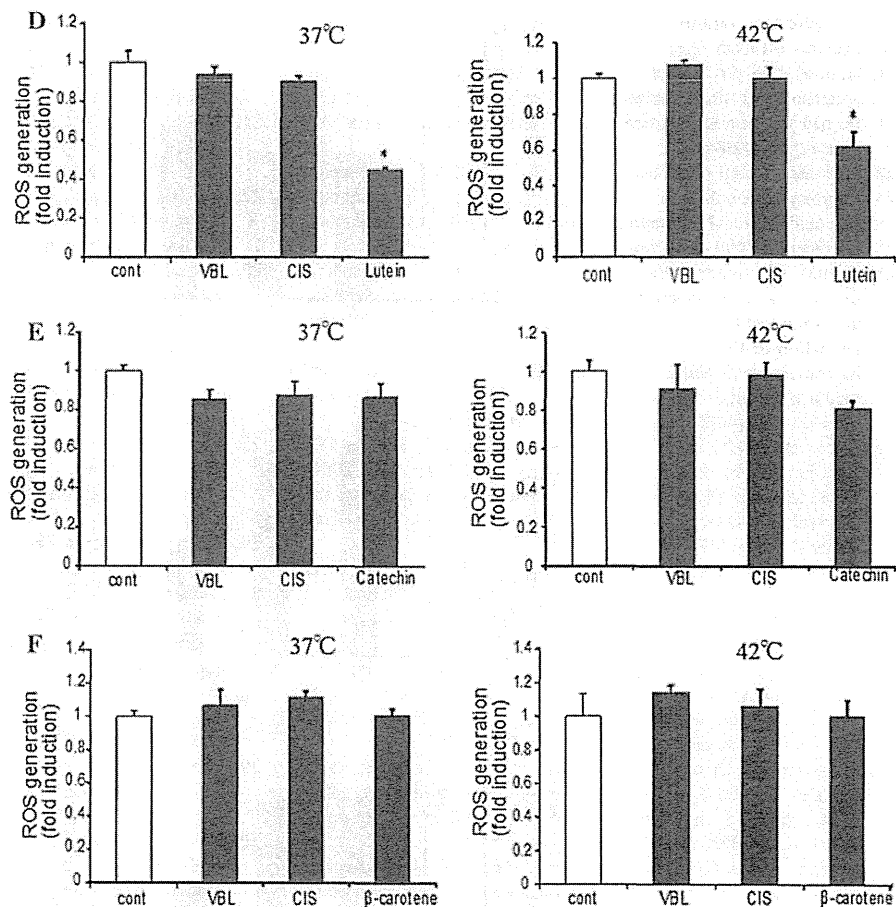Fig. 4 continued

require major attention, at least in terms of ROS production in cancer patients. In particular, ascorbic acid is widely used for multiple purposes, including for viral infection. Accordingly, the current study has suggested that the use of ascorbic acid may be considered carefully by both cancer patients and oncologists. Further, with our findings, the effects of ascorbic acid and its related antioxidants need to be clinically examined in future in cancer patients who are to be treated with chemotherapy and/or hyperthermic therapy.

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PLoS one

Inhibition of EP4 Signaling Attenuates Aortic Aneurysm Formation

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Abstract

Background: Aortic aneurysm is a common but life-threatening disease among the elderly, for which no effective medical therapy is currently available. Activation of prostaglandin E_2 (PGE_2) is known to increase the expression of matrix metalloproteinase (MMP) and the release of inflammatory cytokines, and may thus exacerbate abdominal aortic aneurism (AAA) formation. We hypothesized that selective blocking of PGE_2, in particular, EP4 prostanoid receptor signaling, would attenuate the development of AAA.

Methods and Findings: Immunohistochemical analysis of human AAA tissues demonstrated that EP4 expression was greater in AAA areas than that in non-diseased areas. Interestingly, EP4 expression was proportional to the degree of elastic fiber degradation. In cultured human aortic smooth muscle cells (ASMCs), PGE_2 stimulation increased EP4 protein expression (1.4 ± 0.08-fold), and EP4 stimulation with ONO-AE1-329 increased MMP-2 activity and interleukin-6 (IL-6) production (1.4 ± 0.03- and 1.7 ± 0.14-fold, respectively, $P<0.05$). Accordingly, we examined the effect of EP4 inhibition in an $ApoE^{-/-}$ mouse model of AAA infused with angiotensin II. Oral administration of ONO-AE3-208 (0.01–0.5 mg/kg/day), an EP4 antagonist, for 4 weeks significantly decreased the formation of AAA (45–87% reduction, $P<0.05$). Similarly, $EP4^{+/-}/ApoE^{-/-}$ mice exhibited significantly less AAA formation than $EP4^{+/+}/ApoE^{-/-}$ mice (76% reduction, $P<0.01$). AAA formation induced by periaortic $CaCl_2$ application was also reduced in $EP4^{+/-}$ mice compared with wild-type mice (73% reduction, $P<0.001$). Furthermore, in human AAA tissue organ cultures containing SMCs and macrophages, doses of the EP4 antagonist at 10–100 nM decreased MMP-2 activation and IL-6 production (0.6 ± 0.06- and 0.7 ± 0.06-fold, respectively, $P<0.05$) without increasing MMP-9 activity or MCP-1 secretion. Thus, either pharmacological or genetic EP4 inhibition attenuated AAA formation in multiple mouse and human models by lowering MMP activity and cytokine release.

Conclusion: An EP4 antagonist that prevents the activation of MMP and thereby inhibits the degradation of aortic elastic fiber may serve as a new strategy for medical treatment of AAA.

Introduction

Aortic aneurysm is the 13th leading cause of death in the United States, with roughly 15,000 deaths per year [1]. After rupture occurs, the probability of mortality is greater than 60% [1]. Ultrasonography screening studies of men over 60 years old have shown that a small abdominal aortic aneurysm (AAA), i.e., 3 to 5 cm in diameter, is present in 4% to 5% of patients [2,3]. When patients with a small AAA were followed for up to 6 years, AAA diameter had increased in 55% of patients. The rate of increase in diameter was more than 1 cm per year in 23% of patients, and AAA diameter had expanded to 6 cm in 9% of patients, at which point the risk of rupture significantly increases [3]. Although AAAs typically continue to expand, increasing the likelihood of rupture and consequent mortality, no effective pharmacological therapy to prevent the progression of AAA is currently available.

The hallmarks of AAA are the presence of an inflammatory infiltrate within the vascular wall, which is followed by proteolytic degradation of extracellular matrixes (ECM) [4]. Proinflammatory cytokines play an important role, particularly in the initiation of

aneurysms [1]. Inflammatory mediators such as interleukin-6 (IL-6), IL-1β and monocyte chemoattractant protein-1 (MCP-1) are released in the AAA wall [5,6]. In an experimental AAA model of ApoE$^{-/-}$ mice infused with angiotensin II (AngII), IL-6 and MCP-1 production were both increased [7]. In contrast, the incidence of AAA was decreased after AngII infusion in mice lacking either the IL-6 or MCP-1 receptor CCR2 [7]. Proteolytic enzymes, together with inflammatory mediators, promote extensive structural remodeling of the arterial wall, characterized by the degradation of ECM such as elastic fibers [8]. Activation of proteolytic enzymes, particularly matrix metalloproteinases-2 (MMP-2) and MMP-9 in the tunica media, is considered to be an important cause. These MMPs exacerbate aortic dilatation, as demonstrated in studies using human patients or genetically engineered mice [8,9].

Cyclooxigenase-2 (COX-2)-dependent prostaglandin E$_2$ (PGE$_2$) synthesis is induced during the development of aneurysms [5,10]. PGE$_2$ synthesized by macrophages and smooth muscle cells (SMCs) increases the production of MMPs [11,12] and stimulates the production of cytokines [5]. Selective COX-2 inhibition, as induced by celecoxib or genetic disruption of COX-2, decreased AngII-induced AAA formation in mice [13,14]. Despite these positive findings, however, administration of selective COX-2 inhibitors has increased the frequency of adverse cardiovascular events, as reported in clinical studies [15,16]. Nonetheless, inhibition of pathophysiologic COX-2-dependent PGE$_2$ signaling may still remain an attractive therapeutic strategy.

The present study was designed to examine the hypothesis that the prostanoid receptor, which is downstream of COX-2-dependent PGE$_2$ signaling, plays a critical role in the formation of AAA. We demonstrate that prostanoid receptor EP4 expression was increased in SMCs from human AAA tissue, and that EP4 stimulation enhanced MMP-2 activation and IL-6 production. Further, pharmacological inhibition or genetic disruption of EP4 signaling successfully attenuated AAA formation in mice. We also demonstrate that an EP4 antagonist attenuated MMP-2 activation and IL-6 production in the explants of human AAA.

Materials and Methods

Reagents
Antibody for EP4 was obtained from Cayman chemical (Ann Arbor, MI, USA). Antibodies for α-smooth muscle actin and CD68 were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Dako Cytomation (Glostrup, Denmark), respectively. ONO-AE1-329 and ONO-AE3-208 were kindly provided by the ONO pharmaceutical company (Osaka, Japan).

Human Aortic Samples
We obtained surgical specimens from individuals with AAA. We performed *ex vivo* culture using fresh AAA samples during surgery as described previously [17]. Briefly, tissues were minced to approximately 1 mm thickness, and plated on 24-well plates with 10% FBS/DMEM (Invitrogen, Carlsbad, CA, USA). Media was changed 24 h after plating. We collected some conditioned media after 48 h of incubation as a control for each well. Each well was then treated with ONO-AE1-329 or ONO-AE3-208. Conditioned media 48 h after treatment was obtained and subjected to gelatin zymography and ELISA. To compare the effect of drugs among samples, values for each well obtained from stimulated conditioned media were normalized to values from control conditioned media.

To obtain the primary culture of human aneurysm aortic smooth muscle cells (hAASMCs) from AAA tissue, the medial layer of the AAA was cut into 1- to 2-mm^3 pieces which were placed in the explant culture on uncoated dishes in 10% FBS/DMEM (Invitrogen). Culture medium was changed after 7 days and thereafter every 3 days during a 3- to 4-week period until the specimens became confluent. The purity of the hAASMCs was confirmed by staining with α-smooth muscle actin. When confluent, SMCs were transferred (at passage 2 or 3) onto uncoated 6-well or 96-well plates for immunoblotting, gelatin zymography, and ELISA. Human aortic SMCs (hASMCs) from individuals who died of unrelated causes were obtained from Lonza (Walkersville, MD, USA).

Cell Culture
THP-1cells were obtained from the Health Science Research Resources Bank (Osaka, Japan). We maintained hAASMCs and hASMCs in SmGM-2 containing 5% FBS and growth supplements (Lonza) and maintained THP-1 cells in RPMI1640 (Wako, Osaka, Japan) containing 10% FBS. For differentiation of THP-1 monocytes into adherent macrophages, cells were treated with 100 nM of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 24 h as described previously [18].

AAA Mouse Models
The impact of genetic inhibition of EP4 on AAA formation was examined using the heterozygous EP4 knockout mouse (EP4$^{+/-}$) since homozygous knockout is lethal [18]. AAA was induced by periaoritc application of 0.5 M CaCl$_2$ as described previously [17]. The sham group received saline instead of CaCl$_2$. Aortic morphometry was performed 4 weeks after CaCl$_2$ treatment.

AAA was also induced after crossing EP4$^{+/-}$ [18] with the apolipoprotein E knockout mouse (ApoE$^{-/-}$) (The Jackson Laboratory, Bar Harbor, ME, USA). Briefly, EP4$^{-/-}$ mice with a C57BL/6 genetic background [18] were crossed with ApoE$^{-/-}$ mice with the same genetic background, and the resulting mice (EP4$^{+/-}$/ApoE$^{+/-}$) were intercrossed to generate EP4$^{+/-}$/ApoE$^{-/-}$ mice and their littermate controls (EP4$^{+/+}$/ApoE$^{-/-}$). To induce AAA formation, male EP4$^{+/-}$/ApoE$^{-/-}$ mice and littermate EP4$^{+/+}$/ApoE$^{-/-}$ mice were infused with AngII (1,000 ng/min/kg; Sigma-Aldrich) via an osmotic minipump (Alzet, model 2004, Cupertino, CA, USA) for 4 weeks, as described previously [19].

The effect of pharmacological inhibition of EP4 was examined in ApoE$^{-/-}$ mice infused with AngII. Simultaneously, mice were orally administered ONO-AE3-208 (0.005, 0.01, 0.05, 0.5 mg/kg/day) as a bolus for 4 weeks. At the end of AngII infusion, the mice were sacrificed by an overdose of pentobarbital and were perfusion-fixed with a mixture of 3.7% formaldehyde in PBS at physiological perfusion pressure. Abdominal aorta were photographed to determine their external diameter, and also used for histological analyses. All aortic morphometries were performed by an investigator in a blinded manner. For gelatin zymography, we used freshly isolated aortic tissues at the end of AngII infusion.

Ethics Statement
All protocols using human specimens were approved by the Institutional Review Board at Yokohama City University and all samples were obtained after receiving written informed consent. All animal studies were approved by the Institutional Animal Care and Use Committees of Yokohama City University.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)
Isolation of total RNA and generation of cDNA were performed and RT-PCR analysis was done as described previously [20]. The

primers were designed based on rat nucleotide sequences of human EP1(NM_000955) (5'-GGA TGT ACA CCA AGG GTC CAG-3' and 5'-TCA TGG TGG TGT CGT GCA TC-3'),

human EP2 (NM_000956) (5'-AGG ACT GAA CGC ATT AGT CTC AGA A-3' and 5'-CTC CTG GCT ATC ATG ACC ATC AC-3'), human EP3 variants 1–9,11(NR_028292-4, NM_198714-
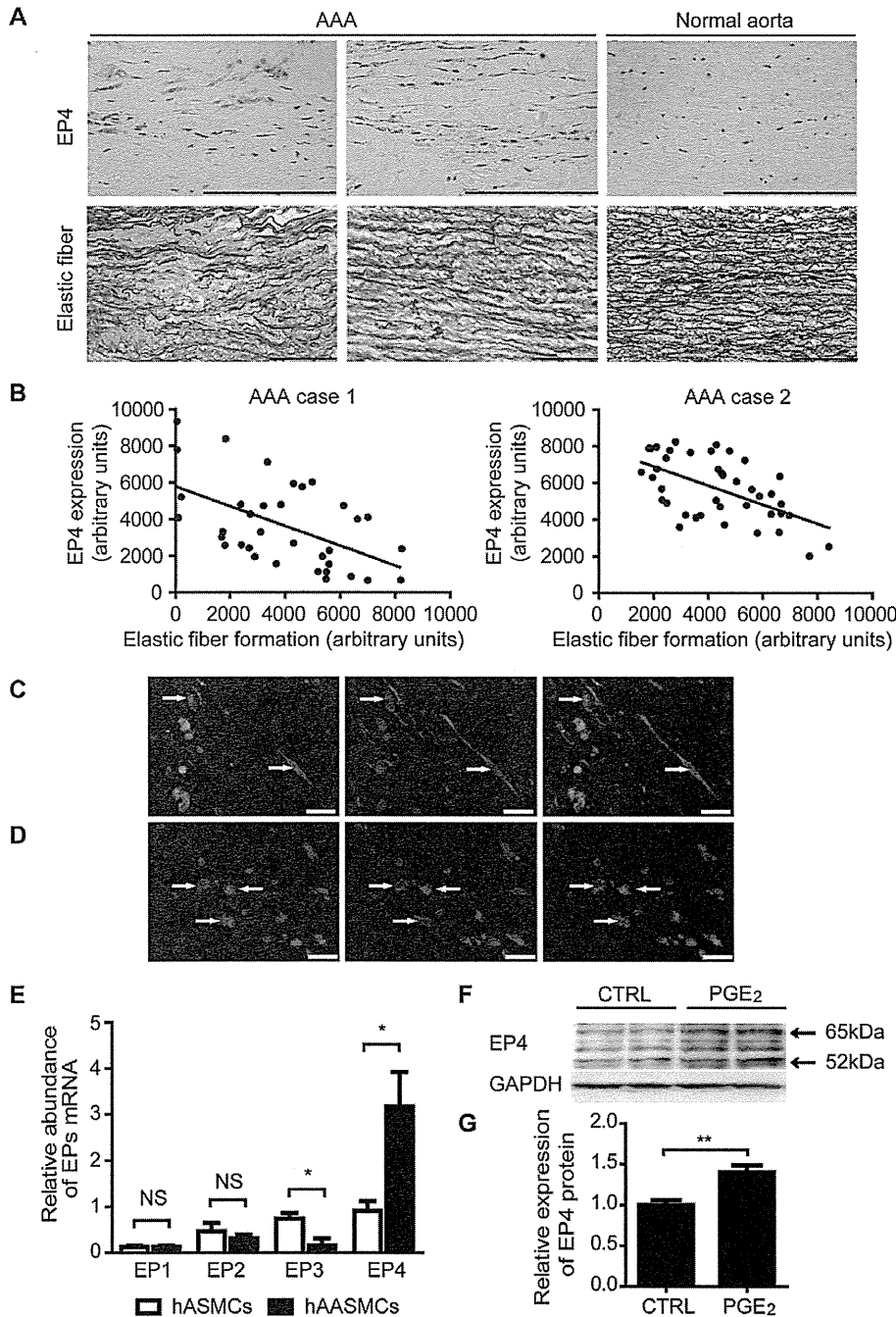

Figure 1. EP4 expression is increased in human AAA tissue. A, Immunohistochemistry for EP4 in human AAA tissues and aortic tissue from individuals who died of unrelated causes (upper panels). Brown areas indicate expression of EP4. Elastica van Gieson-stained aortic tissues (lower panels). Scale bars: 100 µm. B, Representative correlations between EP4 protein expression and elastic fiber formation in human AAA tissues. C, Immunofluorescent staining for EP4 (green, left panel) and α-smooth muscle actin (red, middle panel). Merged image is shown in the right panel. Arrows indicate EP4- and α-smooth muscle actin-positive cells. D, Immunofluorescent staining for EP4 (green, left panel) and CD68 (red, middle panel). Merged image is shown in the right panel. Arrows indicate EP4- and CD68-positive cells. Scale bars: 20 µm. E, Expression of EP1–4 using quantitative RT-PCR in hASMCs and hAASMCs. n = 5. F, Immunoblotting for EP4 and GAPDH in hASMCs incubated in the presence or absence of 1 µM of PGE$_2$ for 72 h. G, Quantification of F. n = 4–5. *, $P<0.05$; **, $P<0.01$; NS, not significant.
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9, NM_001126044) (5'-GGA CTA GCT CTT CGG ATA ACT-3' and 5'-GCA GTG CTC AAC TGA TGT CT-3'), human EP4 (NM_000958) (5'-AAC TTG ATG GCT GCG AAG ACC TAC-3' and 5'-TTC TAA TAT CTG GGC CTC TGC TGT G-3'), and mouse EP4 (5'-TTC CCG CAG TGA TGT TCA TCT-3' and 5'-CGA CTT GCA CAA TAC TAC GAT GG-3'). Each primer set was designed between multiple exons, and PCR products were confirmed by sequencing. The abundance of each gene was determined relative to the 18S transcript.

Immunoblot Analysis

Proteins from whole cells were analyzed by immunoblotting as described previously [20].

Tissue Staining and Immunohistochemistry

Elastic fiber formation was evaluated by elastica van Gieson staining. Immunohistochemical analysis was performed as described previously [20,21]. A color extraction method using Keyence software was performed to quantify elastic fiber formation and expression of EP4.

Gelatin Zymography

MMP activity was examined by gelatin zymography as described previously [17].

ELISA

IL-6 and MCP-1 in conditioned media were measured using ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical Analysis

Data are shown as the mean ± SEM of independent experiments. Unpaired Student's t-test, one-way ANOVA followed by Student-Newman-Keuls multiple comparison test, and Pearson's Correlation Coefficient were used to determine the statistical significance of the data. A value of $P<0.05$ was considered significant.

Results

Prostaglandin E Receptor EP4 Was Up-regulated in Aneurysmal Areas of Human Abdominal Aortas

In human tissue samples obtained from AAA surgeries, we found that EP4 expression and elastic fiber degradation were both enhanced in aneurysmal areas relative to that in normal areas. Indeed, statistical analysis revealed that the correlation was significant between the amount of EP4 expression and the degree of elastic fiber degradation (p<0.0001 to 0.0168) (**Figures 1A and B, and Table 1**).

Previous studies have demonstrated that EP4 is abundantly expressed as primary PGE_2 receptors in macrophages in aneurysmal areas [22]. However, whether or not other cell types such as ASMCs also express EP4 and other subtypes was not determined. We found, by immunohistochemistry of tissue samples, that EP4 was abundantly expressed in both α-smooth muscle actin-positive cells, i.e., ASMCs, (**Figure 1C**) and in CD68-positive cells, i.e., macrophages (**Figure 1D**). EP subtype expression was further characterized in cultured hAASMCs isolated from AAA tissue (**Figure 1E**). We found that EP4 mRNA expression was much greater than that of other EP subtypes such as EP1, EP2, and EP3. In contrast, when hASMCs isolated from normal aorta were examined, EP4 mRNA expression was not increased, suggesting that EP4 was increased only in

Table 1. Correlation between elastic fiber formation and EP4 expression in AAA tissues.

	age	gender	r	number of sampling point	P value
1	76	M	−0.5386	35	0.0008***
2	63	M	−0.5645	41	0.0001***
3	76	M	−0.8000	25	<0.0001***
4	80	M	−0.4607	29	0.011*
5	70	M	−0.5454	39	0.0003***
6	76	M	−0.7571	60	<0.0001***
7	70	M	−0.4333	30	0.0168*
8	89	F	−0.5200	44	0.0003***

r: correlation coefficient; n: number of sampling points.
*, $P<0.05$;
**, $P<0.01$;
***, $P<0.001$.
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aneurysmal ASMCs. When normal hASMCs were stimulated with PGE_2, however, EP4 protein expression was significantly increased (**Figures 1F and G**). Thus, we can tentatively speculate that local production of PGE_2 increased EP4 in the ASMCs in aneurysmal areas, which might play a role in AAA exacerbation.

EP4 Stimulation Increased MMP-2 Activity and IL-6 Production in hAASMCs and Human AAA Tissue Organ Cultures

Previous reports have demonstrated that MMP-2 and MMP-9, which are respectively derived from SMCs and macrophages, play important roles in the progression of aortic aneurysms [9]. We also found that MMP-2 and MMP-9 were both abundant in the supernatants of human AAA tissue organ cultures (**Figure 2A**). We also confirmed that MMP-2 was produced exclusively by hASMCs, and MMP-9 by THP-1 macrophage cells [9]. When hAASMCs or human AAA tissue organ cultures were stimulated with the EP4 agonist ONO-AE1-329, we found that MMP-2 activity was significantly increased in both preparations (**Figure 2B and C**). In contrast, EP4 stimulation did not alter MMP-9 activation in organ cultures (**Figure 2D**). We also examined the effect of EP4 stimulation on cytokines and chemokine because vascular inflammation is another prominent feature of atherosclerotic AAA [1]. We found that EP4 stimulation increased IL-6 production but decreased MCP-1 production in both hASMCs (**Figures 2E and G**) and human AAA tissue organ cultures (**Figures 2F and H**). These findings suggest that enhanced EP4 signaling may increase MMP activity and inflammatory response in AAA.

Genetic Deletion of EP4 Reduced AAA Formation in vivo

Since the above experiments implied that EP4 stimulation has an exacerbating effect on AAA formation, we hypothesized that inhibition of EP4 signaling might have a salutary effect. We therefore examined the effect of genetic disruption of EP4 signaling by using EP4$^{+/−}$ mice, because the total knockout of EP4 is lethal during the neonatal period [18]. EP4 expression in EP4$^{+/−}$ mice was decreased to 43±6% (aorta) and 63±10% (heart), relative to that of wild-type mice (n = 6, $P<0.05$).

When CaCl₂ was applied to the mouse abdominal aorta [17], aneurysmal formation with elastic fiber degradation was induced. However, these changes were significantly decreased in EP4$^{+/-}$ mice (**Figures 3A and B**). In the absence of CaCl₂ application, however, no significant difference between EP4$^{+/-}$ and EP4$^{+/+}$ mice was seen. Similarly, we examined AAA formation in EP4$^{+/-}$ mice crossed with ApoE$^{-/-}$ mice (EP4$^{+/+}$/ApoE$^{-/-}$), with AAA induced by continuous AngII infusion [19]. We found that the incidence of aortic aneurysm formation as well as elastic fiber

degradation was significantly decreased in EP4$^{+/-}$/ApoE$^{-/-}$ mice (**Figures 4A and B**). In the absence of AngII infusion, however, no significant difference between EP4$^{+/-}$/ApoE$^{-/-}$ and EP4$^{+/+}$/ApoE$^{-/-}$ mice was observed. Thus, in two distinct models, EP4 deletion decreased AAA formation.

EP4 Antagonist Reduced AAA Formation in vivo

We also examined the effect of pharmacological inhibition of EP4 by ONO-AE3-208, an EP4 antagonist [23], with AAA
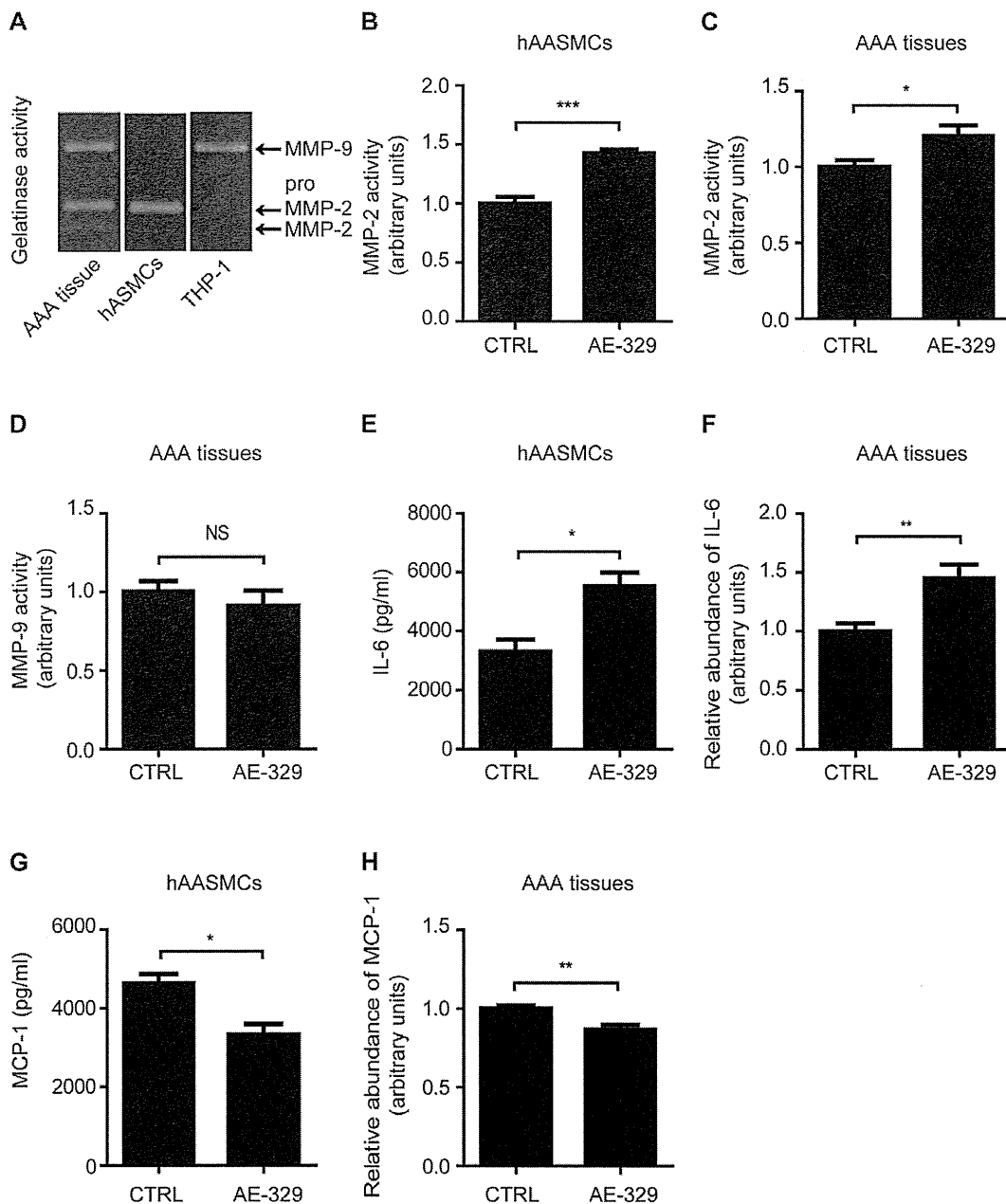

Figure 2. EP4 signaling increased MMP-2 activation and IL-6 production in hAASMCs and human AAA tissues. A, Representative images of gelatin zymography of human AAA tissue, hASMCs, and THP-1 treated with 100 nM of PMA. B, E and G, MMP-2 activation, IL-6, and MCP-1 production in supernatant of hAASMCs treated with or without 1 μM of ONO-AE1-329 (AE1–329) for 48 h, respectively. n = 5–7. C, D, F, and H, MMP-2 and MMP-9 activation, IL-6 and MCP-1 production in supernatant of human AAA tissue organ cultures incubated in the presence or absence of 1 μM of ONO-AE1-329 (AE1–329) for 48 h, respectively. n = 10–11. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; NS, not significant.
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formation induced by AngII infusion in ApoE$^{-/-}$ mice. ONO-AE3-208 (0.005–0.05 mg/kg/day) was administered orally for 4 weeks. We found that elastic fiber degradation and thus AAA formation were inhibited by ONO-AE3-208 in a dose-dependent manner (**Figures 5A, B and C**). MMP-2 and MMP-9 activation were increased by AngII infusion, but activation was decreased in the presence of ONO-AE3-208 (0.05 mg/kg/day) (**Figures 5D and E**).

EP4 Antagonist Inhibited MMP-2 Activation and IL-6 Production in Explants of Human AAA

We further examined the effect of the EP4 antagonist on cytokine and chemokine production in human AAA tissues. ONO-AE3-208 significantly decreased MMP-2 activation in a dose-dependent manner (10^{-8} M to 10^{-7} M) (**Figure 6A**), which was most likely related to ASMCs. MMP-9 activation was unaltered, which was most likely related to macrophages (**Figure 6B**). IL-6 production was decreased in a dose-dependent manner at dosages between 10^{-9} M and 10^{-7} M (**Figure 6C**), but MCP-1 production was unchanged (**Figure 6D**).

Discussion

Our study demonstrated that EP4 expression was increased in the aneurysmal areas of human AAA tissues, both in ASMCs as well as in macrophages in the lesion. Importantly, EP4 expression was not increased in normal human ASMCs, but was induced when normal cells were stimulated by PGE$_2$. When EP4 was stimulated in hAASMCs and AAA tissue organ cultures, both MMP-2 activity and IL-6 production were increased. With these findings in mind, we examined the effect of EP4 inhibition, either by EP4 gene disruption (EP4$^{+/-}$) or the use of an EP4 antagonist (ONO-AE3-208). In various models of AAA, induced by CaCl$_2$ or AngII infusion in ApoE$^{-/-}$ mice, EP4 inhibition significantly decreased AAA formation. Furthermore, the EP4 antagonist inhibited IL-6 production and MMP-2 activation in human AAA tissues, suggesting a mechanism for EP4 antagonist-mediated inhibition of AAA formation. Accordingly, we propose that EP4 inhibition may serve as an effective pharmacological therapy to prevent the exacerbation of AAA in humans.

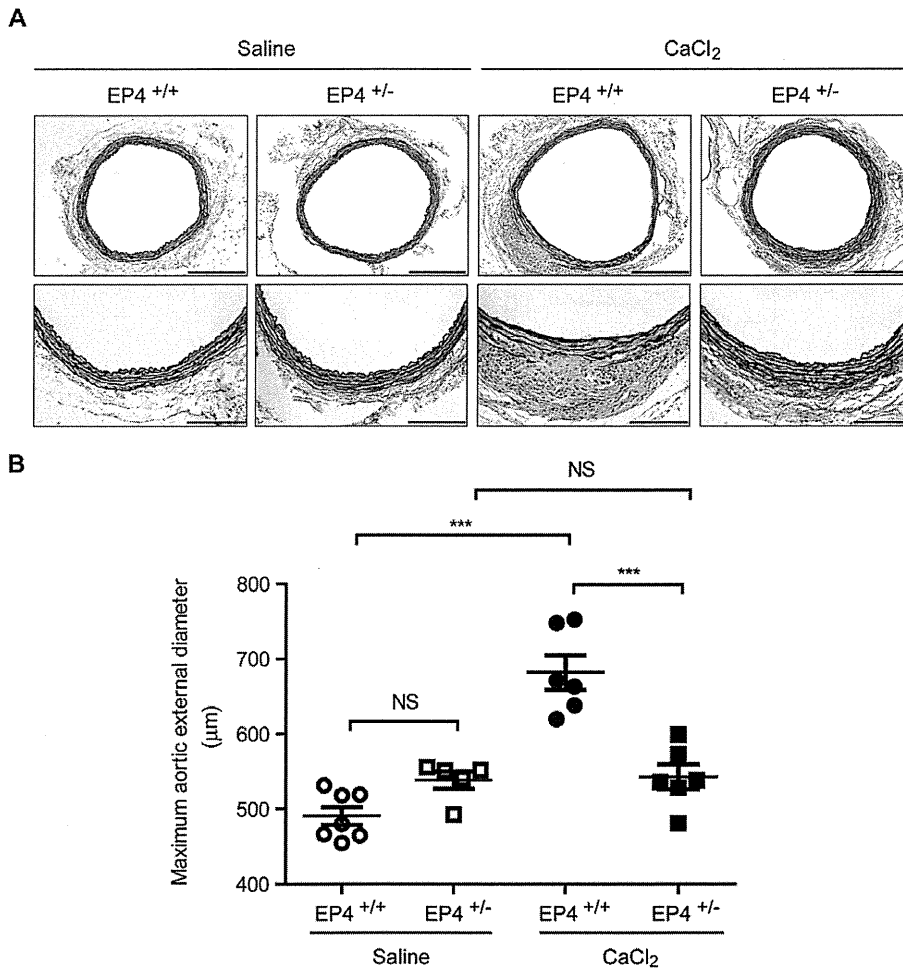Many molecules have been explored as potential targets for a pharmacological therapy of AAA. TGFβ and AngII, for

A

B

Figure 3. CaCl$_2$-induced AAA formation is attenuated in EP4$^{+/-}$ mice. A, Representative images of elastica van Gieson-stained tissue of EP4$^{+/-}$ and EP4$^{+/+}$ mice treated with saline or CaCl$_2$. Lower panels (Scale bars: 100 μm) show higher magnification portions of upper panel images (Scale bars: 200 μm). B, Maximum aortic external diameter of AAA formation induced by CaCl$_2$ in EP4$^{+/-}$ and EP4$^{+/+}$ mice treated with saline or CaCl$_2$. n = 5–7. ***, P<0.001; NS, not significant.
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example, are well known to be increased in AAA. However, it remains controversial whether pharmacological inhibition of these signals can provide effective therapy in AAA [24]. Because it is also well known that COX-2-dependent PGE_2 synthesis is increased, leading to exacerbation of AAA, we hypothesized that this may serve as a possible target for pharmacotherapy as well. Indeed, a previous study demonstrated that COX-2 inhibition by non-steroidal anti-inflammatory drugs prevented AAA exacerbation [5]. Similarly, Gitlin *et al.* showed that COX-2 deficient mice exhibited decreased AngII-induced AAA formation [14]. These findings are in agreement with the fact that PGE_2 is synthesized via COX-2 at high concentration in AAA walls [5,10], so inhibiting it may impede AAA exacerbation.

Because recent clinical studies have shown that COX-2 inhibition *per se* can induce multiple cardiovascular adverse events [15,16], we aimed in this study to inhibit processes further

downstream from the $COX-2/PGE_2$ signal. For PGE_2, there are four receptor subtypes: EP1, EP2, EP3, and EP4 [25]. EP4 is dominantly expressed in macrophages [26], and is a major stimulator of cytokines and proteolytic enzymes production such as MMPs. EP4 is therefore importantly involved in AAA pathophysiology, and many studies have demonstrated that EP4 signaling increases MMP-9 activation in macrophages [27,28,29], leading to exacerbation of AAA [9]. Thus, inhibition of EP4, particularly in macrophages, may be of benefit in preventing AAA. Unexpectedly, however, a very recent study demonstrated that EP4 disruption in bone marrow-derived cells augmented elastin fragmentation and exacerbated AAA formation [30]. Possible reasons for this unfavorable finding may include that EP4 disruption increased MCP-1 because EP4 stimulation can inhibit MCP-1 production in macrophages [31,32]. Consequently, macrophage-selective inhibition of EP4 may not provide an effective therapy for AAA.
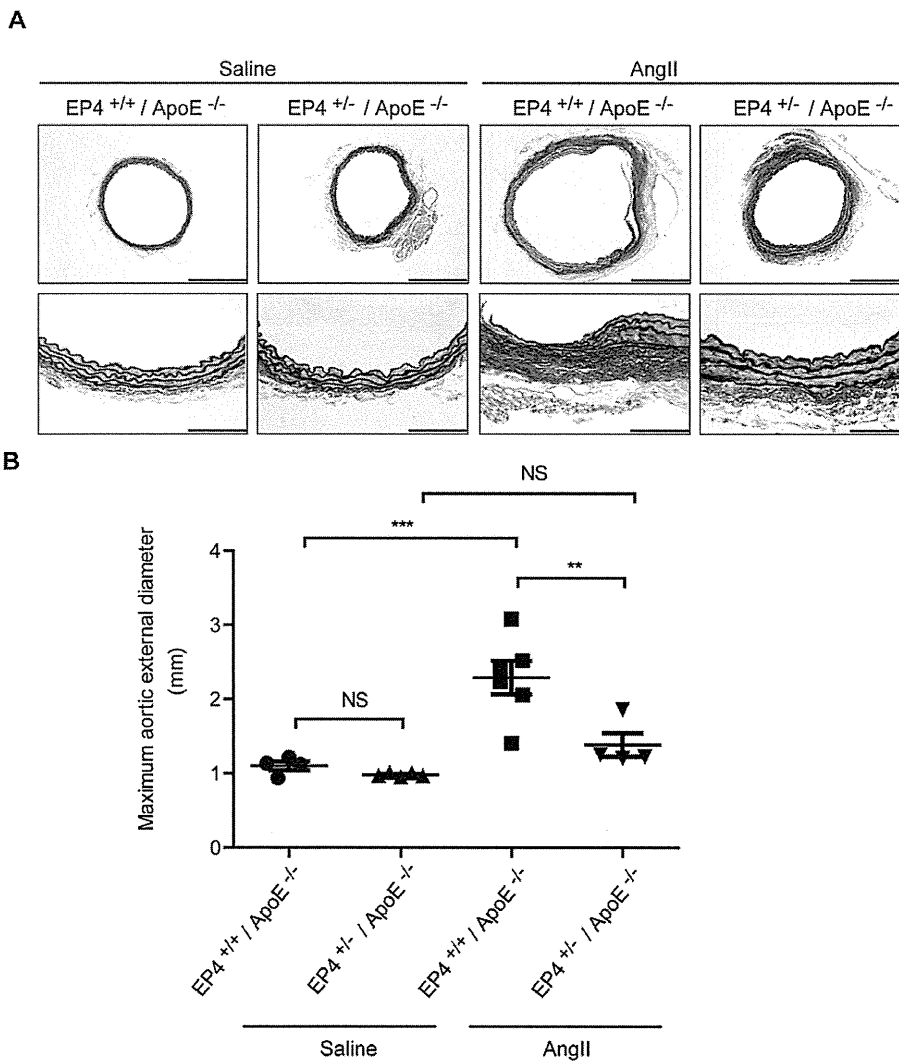
A

B

Figure 4. AngII-induced AAA formation is attenuated in EP4$^{+/-}$/ApoE$^{-/-}$ mice. A, Representative images of elastica van Gieson-stained tissue of EP4$^{+/-}$/ApoE$^{-/-}$ and EP4$^{+/+}$/ApoE$^{-/-}$ mice treated with saline or AngII. Lower panels (Scale bars: 100 μm) show higher magnification portions of upper panel images (Scale bars: 200 μm). B, Maximum aortic external diameter of AAA induced by AngII in EP4$^{+/-}$/ApoE$^{-/-}$ and EP4$^{+/+}$/ApoE$^{-/-}$ mice treated with saline or AngII. n = 4–6. **, $P<0.01$; ***, $P<0.001$; NS, not significant.
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Our study, in contrast, demonstrated the effectiveness of systemic administration of an EP4 antagonist, which inhibits the EP4 signal in all cell types, particularly those with high EP4 expression. Importantly, our study demonstrated, for the first time, that normal ASMCs can increase EP4 expression when stimulated by PGE$_2$. Thus, inflammation in AAA lesions may have increased EP4 expression in ASMCs. The effectiveness of EP4 signaling inhibition in ameliorating AAA exacerbation is also supported by other findings in this study. EP4 stimulation increased IL-6 production and MMP-2 activation in ASMCs, and the use of an EP4 antagonist inhibited IL-6 production and MMP-2 activation in human AAA tissue organ cultures. Although it is known that MMP-2 is mainly expressed in hASMCs [9], PGE$_2$-mediated regulation of MMP-2 has not been demonstrated previously. Here, we demonstrated that EP4 is a potent regulator

of MMP-2 in ASMCs and that this regulation can be indirectly enhanced by IL-6. Our study also indicated that EP4 signaling is a potent inducer of IL-6 production in ASMCs. Because IL-6 *per se* can increase MMP-2 production [33], an EP4 antagonist might indirectly inhibit MMP-2 production by regulating IL-6 in ASMCs as well.

From the view point of pharmacological therapy, when 10 mg/kg/day of ONO-AE3-208 was administered orally as a bolus, the peak plasma concentration was 677 ng/ml (1.7 μM) after 0.25 hours, as shown in a previous study describing a different use [23]. Accordingly, when 0.01 mg/kg/day of ONO-AE3-208 was orally administered in our study, the peak expected plasma concentration in mice was approximately 1.7 nM. Since the Ki value of ONO-AE3-208 was 1.3, 30, 790, and 2,400 nM for EP4, EP3, FP, and TP, respectively [23], our dosages of the EP4
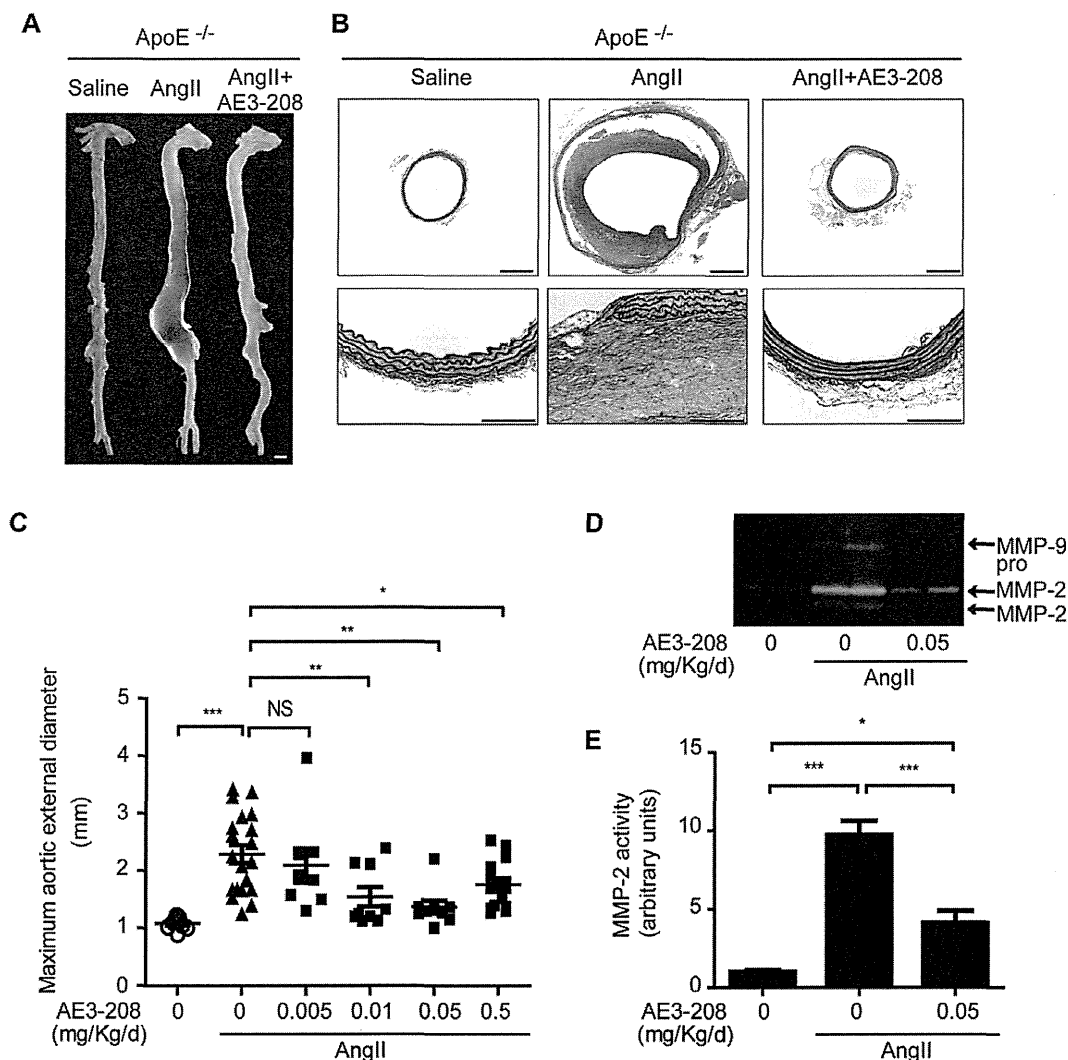

Figure 5. EP4 antagonist attenuated AngII-induced AAA formation in ApoE$^{-/-}$ mice. A, Representative image of aorta of ApoE$^{-/-}$ mice treated with saline, AngII, or AngII+ONO-AE3-208 (AE3-208) (0.05 mg/Kg/d). Scale bar: 1 mm. B, Elastica van Gieson–stained tissue of aortas shown in A. Lower panels (Scale bars: 100 μm) show higher magnification portions of upper panel images (Scale bars: 500 μm). C, Maximum aortic external diameter of AngII-induced AAA formation induced by AngII in ApoE$^{-/-}$ mice treated with saline, AngII or AngII+ONO-AE3-208. n = 8–20. D, Representative images of gelatin zymography of AAA tissues of ApoE$^{-/-}$ mice treated with saline, AngII, or AngII+ONO-AE3-208 (0.05 mg/Kg/d). E, Quantification of D. n = 8–12. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; NS, not significant.
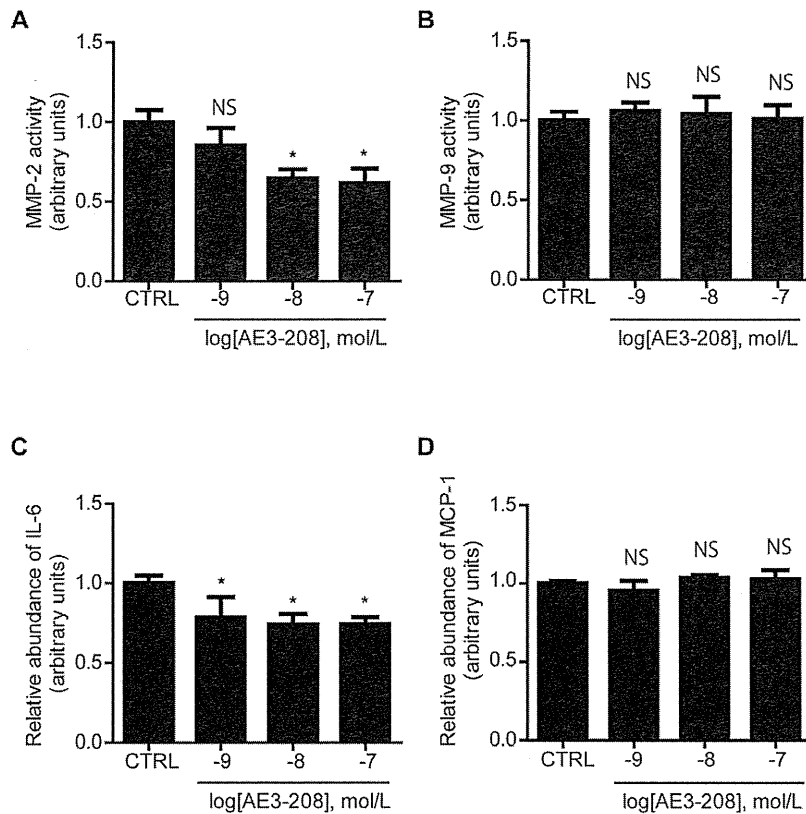doi:10.1371/journal.pone.0036724.g005

Figure 6. EP4 antagonist attenuated MMP-2 activation and IL-6 production in human AAA tissues. A, MMP-2 activity, B, MMP-9 activity, C, IL-6 production, and D, MCP-1 production. Supernatants of human AAA tissue organ cultures incubated in the presence or absence of and increasing concentrations of ONO-AE3-208 (AE3–208). n = 10–20. *, $P<0.05$ vs. control (CTRL); NS, not significant.
doi:10.1371/journal.pone.0036724.g006

antagonist are likely to have inhibited EP4 in a selective manner. Indeed, this EP4 antagonist was effective in 0.01–0.5 mg/kg/day in our mouse study.

In conclusion, this study demonstrated that selective EP4 inhibition was efficacious in inhibiting the exacerbation of AAA formation in a number of mouse models. In particular, pharmacological inhibition of EP4 signaling by an EP4 antagonist was effective at relatively low doses. Although we have not examined the effect of EP4 inhibition on other tissues or organs that also express high EP4, our study suggests, at the very least, that pharmacological EP4 inhibition may serve as a new therapeutic strategy for aneurysmal diseases for which effective medical therapy is currently unavailable.

Author Contributions

Conceived and designed the experiments: UY Y. Ishikawa. Performed the experiments: UY RI MJ Y. Kato OS HJ Y. Ichikawa SK. Analyzed the data: UY RI MJ OS Y. Ichikawa. Contributed reagents/materials/analysis tools: Y. Katayama TF YS SS MM. Wrote the paper: UY Y. Ishikawa. Aided experimental design: SO MS YS HA SS MM SM.

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Prevention of heart failure in mice by an antiviral agent that inhibits type 5 cardiac adenylyl cyclase

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Prevention of heart failure in mice by an antiviral agent that inhibits type 5 cardiac adenylyl cyclase

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[1]Department of Cell Biology and Molecular Medicine, Cardiovascular Research Institute, New Jersey Medical School-University of Medicine and Dentistry of New Jersey, Newark, NJ; [2]Department of Medicine (Cardiology), New Jersey Medical School-University of Medicine and Dentistry of New Jersey, Newark, NJ; [3]Cardiovascular Research Institute, Yokohama City University Graduate School of Medicine, Yokohama, Japan; and [4]Department of Veterinary Medicine, College of Bio-resource Sciences, Nihon University, Fujisawa, Kanagawa, Japan

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Iwatsubo K, Bravo C, Uechi M, Baljinnyam E, Nakamura T, Umemura M, Lai L, Gao S, Yan L, Zhao X, Park M, Qiu H, Okumura S, Iwatsubo M, Vatner DE, Vatner SF, Ishikawa Y. Prevention of heart failure in mice by an antiviral agent that inhibits type 5 cardiac adenylyl cyclase. *Am J Physiol Heart Circ Physiol* 302: H2622–H2628, 2012. First published April 13, 2012; doi:10.1152/ajpheart.00190.2012.—Despite numerous discoveries from genetically engineered mice, relatively few have been translated to the bedside, mainly because it is difficult to translate from genes to drugs. This investigation examines an antiviral drug, which also has an action to selectively inhibit type 5 adenylyl cyclase (AC5), a pharmaceutical correlate of the AC5 knockout (KO) model, which exhibits longevity and stress resistance. Our objective was to examine the extent to which pretreatment with this drug, adenine 9-β-D-arabinofuranoside (Ara-A), favorably ameliorates the development of heart failure (HF). Ara-A exhibited selective inhibition for AC5 compared with the other major cardiac AC isoform, AC6, i.e., it reduced AC activity significantly in AC5 transgenic (Tg) mice, but not in AC5KO mice and had little effect in either wild-type or AC6Tg mice. Permanent coronary artery occlusion for 3 wk in C57Bl/6 mice increased mortality and induced HF in survivors, as reflected by reduced cardiac function, while increasing cardiac fibrosis. The AC5 inhibitor Ara-A significantly improved all of these end points and also ameliorated chronic isoproterenol-induced cardiomyopathy. As with the AC5KO mice, Ara-A increased mitogen/extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK) phosphorylation. A MEK inhibitor abolished the beneficial effects of the AC5 inhibitor in the HF model, indicating the involvement of the downstream MEK-ERK pathway of AC5. Our data suggest that pharmacological AC5 inhibition may serve as a new therapeutic approach for HF.

heart failure; type 5 adenylyl cyclase inhibition; adenine 9-β-D-arabinofuranoside

DESPITE GAINS IN THE TREATMENT of heart failure (HF) with both angiotensin and β-adrenergic receptor (β-AR) blockers, HF still remains a major cause of death and disability. In addition, some patients do not tolerate β-AR blocking therapy (2). It is conceivable that inhibiting mechanisms distal to the β-AR signaling pathway, identified from genetically engineered mouse models, might be a novel approach. While there have

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been numerous potential therapeutic approaches discovered from studies in genetically engineered mice in the past two decades, there are relatively few of these discoveries that have been translated to the bedside, mainly because it is difficult to translate the effects of disrupting a gene in a mouse to therapy in patients with HF.

The goal of this investigation was to examine the extent to which a pharmacological inhibitor of type 5 adenylyl cyclase (AC5), 9-β-D-arabinofuranoside (Ara-A), could mimic the salutary effects observed in the AC5 knockout (KO) mice model, which protects against cardiac stress (10, 11) and increases longevity (15). The first goal was to determine the extent to which Ara-A selectively inhibits AC5. The next goal was to determine whether pretreatment with the pharmacological AC5 inhibitor ameliorates the development of cardiomyopathy and HF following either permanent coronary artery occlusion (CAO) or chronic isoproterenol (ISO) infusion. An additional goal was to determine if the mechanism involved the mitogen/extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway, a key protective signaling pathway in the AC5KO (15). The latter was accomplished by repeating the experiments with permanent CAO in the presence of a specific MEK blocker, U-0126.

The AC5 inhibitor Ara-A is a Food and Drug Administration (FDA)-approved drug, also known as vidarabine (adenine 9-β-D arabinofuranoside), and has been used as an anti-herpes virus drug for many decades. The advantage of identifying Ara-A as a potential drug for HF is that the drug has already been FDA approved and could be rapidly moved to clinical trials.

METHODS

Animal models. Three- to five-month-old male AC5KO (9) (on C57Bl/6 background) and cardiac-specific overexpression of AC5 [AC5 transgenic (Tg)] (7) or AC6 (AC6Tg) (7) (on FVB background) mice were used in this study. In the CAO model, mini-osmotic pumps delivering Ara-A (15 mg·kg^{-1}·day^{-1}), the MEK blocker U-0126 (5 mg·kg^{-1}·day^{-1}) (15), or a combination of U-0126 with Ara-A were subcutaneously implanted 1 wk before the CAO of the left anterior descending artery. Chronic infusion of ISO (Sigma-Aldrich, St. Louis, MO) was performed for 7 days at a dose of 60 mg·kg^{-1}·day^{-1} with or without Ara-A delivered with the mini-osmotic pumps. The dose of Ara-A was selected on the basis of that previously used for viral encephalopathy (13). Animals used in this study were maintained in

□ Vehicle   ■ Ara-A

A Cardiomyocyte Membranes

B Cardiomyocyte Membranes

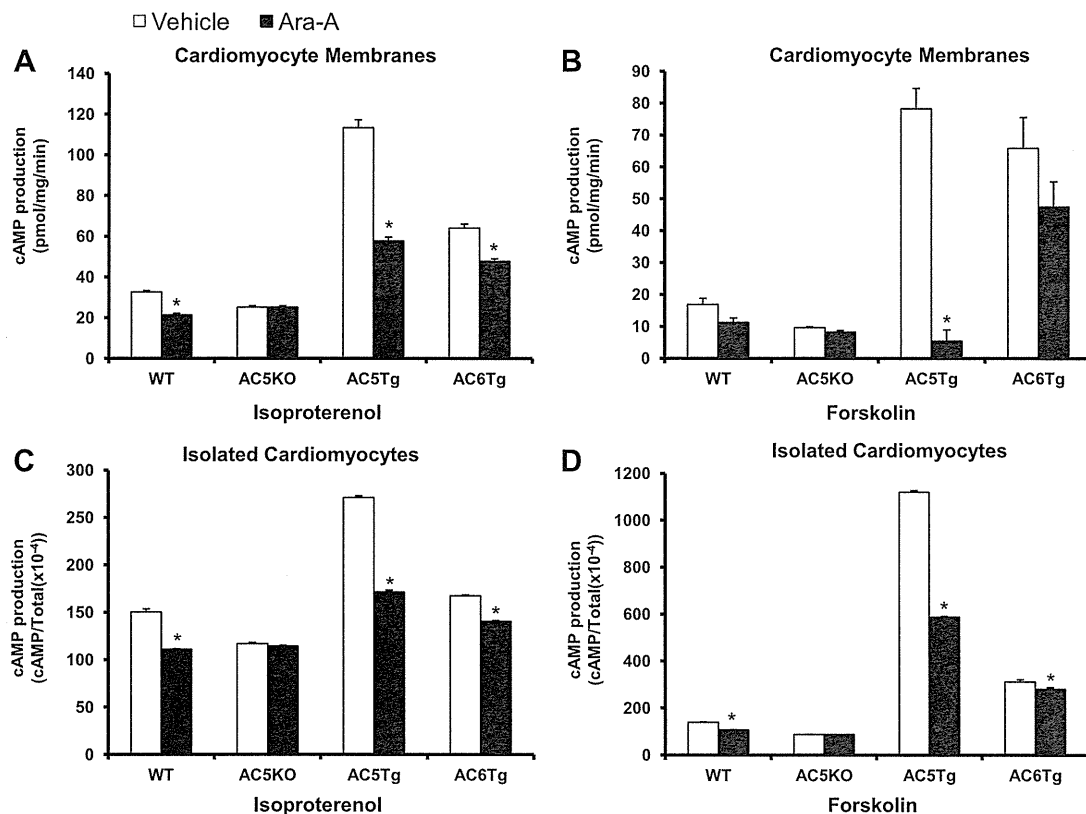**C** Isolated Cardiomyocytes

D Isolated Cardiomyocytes

Fig. 1. Selective inhibition of type 5 adenylyl cyclase (AC5) by adenine 9-β-D-arabinofuranoside (Ara-A) (in vitro). Mouse cardiac membrane preparations were used for A and B. Adult mouse cardiac myocytes were used for C and D. A and B: cAMP reduction with Ara-A (10 μM) was measured in cardiac membrane preparations from myocardium of wild-type (WT), AC5 knockout (KO), AC5 transgenic (Tg), and AC6Tg mice with isoproterenol (ISO) (5 μM, A) or with forskolin (50 μM, B). C and D: cAMP reduction with Ara-A (10 μM) was measured in adult cardiac myocytes from myocardium of WT, AC5KO, AC5Tg, and AC6Tg mice with ISO (C) or with forskolin (D). cAMP reduction by Ara-A was greater in AC5Tg than in WT, was similar between WT and AC6Tg, and was absent in the AC5KO. These data indicate that Ara-A selectively suppresses AC5 enzymatic activity. t-Test: *$P < 0.01$ vs. vehicle; $n = 4$ experiments for A and B and $n = 5$ for C and D.

-◇- Vehicle -■- Ara-A -▲- Metoprolol
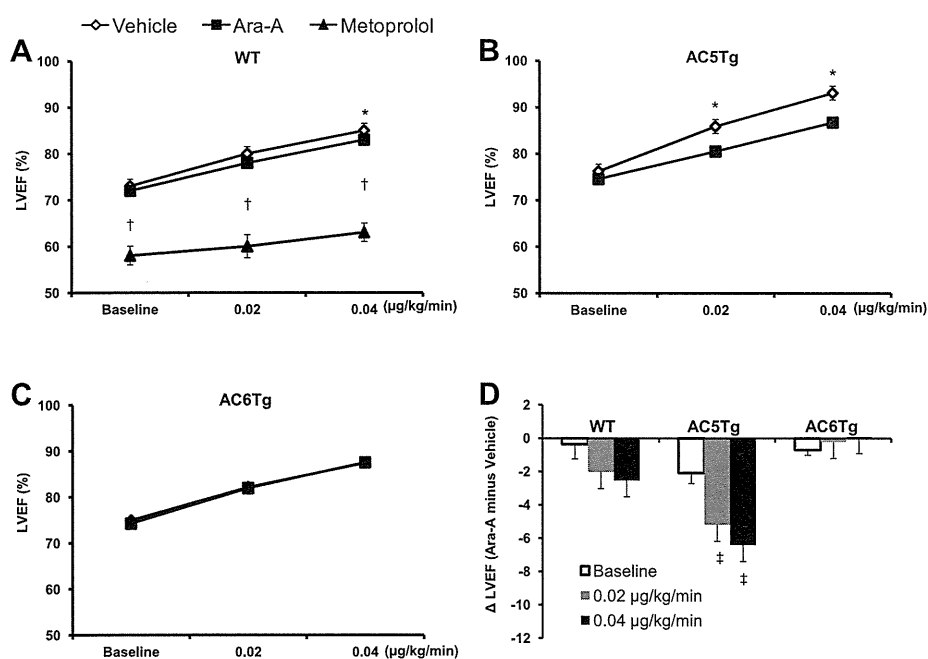
A WT

B AC5Tg

C AC6Tg

D

Fig. 2. Selective inhibition of AC5 by Ara-A (in vivo). Left ventricle ejection fraction (LVEF) was measured in response to ISO challenges in WT (A), AC5Tg (B), and AC6Tg (C) in the presence of either vehicle, Ara-A, or metoprolol ($n = 6$ animals/group). D: negative inotropic effect of Ara-A at baseline and at a dose of 0.02 and 0.04 μg·kg⁻¹·min⁻¹ of ISO. Ara-A decreased ISO-induced increases in LVEF more in AC5Tg mice than WT or AC6Tg mice (A–D). These data show that Ara-A selectively suppresses AC5 in vivo. In contrast to Ara-A, the β-adrenergic receptor (β-AR) blocker metoprolol decreased basal left ventricle (LV) function and also completely blocked the positive inotropic response to ISO (A). t-Test: *$P < 0.05$, Ara-A vs. vehicle; †$P < 0.05$, metoprolol vs. Ara-A or vs. vehicle; and ‡$P < 0.05$ vs. same ISO dose in WT or vs. same ISO dose in AC6Tg.

Table 1. *Postmyocardial infarction cardiomyopathy model*

	Sham ($n = 4$)	Vehicle ($n = 4$)	Ara-A ($n = 12$)	Ara-A + U-0126 ($n = 6$)
Heart rate, beats/min	386 ± 14	517 ± 12‡	520 ± 7	511 ± 13
LV ejection fraction, %	71.6 ± 1.6	42.2 ± 4.6‡	56.9 ± 1.8*	47.9 ± 1.3†
LV end diastolic diameter, mm	3.9 ± 0.2	5.6 ± 0.1‡	4.8 ± 0.1*	5.7 ± 0.2†
LV end systolic diameter, mm	2.6 ± 0.2	4.7 ± 0.2‡	3.6 ± 0.1	4.6 ± 0.1
LV wt/tibial length, mg/mm	5.1 ± 0.4	6.6 ± 0.3‡	7.1 ± 0.2	7.0 ± 0.3
Lung wt/tibial length, mg/mm	7.2 ± 0.3	12.8 ± 0.7‡	10.6 ± 0.4*	15.4 ± 0.6†

Values are means ± SE; n, no. of animals. Ara-A, adenine 9-β-D-arabinofuranoside; LV, left ventricular. $P < 0.05$, Ara-A different from vehicle (*), Ara-A + U-0126 different from Ara-A (†), and vehicle different from sham (‡).

accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, revised 2011). This study has been approved by the University of Medicine and Dentistry Institutional Animal Care and Use Committee.

AC assay. AC activity was measured by a modification of the method of Salomon et al. (10), as we previously described (12). When the AC assays were performed using crude membranes from AC6Tg mice heart, manganese instead of magnesium was used in the assay buffer to obtain maximum enzymatic catalytic activity because AC6 is stimulated more by manganese than by magnesium (16).

Adult cardiac myocytes. Adult cardiac myocytes were isolated from Langendorff-perfused mouse hearts as previously described (14). Enzyme solution containing 1 mg/ml collagenase (type II; Worthington), 0.1 mg/ml protease (type XIV; Sigma), and 10 μM blebbistatin (Toronto Research Chemicals) was perfused in a heart for 15–20 min followed by washing. The heart was removed from the perfusion apparatus and swirled in a culture dish. Ca^{2+} was gradually added to the dish until the concentration reached 1 mM. The cells were filtered with a cell strainer and cultured in DMEM/F-12 medium with 5% horse serum until used for the cAMP accumulation assay.

[³H]adenine labeling and cAMP accumulation assay. cAMP accumulation assays in adult mouse cardiac myocytes were performed as done previously (8). Briefly, cells were incubated with [³H]adenine (3 μCi/ml) for 3 h, and cells were washed and pretreated with 20 mM HEPES-balanced serum-free minimum essential medium containing 0.5 mM 3-isobutyl,1-methylxanthine. After preincubation with Ara-A for 10 min, reactions were started by the addition of 50 μM of the nonspecific AC agonist forskolin or 5 μM of the β_1/β_2-AR agonist ISO. Ten minutes after the addition of forskolin or ISO, reactions were terminated by the addition of 12% (wt/vol) trichloroacetic acid containing 0.25 mM ATP and 0.25 mM cAMP. The [³H]ATP and [³H]cAMP were separated with single acidic alumina columns. The cAMP production was calculated as [³H]cAMP/([³H]cAMP + [³H]ATP) × 10⁴.

ISO challenge. Mice were anesthetized with 2.5% tribromoethanol (0.015 ml/g body wt) injected intraperitoneally, and echocardiography was performed. For acute injection of ISO, a PE-10 catheter was inserted in the right jugular vein, and an ISO solution was injected at the rate of 1 μl/s. For ISO challenge experiments, Ara-A, metoprolol, or their vehicle was administered by miniosmotic pumps from 7 days before the experiment, in a dose 15 and 5 $mg \cdot kg^{-1} \cdot day^{-1}$, respectively.

Histological analyses. Heart specimens were fixed with formalin, embedded in paraffin, and sectioned at 6-μm thickness. Interstitial fibrosis was evaluated by picric acid Sirius red staining and ImagePro-Plus software analysis, as previously described (4, 10).

Western blotting. Western blotting in tissue lysate from the viable region of the left ventricle (LV) was conducted with commercially available antibodies against the phosphorylated form and total MEK or ERK (Cell Signaling). Western blotting was performed as previously described (8).

Data and statistical analysis. All data are reported as means ± SE. Statistical comparisons were calculated using a Student's t-test and ANOVA with Newman-Keuls post hoc comparison test. The groups

passed the normality test and had similar variation. In addition, the Mann-Whitney test confirmed the results from the t-test for the critical data points, e.g., responses of LV ejection fraction (LVEF). Survival curves were compared using the log-rank test and Kaplan-Meier survival analysis. P values of <0.05 were considered significant.

RESULTS

AC5 inhibition decreases cAMP production in the heart. The hearts of AC5Tg mice showed a 10-fold increase in cardiac membrane AC activity using forskolin, indicating that AC5 represented most of the AC activity in the AC5Tg heart, in contrast to the wild-type (WT) or AC6Tg heart, where AC5 expression
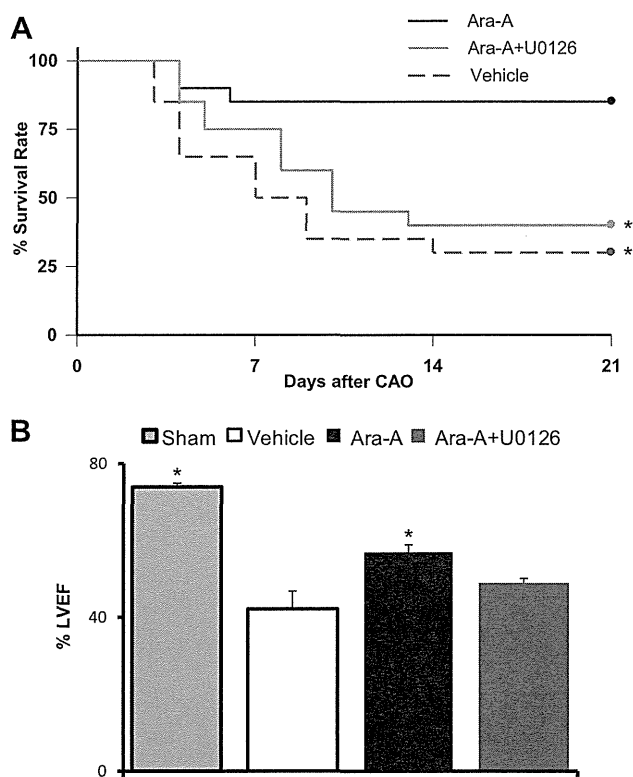

Fig. 3. Ara-A protects against postmyocardial infarction (MI) cardiomyopathy. WT C57Bl/6 mice after permanent coronary artery occlusion (CAO). *A*: follow up for 21 days. Kaplan-Meier graph of post-MI animals treated with Ara-A, Ara-A + U-0126, or vehicle. Ara-A enhanced the survival post-MI in WT C57Bl/6 mice. Log-rank test: *$P < 0.05$ vs. Ara-A, $n = 16$ animals/group (*B*) in survivors from experiment in *A*; LVEF was improved by Ara-A (Ara-A, $n = 12$; Ara-A + U-0126, $n = 5$; and vehicle, $n = 4$). U-0126 blocked the protective effects of Ara-A in terms of mortality and LVEF (*A* and *B*). t-Test: *$P < 0.05$ vs. Ara-A; †$P < 0.05$ vs. vehicle, Ara-A, or Ara-A + U-0126.

represented a relatively minor fraction of total AC. AC5KO mice showed, as expected, the null AC5 expression (9). When cardiac membrane preparations were used, Ara-A reduced cAMP production much more in AC5Tg than in WT, and not in AC5KO (Fig. 1, A and B). When cultured adult cardiac myocytes were used, Ara-A also demonstrated more effective inhibition in AC5Tg than in WT (Fig. 1, C and D). We found that the inhibitory effect was similar in WT and AC6Tg, but significantly less than observed in AC5Tg. These data suggest that Ara-A inhibits AC5, more than AC6, in the heart.

Ara-A attenuates contractile response to β-AR stimulation in AC5Tg, but little in WT. ISO was administered in mice. Ara-A did not reduce baseline LV function and reduced ISO-increased LVEF only slightly (Fig. 2A) in WT mice. In contrast, metoprolol depressed LV function significantly and essentially eliminated the inotropic effects of ISO challenge (Fig. 2A). In AC5Tg mice, the acute ISO challenge increased LVEF even more in the vehicle group, but this increased inotropic effect was not observed in the Ara-A group (Fig. 2B). Thus, the ability of Ara-A to block the inotropic effects of ISO is obvious only when AC5 is overexpressed. The response to ISO challenge in the AC6Tg group was similar to vehicle (Fig. 2C), similarly to the WT group response, further indicating the selectivity of Ara-A for AC5 (Fig. 2, A–D). Therefore, in contrast to metoprolol, Ara-A did not act as a β-AR blocker, i.e., did not depress cardiac function and did not eliminate the inotropic effects of ISO.

Ara-A attenuates the progression of postmyocardial infarction HF. Next, we examined the extent to which Ara-A ameliorated postmyocardial infarction (MI) cardiomyopathy. The post-MI cardiomyopathy model was induced by permanent ligation of the left anterior descending coronary artery, which results in an infarct size of 30–40% of the LV (data not shown). In this model, the LVEF was reduced significantly ($P < 0.05$, 42.2%) compared with sham (71.6%), whereas the LV end-diastolic diameter was increased ($P < 0.05$) from 3.9 in the sham to 5.6 mm in the vehicle-treated post-MI cardiomyopathy group. Lung weight/tibial length, which is an indicator of HF, was increased, $P < 0.05$, in the post-MI cardiomyopathy group (12.8) compared with the sham group (7.2) (Table 1). Ara-A improved LVEF by 38% and reduced LV diastolic end-diastolic diameter by 14% compared with the vehicle group (Fig. 3B and Table 1). Ara-A also significantly improved survival rate compared with vehicle ($P < 0.05$, log-rank test) (Fig. 3A), and reduced, $P < 0.05$, intestinal fibrosis (Fig. 4, A and B). At autopsy, the cause of death in the mice that died was either due to cardiac rupture or HF.

MEK-ERK pathway mediates the salutary effects of Ara-A. Administration of Ara-A increased the phosphorylation of MEK, ERK1, and ERK2 in WT mouse hearts (Fig. 5A) and in the post-MI cardiomyopathy model (Fig. 5B). U-0126, a MEK inhibitor, inhibited basal and Ara-A-induced ERK phosphorylation, suggesting that U-0126 indeed inhibits ERK signaling in the heart in vivo, and Ara-A activates ERK via MEK phosphorylation. U-0126 inhibited activity of MEK, but not phosphorylation itself, which is consistent with a previous report by Favata et al. (4). We found that U-0126 abolished the salutary effects of Ara-A in terms of survival (Fig. 3A), preservation of LV function (Fig. 3B), and histological evidence of fibrosis (Fig. 4, A and B).
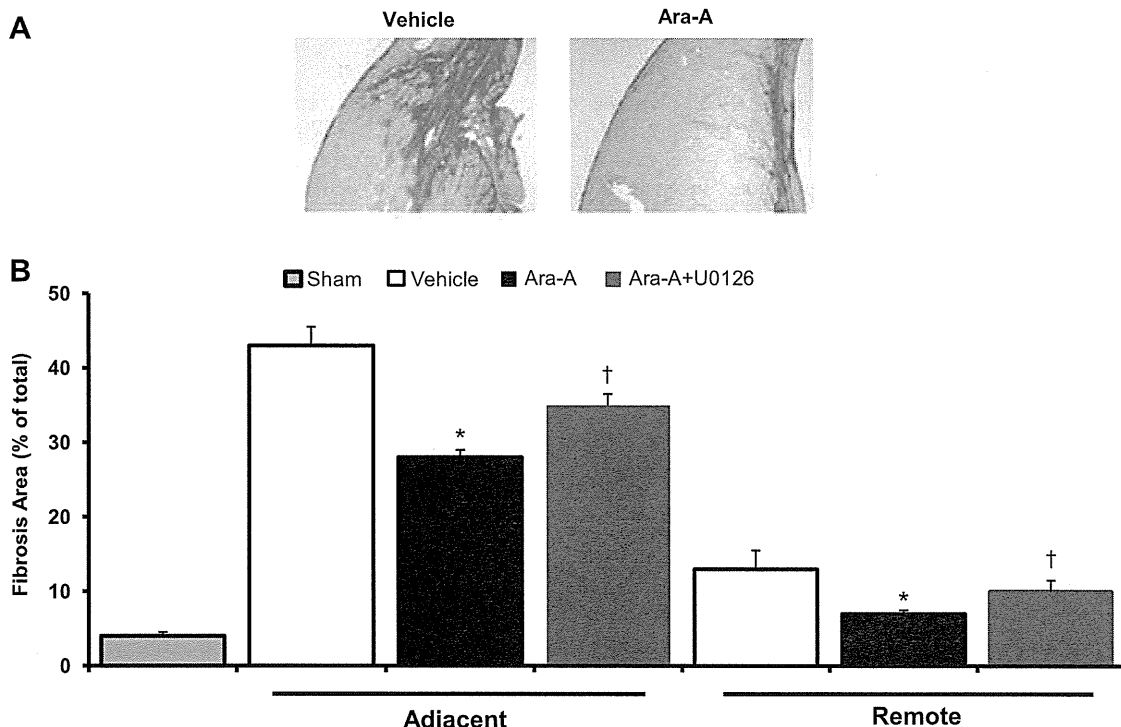

Fig. 4. Ara-A protects against cardiac fibrosis post-MI. WT C57Bl/6 mice after permanent CAO. *A*: representative images of fibrosis adjacent to infarcted myocardium with picric acid Sirius red (PASR) staining from animals treated with vehicle or Ara-A; $n = 16$ animals/group. *B*: fibrosis was increased post-MI both adjacent and remote from the infarcted area and was partially protected by Ara-A. U-0126 blocked this protection with Ara-A; vehicle group, $n = 4$; Ara-A group, $n = 10$; and Ara-A + U-0126, $n = 6$ animals. t-Test: *$P < 0.05$ vs. vehicle; †$P < 0.05$ vs. Ara-A within the respective zone (either adjacent or remote).
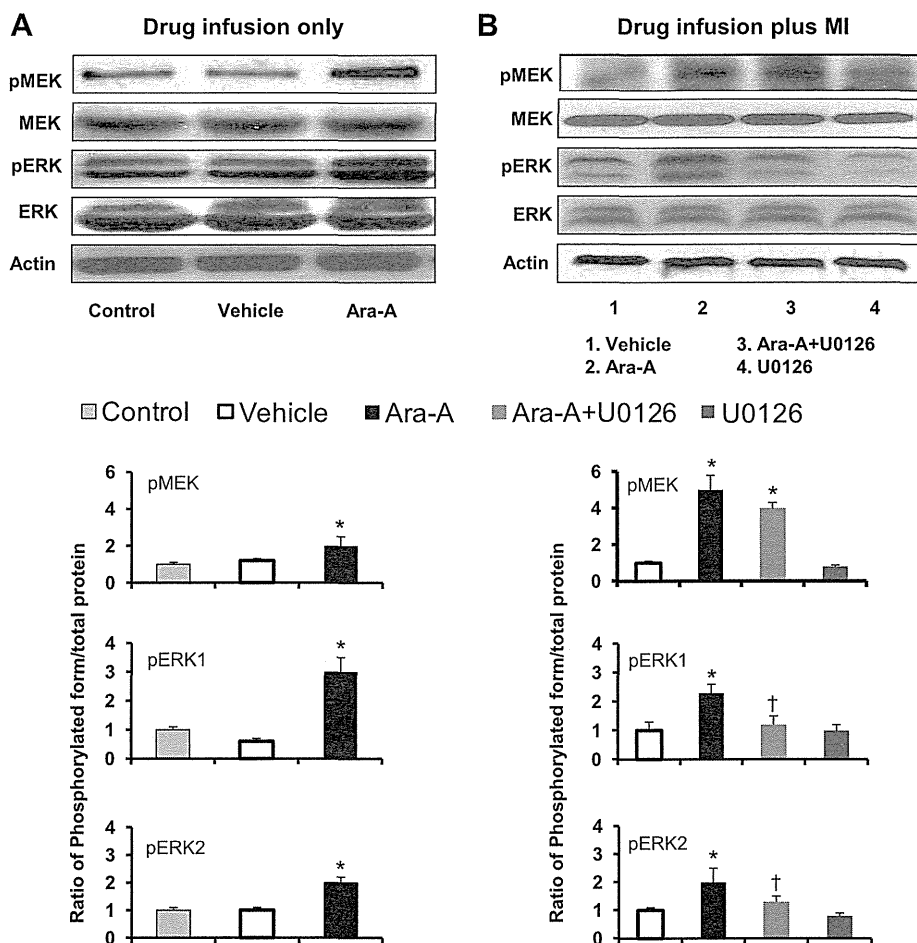
Fig. 5. Mitogen/extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling is activated by Ara-A. A: Western blot analyses showed that Ara-A increased phosphorylation of MEK and ERK in the mice hearts with chronic infusion of Ara-A for 6 days. Control mice did not undergo any pump implantation. B: animals treated with vehicle, Ara-A, and Ara-A + U-0126 post-MI and sham were evaluated for MEK/ERK activation by Western blot. Ara-A increased ERK phosphorylation, which was inhibited by U-0126. U-0126 selectively inhibited ERK phosphorylation but not phosphorylation of MEK. The numbers at the base of B refer to different groups; $n = 4$ animals/group. t-Test: $*P < 0.05$ vs. vehicle or vs. control; $^{†}P < 0.05$ vs. Ara-A.

Ara-A preserves cardiac function with chronic catecholamine stress. We next examined whether the AC5 inhibitor, Ara-A, attenuates cardiac dysfunction induced by excessive catecholamine stress with chronic ISO infusion. Survival rate during chronic ISO infusion was higher in the Ara-A group than in the vehicle group ($P < 0.05$, log-rank test) (Fig. 6A). Ara-A showed preserved contractile function as measured by LVEF (Fig. 6B), suggesting that Ara-A protects against ISO-induced cardiac dysfunction. These data indicate that Ara-A retards the progression of ISO-induced cardiomyopathy.

DISCUSSION

There are many discoveries in genetically altered mice that cannot be applied clinically, since there is no pharmacological counterpart that can be given to animals or patients with cardiovascular disease. The major finding of this investigation is that a drug, which has been commercially available for decades, but only as an anti-viral agent, has potent and selective AC5 inhibitory properties and that this drug ameliorates the progression of cardiomyopathy in animals induced with either chronic ISO or MI. With both interventions, Ara-A demonstrated increased survival, preserved contractile dysfunction, and reduced cardiac interstitial fibrosis.

First, it was important to demonstrate that Ara-A impairs AC5 activity selectively, both in vitro and in vivo. AC5Tg showed enhanced cAMP production compared with WT, and

the effects of Ara-A were greater in AC5Tg than that either in WT or in AC6Tg, which represents the other major AC isoform in the heart, indicating a high selectivity for AC5. This was supported by the data showing that Ara-A does not inhibit cAMP production in AC5KO and inhibits cAMP almost identically in AC6Tg and WT. We also examined this inhibitor in vivo and demonstrated in parallel experiments that Ara-A reduced ISO-stimulated LVEF more in AC5Tg than WT, and reduced the ISO response minimally in AC6Tg, similar to that in WT. If Ara-A was a nonselective AC inhibitor, then it should have shown enhanced AC inhibition with overexpressed AC6 as it did with overexpressed AC5. However, although we demonstrated relatively specific selectivity for AC5, and the MEK/ERK pathway, this does not mean that the inhibitor may also have other actions as well.

One could argue that Ara-A exerts its beneficial effect in ameliorating the extent of HF, simply acting as another β-AR blocker, rather than specifically on AC5, since AC5 is involved in β-AR signal transduction. We do not subscribe to this view for several reasons. First, Ara-A reduced cAMP production relatively modestly in response to ISO in WT mice in vitro and in vivo, particularly compared with the effects of metoprolol, which essentially abolished the inotropic response to ISO. Ara-A inhibited the inotropic response to ISO significantly only in the presence of elevated AC5, as in the AC5Tg, which was still less of a negative inotropic effect than metoprolol.
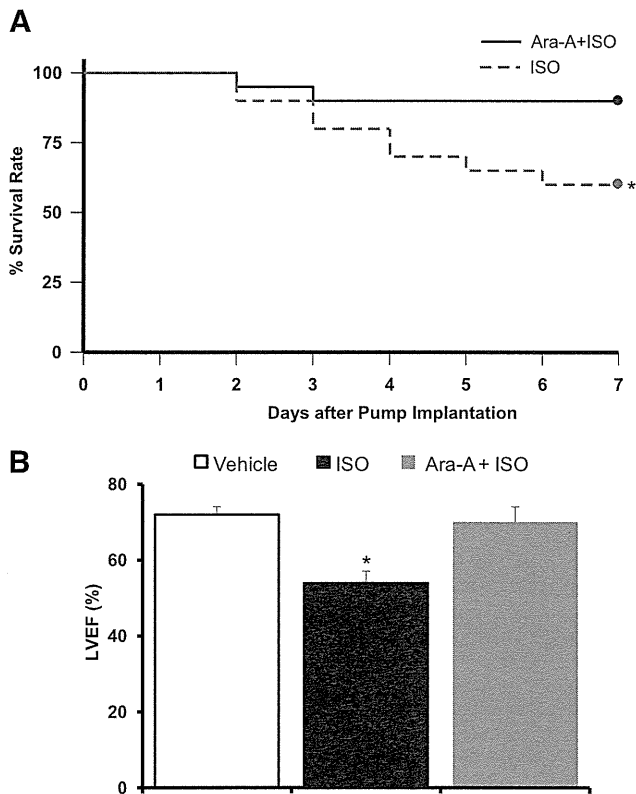
A

B

Fig. 6. AC5 inhibition attenuates chronic ISO-induced heart failure (HF). WT C57Bl/6 mice for 7 days of ISO infusion. *A*: Ara-A increased the survival rate during the period of chronic ISO infusion; $n = 32$ for ISO and $n = 18$ for the ISO + Ara-A group. *B*: Ara-A prevented LVEF dysfunction induced by chronic ISO; ISO, $n = 28$; ISO + Ara-A, $n = 16$ and vehicle, $n = 4$. t-Test: $*P < 0.05$ vs. vehicle or vs. Ara-A + ISO.

demonstrates that the MEK-ERK pathway is also involved in the protection afforded in the heart by Ara-A during the development of HF and cardiac remodeling induced by MI, as evidenced by the increase in MEK-ERK signaling with Ara-A and the blockade of the salutary effects of the AC5 inhibition with the MEK inhibitor U-0126.

It is well recognized that acutely administered ISO improves LV function, whereas chronic ISO induces LV dysfunction and eventually HF along with increased mortality (1, 14). Ara-A also preserved cardiac function and reduced mortality with chronic ISO in the current study. Thus, it is tempting to speculate that pharmacological inhibition of AC5 could enhance survival in HF patients and preserve their cardiac function.

An underlying assumption of the current study is that the induction of HF, either by chronic ISO or chronic MI, induces upregulation of AC5 in the heart. Indeed this was found in a prior study in the chronic ISO model (9). This was more difficult to demonstrate in the chronic MI model in the mouse, since there is so little salvaged myocardium adjacent to the infarct. Accordingly, we examined a rat model of chronic MI and analyzed AC5 protein content, using a specific AC5 antibody (5), in myocardial samples, both adjacent and remote to the infarct. The upregulation of AC5 in the remote zone was only modest, but was more striking in the adjacent tissue (Fig. 7).

In summary, AC5 inhibition with Ara-A could be a new approach to the treatment of HF. In addition to its favorable action on halting the progression to HF due to cardiomyopathy following either permanent CAO or chronic ISO, it exerts little cardiac depression, potentially making the drug more tolerable for patients with compromised cardiac function. Importantly, since the drug studied is already FDA approved, the time from bench to bedside may be accelerated.

Furthermore, the AC5 KO mouse does not show a decreased heart rate and only minimally reduced LV function (7), which is not consistent with the actions of a β-blocker. In support of this, heart rate was not lower in animals with chronic MI treated with Ara-A (Table 1).

Ara-A was previously found to inhibit AC5 activity by the computer-based drug screening system, and its inhibition was confirmed in in vitro AC assays (8). The major finding of the current investigation was to demonstrate that pretreatment with this drug ameliorated the development of HF through the MEK-ERK pathway. Thus, an anti-herpes viral drug could be used in the treatment of HF through mechanisms that have never been considered previously, i.e., inhibition of AC5. Because the animals that died after the intervention most likely suffered more severe LV dysfunction, and more animals died without treatment, then it could be argued that the salutary effects of Ara-A with both chronic ISO and post-MI may be underestimated since the mortality was also reduced.

It is important to point out that the cellular mechanism mediating the beneficial effects of Ara-A does not involve simple β-AR blockade, but rather involves MEK-ERK signaling. The link between reduced AC5 as in the AC5KO mouse and the increase in Raf-1-MEK-ERK signaling was elucidated in a prior study from our laboratory demonstrating that, in the AC5KO mouse, this pathway was involved in enhanced longevity in this mouse model (15). The current investigation
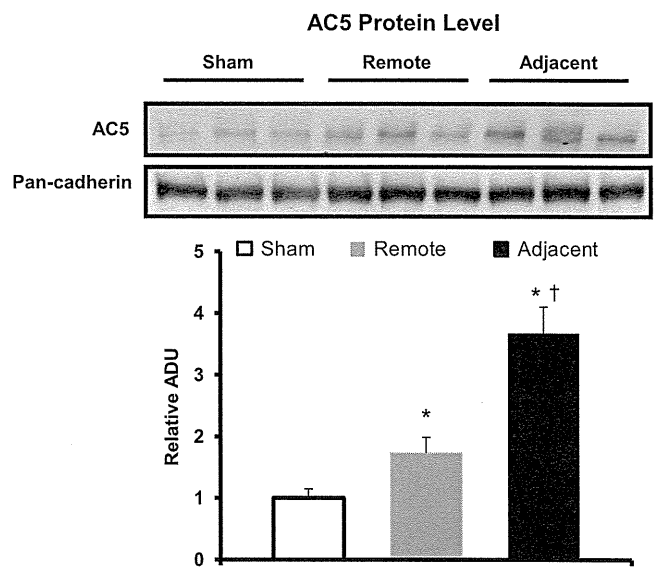
AC5 Protein Level

Fig. 7. AC5 protein levels are increased adjacent to infarct. Western blotting demonstrated that AC5 protein levels, assessed with a specific AC5 antibody (5), were significantly upregulated adjacent to the infarct in the chronic CAO model and less so in the remote zone. In this experiment, we used a rat model because there was not enough tissue adjacent to the infarct in the mouse chronic MI model. $*P < 0.05$ from sham; $^{\dagger}P < 0.05$ from remote.

DISCLOSURES

No conflicts of interest are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: K.I., C.B., E.B., T.N., L.Y., D.E.V., S.F.V., and Y.I. conception and design of research; K.I., C.B., M. Uechi, E.B., T.N., M. Umemura, L.L., S.G., L.Y., M.P., H.Q., S.O., M.I., D.E.V., S.F.V., and Y.I. analyzed data; K.I., C.B., M. Uechi, E.B., T.N., M. Umemura, S.G., L.Y., M.P., H.Q., S.O., M.I., D.E.V., S.F.V., and Y.I. interpreted results of experiments; K.I., C.B., T.N., M. Umemura, S.G., L.Y., M.P., H.Q., M.I., D.E.V., S.F.V., and Y.I. drafted manuscript; K.I., C.B., M. Uechi, E.B., L.Y., M.P., H.Q., M.I., D.E.V., S.F.V., and Y.I. edited and revised manuscript; K.I., C.B., E.B., L.Y., D.E.V., S.F.V., and Y.I. approved final version of manuscript; C.B., M. Uechi, E.B., T.N., M. Umemura, L.L., S.G., X.Z., M.P., H.Q., S.O., and M.I. performed experiments; C.B., M. Uechi, E.B., T.N., M. Umemura, L.L., S.G., L.Y., M.P., H.Q., S.O., and M.I. prepared figures.

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