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

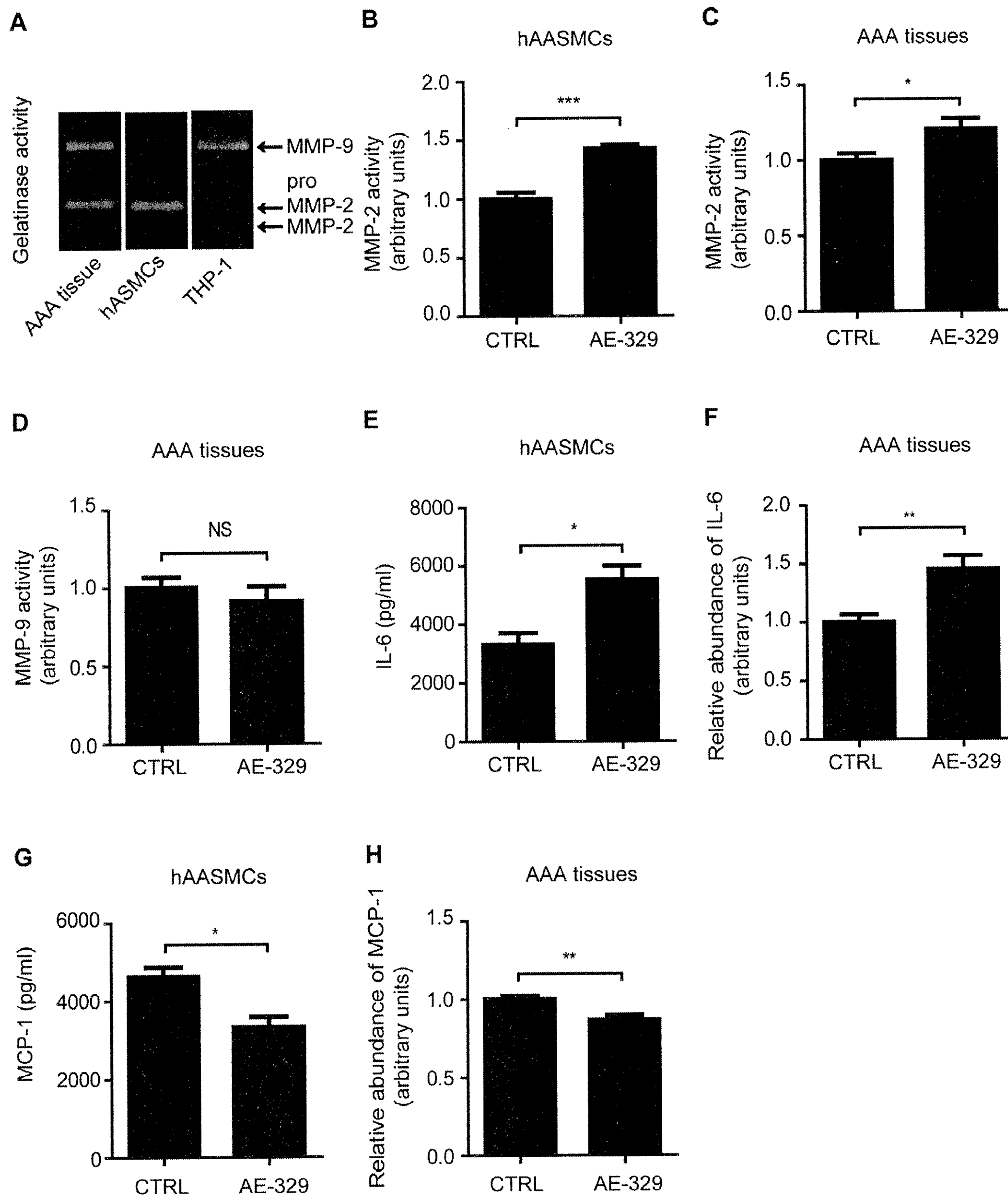


Figure 2. EP4 signaling increased MMP-2 activation and IL-6 production in hAASMCs and human AAA tissues. A, Representative images of gelatin zymography of human AAA tissue, hASMCs, and THP-1 treated with 100 nM of PMA. B, E and G, MMP-2 activation, IL-6, and MCP-1 production in supernatant of hAASMCs 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.
doi:10.1371/journal.pone.0036724.g002

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

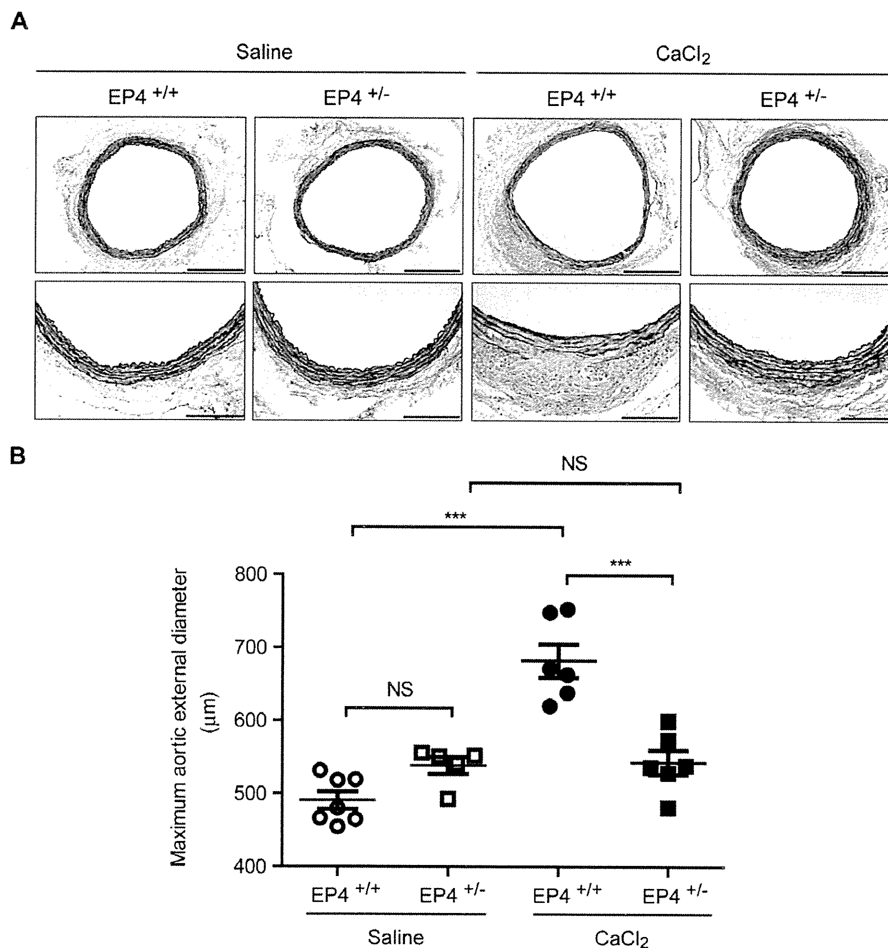
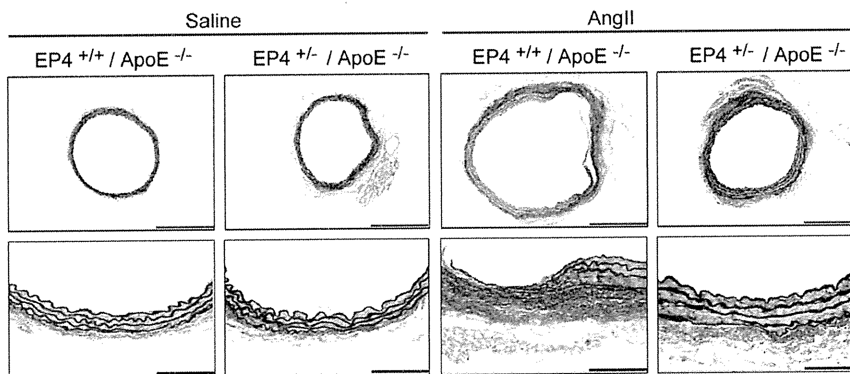


Figure 3. $CaCl_2$-induced AAA formation is attenuated in EP4$^{+/-}$ mice. A, Representative images of elastica van Gieson-stained tissue of EP4$^{+/-}$ and EP4$^{+/+}$ mice treated with saline or $CaCl_2$. Lower panels (Scale bars: 100 μm) show higher magnification portions of upper panel images (Scale 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.
doi:10.1371/journal.pone.0036724.g003

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

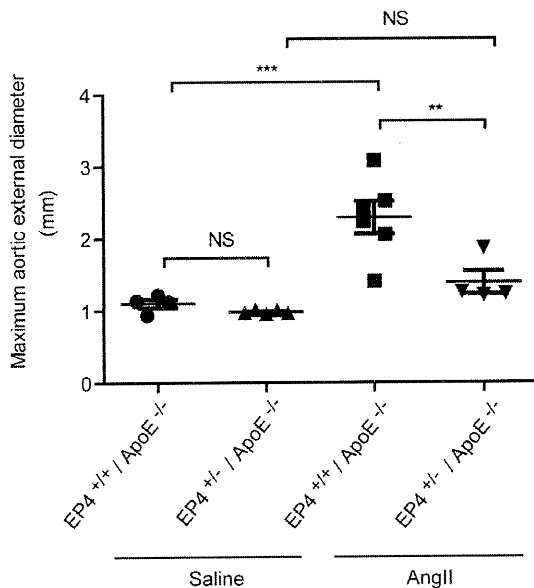


Figure 4. AngII-induced AAA formation is attenuated in $EP4^{+/-}/ApoE^{-/-}$ mice. A, Representative images of elastica van Gieson-stained tissue of $EP4^{+/-}/ApoE^{-/-}$ and $EP4^{+/+}/ApoE^{-/-}$ mice treated with saline or AngII. Lower panels (Scale bars: 100 μm) show higher magnification portions 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.
doi:10.1371/journal.pone.0036724.g004

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

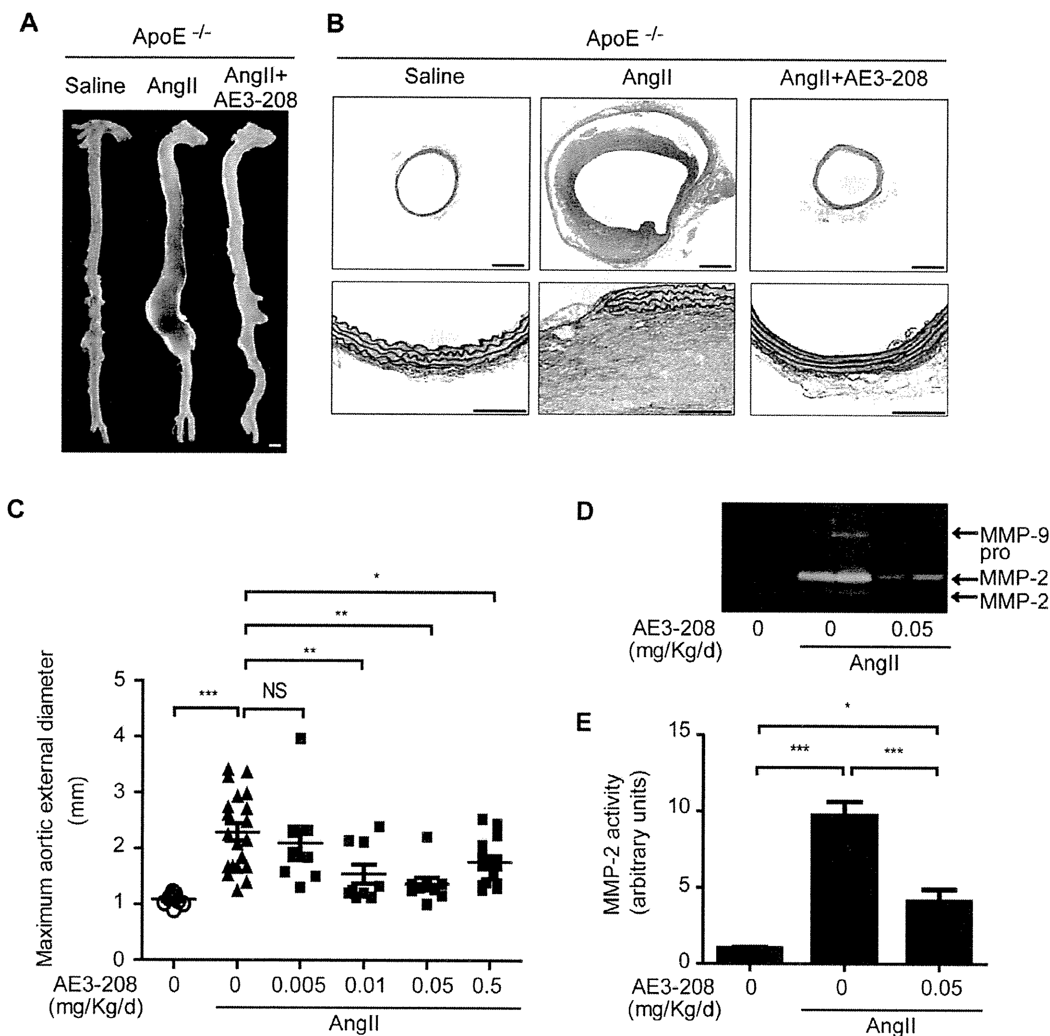


Figure 5. EP4 antagonist attenuated AngII-induced AAA formation in ApoE$^{-/-}$ mice. A, Representative image of aorta of ApoE$^{-/-}$ mice treated with saline, AngII, or AngII+ONO-AE3-208 (AE3–208) (0.05 mg/Kg/d). Scale bar: 1 mm. B, Elastica van Gieson–stained tissue of aortas shown in A. Lower panels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

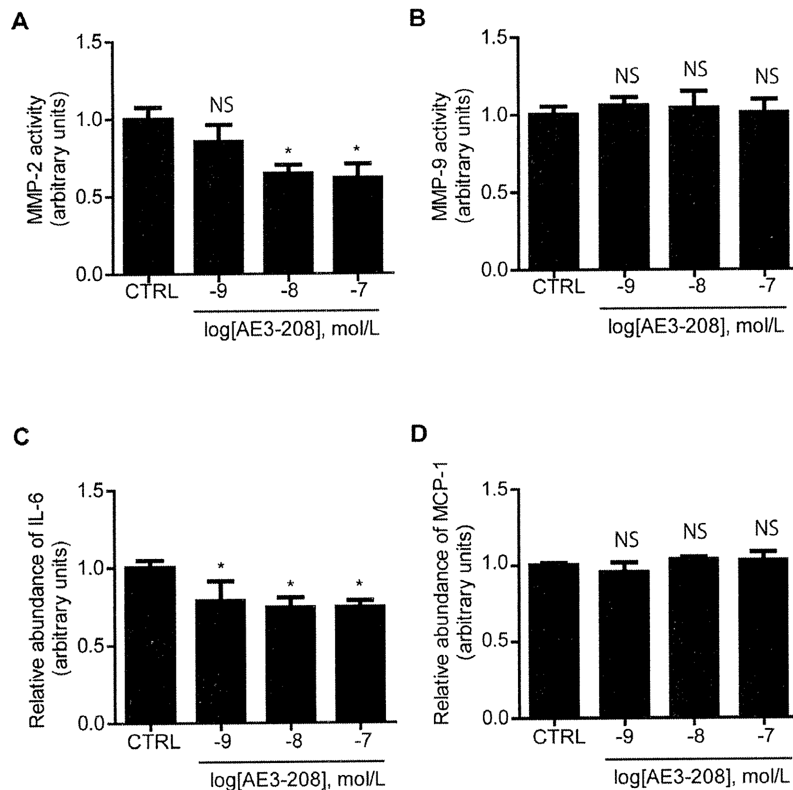


Figure 6. EP4 antagonist attenuated MMP-2 activation and IL-6 production in human AAA tissues. A, MMP-2 activity, B, MMP-9 activity, C, IL-6 production, and D, MCP-1 production. Supernatants of human AAA tissue organ cultures incubated in the presence or absence of and increasing concentrations of ONO-AE3-208 (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.

Acknowledgments

The authors would like to express their gratitude to Professor Shu Narumiya (Department of Pharmacology, Kyoto University Faculty of Medicine) for kindly providing $EP4^{+/-}$ mice used in this study. We thank Yuka Sawada and Shiori Inoue for the histological analysis.

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.

References

1. Annambhotla S, Bourgeois S, Wang X, Lin PH, Yao Q, et al. (2008) Recent advances in molecular mechanisms of abdominal aortic aneurysm formation. World J Surg 32: 976–986.
2. Collin J, Araujo L, Walton J, Lindsell D (1988) Oxford screening programme for abdominal aortic aneurysm in men aged 65 to 74 years. Lancet 2: 613–615.
3. Scott RA, Ashton HA, Kay DN (1991) Abdominal aortic aneurysm in 4237 screened patients: prevalence, development and management over 6 years. Br J Surg 78: 1122–1125.
4. Freestone T, Turner RJ, Coady A, Higman DJ, Greenhalgh RM, et al. (1995) Inflammation and matrix metalloproteinases in the enlarging abdominal aortic aneurysm. Arterioscler Thromb Vasc Biol 15: 1145–1151.
5. Walton LJ, Franklin IJ, Bayston T, Brown LC, Greenhalgh RM, et al. (1999) Inhibition of prostaglandin E2 synthesis in abdominal aortic aneurysms: implications for smooth muscle cell viability, inflammatory processes, and the expansion of abdominal aortic aneurysms. Circulation 100: 48–54.
6. Treska V, Kocova J, Boudova L, Neprasova P, Topolcan O, et al. (2002) Inflammation in the wall of abdominal aortic aneurysm and its role in the symptomatology of aneurysm. Cytokines Cell Mol Ther 7: 91–97.
7. Tieu BC, Lee C, Sun H, Lejeune W, Recinos A, 3rd, et al. (2009) An adventitial IL-6/MCP1 amplification loop accelerates macrophage-mediated vascular inflammation leading to aortic dissection in mice. J Clin Invest 119: 3637–3651.
8. Guo DC, Papke CL, He R, Milewicz DM (2006) Pathogenesis of thoracic and abdominal aortic aneurysms. Ann N Y Acad Sci 1085: 339–352.
9. Longo GM, Xiong W, Greiner TC, Zhao Y, Fiotti N, et al. (2002) Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms. J Clin Invest 110: 625–632.
10. Holmes DR, Wester W, Thompson RW, Reilly JM (1997) Prostaglandin E2 synthesis and cyclooxygenase expression in abdominal aortic aneurysms. J Vasc Surg 25: 810–815.

11. Khan KM, Howe LR, Falcone DJ (2004) Extracellular matrix-induced cyclooxygenase-2 regulates macrophage proteinase expression. J Biol Chem 279: 22039–22046.
12. Corcoran ML, Stetler-Stevenson WG, Brown PD, Wahl LM (1992) Interleukin 4 inhibition of prostaglandin E2 synthesis blocks interstitial collagenase and 92-kDa type IV collagenase/gelatinase production by human monocytes. J Biol Chem 267: 515–519.
13. King VL, Trivedi DB, Gitlin JM, Loftin CD (2006) Selective cyclooxygenase-2 inhibition with celecoxib decreases angiotensin II-induced abdominal aortic aneurysm formation in mice. Arterioscler Thromb Vasc Biol 26: 1137–1143.
14. Gitlin JM, Trivedi DB, Langenbach R, Loftin CD (2007) Genetic deficiency of cyclooxygenase-2 attenuates abdominal aortic aneurysm formation in mice. Cardiovasc Res 73: 227–236.
15. Ray WA, Stein CM, Daugherty JR, Hall K, Arbogast PG, et al. (2002) COX-2 selective non-steroidal anti-inflammatory drugs and risk of serious coronary heart disease. Lancet 360: 1071–1073.
16. McGettigan P, Henry D (2006) Cardiovascular risk and inhibition of cyclooxygenase: a systematic review of the observational studies of selective and nonselective inhibitors of cyclooxygenase 2. JAMA 296: 1633–1644.
17. Yoshimura K, Aoki H, Ikeda Y, Fujii K, Akiyama N, et al. (2005) Regression of abdominal aortic aneurysm by inhibition of c-Jun N-terminal kinase. Nat Med 11: 1330–1338.
18. Segi E, Sugimoto Y, Yamasaki A, Aze Y, Oida H, et al. (1998) Patent ductus arteriosus and neonatal death in prostaglandin receptor EP4-deficient mice. Biochem Biophys Res Commun 246: 7–12.
19. Daugherty A, Manning MW, Cassis LA (2000) Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. J Clin Invest 105: 1605–1612.
20. Yokoyama U, Minamisawa S, Adachi-Akahane S, Akaike T, Naguro I, et al. (2006) Multiple transcripts of Ca2+ channel alpha1-subunits and a novel spliced variant of the alpha1C-subunit in rat ductus arteriosus. Am J Physiol Heart Circ Physiol 290: H1660–1670.
21. Yokoyama U, Minamisawa S, Quan H, Ghatak S, Akaike T, et al. (2006) Chronic activation of the prostaglandin receptor EP4 promotes hyaluronan-mediated neointimal formation in the ductus arteriosus. J Clin Invest 116: 3026–3034.
22. Bayston T, Ramessur S, Reise J, Jones KG, Powell JT (2003) Prostaglandin E2 receptors in abdominal aortic aneurysm and human aortic smooth muscle cells. J Vasc Surg 38: 354–359.
23. Kabashima K, Saji T, Murata T, Nagamachi M, Matsuoka T, et al. (2002) The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut. J Clin Invest 109: 883–893.
24. Wang Y, Ait-Oufella H, Herbin O, Bonnin P, Ramkhelawon B, et al. (2010) TGF-beta activity protects against inflammatory aortic aneurysm progression and complications in angiotensin II-infused mice. J Clin Invest 120: 422–432.
25. Woodward DF, Jones RL, Narumiya S (2011) International Union of Basic and Clinical Pharmacology. LXXXIII: classification of prostanoid receptors, updating 15 years of progress. Pharmacol Rev 63: 471–538.
26. Nataraj C, Thomas DW, Tilley SL, Nguyen MT, Mannon R, et al. (2001) Receptors for prostaglandin E(2) that regulate cellular immune responses in the mouse. J Clin Invest 108: 1229–1235.
27. Steenport M, Khan KM, Du B, Barnhard SE, Dannenberg AJ, et al. (2009) Matrix metalloproteinase (MMP)-1 and MMP-3 induce macrophage MMP-9: evidence for the role of TNF-alpha and cyclooxygenase-2. J Immunol 183: 8119–8127.
28. Pavlovic S, Du B, Sakamoto K, Khan KM, Natarajan C, et al. (2006) Targeting prostaglandin E2 receptors as an alternative strategy to block cyclooxygenase-2-dependent extracellular matrix-induced matrix metalloproteinase-9 expression by macrophages. J Biol Chem 281: 3321–3328.
29. Cipollone F, Fazia ML, Iezzi A, Cuccurullo C, De Cesare D, et al. (2005) Association between prostaglandin E receptor subtype EP4 overexpression and unstable phenotype in atherosclerotic plaques in human. Arterioscler Thromb Vasc Biol 25: 1925–1931.
30. Tang EH, Shvartz E, Shimizu K, Rocha VZ, Zheng C, et al. (2011) Deletion of EP4 on bone marrow-derived cells enhances inflammation and angiotensin II-induced abdominal aortic aneurysm formation. Arterioscler Thromb Vasc Biol 31: 261–269.
31. Takayama K, Garcia-Cardena G, Sukhova GK, Comander J, Gimbrone MA, Jr., et al. (2002) Prostaglandin E2 suppresses chemokine production in human macrophages through the EP4 receptor. J Biol Chem 277: 44147–44154.
32. Hishikari K, Suzuki J, Ogawa M, Isobe K, Takahashi T, et al. (2009) Pharmacological activation of the prostaglandin E2 receptor EP4 improves cardiac function after myocardial ischaemia/reperfusion injury. Cardiovasc Res 81: 123–132.
33. Kossakowska AE, Edwards DR, Prusinkiewicz C, Zhang MC, Guo D, et al. (1999) Interleukin-6 regulation of matrix metalloproteinase (MMP-2 and MMP-9) and tissue inhibitor of metalloproteinase (TIMP-1) expression in malignant non-Hodgkin's lymphomas. Blood 94: 2080–2089.

Circulation Journal

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

Journal:	*Circulation Journal*
Manuscript ID:	CJ-12-0215.R2
mstype:	Experimental Investigation
Date Submitted by the Author:	n/a

Complete List of Authors:	Ichikawa, Yasuhiro; Yokohama City University, Cardiovascular Research Institute Yokoyama, Utako; Yokohama City University, Cardiovascular Research Institute Iwamoto, Mari; Yokohama City University, Department of Pediatrics Oshikawa, Jin; Yokohama City University, Department of Medical Science and Cardiorenal Medicine Okumura, Satoshi; Yokohama City University, Cardiovascular Research Institute Sato, Motohiko; Yokohama City University, Cardiovascular Research Institute Yokota, Syumpei; Yokohama City University, Department of Pediatrics Masuda, Munetaka; Yokohama City University, Department of Surgery Asou, Toshihide; Kanagawa Children's Medical Center, Department of Cardiovascular Surgery Ishikawa, Yoshihiro; Yokohama City University, Cardiovascular Research Institute

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

SCHOLARONE™
Manuscripts

Japanese Circulation Society

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

Yasuhiro Ichikawa MD [1,2], Utako Yokoyama MD, PhD [1], Mari Iwamoto MD, PhD [2], Jin Oshikawa MD, PhD [3], Satoshi Okumura MD, PhD [1], Motohiko Sato MD, PhD [1], Shumpei Yokota MD, PhD [2], Munetaka Masuda MD, PhD [4], Toshihide Asou MD, PhD [5], Yoshihiro Ishikawa MD, PhD [1]

1) Cardiovascular Research Institute, Yokohama City University
2) Department of Pediatrics, Yokohama City University
3) Department of Medical Science and Cardiorenal Medicine, Yokohama City University
4) Department of Surgery, Yokohama City University
5) Department of Cardiovascular Surgery, Kanagawa Children's Medical Center

For Review Only

Short title: PDE3 Inhibitors Dilate the DA Without Remodeling.

Grants: This work was supported by grants from the Ministry of Health, Labor, and Welfare of Japan, a Grant-in-Aid for Scientific Research on Innovative Areas (22136009 to Y. Ishikawa and 23116541 to U. Yokoyama), the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Y. Ichikawa, U. Yokoyama, M. Masuda and Y. Ishikawa), the Kitsuen Kagaku Research Foundation (Y. Ishikawa), the Yokohama Foundation for Advanced Medical Science (Y. Ichikawa and U. Yokoyama), the Takeda Science Foundation (U. Yokoyama and Y. Ishikawa), the Miyata Cardiac Research Promotion Foundation (U. Yokoyama), and the Mochida Memorial Foundation for Medical and Pharmaceutical Research (U. Yokoyama).

Correspondence to: Utako Yokoyama or Yoshihiro Ishikawa, Cardiovascular Research Institute, Yokohama City University, 3-9 Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236-0004, Japan
Fax:+81-45-787-1470
Telephone:+81-45-787-2575
Email: utako@yokohama-cu.ac.jp or yishikaw@med.yokohama-cu.ac.jp

Total word count: 5812 words (7 figures and 1 table)

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

For Review Only

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.

For Review Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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.

For Review Only

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

was purchased from Roche Diagnostics (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Equitech-Bio (Kerrville, TX).

Primary culture of rat smooth muscle cells (SMCs)

Vascular SMCs in primary culture were obtained from the DA (DASMCs), the aorta (ASMCs), and the pulmonary arteries (PASMCs) of Wistar rats on the 21st day of gestation. Isolation of DASMCs and ASMCs has been described previously [17]. To obtain PASMCs, the branch extralobular pulmonary arteries were dissected, cleaned from adherent tissue, and cut into small pieces. The tissues were transferred to a 1.5-ml centrifuge tube that contained 800 μl of collagenase-dispase enzyme mixture (1.5 mg/ml collagenase-dispase, 0.5 mg/ml of elastase type II-A, 1 mg/ml of trypsin inhibitor type I-S, and 2 mg/ml of bovine serum albumin fraction V in HBSS). Digestion was carried out at 37°C for 15 min. Cell suspensions were then centrifuged, and the medium was changed to a collagenase II enzyme mixture (1 mg/ml collagenase II, 0.3 mg/ml trypsin inhibitor type I-S, and 2 mg/ml bovine serum albumin fraction V in HBSS). After 12 min of incubation at 37°C, cell suspensions were transferred to growth medium in 35-mm poly-L-lysine-coated dishes in a moist tissue culture incubator at 37°C in 5% CO_2-95% ambient mixed air. The growth medium contained DMEM with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. We confirmed that >99% of cells were positive for α-smooth muscle actin and exhibited typical "hill-and-valley" morphology. Expression levels of PDE3, EP4, and prostacyclin (IP) receptor mRNAs in DASMCs, ASMCs, and PASMCs are shown in the Supplemental Fig. S1.

Human tissues from patients with CHDs

We obtained eight neonatal DAs and adjacent aortas during cardiac surgery in children between 0 days and 1 month of age. All excised tissue was fixed in 4% paraformaldehyde within 3 hours. The DA tissues were obtained from the Yokohama City University Hospital and Kanagawa Children's Medical Center. The study was

For Review Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

approved by the human subject committees at both Yokohama City University and Kanagawa Children's Medical Center. Detailed patient information is summarized in Table 1.

RNA isolation and quantitative RT-PCR

Pooled vascular tissues were obtained from Wistar rats on the 21st day of gestation. After excision, tissues were frozen in liquid nitrogen and stored at -80°C. The total RNA was isolated from the tissues using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and from the cultures using Trizol reagent (Invitrogen, Carlsbad, CA). The primers were designed based on the rat nucleotide sequences of PDE3a (NM_017337) (5'- CGC CTG AGA AGA AGT TTG C -3' and 5'- AGA CAG CAT AGG ACG AAG TGA AG -3'), PDE3b (NM_017229.1) (5'- TCC AAA GCA GAG GTC ATC ATC -3' and 5'- GTA TCA AGA AAT CCT ACG GGT GA -3'), EP4 (NR_032076.3) (5'- CTC GTG GTG CGA GTG TTC AT -3' and 5'- AAG CAA TTC TGA TGG CCT GC -3'), and IP (NM_00177644.1) (5'- GGG CAC GAG AGG ATG AAG -3' and 5'- GGG CAC ACA GAC AAC ACA AC -3'). Reverse transcription polymerase chain reaction (PCR) was performed using a PrimeScript RT reagent Kit (TaKaRa Bio, Tokyo, Japan) and real-time PCR was performed using SYBR Green (Applied Biosystems, Foster City, CA). The abundance of each gene was determined relative to that in 18S ribosomal RNA.

For Review Only

Rapid whole-body freezing method

To study the in situ morphology and inner diameter of the neonatal DA, a rapid whole-body freezing method was used as previously described [2]. Fetuses on the 21st day of gestation were delivered by cesarean section and intraperitoneally injected immediately after birth with milrinone (10 mg/kg, 1 mg/kg, 0.1 mg/kg), olprinone (5 mg/kg, 0.5 mg/kg, 0.05 mg/kg), or PGE_1 (10 μg/kg). The rat pups were frozen in liquid nitrogen at 0, 0.5, 1, 2, 4, 6, 8, and 12 hours after injection. The frozen thoraxes were

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

then cut on a microtome, and the inner diameter of each DA was measured.

Determination of respiratory rate

Fetuses on the 21st day of gestation were delivered by cesarean section and intraperitoneally injected 0 or 2 hours after birth with milrinone (10 mg/kg, 1 mg/kg), olprinone (5 mg/kg, 0.5 mg/kg), or PGE_1 (10 μg/kg). We measured the respiratory rate by counting the movements of the rat thorax.

Quantitation of hyaluronan (HA)

The amount of HA in the cell culture supernatant was measured according to the latex agglutination method as previously described [1].

SMC migration assay

The migration assay was performed using 24-well transwell culture inserts with polycarbonate membranes (8-μm pores) (Corning Inc., Corning, NY) as previously described [1]. Cells were stimulated with milrinone (10 μM), PGE1 (1 μM), PDEF-BB (10 ng/ml), HA (200 ng/ml), or milrinone+HA for 3 days.

Cell proliferation assay

SMCs were cultured on 24-well plates at 1×10^5 cells per well in DMEM supplemented with 10% FBS. After various treatments over 3 days, 500 μl of 1 mg/ml MTT solution was added to each well and incubated for 2 hours. The supernatants were aspirated, and the formazan crystals in each well were solubilized with 0.05 M HCl (500 μl). Each solution (100 μl) was placed in a 96-well plate. SMC proliferation was measured based on absorbance at 570 nm using a microplate reader.

Immunohistochemistry

Immunohistochemical analysis was performed as previously described [1, 18]. Rabbit polyclonal anti-PDE3A antibody (sc-20792) and goat polyclonal anti-PDE3b antibody (sc-11835) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A color extraction method using BIOREVO bz-9000 (KEYENCE, Osaka, Japan) was performed

For Review Only

to quantify the expression of PDE3s in the DAs and the aortas of case 1, 4, 5, and 8 (Table 1). Eighteen fields in the smooth muscle layer of the DA and the aorta respectively were examined in four cases. Diaminobenzidine (DAB)-stained colors, PDE3a-positive or PDE3b-positive areas, were extracted and counted on the screen.

Cyclic AMP Production Measured by Radioimmunoassay

Measurement of cAMP accumulation in DASMCs was performed as previously described [2, 19]. Briefly, DASMCs grown on 24-well plates were serum-starved for 24 h and assayed for cAMP production after a 10- or 20-min period of incubation with 10 μM of milrinone. Reactions were terminated by aspiration of the media and the addition of 300 μl of ice-cold trichloroacetic acid (7.5%) to each well. Forty microliters of each sample were acetylated and incubated with ^{125}I-cAMP (Perkin Elmer, Waltham, MA) and 50 μl of rabbit anti-cAMP antibody (diluted 1:3000, Millipore, Billerica, MA) overnight at 4°C. Each mixture was then incubated with 50 μl of goat anti-rabbit antibody with magnetic beads (Qiagen, Valencia, CA) for 1 h. Separation of bound antibodies from free antibodies was achieved by filtration, and bound radioactivity was counted. Production of cAMP was normalized to the amount of protein per sample.

For Review Only

Statistics

Data are presented as means ± standard error of the mean (SEM) of independent experiments. Statistical analysis was performed between two groups by unpaired two-tailed Student's *t* test or unpaired *t* test with Welch's correction, and among multiple groups by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A *p* value of <0.05 was considered significant.

Results

Messenger RNA of PDE3 isoforms was highly expressed in rat DA

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

We first examined whether the target molecule of PDE3 inhibitors is highly expressed in the DA. We measured the mRNA expression levels of PDE3s using quantitative RT-PCR in the rat DA, aorta, and pulmonary arteries (PA) on the 21st day of gestation (Figure 1). Expression of PDE3a mRNA was higher in the DA than in the PA. Expression of PDE3b mRNA was higher in the DA than in the aorta or the PA. We also confirmed that EP4 mRNA was more highly expressed in the DA than in the aorta or the PA. Thus, PDE3 isoforms were abundantly expressed in the DA relative to the PA.

Vasodilatory effects of PDE3 inhibitors on rat DA in vivo

PDE3 inhibitors are widely used in neonates and children with low cardiac output following myocarditis and cardiovascular surgery for congenital heart disease [20, 21]. We examined whether milrinone or olprinone dilated the DA using the rapid whole-body freezing method in rat neonates. Neonates were injected with one of these drugs immediately after birth to mimic the vasodilatory treatment currently used in DA-dependent congenital heart diseases.

Intraperitoneal injection of PGE_1 (10 μg/kg, the amount that is intravenously administered daily as a clinical maintenance dose) induced maximal dilatation of the DA for 30 min, but this effect was completely lost within 2 h after injection (Figure 2A). A single intraperitoneal administration of 10 mg/kg of milrinone maintained maximal dilation of the DA for up to 12 h (Figures 2B, 2C). 1 mg/kg of milrinone, the amount that is intravenously administered daily as a clinical maintenance dose, maintained maximal dilatation for 2 h, after which DA closure occurred at 4 h after injection. 0.1 mg/kg of milrinone did not affect DA tone. Both 5 mg/kg and 0.5 mg/kg of olprinone, the latter of which is suitable for daily intravenous administration as a clinical maintenance dose, induced maximal dilatation for 1 h after injection (Figures 2D, 2E). 0.05 mg/kg of olprinone did not dilate the DA. Thus, both milrinone and olprinone produced dose-dependent vasodilatory effects (Figure 2F), but those of milrinone lasted

For Review Only

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

longer.

PDE3 inhibitors did not induce respiratory distress

Since respiratory distress is a major adverse effect of PGE_1 [22], we examined whether PDE3 inhibitors cause respiratory distress. We counted the respiratory rate of rat neonates administered milrione, olprinone, or PGE_1. When rat neonates were administered each drug immediately after birth, PGE_1 significantly reduced the respiratory rate at 15 or 30 minutes after injection, whereas milrinone (1 and 10 mg/kg) and olprinone (0.5 and 5 mg/kg) did not induce respiratory distress up to 8 h after injection compared to the saline control (Figure 3A). To exclude the possibility that neonates administrated PGE_1 had a congenital respiratory problem, we examined the effect of drugs using a different injection timing. We confirmed that all rat neonates established normal breathing 1 h after birth, and then administrated each drug. PGE_1 significantly reduced the respiratory rate up to 1 h after injection. On the other hand, milrinone (10 mg/kg) and olprinone (5 mg/kg) did not affect the respiratory rate compared to the control (Figure 3B). These data suggest that PDE3 inhibitors did not cause respiratory distress.

Milrinone did not promote HA production or SMC migration and proliferation

Although it was previously suggested that PDE3 inhibitors induced vasodilation of the DA, it remained unknown whether they also induced IT formation, a key process in the anatomical closure of the DA. It is known that PGEs stimulate HA production along with increased DASMC migration through the action of HA as a potent trigger of cell migration. This is the major mechanism underlying the increase in intimal thickening induced by PGEs [1, 2, 5].

We thus examined whether a PDE3 inhibitor, milrinone, regulated HA production or SMC migration. First, we confirmed cAMP production in the presence of milrinone. Milrinone significantly increased cAMP accumulation in DASMCs at a

For Review Only

1
2
3
4
5 dosage of 10 μM, which also induced marked dilatation of DA explants [16] (Figure 4A).
6
7 However, the same dosage of milrinone (10 μM) did not induce HA production in
8
9 DASMCs (Figure 4B). We also confirmed that the PDE3 inhibitor cilostazol did not
10
11 induce HA production in DASMCs. Similarly, PGE_1 (1 μM) induced DASMC migration;
12
13 however, milrinone did not increase DASMC migration, as determined by the Boyden
14
15 chamber method (Figure 5A). The cells used for these tests were sufficiently stimulated
16
17 with PGE_1 to induce HA production and with PDGF-BB to induce migration. Next, we
18
19 examined the effects of a PDE3 inhibitor on SMC proliferation, because SMC
20
22 proliferation plays a role in IT formation of the DA [23,24]. Milrinone and PGE_1 did not
23
24 increase DASMC proliferation, as determined by MTT assays, in the presence of 0 or
25
26 10% FBS (Figure 5B). Moreover, we found that milrinone did not enhance HA-mediated
27
28 migration in DASMCs (Figure 6A). Milrinone also did not affect proliferation in
29
30 HA-treated DASMCs (Figure 6B). Similarly, in ASMCs and PASMCs, neither milrinone
31
32 nor PGE_1 increased HA production or cell migration and proliferation (Figures 4B, 5A
33
34 and 5B). These findings suggest that PDE3 inhibitors do not promote HA production or
35
36 cell migration or proliferation, although they do produce cAMP and dilate the DA.
37
38 ***PDE3a and PDE3b were highly expressed in the smooth muscle layer in human DA***
39
40 ***tissues***
41
43 The expression pattern of PDE3s in the human DA remains unknown. We examined
44
45 PDE3a and PDE3b protein expression in the DA of eight patients with various CHDs,
46
47 such as interruption of the aortic arch, complex aortic coarctation, hypoplastic left
48
49 ventricle, and asplenia. The DA of all patients showed a strong immunoreaction for both
50
51 PDE3a and PDE3b. Representative images are shown in Figure 7A. It has been
52
53 demonstrated that PDE3a and PDE3b are abundantly expressed in the rat and human
54
55 aorta [25,26]. The expression of PDE3a and PDE3b in the DAs was equivalent to that in
56
57 the adjacent aortas (Figure 7B). This demonstrates that PDE3s are abundantly
58
59
60

For Review Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

expressed in human patients with CHDs of the type that may require long-term vasodilatotherapy prior to surgery.

Discussion

The present study has demonstrated that the PDE3 inhibitors milrinone and olprinone dilate the DA without causing apnea and have a longer duration of action than PGE_1. These findings are expected to apply to human patients, given that PDE3s are abundantly expressed in the DA tissue of infants with CHD. Importantly, this study has shown for the first time that these PDE3 inhibitors do not promote HA production, cell migration, and cell proliferation in the DASMC, processes which potently induce intimal thickening and thus DA closure [1]. The PDE3 inhibitors are very unlikely to produce these unfavorable effects when used as DA dilators. Furthermore, these PDE3 inhibitors are already used in humans for other purposes [9, 10, 13, 14]. Accordingly, they may serve as useful alternatives to PGE_1, the current means of keeping the DA patent.

PGE_1 increases the production of cAMP by activating G protein and adenylyl cyclase [1, 2, 27]. In contrast, milrinone increases the concentration of cAMP by inhibiting its breakdown [7]. Although both drugs increase cAMP and dilate the DA, PGE_1 induces HA production and subsequent migration in DASMCs while milrinone does not. We do not know the molecular mechanism underlying this difference between PGE_1 and the PDE inhibitors. It can be tentatively speculated, however, that they differ in terms of intracellular localization and thus in terms of coupling with other molecules, as recent studies have suggested [28]. Regardless of the mechanisms involved, it is known that PGE_1 and PGE_2 both increase cAMP production and induce HA production via increased expression of HA synthase 2 [1, 5], and we found that a PDE4 inhibitor, rolipram,

For Review Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

did not induce HA production (Figure 4B). Alternatively, increases in cGMP, which is also induced by milrinone, may play a role. These issues need to be further investigated in future studies.

Previous studies effectively demonstrated the vasodilatory effects of the PDE3 inhibitors milrinone, amrinone and cilostazol on the rat or sheep DA that were contracted by indomethacin [15, 16]. In contrast, we have evaluated the effects of PDE3 inhibitors in the absence of indomethacin to examine the effects of PDE3 inhibitors in more relevant clinical settings. We also found, for the first time, that olprinone, a relatively new PDE3 inhibitor, dilates the DA. Our finding that these PDE3 inhibitors do not increase HA production is also novel, as this question had not been investigated previously.

The present study shows that milrinone does not induce SMC migration and proliferation in the DA (Figures 5, 6). Our findings are, at least in part, consistent with those obtained using vascular SMCs from non-DA vessels. PDE3 inhibitors have elsewhere been shown to reduce proliferation and migration of vascular SMCs and to decrease the accumulation of synthetic/activated vascular SMCs in the intimal layers of damaged blood vessels [7, 29, 30]. Similarly, in peripheral pulmonary arteries, PDE3 and PDE4 inhibition do not promote PASMC migration [31]. Furthermore, PDE3a deficiency caused G0/G1 cell cycle arrest in PDE3a knockout mice [8].

PGE_1 is currently the sole DA dilator, however, PGE_1-induced apnea or respiratory distress was noted in 18% of patients with congenital heart disease [32]. Respiratory depression was particularly common in infants weighing less than 2.0 kg at birth who received PGE_1 therapy (42%) [22]. The present study showed that milrinone and olprinone did not induce respiratory distress in rat neonates (Figure 3). Furthermore, no patient who caused apnea or respiratory distress with PDE3 inhibitors was reported in the previous clinical reports [9, 10, 13, 14]. Therefore, the PDE3 inhibitors are very

For Review Only