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-

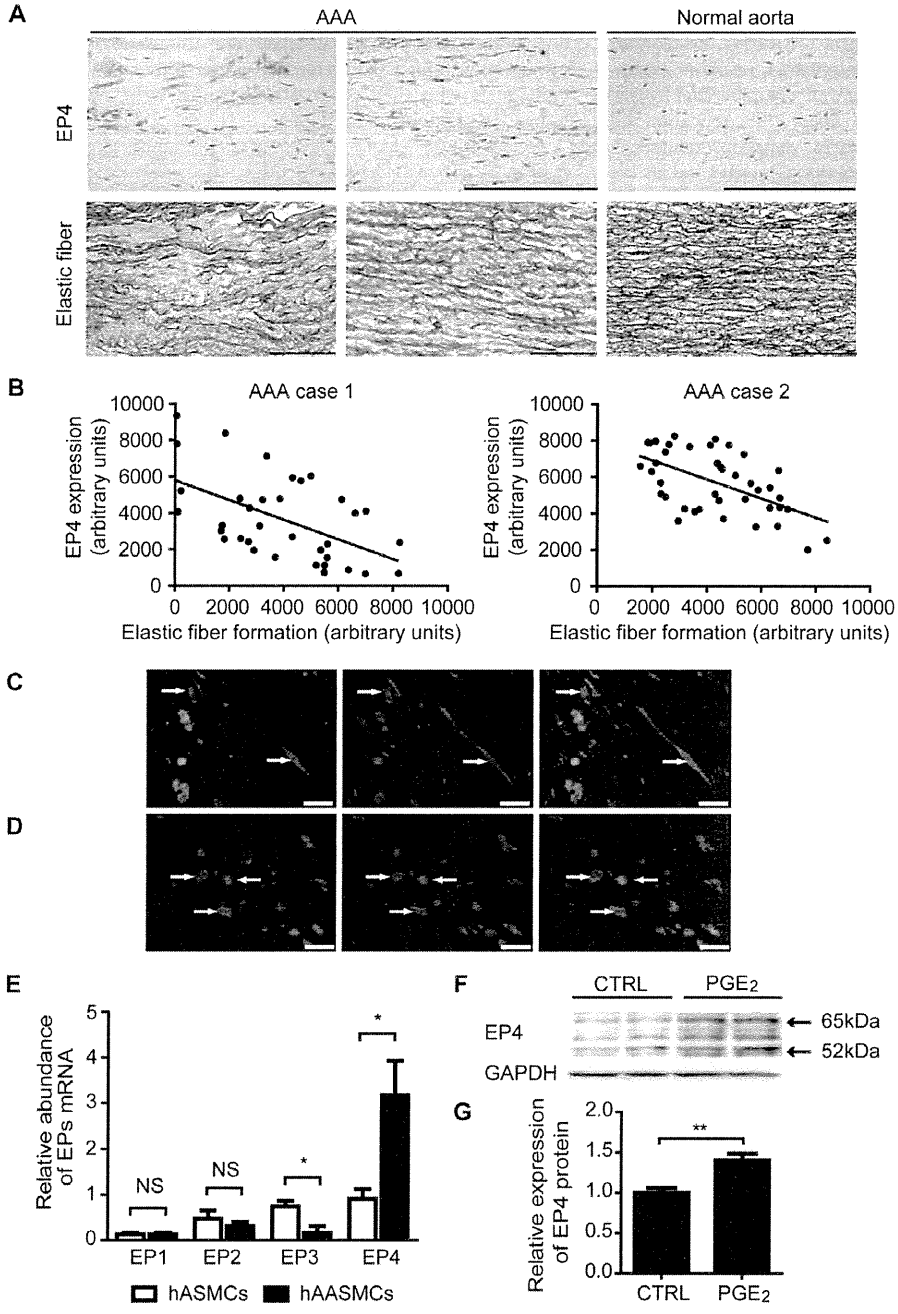

Figure 1. EP4 expression is increased in human AAA tissue. A, Immunohistochemistry for EP4 in human AAA tissues and aortic tissue from individuals who died of unrelated causes (upper panels). Brown areas indicate expression of EP4. Elastica van Gieson-stained aortic tissues (lower panels). Scale bars: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

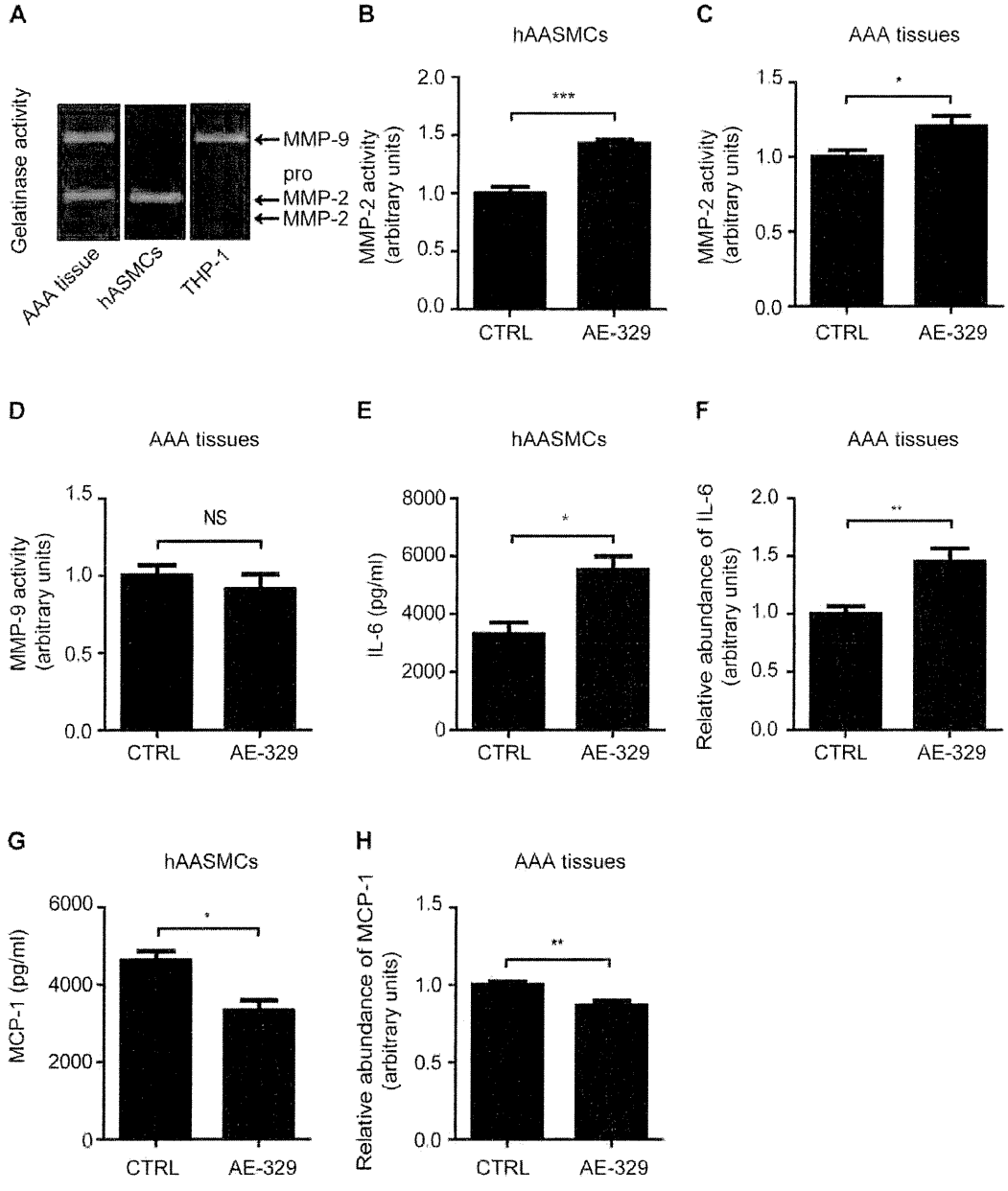

Figure 2. EP4 signaling increased MMP-2 activation and IL-6 production in hAASMCs and human AAA tissues. A, Representative images of gelatin zymography of human AAA tissue, hASMCs, and THP-1 treated with 100 nM of PMA. B, E and G, MMP-2 activation, IL-6, and MCP-1 production in supernatant of hAASMCs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

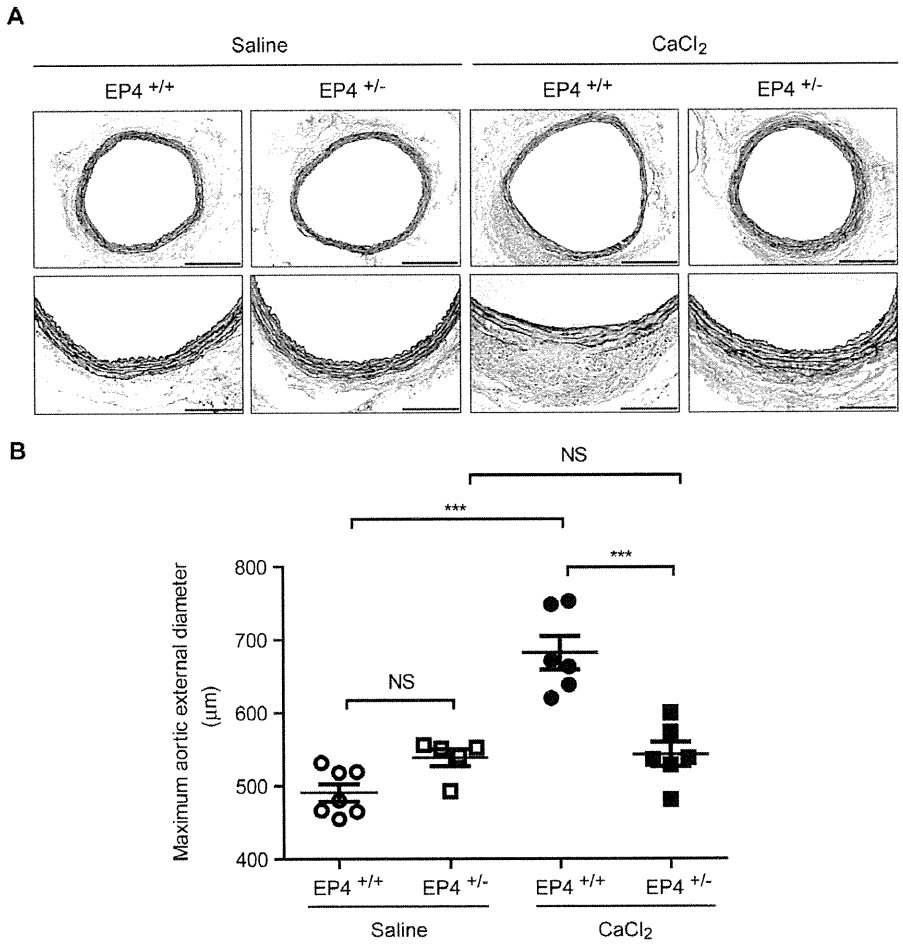

Figure 3. $CaCl_2$-induced AAA formation is attenuated in EP4$^{+/-}$ mice. A, Representative images of elastica van Gieson-stained tissue of EP4$^{+/-}$ and EP4$^{+/+}$ mice treated with saline or $CaCl_2$. Lower panels (Scale bars: 100 μm) show higher magnification portions of upper panel images (Scale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

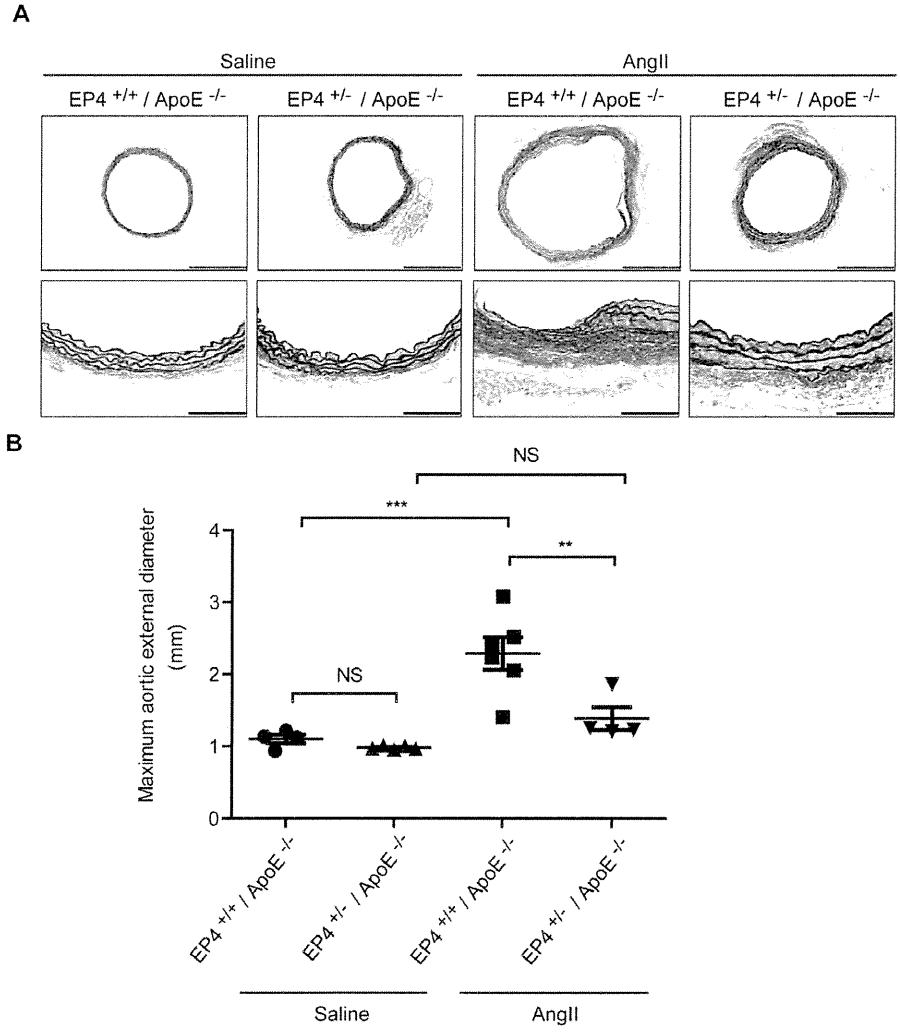

Figure 4. AngII-induced AAA formation is attenuated in $EP4^{+/-}/ApoE^{-/-}$ mice. A, Representative images of elastica van Gieson-stained tissue of $EP4^{+/-}/ApoE^{-/-}$ and $EP4^{+/+}/ApoE^{-/-}$ mice treated with saline or AngII. Lower panels (Scale bars: 100 μm) show higher magnification portions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

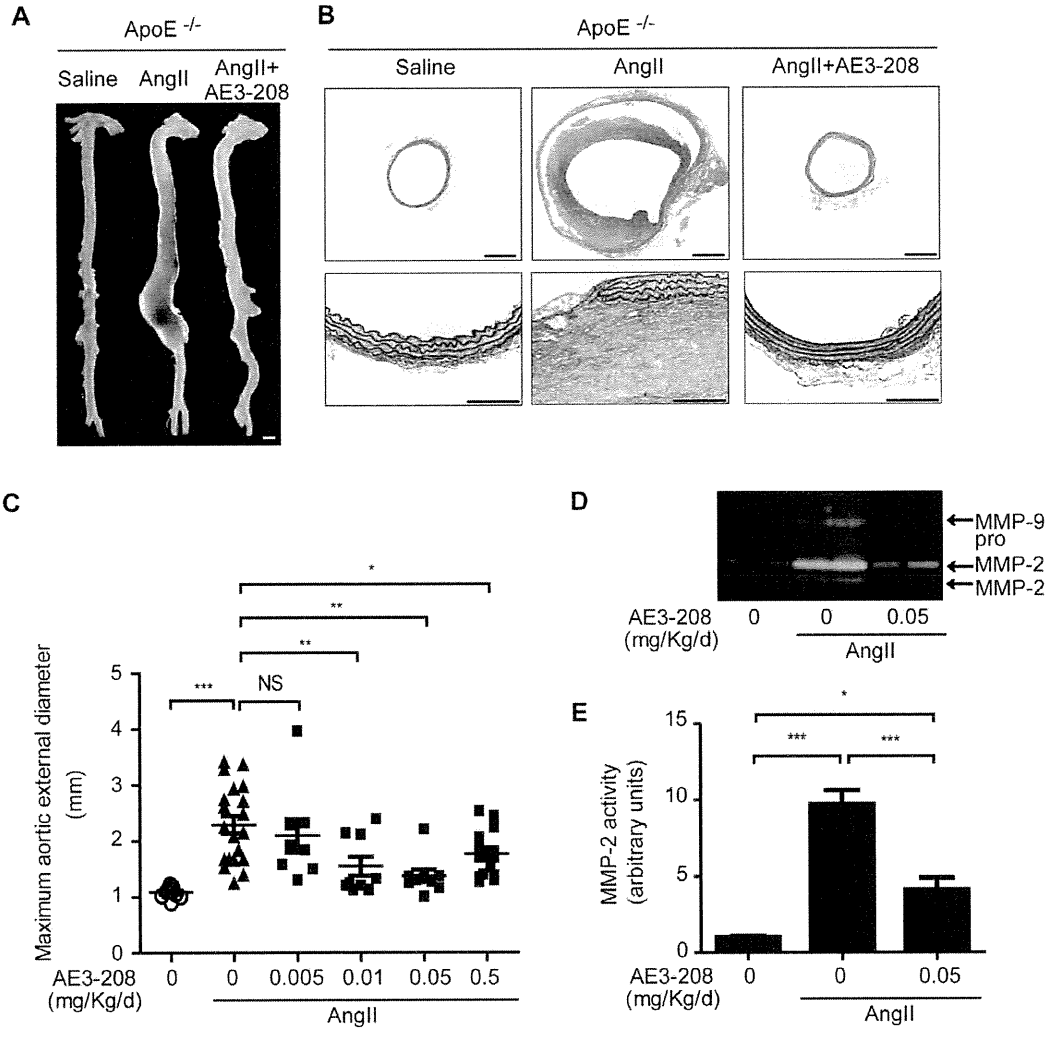

Figure 5. EP4 antagonist attenuated AngII-induced AAA formation in ApoE$^{-/-}$ mice. A, Representative image of aorta of ApoE$^{-/-}$ mice treated with saline, AngII, or AngII+ONO-AE3-208 (AE3–208) (0.05 mg/Kg/d). Scale bar: 1 mm. B, Elastica van Gieson–stained tissue of aortas shown in A. Lower panels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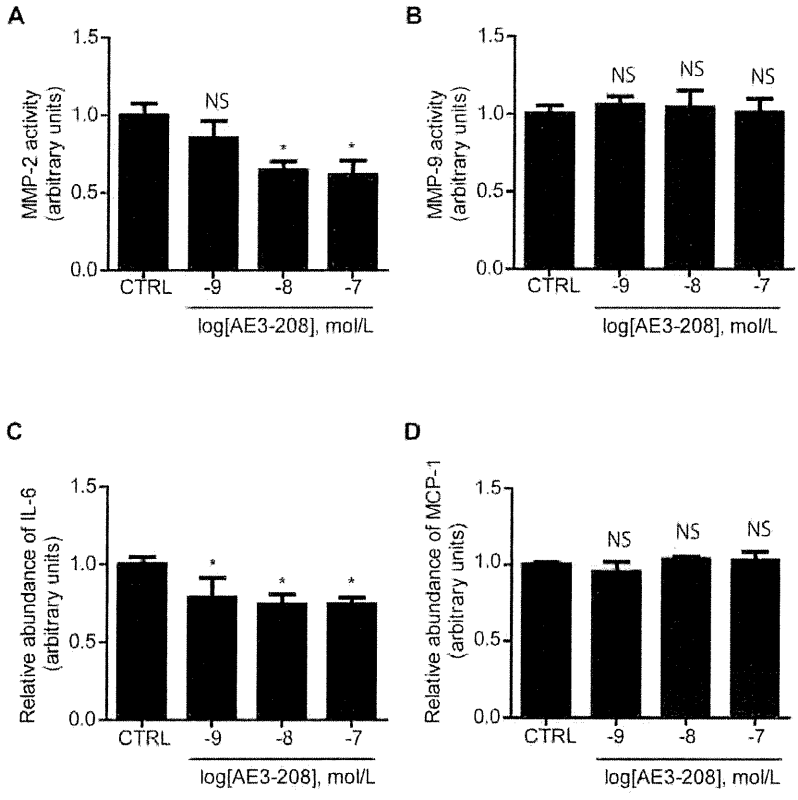

Figure 6. EP4 antagonist attenuated MMP-2 activation and IL-6 production in human AAA tissues. A, MMP-2 activity, B, MMP-9 activity, C, IL-6 production, and D, MCP-1 production. Supernatants of human AAA tissue organ cultures incubated in the presence or absence of and increasing concentrations of ONO-AE3-208 (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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Sarcalumenin is essential for maintaining cardiac function during endurance exercise training

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Jiao Q, Bai Y, Akaike T, Takeshima H, Ishikawa Y, Minamisawa S. Sarcalumenin is essential for maintaining cardiac function during endurance exercise training. *Am J Physiol Heart Circ Physiol* 297: H576–H582, 2009. First published June 5, 2009; doi:10.1152/ajpheart.00946.2008.—Sarcalumenin (SAR), a Ca^{2+}-binding protein located in the longitudinal sarcoplasmic reticulum (SR), regulates Ca^{2+} reuptake into the SR by interacting with cardiac sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a). We have previously demonstrated that SAR deficiency induced progressive heart failure in response to pressure overload, despite mild cardiac dysfunction in sham-operated SAR knockout (SARKO) mice (26). Since responses to physiological stresses often differ from those to pathological stresses, we examined the effects of endurance exercise on cardiac function in SARKO mice. Wild-type (WT) and SARKO mice were subjected to endurance treadmill exercise training (~65% of maximal exercise ability for 60 min/day) for 12 wk. After exercise training, maximal exercise ability was significantly increased by 5% in WT mice ($n = 6$), whereas it was significantly decreased by 37% in SARKO mice ($n = 5$). Cardiac function assessed by echocardiographic examination was significantly decreased in accordance with upregulation of biomarkers of cardiac stress in SARKO mice after training. After training, expression levels of SERCA2a protein were significantly downregulated by 30% in SARKO hearts, whereas they were significantly upregulated by 59% in WT hearts. Consequently, SERCA2 activity was significantly decreased in SARKO hearts after training. Furthermore, the expression levels of other Ca^{2+}-handling proteins, including phospholamban, ryanodine receptor 2, calsequestrin 2, and sodium/calcium exchanger 1, were significantly decreased in SARKO hearts after training. These results indicate that SAR plays a critical role in maintaining cardiac function under physiological stresses, such as endurance exercise, by regulating Ca^{2+} transport activity into the SR. SAR may be a primary target for exercise-related adaptation of the Ca^{2+} storage system in the SR to preserve cardiac function.

treadmill; calcium uptake; heart failure; excitation-contraction coupling

ENDURANCE EXERCISE IS ONE of the most common physiological stresses affecting the homeostasis of the whole body. Adaptations to chronic endurance exercise result in functional and structural changes in the heart (19, 31, 33); for example, after chronic endurance exercise training, it has been shown that resting heart rate is decreased and that maximal stroke volume is increased, since myocardial contractile function is enhanced and left-ventricular cavity dimension is augmented (2, 14, 25). A growing body of evidence has demonstrated that the regulation of intracellular Ca^{2+} through the sarcoplasmic reticulum (SR) plays a critical role in maintaining cardiac function under both physiological and pathological stresses (5, 7, 17). In particular, rapid transport of Ca^{2+} from the cytosol to the SR via the cardiac sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) is a critical determinant for the maintenance of Ca^{2+} storage in the SR. Therefore, it is extremely important for us to understand the effect of endurance exercise training on SERCA2a function and thus on the Ca^{2+} storage system in the heart. In this regard, a considerable number of previous studies on animals have demonstrated that endurance exercise training increases the expression and/or activity of SERCA2a in the heart, resulting in enhanced cardiac function of the healthy (9, 10, 20, 22, 30, 35) or pathological heart (6, 15, 21, 24, 34, 39).

Sarcalumenin (SAR) is an SR luminal glycoprotein responsible for Ca^{2+} buffering in skeletal and cardiac muscles (13, 16). SAR is predominantly found in the longitudinal SR, where SERCA and phospholamban (PLN) are also located. Our laboratory's previous study has demonstrated that SAR interacts with SERCA2 to enhance the protein stability of SERCA2a, and that it facilitates Ca^{2+} sequestration into the cardiac SR (26). Although young sedentary SAR knockout (SARKO) mice exhibit only mild impairments in Ca^{2+} transient and cardiac function (38), we have recently demonstrated that SAR deficiency induced progressive heart failure in response to pressure overload (26), indicating that SAR plays a critical role in adapting to pathological stresses, such as pressure overload in the heart. We found that SAR is essential for maintaining SERCA2a expression and activity in the pressure-overloaded heart. However, it has recently been reported that skeletal muscle from SARKO mice is highly resistant to fatigue compared with that from wild-type (WT) mice (40); this fatigue resistance of SARKO skeletal muscle is likely due to enhanced store-operated Ca^{2+} entry (SOCE) induced by upregulated expression of mitsugumin 29 (MG29), a synaptophysin-related membrane protein that is not expressed in the heart. In addition, it is known that the heart often responds differently to physiological stresses, such as endurance exercise, than to pathological stresses, such as pressure overload. Therefore, it remains unknown whether SAR also plays a role in maintaining cardiac function when the heart is exposed to physiological stresses, such as endurance exercise. To clarify the mode of action of SAR in the heart under a physiological stress, such as endurance exercise training, we investigated the

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impact of SAR deficiency on the expression and activity of SERCA2a in the heart and on cardiac function after endurance exercise training.

MATERIALS AND METHODS

Animal preparation. Generation of SARKO mice has been described previously (38). SARKO and C57BL/6J WT mice (8–10 wk of age) were bred at Yokohama City University. All mice used in the present study came from the same genetic background. All animal care and study protocols were approved by the Animal Ethics Committees of Yokohama City University School of Medicine and Waseda University, and the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication No. 85–23, revised 1996).

Maximal exercise ability and treadmill endurance exercise training. Mice were randomized into four groups: sedentary WT (SED-WT) and sedentary SARKO (SED-SARKO) mice, and WT (ET-WT) and SARKO (ET-SARKO) mice subjected to endurance exercise training.

Animals ran on a rodent motor-driven treadmill (MANUAL, LE 8700 series, Panlab, Barcelona, Spain) with adjustable belt speed (0–150 cm/s). The treadmill apparatus was equipped with adjustable-amperage (0–2 mA) shock bars at the rear of the belt, through which mild electrical stimulation (grid shock <1 mA) was applied to encourage the mice to run. A detector located above the shock grid measured the number of shock stimuli received by each mouse.

First, mice were acclimated to the treadmill via three 15-min running sessions with mild shock stimulation and a belt speed of 30 cm/s. After acclimation, all mice underwent a treadmill exercise test to determine their exercise ability before the endurance exercise training described below; a similar assessment was made during and after training for comparison purposes. The belt speed of the treadmill was set to 30 cm/s at the beginning of each test. It was then increased linearly by 2 cm/s every 30 s until the mice could not continue to run regularly on the treadmill, or until they had rested on the shock grid more than three times. The final belt speed achieved by each mouse was considered to be that mouse's maximal exercise ability. Maximal exercise ability was determined by averaging the maximal belt speeds of at least three measurements for each mouse; there was an intermission of at least 1 h between each measurement. Workloads of endurance exercise training were then adjusted for each mouse in accordance with its maximal exercise ability.

Before the start of each exercise training session, each mouse performed a 5-min warm-up at 40% of its maximal speed. ET-WT and ET-SARKO mice then ran on the treadmill (at 0° inclination) at 65% of their maximal speeds for 60 min/day, 5 days/wk, for 12 wk. Each mouse's maximal exercise ability was reevaluated every 4 wk, and each mouse's workload was adjusted again based on its current maximal speed (Supplemental Fig. 1). (The online version of this article contains supplemental data.) For sedentary mice, running skill was maintained by treadmill running for 15 min at 0° inclination at a belt speed of 30 cm/s, 3 days/wk.

Citrate synthase activity. As a marker for endurance training, the myocardial citrate synthase (CS) activity was measured at 37°C in the presence of 0.2% Triton X-100 with 20 μg protein sample, as previously described (27, 32). CS activity was also measured in soleus muscle homogenates to assess the efficacy of endurance exercise training.

Cardiac function assessed by echocardiography. Mice were anesthetized with an intraperitoneal injection of Avertin (250 μg/g) and subjected to echocardiography, as described in our laboratory's previous publications (28, 38). Since we have observed that the heart rates of mice decrease after intraperitoneal injection of Avertin, reaching stable minimal levels around 15–20 min after injection (Supplemental Fig. 2), we obtained the echocardiographic data around 15–20 min after injection of Avertin. After the final assessment of cardiac function after endurance training, heart and skeletal (soleus) muscles were immediately placed in chilled phosphate-buffered saline to remove all residual blood. Hearts were then weighed, and left ventricles were immediately frozen in liquid nitrogen and stored at −80°C.

Quantitative RT-PCR analysis. Total RNA was isolated from various tissues using TRIzol reagent (Invitrogen, Carlsbad, CA), as recommended by the manufacturer. Generation of cDNA and RT-PCR analysis was performed as described previously (36, 37). The primers for PCR amplification were designed based on the mouse nucleotide sequences of atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP). The mRNA levels of interest were normalized to mouse glyceraldehyde-3-phosphate dehydrogenase.

Immunoblot analysis. We prepared protein samples from the left ventricular tissues of the sedentary and trained mice, which had been immediately frozen and stored at −80°C after death of the animals. Immunoblot analyses were performed as described previously (26, 36). Briefly, tissues were defrosted to 0°C and homogenized in a chilled homogenization buffer [in mM: 50 Tris (pH 8.0), 1 EDTA, 1 EGTA, 1 dithiothreitol, and 200 sucrose] with protease inhibitors (Complete Mini, Roche, Basel, Switzerland). Protein content was determined using the Coomassie Plus protein assay (Pierce Chemical, Rockford, IL), and BSA (0.1–1 mg/ml) was used as a standard. The protein samples (20 μg) were separated in the same gel by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). When the molecular size of target proteins was different, polyvinylidene difluoride membranes were cut in accordance with their size. When the molecular size of target proteins was similar, we reused the same membrane for a different antibody after washing the membrane with a stripping buffer [in mM: 62.5 Tris (pH 8.0), 100 2-mercaptoethanol, and 2% SDS]. Antibodies used in the present study are shown in Supplemental Table 1. After application of a secondary antibody, quantification of the target signals was performed using the LAS-3000 imaging system (FUJIFILM, Tokyo, Japan). The protein levels of interest were normalized to rat β-actin. For reuse, a membrane was washed with a stripping buffer at 55°C for 10 min and was washed three times with 0.1% Tris buffered saline-Tween 20 buffer.

SR Ca^{2+}-ATPase assay. SR Ca^{2+}-ATPase activity was measured in triplicate spectrophotometrically at 37°C, as described previously with some modifications (18). Briefly, using 5 μg of SR protein from mice heart tissues, the reaction was carried out at 37°C in a reaction medium [in mM: 30 TES, 100 KCl, 5 NaN_3, 5 $MgCl_2$, 0.5 EGTA, and 4 ATP, with or without 0.5 $CaCl_2$]. The reaction medium was preincubated at 37°C for 5 min. The reaction was started at 37°C by adding SR protein to the medium. After 5 min, the reaction was stopped by adding 0.5 ml of ice-cold 10% trichloroacetic acid solution, and the mixture was placed on ice. Inorganic phosphate was measured by using U2001 (Hitachi), as described previously (8). Ca^{2+}-ATPase activity was calculated by subtracting the ATPase activity in the presence of 0.5 mM EGTA (no added Ca^{2+}) from the activity in the presence of 0.5 mM $CaCl_2$.

Statistical analysis. All values are expressed as means ± SE. Comparisons of data from multiple groups were performed by unpaired ANOVA followed by the Student Newman-Keuls post hoc test. Statistical significance was defined as $P < 0.05$.

RESULTS

Effects of endurance exercise training on exercise ability in SARKO mice. Before the start of endurance exercise training, exercise ability was examined in WT and SARKO mice by a treadmill-based exercise stress test, described above. Maximal exercise ability, as evaluated by maximal belt speed, was lower in SARKO mice (n = 16, 65.0 ± 3.6 cm/s) than in WT mice

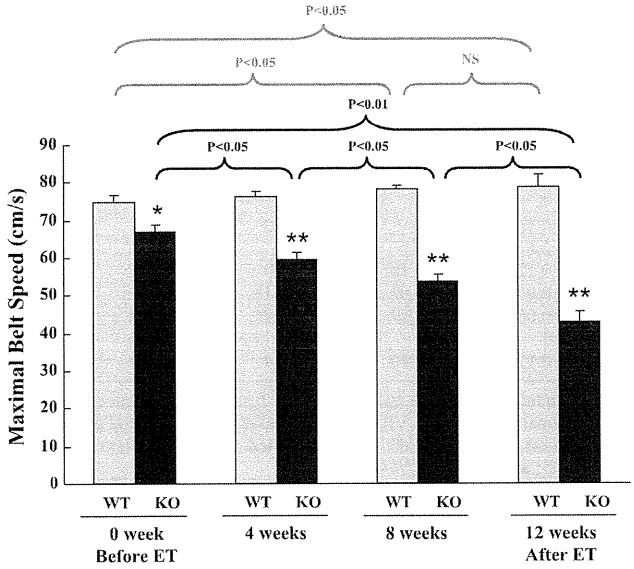

Fig. 1. The effect of endurance exercise training (ET) on maximal exercise ability in sarcalumenin (SAR) knockout (SARKO) mice. Maximal exercise ability, as evaluated by maximal belt speed, is already lower in SARKO mice than in wild-type (WT) mice before ET. During ET, maximal exercise ability gradually increased in WT mice, whereas it actually decreased in SARKO mice in a time-dependent manner. Maximal exercise ability after ET was significantly increased in WT mice, whereas it was actually decreased in SARKO mice compared with their maximal exercise ability before the training. Values are means ± SE; n = 6 and 5 for WT and knockout (KO), respectively.

(n = 16, 74.1 ± 2.1 cm/s), although it did not reach a statistical significance (P = 0.059). As expected, maximal exercise ability in sedentary animals (SED-WT and SED-SARKO mice) did not significantly change during the 12-wk training period (data not shown). In ET-WT mice, maximal exercise ability gradually increased during endurance exercise training, whereas, in ET-SARKO mice, it gradually decreased (Fig. 1). Whenever a change in a mouse's maximal exercise ability was detected by a regular treadmill test, that mouse's training workload was adjusted based on its current maximal speed (Supplemental Fig. 1). Maximal exercise ability after endurance exercise training significantly increased by 5% in ET-WT mice, whereas it actually decreased by 37% in ET-SARKO mice compared with their ability measured before the training regime began (Fig. 1).

Exercise training did not improve CS activity in SARKO mice. We observed no difference between WT and SARKO mice in terms of CS activity of skeletal or cardiac muscle at a basal condition. After the endurance exercise training, ET-WT mice exhibited increased CS activity of soleus muscle (Fig. 2*A*), indicating an appropriate effect of the training program on working muscles. In accordance, they also exhibited increased CS activity of cardiac muscle (Fig. 2*B*), which is consistent with several previous studies (1, 20), although most of previous studies have demonstrated that CS activity is not increased or little increased by endurance exercise in rodent hearts (4, 19). In ET-SARKO mice, on the other hand, CS activity was not increased in either soleus or cardiac muscle (Fig. 2).

Endurance exercise training resulted in cardiac dysfunction in SARKO mice. To examine the effect of endurance exercise training on cardiac function, we investigated it using transthoracic echocardiography. Before endurance exercise training, all parameters listed in Table 1 were similar between WT and SARKO mice, including body weight, heart rate, left ventricular fractional shortening, thickness of myocardial walls, and ejection time. After endurance exercise training, left ventricular fractional shortening was significantly decreased in ET-SARKO mice, whereas it was not changed in ET-WT mice (Table 1). As we expected, the diameter of the end-diastolic left ventricular chamber was significantly increased in ET-SARKO mice. Furthermore, ejection time was significantly prolonged in ET-SARKO mice, and their heart rate corrected velocity of circumferential fiber shortening was significantly lower (Table 1).

Biomarkers of cardiac stress were increased in ET-SARKO hearts. To examine the effect of endurance exercise training on the myocardium itself, we measured molecular markers of

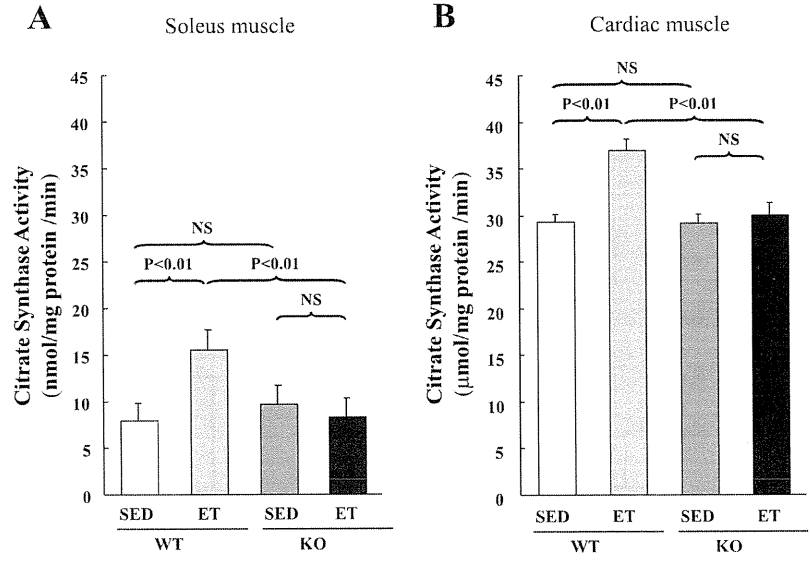

Fig. 2. Citrate synthase (CS) activity after ET. After ET, CS activity of soleus muscle (*A*) and cardiac muscle (*B*) was increased in ET-WT mice, but not in ET-SARKO mice. Values are means ± SE; n = 5 for each group. SED, sedentary; NS, not significant.

Table 1. *Cardiac function after endurance exercise training*

	SED-WT		ET-WT		SED-SARKO		ET-SARKO	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
n	10	10	6	6	10	10	6	5
BW, g	24.2±1.3	32.6±1.9	26.3±1.7	31.1±1.3	23.3±1.0	27.3±1.1	22.6±1.2	26.3±1.8
HR, beats/min	464±15	474±15	438±18	436±18	430±12	409±14	449±24	425±14
LV weight, mg		115±9		111±7		93±6		86±7
LV-to-BW weight ratio, mg/g		3.55±0.25		3.58±0.15		3.38±0.12		3.26±0.15
LV FS, %	35.6±1.1	35.5±1.0	34.7±1.2	34.9±2.6	37.5±1.3	34.0±1.2	38.6±2.7	28.4±1.1[a,c,d]
LVIDd, mm	4.11±0.08	4.12±0.11	4.03±0.06	4.25±0.12	3.88±0.10	4.02±0.09	3.84±0.12	4.14±0.19[a]
IVSTd, mm	0.77±0.03	0.79±0.05	0.77±0.02	0.71±0.05	0.76±0.02	0.67±0.01[b]	0.76±0.02	0.66±0.02[b]
LVPWTd, mm	0.76±0.03	0.75±0.04	0.76±0.04	0.76±0.06	0.72±0.07	0.65±0.04[a]	0.69±0.02	0.67±0.02[a,c]
Ejection time, ms	60±1	60±1	64±2	66±3	64±2	65±2	58±2	69±3[a]
Vcfc, circumferences/s	2.14±0.06	2.13±0.07	2.12±0.10	2.14±0.17	2.21±0.10	2.04±0.14	2.44±0.18	1.56±0.05[b,c,d]

Values are means ± SE; *n*, no. of mice. SED, sedentary; WT, wild-type mice; ET, endurance exercise training; SARKO, sarcalumenin-knockout mice; Pre, before ET; Post, after ET; BW, body weight; HR, heart rate; LV, left ventricle; FS, fractional shortening; LVIDd, LV internal dimensions at end diastole; IVSTd, interventricular septum thickness at end diastole; LVPWTd, LV posterior wall thickness at end diastole; Vcfc, corrected velocity of circumferential fiber shortening. Significant difference vs. Pre: [a]$P < 0.05$ and [b]$P < 0.01$; vs. WT: [c]$P < 0.05$; and vs. SED: [d]$P < 0.05$.

cardiac stress, such as ANF and BNP mRNAs. These were significantly upregulated in ET-SARKO mice (Fig. 3). Endurance training did not affect the expression of ANF and BNP mRNAs in ET-WT mice.

Significant reductions in the expression of Ca^{2+} handling proteins in ET-SARKO mice. Since the expression levels of SERCA2a and other Ca^{2+} handling proteins are critical for the regulation of cardiac function, we examined them by Western blot analyses (Fig. 4, Table 2). Consistent with our laboratory's previous report (26, 38), the expression levels of SERCA2a and total PLN were significantly downregulated in SED-SARKO mice compared with those in SED-WT mice. After endurance exercise training, the expression level of SERCA2a protein was significantly increased by 59% in ET-WT mice, whereas it was reduced by 30% in ET-SARKO mice compared with sedentary mice of each group's respective genotype. Endurance exercise training also resulted in a further significant downregulation of both total and phosphorylated PLN proteins in ET-SARKO mice, but not in ET-WT mice. The SERCA2a-to-PLN protein ratio was significantly decreased in ventricular muscles of ET-SARKO mice (Table 2). The ratio of phosphorylated threonine 17 PLN to total PLN protein was significantly lower in ET-SARKO than in ET-WT, but that of serine 16 to total PLN protein was not (Table 2). It should be noted that intraperitoneal injection of Avertin did not affect the phosphorylation status of serine 16 and threonine 17 in PLN (Supplemental Fig. 2).

The expression levels of calsequestrin 2 (CSQ2) and ryanodine receptor type 2 (RyR2) proteins in SED-SARKO mice were comparable to those in SED-WT mice, while those of sodium/calcium exchanger 1 (NCX1) protein were even higher in SED-SARKO mice than in SED-WT mice. After the endurance exercise training, all of these proteins were significantly downregulated in ET-SARKO mice, but not in ET-WT mice (Fig. 4, Table 2). Overall, in addition to SERCA2, all other Ca^{2+} handling proteins that we examined were downregulated in ET-SARKO mice after endurance exercise training.

Significant reduction in SERCA2a activity in ET-SARKO mice. As measured in myocardial homogenates, maximal Ca^{2+}-ATPase activity was lower in SED-SARKO mice than in SED-WT mice (Fig. 5). After the endurance exercise training, maximal Ca^{2+}-ATPase activity was further significantly decreased in ET-SARKO mice, whereas it was significantly increased in ET-WT mice. This result was consistent with the change in the ratio of SERCA2a to PLN protein expression shown in Table 2.

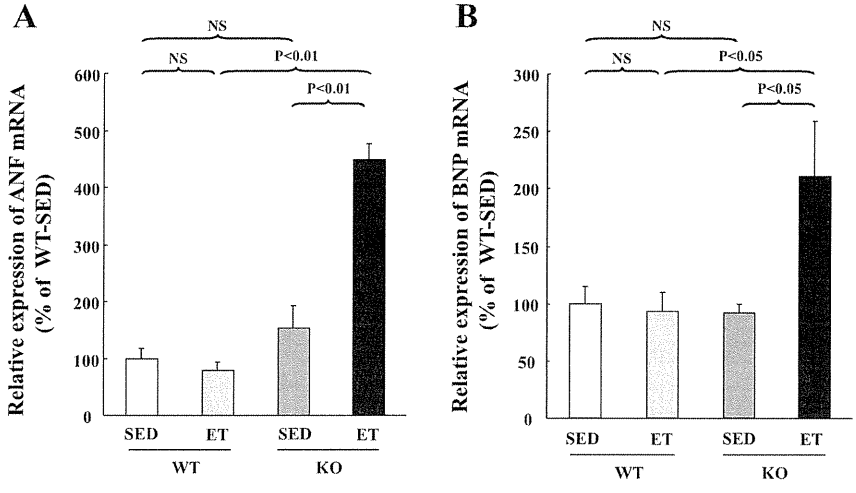

Fig. 3. Upregulation of atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) mRNAs in ET-SARKO mice. Quantitative RT-PCR analyses revealed that the expression levels of ANF (*A*) and BNP (*B*) mRNAs were significantly upregulated in the ventricles of ET-SARKO mice. The expression levels observed in SED-WT mice were set as 100% as a control. mRNA expression was normalized by GAPDH. Values are means ± SE; $n = 5$ for each group.

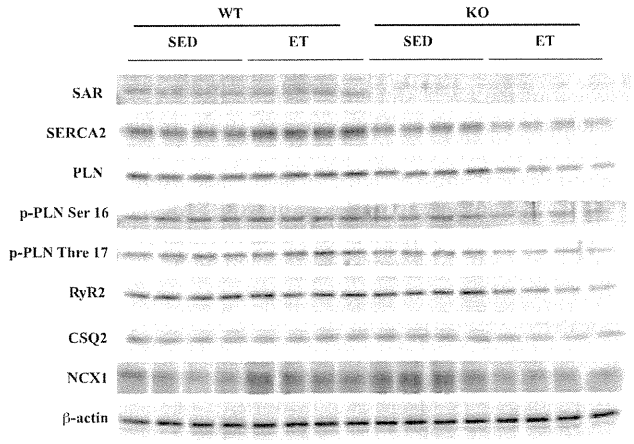

Fig. 4. The expression of Ca^{2+} handling proteins after ET. The expression levels of sarcalumenin (SAR), sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2 (SERCA2), phospholamban (PLN), phosphorylated PLN (p-PLN), ryanodine receptor 2 (RyR2), calsequestrin 2 (CSQ2), and sodium/calcium exchanger 1 (NCX1) proteins were quantified in hearts isolated from SED and ET mice. Protein expression was normalized by β-actin.

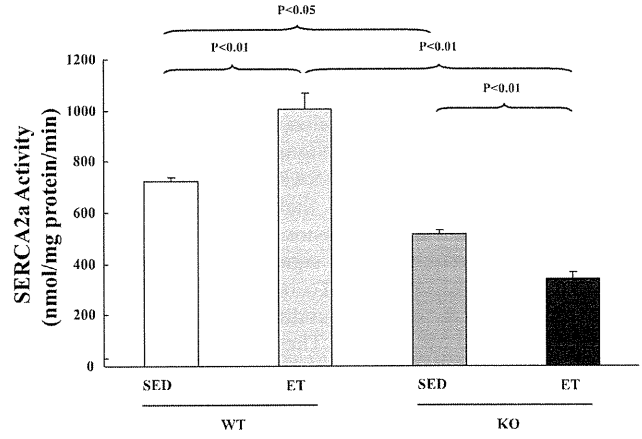

Fig. 5. SERCA2a activity after ET. SERCA2a activity was increased in ET-WT, whereas it was decreased in ET-SARKO after ET. Values are means ± SE; $n = 5$ for each group.

DISCUSSION

The most striking finding in the present study is that long-term (12 wk) endurance exercise training induced a significant cardiac dysfunction in mice that harbor systemic ablation of the SAR gene. Along the same lines, we have recently demonstrated that SARKO mice failed to adapt to pressure-overloaded stress induced by transverse aortic constriction (26), whereas sedentary young SARKO mice exhibit mild cardiac dysfunction (38). Since exercise is one of the most common physiological stresses, the present data indicate that SAR plays an important role in preserving cardiac function during adaptation to not only pathological, but also physiological, stresses.

It should be noted that the absolute training intensity undertaken by SARKO mice was significantly lower than that undertaken by WT mice (Supplemental Fig. 1), because the intensity of each mouse's exercise regime was determined on the basis of that mouse' maximal exercise ability. Accordingly, CS activity in soleus muscle after endurance exercise training was significantly lower in ET-SARKO mice than in ET-WT mice (Fig. 2). Since skeletal muscle CS activity is a marker for mitochondrial content (a hallmark of endurance exercise) and muscle oxidative capacity, this result indicates that our exercise training program is sufficient to enhance the exercise ability of WT mice, but insufficient to enhance that of SARKO mice. Although this may explain a number of the negative effects on SARKO mice that were caused by exercise training in the present study, it is, nevertheless, very difficult to explain why ET-SARKO mice exhibited progressive cardiac dysfunction. We assume that inadequate adaptation to endurance exercise in ET-SARKO mice caused impaired cardiac function, the primary insult, which, secondarily, resulted in a number of negative effects on SARKO mice caused by training.

Table 2. *The expression of calcium handling proteins after endurance exercise training*

	SED-WT	ET-WT	SED-SARKO	ET-SARKO
SAR	100±5	108±9		
SERCA2	100±10	159±13§	74±4*	52±6†‡
PLN	100±6	123±8	83±2*	71±2†§
p-PLN Ser 16	100±3	120±11	95±5	82±3†‡
p-PLN Thre 17	100±4	112±7	92±6	78±4†‡
SERCA2/PLN	100±5	132±12‡	94±4	75±10†‡
p-PLN Ser 16/PLN	100±4	98±8	113±4	116±8
p-PLN Thre 17/PLN	100±1	98±4	93±2	88±2*
RyR2	100±5	100±10	97±6	68±8*‡
CSQ2	100±7	101±4	99±3	83±6*‡
NCX1	100±10	139±11‡	124±3*	92±11*‡

Values are means ± SE; $n = 5$ mice for each group. The expression level in SED-WT mice was referred to 100% as a control. Protein expression was normalized by β-actin. SAR, sarcalumenin; SERCA2, sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2; PLN, phospholamban; p-PLN: phosphorylated phospholamban; RyR2, ryanodine receptor 2; CSQ2, calsequestrin 2; NCX1, sodium/calcium exchanger 1. Significant difference vs. WT: *$P < 0.05$ and †$P < 0.01$; vs. SED: ‡$P < 0.05$ and §$P < 0.01$.

The mechanism by which endurance exercise induced progressive cardiac dysfunction in SARKO mice is a critical question. One observation that may be relevant to this question is the significant decrease in the expression and activity of SERCA2a in ET-SARKO mice. A number of previous studies have reported that endurance exercise training increased the expression and/or activity of SERCA2a in healthy (9, 10, 20, 22, 30, 35) or diseased rodents (6, 21, 24, 34, 39); similarly, we found that the expression and activity of SERCA2a increased after endurance exercise training in control mice. Yet other studies have demonstrated that endurance exercise training does not change the expression and/or activity of SERCA2a (3, 4) or Ca^{2+} transients (12) in rodents. It is worth noting that these conflicting results may have their origins in such factors as differences in species, exercise protocols, and/or condition of the subjects; few studies, however, have shown that endurance exercise decreases the expression and/or activity of SERCA2a. Therefore, our results found in ET-SARKO mice were so remarkable that it is very important to investigate why SAR deficiency caused the significant reduction in the expression and activity of SERCA2a under endurance exercise training.

Our laboratory's recent study has demonstrated that SAR interacts with SERCA2 to enhance the protein stability of SERCA2a (26). Since exercise training usually increases pro-

tein synthesis and degradation in muscle (11, 23), we assume that endurance exercise training also increased the turnover rate of SERCA2a protein. Then we postulate that SAR deficiency induced a progressive degradation of SERCA2a protein due to impaired protein stabilization under endurance exercise training and resulted in the significant decrease in the expression of SERCA2a in ET-SARKO mice. Importantly, the present study demonstrated that endurance exercise training slightly increased the expression levels of SAR protein in WT hearts, in accordance with a significant increase in the expression of SERCA2a protein. To our knowledge, this is the first report to show the effect of endurance exercise training on the expression of SAR protein. These data suggest that SAR is a key regulatory protein to maintain the expression level of SERCA2a protein under pathophysiological stresses. In addition, the ratios of SERCA2a to PLN protein and phosphorylated threonine 17 PLN to total PLN protein were significantly decreased in the ventricular muscles of ET-SARKO mice, indicating that SERCA2a activity was inhibited by PLN more in ET-SARKO mice than in other groups. Taken together, this evidence shows that SAR deficiency induced a significant reduction in SERCA2a activity and deterioration of the Ca^{2+} storage system in the SR under endurance exercise stress, which is very likely to play a primary role in the exercise-induced cardiac dysfunction exhibited by ET-SARKO mice.

Interestingly, in addition to the decreases in the SERCA2a and PLN proteins that interact with SAR in the longitudinal SR, other Ca^{2+} handling proteins, such as RyR2, CSQ2, and NCX1, were also significantly downregulated in ET-SARKO mice, which has not been investigated in pressure-overloaded SARKO hearts (26). These abnormalities probably contribute to the further impairment of cardiac function during endurance exercise training. We assume that the downregulation of RyR2, CSQ2, and NCX1 could be a secondary phenomenon that occurs under physiological stress conditions, as SAR does not directly interact with these proteins. The mechanism of these discrepant responses to different stresses in SARKO mice is currently not clear; it is an important question that should be addressed in future studies.

In one way, the results of the present study somehow contradict those of a recent report by Zhao et al. (40), which showed that skeletal muscles from SARKO mice are highly resistant to fatigue compared with those from WT mice. The same authors have also demonstrated that SOCE was promoted in SARKO skeletal muscle by the upregulation of MG29 (40). They proposed that the promotion of SOCE played a role in making skeletal muscle more fatigue resistant (40). In the present study, however, we did not detect any expression of MG29 protein in either WT or SARKO hearts, before or after exercise training, although we used the same membranes for our Western blot analyses (data not shown). This observation is consistent with a previous study (29). Currently, we cannot explain the exact reason for the disagreement between the results of Zhao et al. (40) and our own. A possible explanation is the difference in the exercise programs our two groups used to evaluate the exercise performance of SARKO mice. Further investigation is needed to clarify whether a defect of MG29 may cause the negative responses to exercise in SARKO cardiac muscle cells.

In conclusion, we found that cardiac function and maximal exercise ability were significantly impaired in SARKO mice after endurance treadmill exercise training. These impairments were due, at least in part, to a significant downregulation of SERCA2a and other Ca^{2+} handling proteins and to a deterioration of the Ca^{2+} storage system in the SARKO heart under endurance exercise. Thus present study indicates that SAR plays a critical role in maintaining cardiac function under physiological stresses, such as endurance exercise, by regulating Ca^{2+} transport activity into the SR. SAR may be a primary target for exercise-related adaptation of the Ca^{2+} storage system in the SR to preserve cardiac function.

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ORIGINAL PAPER

Effect of ascorbic acid on reactive oxygen species production in chemotherapy and hyperthermia in prostate cancer cells

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Abstract Cellular reactive oxygen species (ROS) production is increased by both temperature and anticancer drugs. Antioxidants are known to suppress ROS production while cancer patients may take them as dietary supplement during chemotherapy and hyperthermic therapy. We examined changes in ROS production in prostate cancer cells in the presence of various anticancer drugs and antioxidants at different temperatures. ROS production was increased with temperature in cancer cells, but not in normal cells; this increase was potently inhibited by ascorbic acid. ROS production was also increased in the presence of some anticancer drugs, such as vinblastine, but not by others. Dietary antioxidant supplements, such as β-carotene, showed variable effects. Ascorbic acid potently inhibited ROS production, even in the presence of anticancer drugs, while β-carotene showed no inhibition. Accordingly, our results suggest that cancer patients should carefully choose antioxidants during their cancer chemotherapy and/or hyperthermic therapy.

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Keywords Reactive oxygen species · Prostate cancer cells · Hyperthermia · Ascorbic acid · Anti-oxidants · Anti-cancer drugs

Introduction

Physiology of cancer cells has been extensively studied, and the understanding of mechanisms for their rapid growth and proliferation has been advanced in the past decade [1–3]. Accordingly, various therapeutic strategies in cancer treatment have been developed [1, 4]. Although surgical removal of the cancer tissue is still the golden standard for complete cure, it is not always feasible in cases with advanced or metastatic cancer. Surgical stress may be too large for geriatric and/or exhausted patients. In such cases, combination of various therapeutic strategies has been recommended. Among such strategies, hyperthermic therapy may be applied on the top of the conventional cancer chemotherapy or radiation therapy [5, 6]. Although it may not achieve complete remission of cancer by itself, clinical studies have demonstrated that the survival and quality of life may be significantly improved [3, 7].

Molecular mechanism of hyperthermic therapy includes the overstimulation metabolism of rapidly proliferating cancer cells, leading to the induction of apoptosis [8]. Increased production of reactive oxygen species (ROS) from mitochondria may also be involved [9]. Because ROS production may be increased in the presence of anticancer drugs on their own, the combination of chemotherapy and hyperthermic therapy will synergistically increase ROS production, leading to effective cancer cell death [6]. However, ROS production is inhibited in the presence of various antioxidants [10]. In this regard, various antioxidants, which are also used as dietary supplements, may interfere with the

efficacy of such chemotherapy and/or hyperthermic therapy. Unfortunately, however, evaluation of the effect of such antioxidants in the combination of cancer chemotherapy has not been well performed [11, 12]. Ascorbic acid, for example, is often used as a dietary supplement. Because ascorbic acid may improve immunity or peripheral circulation [13], people, including cancer patients, take this antioxidant. However, the use of ascorbic acid in cancer patients remains controversial; ascorbic acid may enhance [10] or suppress [13] the efficacy of chemotherapy.

In this study, we examined the effect of temperature, anticancer drugs, and antioxidants on ROS production. We used MAT-Lu prostate cancer cells since hyperthermia therapy has often been applied to prostatic cancer patients [14, 15], and thus it is necessary to evaluate the effect of hyperthermia on this cancer cell type. We demonstrated their effect on ROS production, and make potential suggestions for future use of antioxidants in cancer patients.

Materials and methods

Materials

We used the following anticancer drugs; vinblastine (VBL) (Nihon Kayaku, Japan), cisplatin (CIS), (Pfizer, Japan), adriamycin (ADR), (Wako, Japan), docetaxel (DTX), (Sanofi Aventis, Japan). Similarly, as antioxidants, we used *N*-acetyl-cysteine (NAC), (Sigma, Japan), retinoic acid (Sigma), quercetin (Sigma), catechin (Wako), lutein (Sigma), β-carotene (Sigma), and ascorbic acid (Wako).

Cell culture

Rat prostatic adenocarcinoma cells (R3327-MAT-Lu) were cultured in RPMI-1640 medium supplemented with 10% FBS and 250 nM dexamethasone, which were kindly provided by Dr. J. T. Isaacs (Johns Hopkins University, MD, USA). Cells were incubated at 37°C in 5% CO_2. In some experiments, cells were incubated at 42°C as hyperthermic treatment (see below). Rat cardiac fibroblasts were isolated from adult rats (250–300 g, male) by using a modification of published methods [16]. Fibroblasts were separated from cardiac myocytes by gravity separation and grown to confluence on 10-cm cell culture dishes at 37°C with 90% air with 10% CO_2 in growth media (DMEM with 10% FBS, 1% penicillin, and 1% streptomycin).

Hyperthermic stress and measurement of reactive oxygen species

Cells were plated in 24-well culture plates (5.0×10^4 cells/well) overnight. Cells were then treated with various agents, including anticancer drugs, at 37°C for 3 h. For hyperthermic treatment, cells were further incubated in the presence or absence of various reagents at 42°C for 1 h. The intracellular ROS level was then measured using a fluorescent dye 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Life technologies, Japan) as previously described [17]. In the presence of oxidant, DCFH is converted into the highly fluorescent 2′,7′-dichlorofluorescein. Cells were first washed with PBS, and serum-free DMEM containing 10 μM DCFH-DA was added to each well. Cells were then incubated at 37°C for 45 min. ROS production was measured using a microplate reader equipped with a spectrofluorometer (PerkinElmer ARVO MX, Japan) at an emission wavelength of 538 nm and extinction wavelength of 485 nm.

Statistical analysis

Data are expressed as means ± SEM. Data was analyzed by one-way ANOVA followed by Tukey post hoc using Graph-pad Prism software. Statistical significance was set at $p < 0.05$.

Results

Effect of temperature on ROS generation

It is known that cancer cells exhibit higher metabolism than normal cells. High metabolic rate may be reflected by increased ROS generation, in particular, upon hyperthermia. Accordingly, we compared the effect of temperature on ROS production between MAT-Lu prostate cancer cells and normal fibroblasts obtained from the cardiac tissue. It is known that fibroblasts grow rapidly and thus possess high metabolic rate in comparison to other normal cell types.

As shown in Fig. 1a, ROS production was lower at 32°C than at 37°C while it was higher at 42°C. Thus, ROS production was increased in a temperature-dependent manner, at least in prostate cancer cells. In contrast, ROS production in cardiac fibroblasts was not increased at 42°C in comparison to that at 37°C (Fig. 1b). Thus, ROS production by hyperthermia was increased only in cancer cells.

Effect of ascorbic acid on ROS production

We then examined the effect of ascorbic acid, which has been used in cancer treatment as part of chemotherapy, but is also known as a major antioxidant. In the presence of an increasing concentration of ascorbic acid (10 μM–100 mM), ROS production was decreased in a concentration-dependent manner at 37°C (Fig. 1c). Similar inhibition was observed at 42°C. Thus, ascorbic acid potently inhibited the production of ROS.

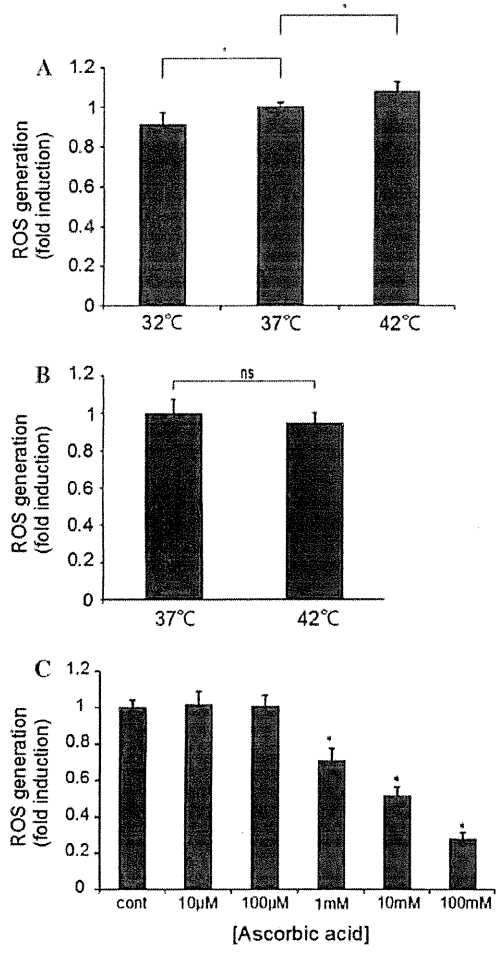

Fig. 1 ROS production in cancer cells and normal cells at different temperatures. **a** ROS production in cancer cells at 32, 37, and 42°C. Prostate cancer cells were incubated at different temperatures, followed by determination of ROS production (mean ± SEM; $n = 4$, $^*p < 0.05$). **b** ROS production in cardiac fibroblasts at 37 and 42°C. Cardiac fibroblasts were incubated at different temperatures similarly, followed by determination of ROS production (mean ± SEM; $n = 4$, $^*p < 0.05$). **c** ROS production was determined with cancer cells in the presence of an increasing concentration of ascorbic acid (10 μM–100 mM). Prostate cancer cells were incubated at 37°C, followed by determination of ROS production (mean ± SEM; $n = 4$, $^*p < 0.05$)

Effect of anticancer drugs on ROS production

Anticancer drugs may induce cytotoxicity through various mechanisms. We examined the effect of these anticancer drugs, which have been widely used in many cancer cell types, including prostate cancer, on ROS production. We first determined the EC_{50} values of these drugs in prostate cancer cells, which were 200 nM for VBL, 15 μM for CIS, 7.5 μM for ADR, and 1 mM for DTX. When prostate cancer cells were incubated with these drugs at the EC_{50} value concentration, ROS production was slightly, but significantly, increased with VBL and CIS, but not with DTX and ADR at 37°C (Fig. 2a). When hyperthermic treatment at 42°C was added, ROS production by VBL and CIS became even greater (Fig. 2a). Thus, hyperthermia by itself can increase ROS production, which is further enhanced in the presence of certain anticancer drugs.

We then examined the effect of ascorbic acid in the presence of anticancer drugs. ROS production was potently inhibited by 1 mM ascorbic acid in the presence of any anticancer drugs (Fig. 2b). ROS production at 37°C was similar among these anticancer drugs. However, when hyperthermic treatment at 42°C was added, ROS production was significantly greater with VBL (Fig. 2b). Thus, ascorbic acid may negate ROS production induced by certain anticancer drugs at 37°C; however, it cannot negate ROS production of VBL at 42°C. Accordingly, anticancer drug-induced ROS enhancement may be retained in hyperthermia for VBL, but not others.

Effect of ascorbic acid on ROS production by Resovist

Resovist is super-paramagnetic iron oxide nanoparticle that has been used as MRI contrast agent. Because of its magnetic property, similar compounds have been used as source of heat production in hyperthermic therapy. We found that the ROS production was increased in the presence of 10 μM Resovist at 37°C, suggesting that Resovist can produce ROS with cancer cells. When ascorbic acid was added, ROS production was negated or instead decreased (Fig. 3). Thus, ascorbic acid could potently inhibit ROS production induced by Resovist.

Effect of various antioxidants on ROS production

Patients may take various dietary supplements during cancer chemotherapy. In some cases, patients may take supplementary antioxidants on the top of anticancer drugs. We thus examined the effect of these antioxidants and related drugs, namely, *N*-acetyl cysteine (NAC), retinoic acid, quercetin, catechin, lutein, and β-carotene, on ROS production. We used these antioxidants at concentrations as previously demonstrated to be effective in various assays [11, 18, 19]. We examined their effect on VBL and CIS, which increased ROS production in the above assays.

As shown in Fig. 4a–f, these antioxidative compounds exhibited various degrees of antioxidative effects. NAC showed the most potent inhibition on ROS production; ROS production was decreased by a quarter in prostate cancer cells. VBL or CIS did not further increase ROS production in the presence of NAC at either 37 or 42°C, suggesting the ROS production by these anticancer drugs was completely suppressed by NAC. Thus, NAC showed