別紙４

研究成果の刊行に関する一覧表

書籍

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		総監訳　泉井亮 監訳　河南洋、 久保川　学 訳　青木史暁、 赤池紀生、石川義弘、 石山延吉、上田陽一、 上野伸哉、大野忠雄、 河原克雅、河南洋、 北村竜一、久保川学、 桑木共之、小島至、 佐々木和彦、 高瀬堅吉、槌田成紀、 照井直人、中村晃、 花森隆充、 福田康一郎、藤原広明、船橋利也、 山崎将生、山田聡子、 山本頼綱、泉井亮	ボロン・プルーペ　生理学	西村書店	東京	2011	14-24章

雑誌

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[IV]

研究成果の刊行物・別刷

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.

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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-

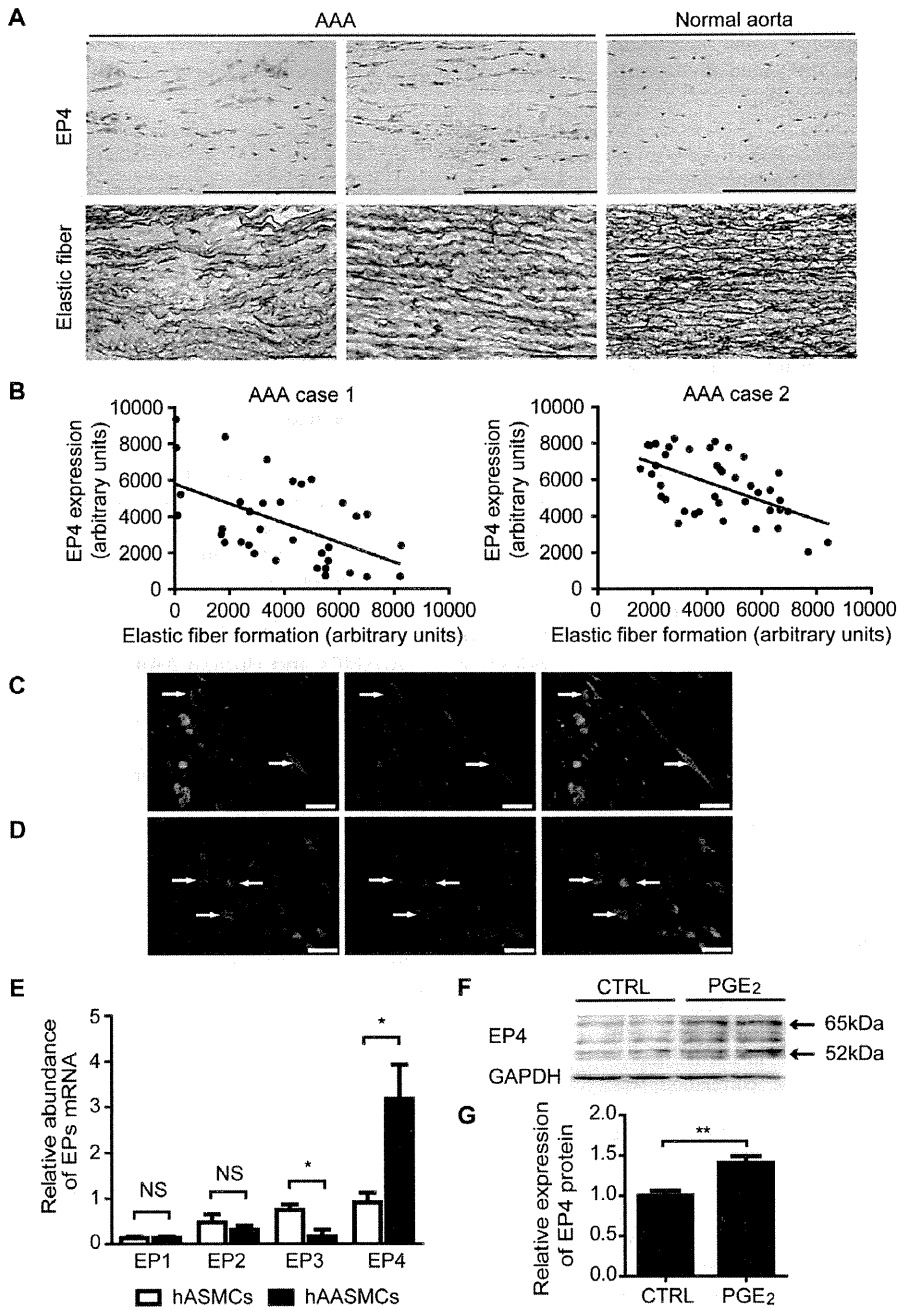

Figure 1. EP4 expression is increased in human AAA tissue. A, Immunohistochemistry for EP4 in human AAA tissues and aortic tissue from individuals who died of unrelated causes (upper panels). Brown areas indicate expression of EP4. Elastica van Gieson-stained aortic tissues (lower panels). Scale bars: 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 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.

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.
*, $P<0.05$;
**, $P<0.01$;
***, $P<0.001$.
doi:10.1371/journal.pone.0036724.t001

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 $CaCl_2$ 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 $CaCl_2$ 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

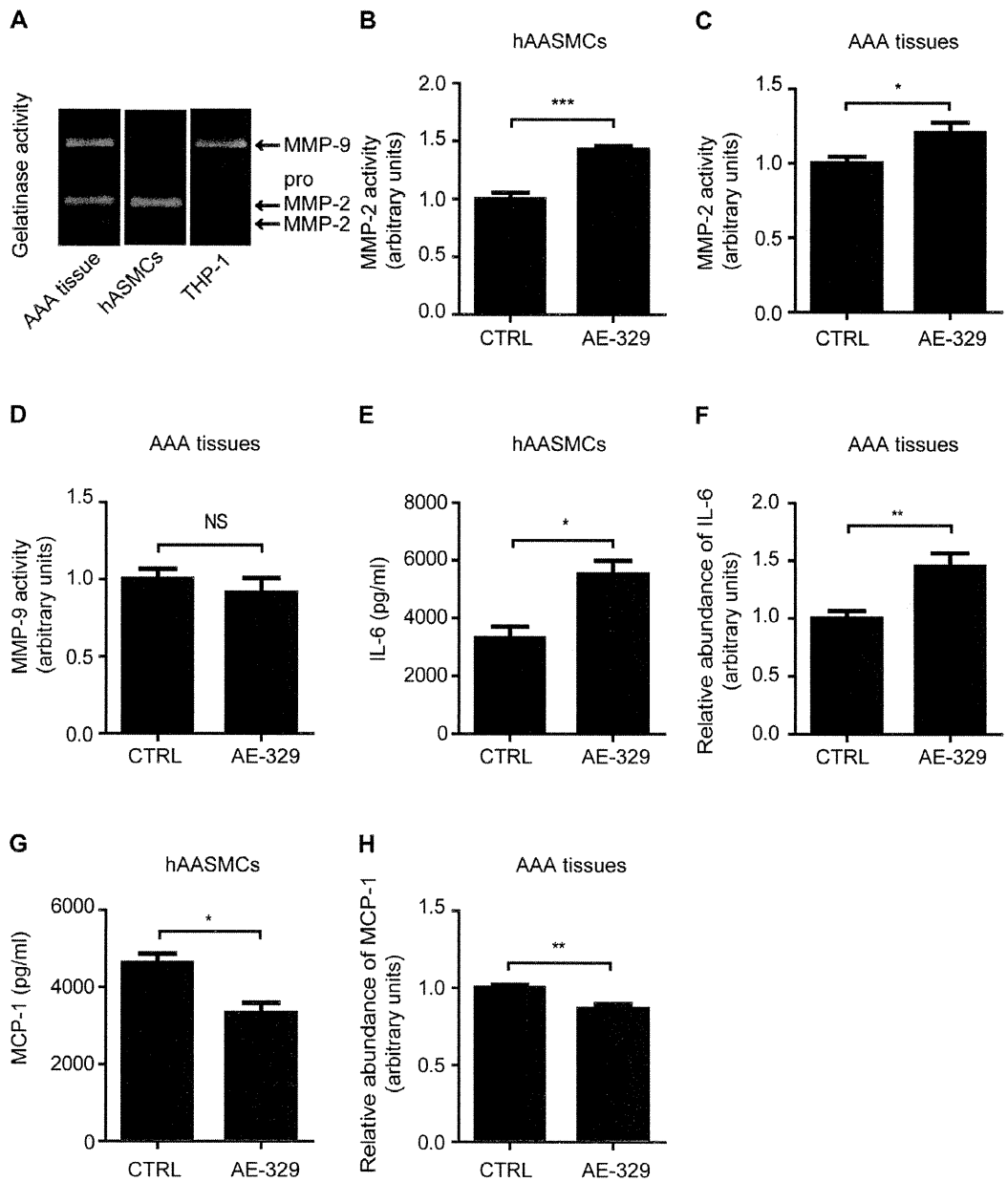

Figure 2. EP4 signaling increased MMP-2 activation and IL-6 production in hAASMCs and human AAA tissues. A, Representative images of gelatin zymography of human AAA tissue, hASMCs, and THP-1 treated with 100 nM of PMA. B, E and G, MMP-2 activation, IL-6, and MCP-1 production in supernatant of hAASMCs 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

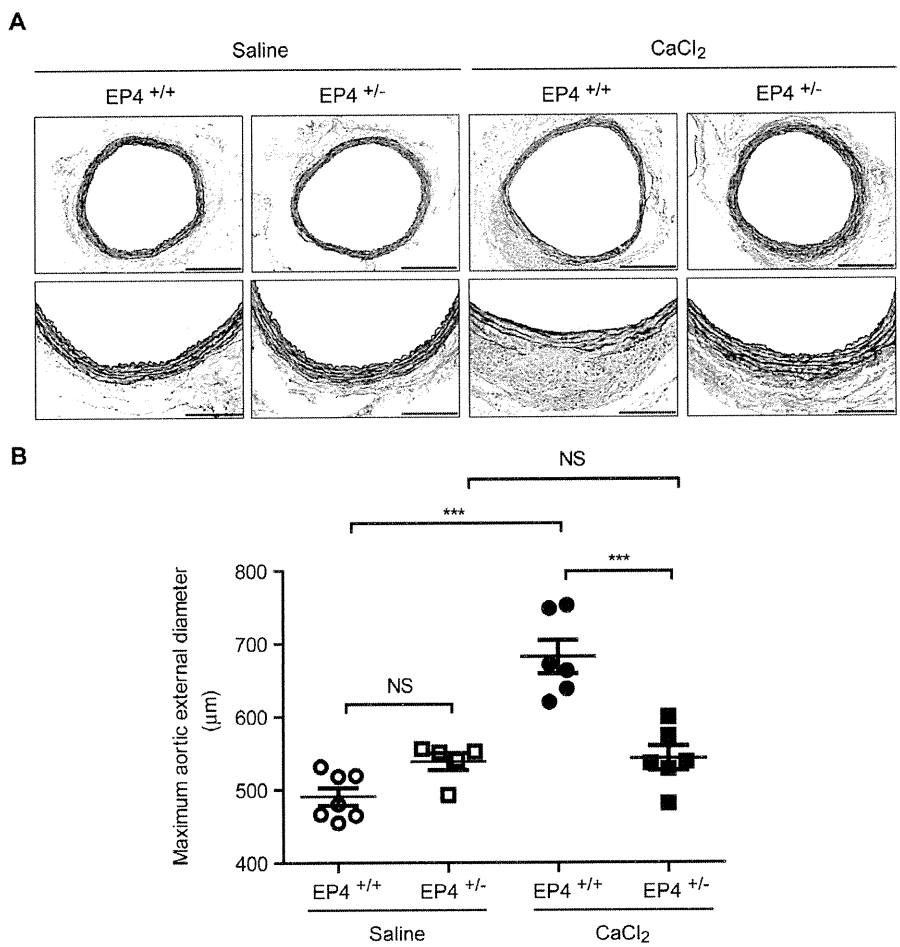

Figure 3. $CaCl_2$-induced AAA formation is attenuated in EP4$^{+/-}$ mice. A, Representative images of elastica van Gieson-stained tissue of EP4$^{+/-}$ and EP4$^{+/+}$ mice treated with saline or $CaCl_2$. Lower panels (Scale bars: 100 μm) show higher magnification portions of upper panel images (Scale 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.

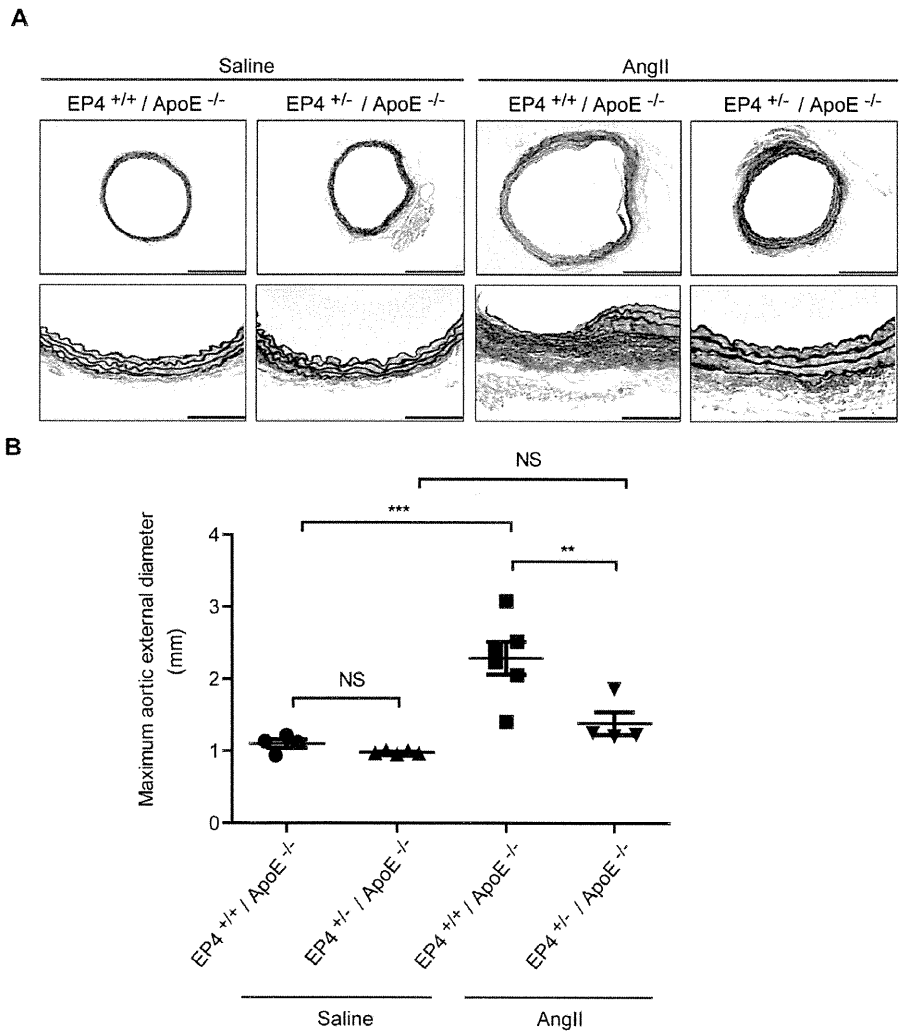

Figure 4. AngII-induced AAA formation is attenuated in $EP4^{+/-}/ApoE^{-/-}$ mice. A, Representative images of elastica van Gieson-stained tissue of $EP4^{+/-}/ApoE^{-/-}$ and $EP4^{+/+}/ApoE^{-/-}$ mice treated with saline or AngII. Lower panels (Scale bars: 100 μm) show higher magnification portions 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

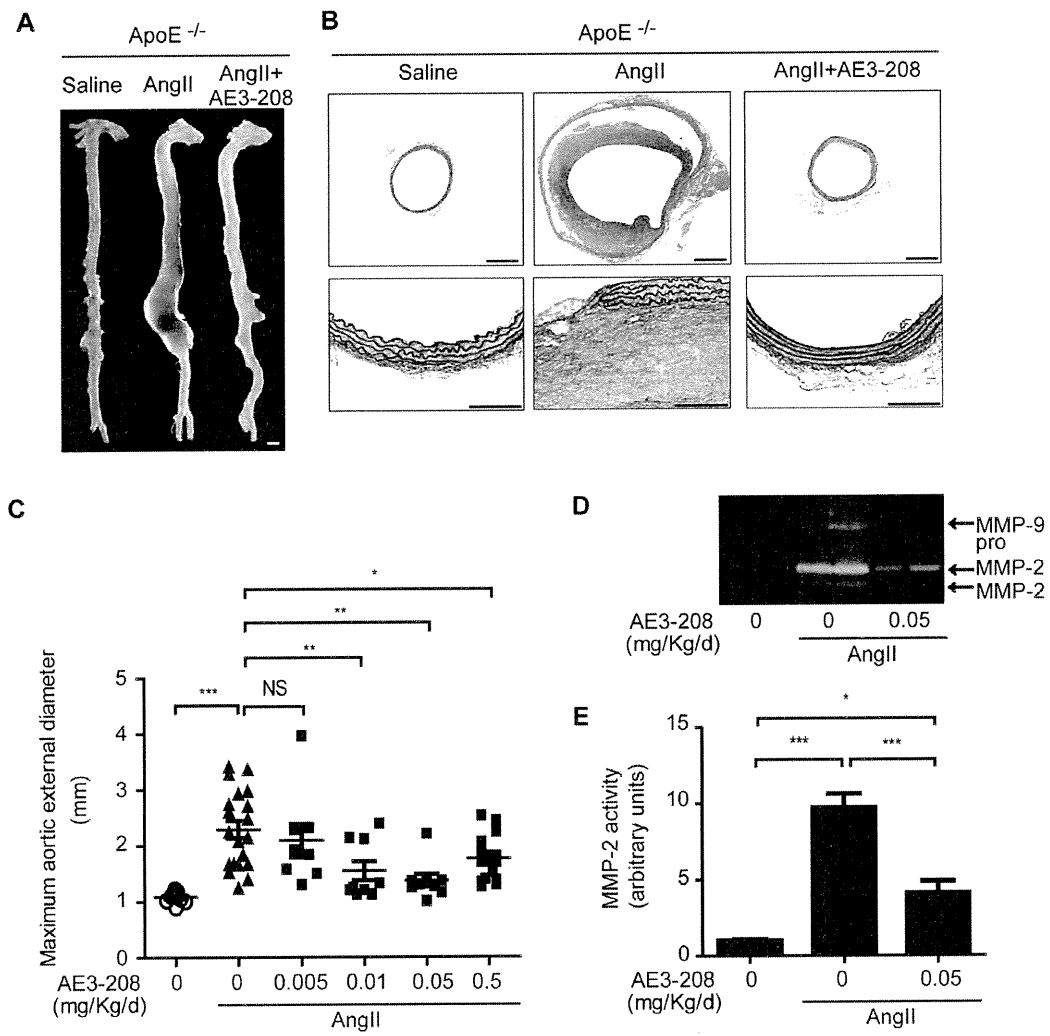

Figure 5. EP4 antagonist attenuated AngII-induced AAA formation in ApoE$^{-/-}$ mice. A, Representative image of aorta of ApoE$^{-/-}$ mice treated with saline, AngII, or AngII+ONO-AE3-208 (AE3–208) (0.05 mg/Kg/d). Scale bar: 1 mm. B, Elastica van Gieson–stained tissue of aortas shown in A. Lower panels (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

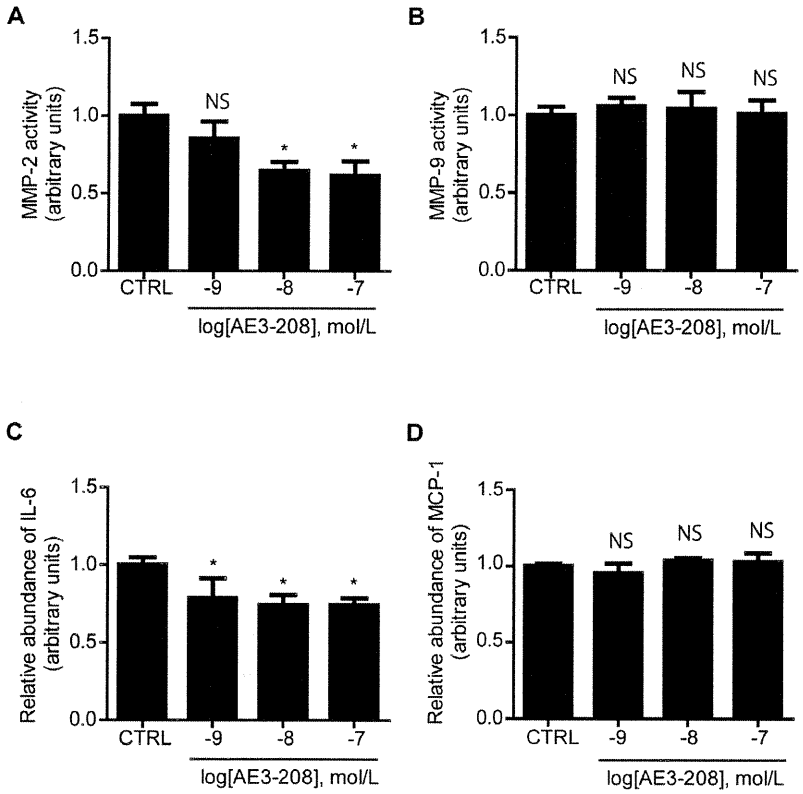

Figure 6. EP4 antagonist attenuated MMP-2 activation and IL-6 production in human AAA tissues. A, MMP-2 activity, B, MMP-9 activity, C, IL-6 production, and D, MCP-1 production. Supernatants of human AAA tissue organ cultures incubated in the presence or absence of and increasing concentrations of ONO-AE3-208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

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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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Circulation Journal

Inhibition of Phosphodiesterase Type 3 Dilates the Rat Ductus Arteriosus Without Inducing Intimal Thickening

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Keywords:	Ductus arteriosus, Milrinone, Phosphodiesterase, congenital heart disease
Category:	Congenital heart desease/Child cardiovascular disease

Inhibition of Phosphodiesterase Type 3 Dilates the Rat Ductus Arteriosus Without Inducing Intimal Thickening

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Short title: PDE3 Inhibitors Dilate the DA Without Remodeling.

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Abstract

Background: Prostaglandin E_1 (PGE_1), via cAMP, dilates the ductus arteriosus (DA). For patients with DA-dependent congenital heart diseases (CHDs), PGE_1 is the sole DA dilator that is used until surgery. However, PGE_1 has a short duration of action and frequently induces apnea. Most importantly, PGE_1 increases hyaluronan (HA) production, leading to intimal thickening (IT) and eventually DA stenosis after long-term use. In this study, therefore, we explored potential DA dilators, such as phophodiesterase 3 (PDE3) inhibitors, as alternatives to PGE_1.

Methods and Results: Expression levels of PDE3a and PDE3b mRNAs in rat DA tissues were higher than those in the PA. Intraperitoneal injection of milrinone (10 or 1 mg/kg) or olprinone (5 or 0.5 mg/kg) induced maximal dilatation of the DA lasting for up to 2 hours in rat neonates. In contrast, vasodilation induced by PGE_1 (10 μg/kg) was diminished within 2 hours. No respiratory distress was observed with milrinone or olprinone. Most important, milrinone did not induce HA production, cell migration, or proliferation when applied to cultured rat DA smooth muscle cells. Further, high expression levels of both PDE3a and PDE3b were demonstrated in the human DA tissues of CHD patients.

Conclusions: Because PDE3 inhibitors induced longer-lasting vasodilation without causing apnea or HA-mediated IT, they may be good alternatives to PGE_1 for patients with DA-dependent CHDs.

Word count: 217 words (abstract)

Key words: Ductus arteriosus, Milrinone, Phosphodiesterase, Congenital heart disease

Introduction

The ductus arteriosus (DA), the fetal arterial connection between the pulmonary artery and the descending aorta, is essential to maintain fetal life in utero. The DA closes after birth by two different mechanisms, namely, vasoconstriction and intimal thickening (IT) [1-3]. During the first few hours after birth, acute vasoconstriction occurs as a result of smooth muscle contraction in the DA. This is triggered by increased oxygen tension, due to the initiation of spontaneous breathing, and decreased circulating prostaglandin E_2 (PGE_2), due to disconnection from the placenta [3]. This functional vasoconstriction, however, must be preceded by intimal thickening of the DA, because vascular remodeling, including intimal thickening, is critical for anatomical closure of the DA.

The intimal thickening of DA is a result of many cellular processes, such as an increase in smooth muscle cell (SMC) migration and proliferation, the production of hyaluronan (HA) under the endothelial layer, and decreased elastin fiber assembly [1, 3, 4]. We have previously demonstrated that PGEs promoted HA production via cAMP/protein kinase A and subsequent SMC migration, resulting in intimal thickening of the DA during the late gestational period [1, 4, 5].

In patients with DA-dependent congenital heart diseases (CHDs), such as pulmonary atresia with intact ventricular septum or arch anomalies (coarctation of aorta or interruption of aortic arch), however, patent DA after birth is essential for survival. PGE_1 is widely used to keep the DA open as it increases intracellular cAMP and thus dilates the DA. However, PGE_1 induces hyaluronan (HA)-mediated intimal thickening and thus DA stenosis after prolonged use [6]. The fact that it induces only a very short duration of vasodilation, together with its severe adverse effects, such as apnea, respiratory distress, and hypotension, present additional problems, making it difficult for some patients with CHD to continue the use of PGE_1 until surgery. As such, possible alternatives to PGE_1 need to be explored.

Phosphodiesterases (PDEs), which catalyze the hydrolysis of cAMP/cGMP, constitute a superfamily of at least 11 gene families (PDE1–PDE11) [7]. The two PDE3 subfamilies, PDE3A and PDE3B, are encoded by closely related genes [8], and both hydrolyze cAMP. PDE3 inhibitors have been approved by the U.S. Food and Drug Administration (FDA) for use as vasodilators as well as in heart failure. Two of these are milrinone and olprinone, which are widely used to treat heart failure [9-12] and persistent pulmonary hypertension in neonates [13, 14]. Previous studies have shown that the PDE3 inhibitors milrinone, amrinone, and cilostazol counteract indomethacin-induced DA contraction [15, 16]. Thus, PDE3 inhibitors alone may be sufficient to dilate the DA. Nevertheless, it remains undetermined whether they induce intimal thickening, which is a major problem with PGE_1, via HA production, cell migration, or cell proliferation. In the current study, we investigated the role of PDE3 inhibitors in DA vascular remodeling and vasodilation with a view to their potential use as alternatives to the current PGE therapy.

Materials and Methods

Animals and materials

Timed pregnant Wistar rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All animal studies were approved by the institutional animal care and use committees of Yokohama City University. Milrinone, PDGF-BB, MTT, trichloroacetic acid, and 10% buffered formalin were obtained from Wako (Osaka, Japan). Olprinone, cilostazol, rolipram, PGE_1, PGE_2, elastase type II, trypsin inhibitor, bovine serum albumin V, poly-L-lysine, penicillin-streptomycin solution, acetic anhydride, triethylamine, Dulbecco's modified Eagle's medium (DMEM), and Hank's balanced salt solution (HBSS) were purchased from Sigma-Aldrich (St Louis, MO). Collagenase II was purchased from Worthington Biochemical Corp. (Lakewood, NJ). Collagenase/dispase