model. We induced AAA by applying $CaCl_2$ directly onto the aorta, promoting acute inflammation and tissue destruction in the aortic wall. Although olmesartan seemed not to affect AAA onset in either wild-type mice or *Opg*-KO mice at 1 week after AAA induction (Figure S2), it prevented AAA progression occurring up to 6 weeks after AAA induction (Figures 1 and 2). Thus, the protective effect of olmesartan against AAA progression in *Opg*-KO mice may be attributed not to the inhibition of acute inflammation induced by $CaCl_2$ application to the aorta but, rather, to attenuation of the subsequent progression phase related to the Trail-induced Jnk-Mmp9 pathway. Progressive tissue degradation is a characteristic pathological event also observed in human AAA,[37] and the Jnk-Mmp9 pathway is activated in human AAA.[18] Although many pharmacological therapies have been shown to inhibit acute AAA formation in animal models, none have been effective against AAA progression in humans.[4] Our findings indicate that ARBs may attenuate one of the pathways that aggravate AAA, highlighting their potential as therapeutic agents against human AAA that normally progresses in a chronic manner.

ARBs have been reported to attenuate aortic enlargement in a mouse model of Marfan syndrome.[38] Because loss of the angiotensin II type 2 receptor abolished the attenuating effect of ARBs, it was concluded that activation of the angiotensin II type 2 receptor, which can subsequently downregulate mitogen-activated protein kinases, was required for the inhibition of aortic enlargement by ARBs.[38] However, a recent study reported that an angiotensin II type 2 receptor agonist did not have the same attenuating effect.[39] Thus, it is possible that although the existence of the angiotensin II type 2 receptor is essential, the binding of the receptor to its ligand is not required for the beneficial effect of ARBs in the mouse model of Marfan syndrome. Interestingly, the angiotensin II type 2 receptor has been reported to interact functionally with the Mas receptor, a receptor for angiotensin (1–7), and affect angiotensin (1–7) signaling.[40,41] Angiotensin (1–7) has also been suggested to act in an inhibitory manner with respect to the activation of mitogen-activated protein kinases.[42–44] Thus, we speculate that, in the attenuation of aortic dilatation in the Marfan syndrome mouse model by ARBs, the angiotensin II type 2 receptor might not act as a receptor for its ligands. Rather, the angiotensin II type 2 receptor may facilitate Mas receptor-mediated angiotensin (1–7) signaling, which is upregulated by ARBs. In future studies, it will be informative to focus not only on the angiotensin II type 2 receptor, but also on angiotensin (1–7), to study the therapeutic effects of ARBs in aneurysmal diseases, including AAA or aortic dilatation observed in Marfan syndrome.

The protective effect of ARBs against AAA progression was attributed to a molecular mechanism involving Trail and the downstream Jnk-Mmp9 pathway. It is unclear whether the same therapeutic effects of ARBs against AAA would also be observed in other AAA models and human AAA. However, because the Jnk pathway is likely one of the responsible signaling pathways for AAA development in both humans and mice,[18] inhibiting Jnk activation might also be an effective strategy to limit AAA progression. In this regard, angiotensin (1–7) activates a phosphatase that targets mitogen-activated protein kinases, including Jnk,[44] and reduces Jnk phosphorylation under various stimulation conditions apart from Trail.[28,29] This suggests that angiotensin (1–7) may be effective against AAA involving Jnk activation, regardless of Trail involvement, and may serve as a molecule that can attenuate the progression of human AAA, a complex multifactorial disease.

The importance of angiotensin (1–7) in the protective effect of olmesartan against AAA progression suggests that angiotensin (1–7) can be used as a surrogate marker for ARB efficacy against AAA. Interestingly, a previous clinical study reported that the increase in angiotensin (1–7) levels by ARB treatment may differ by type of ARB.[23] Another study suggested that the ACE2–angiotensin (1–7)–Mas receptor axis is modulated by ACE2 gene polymorphisms.[45] Thus, the effectiveness of ARBs against AAA via angiotensin (1–7) upregulation might differ by ARB type or the particular individual. In such situations, circulating angiotensin (1–7) levels could be a useful indicator of the therapeutic effects of ARBs against AAA. To address this possibility, experiments testing the relationship between blood angiotensin (1–7) levels and AAA growth rate in patients with AAA taking ARBs will be needed. Clarifying the role of angiotensin (1–7) in human AAA may also help resolve discrepancies on the efficacy of ARBs against human AAA.

In conclusion, the present study found that olmesartan, an ARB, prevents AAA progression in *Opg*-KO mice, likely via attenuation of the Trail-Jnk-Mmp9 pathway by upregulating angiotensin (1–7). Our findings highlight ARBs as promising agents in AAA treatment. Clinical studies that investigate the importance of angiotensin (1–7) in the therapeutic effects of ARBs against AAA are warranted.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Data S1

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Supplemental Material

Table S1. Sequences of used primers.

Genes	Sequence	product size (bp)
Ace2	forward 5'- GCACTCTCAGCAGACAAGAACAA-3' reverse 5'- ATTTCATCCAATCCTGGCTCAAGT-3'	134
G3pdh	forward 5'- ACCACAGTCCATGCCATCAC-3' reverse 5'- TCCACCACCCTGTTGCTGTA-3'	452
Mmp9	forward 5' -GCCCTGGAACTCACACGACA-3' reverse 5'-TTGGAAACTCACACGCCAGAAG-3'	85

Table S2. Systolic blood pressure at six weeks after AAA induction.

Group		n	Systolic blood pressure (mmHg)	*p*-Value (Steel's multiple comparison test)
Opg-KO	AAA	5	104.40 ± 7.39	N/A
	AAA + Olm	6	93.67 ± 5.34	0.080
	AAA + Olm + A779	6	91.17 ± 8.75	0.097

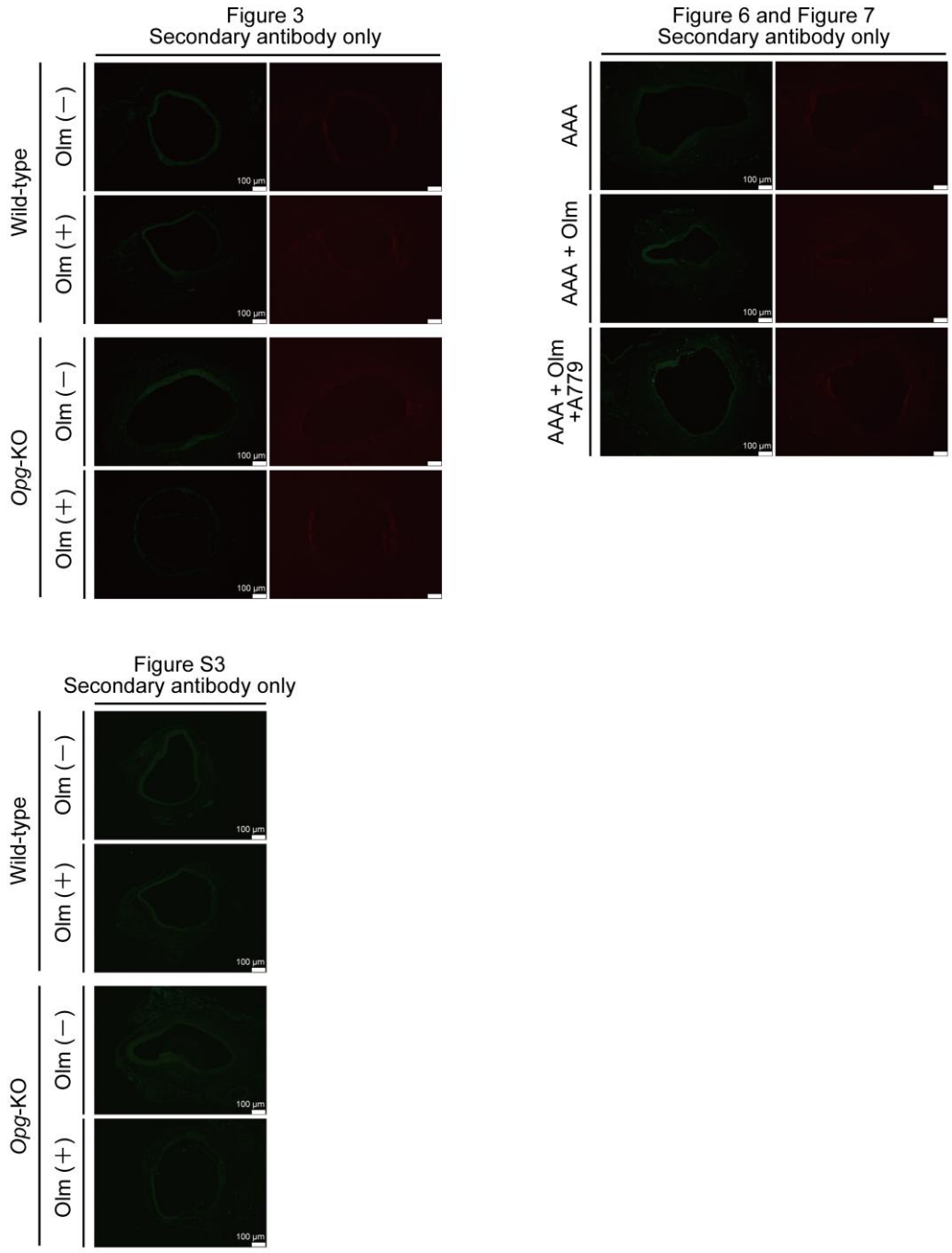

Figure S1. Staining controls without primary antibodies of fluorescence immunohistochemistry for Figures 3, 6, 7 and S3.

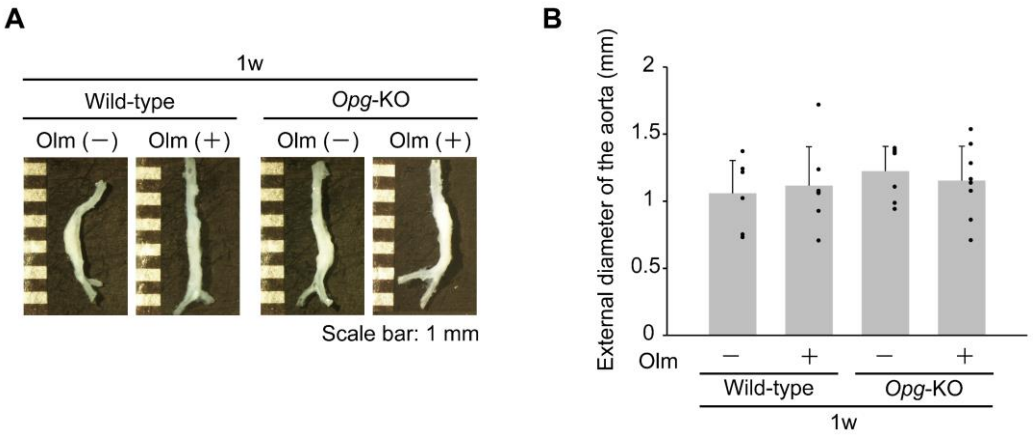

Figure S2. Olmesartan does not affect dilatation at the early phase in the $CaCl_2$-induced mouse AAA model.

(A) Representative harvested aortae at one week (w) after AAA induction in wild-type and *Opg*-KO mice, with or without olmesartan (Olm) administration. (B) External aortic diameter measurements at one week. Data are shown as mean ± SD (n = 6 to 8). Kruskal-Wallis test with Steel-Dwass post-hoc analysis was used for multiple comparisons between groups.

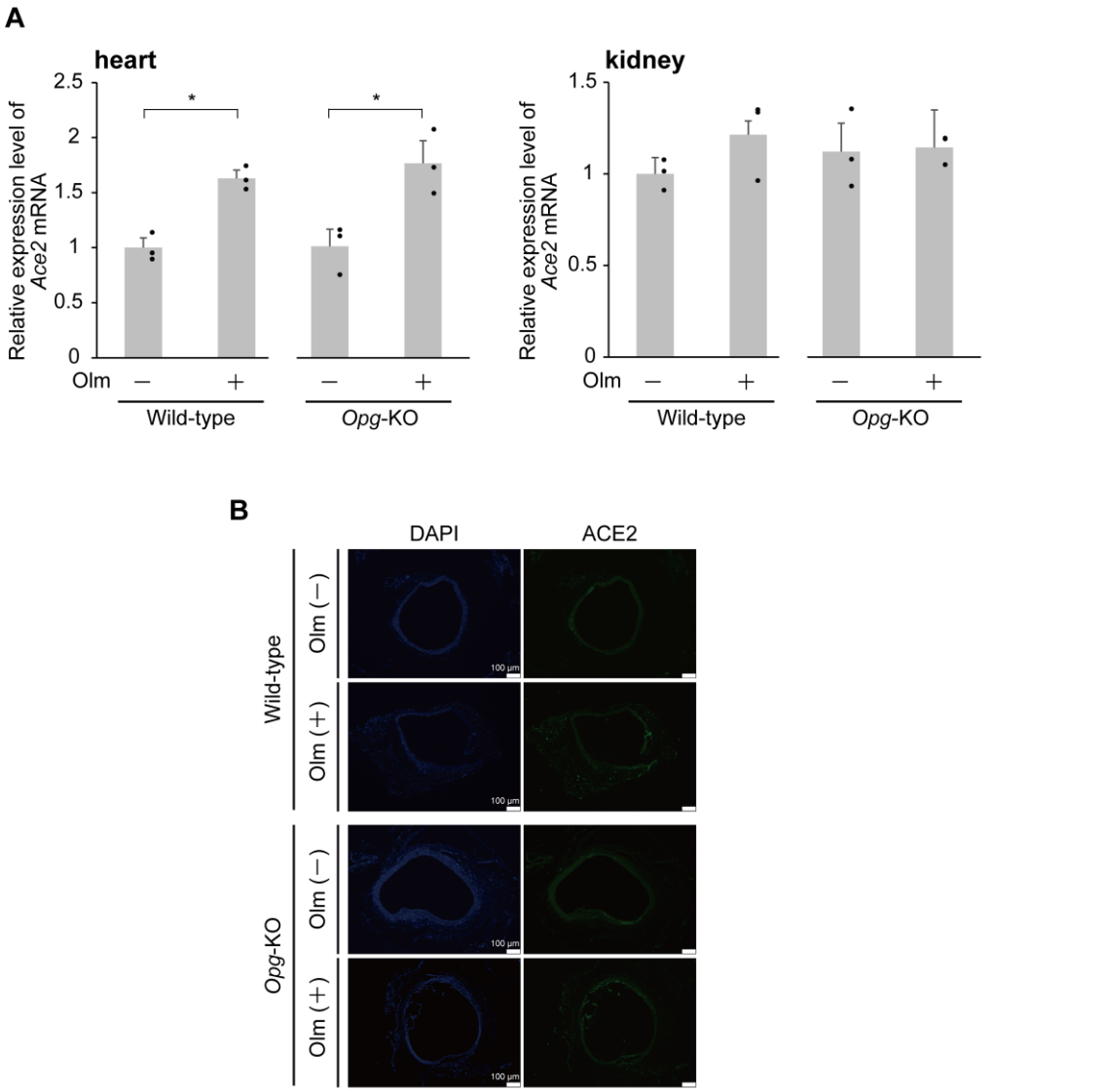

Figure S3. Olmesartan increases ACE2 expression in hearts

(A) Relative expression levels of *Ace2* mRNA quantified with qPCR in the hearts and the kidneys at one week after AAA induction in wild-type and *Opg*-KO mice, with or without olmesartan (Olm) administration. Data are shown as mean ± SD (n = 3). *p<0.05. Two-tailed unpaired Student's t-test was used for comparisons between groups. (B) Representative images of immunofluorescent staining for ACE2 (green) at six weeks after AAA induction in the aortae of wild-type and *Opg*-KO mice, with or without olmesartan (Olm) administration. Nuclei are stained with DAPI (blue).

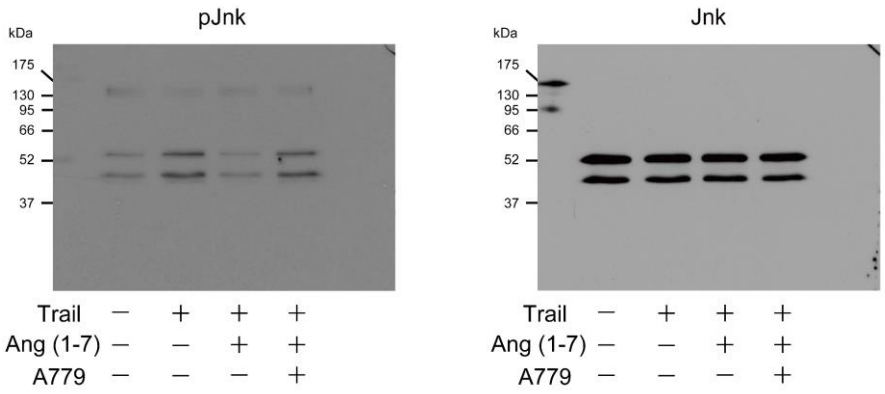

Figure S4. Full-size blot images for Figure 4A