ORIGINAL ARTICLE

Xanthine Oxidase Inhibition by Febuxostat in Macrophages Suppresses Angiotensin II-Induced Aortic Fibrosis

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BACKGROUND
Several reports from basic researches and clinical studies have suggested that xanthine oxidase (XO) inhibitors have suppressive effects on cardiovascular diseases. However, the roles of a XO inhibitor, febuxostat (FEB), in the pathogenesis of vascular remodeling and hypertension independent of the serum uric acid level remain unclear.

METHODS
To induce vascular remodeling in mice, angiotensin II (Ang II) was infused for 2 weeks with a subcutaneously implanted osmotic minipump. FEB was administered every day during Ang II infusion. Aortic fibrosis was assessed by elastica van Gieson staining. Mouse macrophage RAW264.7 cells (RAW) and mouse embryonic fibroblasts were used for *in vitro* studies.

RESULTS
FEB suppressed Ang II-induced blood pressure elevation and aortic fibrosis. Immunostaining showed that Ang II-induced macrophage infiltration in the aorta tended to be suppressed by FEB, and XO was mainly colocalized in macrophages, not in fibroblasts. Transforming growth factor-β1 (TGF-β1) mRNA expression was induced in the aorta in the Ang II alone group, but not in the Ang II + FEB group. Ang II induced α-smooth muscle actin-positive fibroblasts in the aortic wall, but FEB suppressed them. XO expression and activity were induced by Ang II stimulation alone but not by Ang II + FEB in RAW. FEB suppressed Ang II-induced TGF-β1 mRNA expression in RAW.

CONCLUSIONS
Our results suggested that FEB ameliorates Ang II-induced aortic fibrosis *via* suppressing macrophage-derived TGF-β1 expression.

Keywords: angiotensin II; aortic fibrosis; blood pressure; febuxostat; hypertension; macrophage.

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Febuxostat (FEB), a xanthine oxidase (XO) inhibitor, is often used in patients with hyperuricemia. XO produces uric acid and hydrogen peroxide from xanthine or hypoxanthine. Thus, XO inhibitors suppress hydrogen peroxide production while also reducing uric acid synthesis. Several reports have suggested that XO inhibitors have suppressive effects on several animal models of cardiovascular diseases. FEB was shown to inhibit plaque formation in the aortae in apolipoprotein E-deficient mice.[1] FEB was also found to suppress renal interstitial inflammation and fibrosis in unilateral ureteral obstructive nephropathy,[2] and another study reported that FEB ameliorated doxorubicin-induced cardiotoxicity in rats.[3] These reports suggest that FEB suppresses inflammatory cell accumulation, inflammatory cytokine expression, and kidney or cardiac fibrosis. In addition, several clinical studies have suggested that XO inhibitors prevent cardiovascular events[4,5] and have the beneficial effects on chronic kidney disease progression and blood pressure in patients with chronic kidney disease with hyperuricemia.[6] However, the roles of FEB in the pathogenesis of vascular remodeling and hypertension independent of the serum uric acid level remain unclear.

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Many reports have suggested that patients with hyperuricemia are at risk for cardiovascular diseases, hypertension, and chronic kidney disease.[7–9] Indeed, some reports have demonstrated the contribution of uric acid to endothelial cell dysfunction.[10–12] However, because uric acid itself has anti-oxidative activity[13] and because hypouricemia decreases renal hemodynamics,[14] a low serum uric acid level can negatively affect the cardiovascular system. On the other hand, several recent reports have suggested that XO acts as an inflammation activator in endothelial cells or macrophages. XO was shown to induce interleukin (IL)-1β secretion *via* NLRP3 inflammasome activation in macrophages,[15] and angiotensin II (Ang II) was shown to induce XO expression in endothelial cells or macrophages.[16,17] Thus, based on these reported beneficial effects of XO inhibitors or FEB on cardiovascular events, XO inhibitors may have beneficial effects independent of the level of uric acid.

In this study, we used a mouse model of hypertension induced by Ang II characterized by aortic fibrosis and macrophage accumulation[18] to investigate the effects of a XO inhibitor, FEB, on the pathogenesis of vascular remodeling independent of the level of uric acid. We found that FEB has beneficial effects on vascular fibrosis and hypertension. Because accumulated macrophages in aortae following Ang II administration expressed high levels of XO, the major target cells of FEB may be macrophages. Our results suggested that FEB inhibited Ang II-induced transforming growth factor (TGF)-β1 expression in macrophages and suppressed fibrotic processes in aortae. FEB may be potentially effective against hypertension accompanied by aortic stiffness in clinical practice *via* its additive effects outside of ameliorating hyperuricemia.

MATERIALS AND METHODS

Details for Materials and Methods section are in the online-only Supplementary Data.

Mouse model of Ang II-induced vascular remodeling

This study was carried out in accordance with the recommendations of "the guidelines for animal experimentation administered by Tokushima University, The Animal Care and Use Committee for Tokushima University." The protocol was approved by the "The Animal Care and Use Committee for Tokushima University." C57BL/6J male mice (8 weeks old) were purchased from CLEA Japan (Tokyo, Japan). The animals were housed in a temperature-controlled room at 25 °C under a 12-hour light/dark cycle, with free access to food and water. Ang II (A9525; Sigma–Aldrich) dissolved in normal saline was continuously infused at 2.0 mg/kg/d for 2 weeks with a subcutaneously implanted osmotic minipump (Alzet model 2002; Alza, Mountain View, CA); the dose used in this study was based on the results of another study[19] and can induce vascular remodeling with blood pressure elevation. Mice without Ang II administration were sham-operated (skin incision). FEB (F0847; Tokyo Chemical Industry, Tokyo, Japan) (10 mg/kg/day, p.o.) suspended in 1% carboxymethylcellulose-normal saline was administered every day during Ang II infusion. The mice were divided into 4 groups: control, Ang II, FEB, and Ang II + FEB (n = 10–15). Systolic blood pressure was measured noninvasively with a computerized tail-cuff system (BP-98A; Softron, Tokyo, Japan) (n = 7–9). Two weeks after Ang II infusion, the mice were anesthetized with an intraperitoneal injection of both pentobarbital sodium (Somnopentyl; Kyoritsu Seiyaku, Tokyo, Japan) (dosage: 50 mg/kg) and xylazine (Selactar; Bayer, Osaka, Japan) (dosage: 10 mg/kg), and 1% lidocaine hydrochloride (Xylocaine; AstraZeneca, London, UK) was injected subcutaneously for local analgesia. The absence of a pedal withdrawal reflex was checked frequently to ensure the adequacy of anesthesia, and more anesthetic was injected if the mice exhibited any signs of pain during the surgery. Aortae were isolated for further analysis at the end of the experiments (qRT-PCR, histomorphology, and immunofluorescence). Blood samples were incubated at 37 °C for 30 minutes and centrifuged at 5,000 rpm for 2 minutes. The supernatant serum was used to measure the XO activity and uric acid.

Cell culture

Rat aortic smooth muscle cells were isolated as previously described.[20] Mouse embryonic fibroblasts were isolated as previously described,[21] and we checked that almost of the isolated cells were fibroblasts by immunofluorescence with fibroblast-specific protein (FSP) 1 antibody. Rat aortic smooth muscle cells and mouse embryonic fibroblasts were cultured in DMEM (08459-35; Nacalai Tesque, Kyoto, Japan). RAW264.7 mouse macrophage cells were purchased from ATCC (Manassas, VA) and were cultured in RPMI1640 (30264-56; Nacalai Tesque). All cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. FEB (100 nM) was added 1 hour prior to Ang II stimulation. For western blotting and MTT assays, the culture medium was changed to serum-free medium 2 days before the stimulation.

Statistical analysis

We tested for normality and equal variance before parametric data analysis. Data are presented as the mean ± SEM. Statistical significance was assessed by 2-way analysis of variance followed by Bonferroni post-hoc testing for multiple comparisons; values of $P < 0.05$ or $P < 0.01$ were considered significant. The number of individual experiments is indicated by n. For the gene expression analysis with Genevestigator, Mann–Whitney's U-test was used for statistical analysis.

RESULTS

FEB suppressed Ang II-induced aortic fibrosis but not medial thickening in mice

Ang II administration did not affect serum XO activity or serum uric acid level. FEB administration suppressed serum XO activity and serum uric acid level in sham-operated as

well as Ang II-infused mice (Figure 1a and Supplementary Figure 1), confirming that the FEB treatment produced its pharmacological effects in mice irrespective of Ang II infusion. FEB did not affect the Ang II-induced heart weight increase (Figure 1b) but suppressed Ang II-induced systolic blood pressure elevation (Figure 1c).

Elastica van Gieson staining showed that FEB suppressed Ang II-induced aortic perivascular fibrosis (red-stained area) but not medial thickening in mice (Figure 1d and 1e). These results suggested that the suppressive effects of FEB on Ang II-induced aortic fibrosis and blood pressure elevation were independent of the circulating uric acid level.

Since smooth muscle cell proliferation is linked to medial thickening in aortae,[22] we also assessed the effects of FEB on aortic smooth muscle cells *in vitro* (Supplementary Figure 2A). FEB did not affect Ang II-induced VSMC proliferation. In

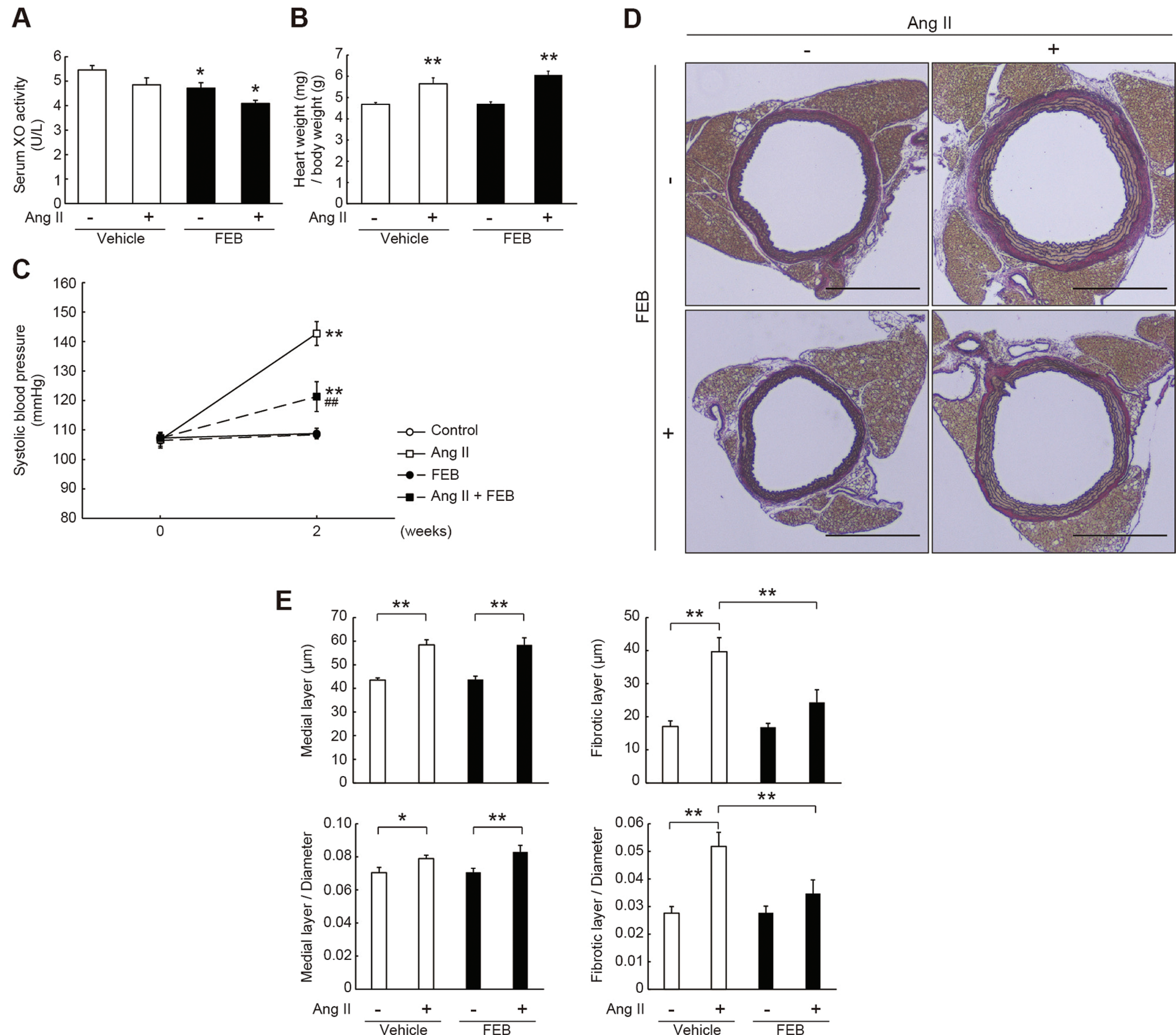

Figure 1. The effects of FEB on Ang II-induced aortic fibrosis and medial thickening. (**a**) FEB administration (10 mg/kg/day, 2 weeks) significantly suppressed serum XO activity in mice. Statistical significance was assessed by 2-way ANOVA, and multiple comparisons were made by Bonferroni's *t*-test. Means ± SEM, *$P < 0.05$ vs. vehicle administration. ($n = 6–8$) (**b**) FEB did not affect Ang II-induced heart weight elevation adjusted for body weight. Statistical significance was assessed by 2-way ANOVA, and multiple comparisons were made by Bonferroni's *t*-test. Means ± SEM, **$P < 0.01$ vs. without Ang II. ($n = 7–9$) (**c**) FEB suppressed Ang II-induced systolic blood pressure elevation. Statistical significance was assessed by 2-way ANOVA, and multiple comparisons were made by Bonferroni's t-test. Means ± SEM, **$P < 0.01$ vs. without Ang II, ##$P < 0.01$ vs. with Ang II alone. ($n = 7–9$) (**d**) EVG staining showed that FEB suppressed Ang II-induced aortic fibrosis (stained red), but not Ang II-induced medial thickening. Scale bar = 0.5 mm. (**e**) Four graphs indicate medial layer (μm), fibrotic layer (μm), medial layer to the diameter, and fibrotic layer to the diameter. Statistical significance was assessed by 2-way ANOVA, and multiple comparisons were made by Bonferroni's t-test. Means ± SEM, *$P < 0.05$, **$P < 0.01$. ($n = 10–15$). Abbreviations: Ang II, angiotensin II; ANOVA, analysis of variance; EVG, elastica van Gieson; FEB, febuxostat; XO, xanthine oxidase.

addition, FEB did not affect Ang II-induced ERK1/2 and AKT phosphorylation, in rat aortic smooth muscle cells (Supplementary Figure 2B,C). These results agreed well with the effect of FEB on Ang II-induced medial thickening.

Under FEB administration, Ang II did not induce vascular inflammation in mouse aortae

To determine how FEB suppressed Ang II-induced aortic fibrosis, we examined inflammation in aortae. F4/80 immunofluorescence staining showed that Ang II induced macrophage accumulation in aortae, whereas it was attenuated by the FEB treatment (Figure 2a). F4/80 mRNA expression was also induced in aortae in mice treated with Ang II alone but not in mice treated with Ang II and FEB (Figure 2b). The mRNA expression levels of monocyte chemotactic protein (MCP)-1, IL-1β and lysozyme M (LysM) were increased in aortae in the Ang II alone group but not in the Ang II and FEB group (Figure 2c). These results suggest that under FEB administration, Ang II does not induce inflammation in aortae.

FEB mainly affected macrophages accumulating in the aortae following Ang II infusion

To determine the target cells of FEB, we used XO immunofluorescence staining of the aorta sections from mice treated with Ang II. The results indicated that cells highly expressing XO were scattered about the adventitia, but the medial area and endothelium had very low levels of XO expression (Figure 3a and Supplementary Figure 3). Some of the XO-expressing cells colocalized with F4/80 (Figure 3a), but not with fibroblast-specific protein (FSP) 1 (Supplementary Figure 3). These results suggest that XO is highly expressed in macrophages and that FEB mainly modulates the function of macrophages accumulating in the aortae upon Ang II infusion.

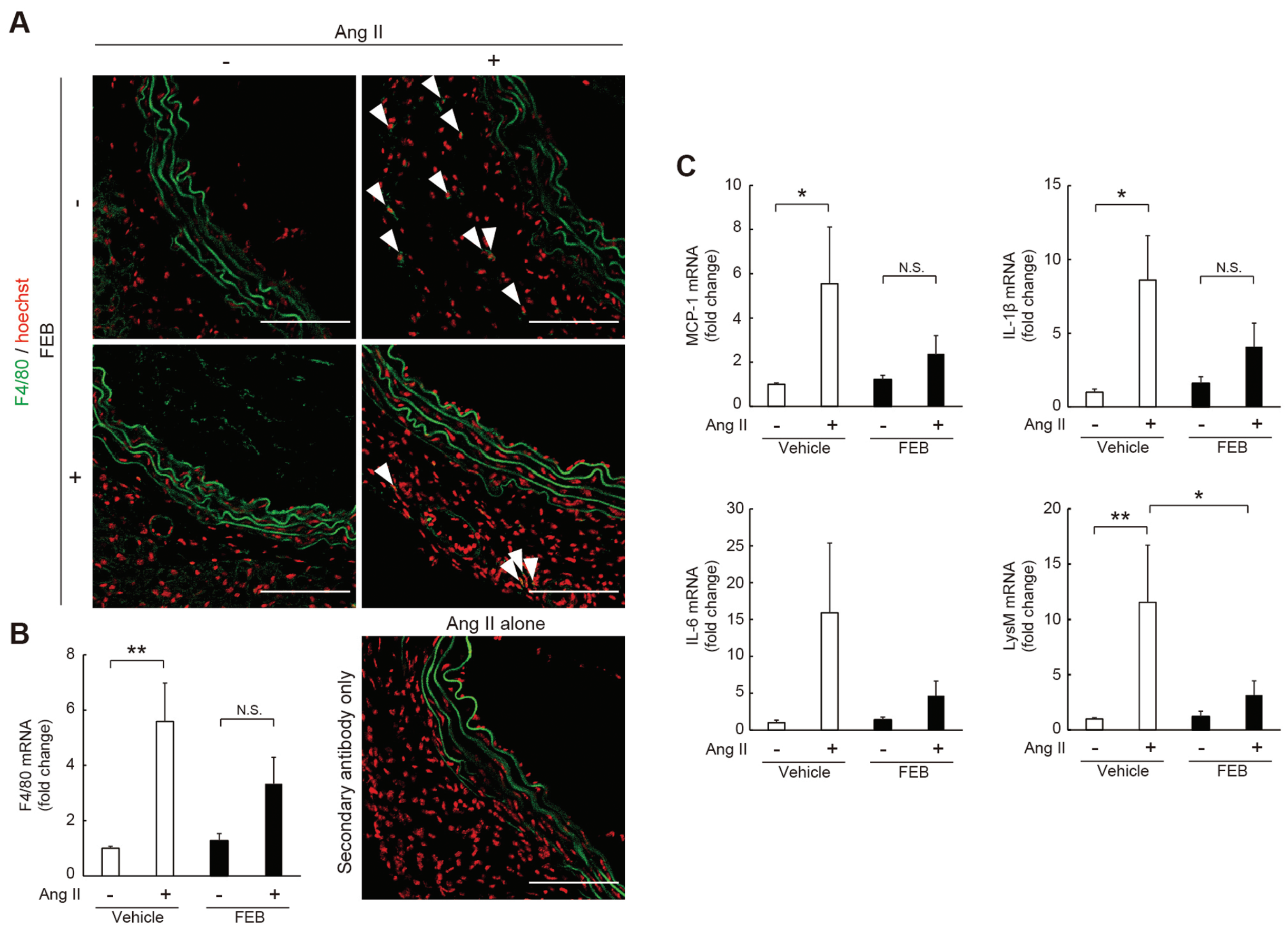

Figure 2. The effects of FEB on Ang II-induced macrophage accumulation and inflammation in aortae. (**a**) F4/80 immunofluorescence staining showed that Ang II induced macrophage accumulation (white arrowheads), but not so much under the FEB administration in the aortic adventitia. Scale bar = 0.1 mm. (**b**) F4/80 mRNA expression was induced by Ang II, but not under the FEB administration in the aortae. Statistical significance was assessed by 2-way ANOVA, and multiple comparisons were made by Bonferroni's t-test. Means ± SEM, $^{**}P < 0.01$. ($n = 8–11$) (**c**) the mRNA expression levels of MCP-1, IL-1β, IL-6, and LysM in the aortae. Statistical significance was assessed by 2-way ANOVA, and multiple comparisons were made by Bonferroni's t-test. Means ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$. ($n = 8–11$). Abbreviations: Ang II, angiotensin II; ANOVA, analysis of variance; FEB, febuxostat; IL, interleukin; LysM, lysozyme M; MCP-1, monocyte chemoattractant protein-1.

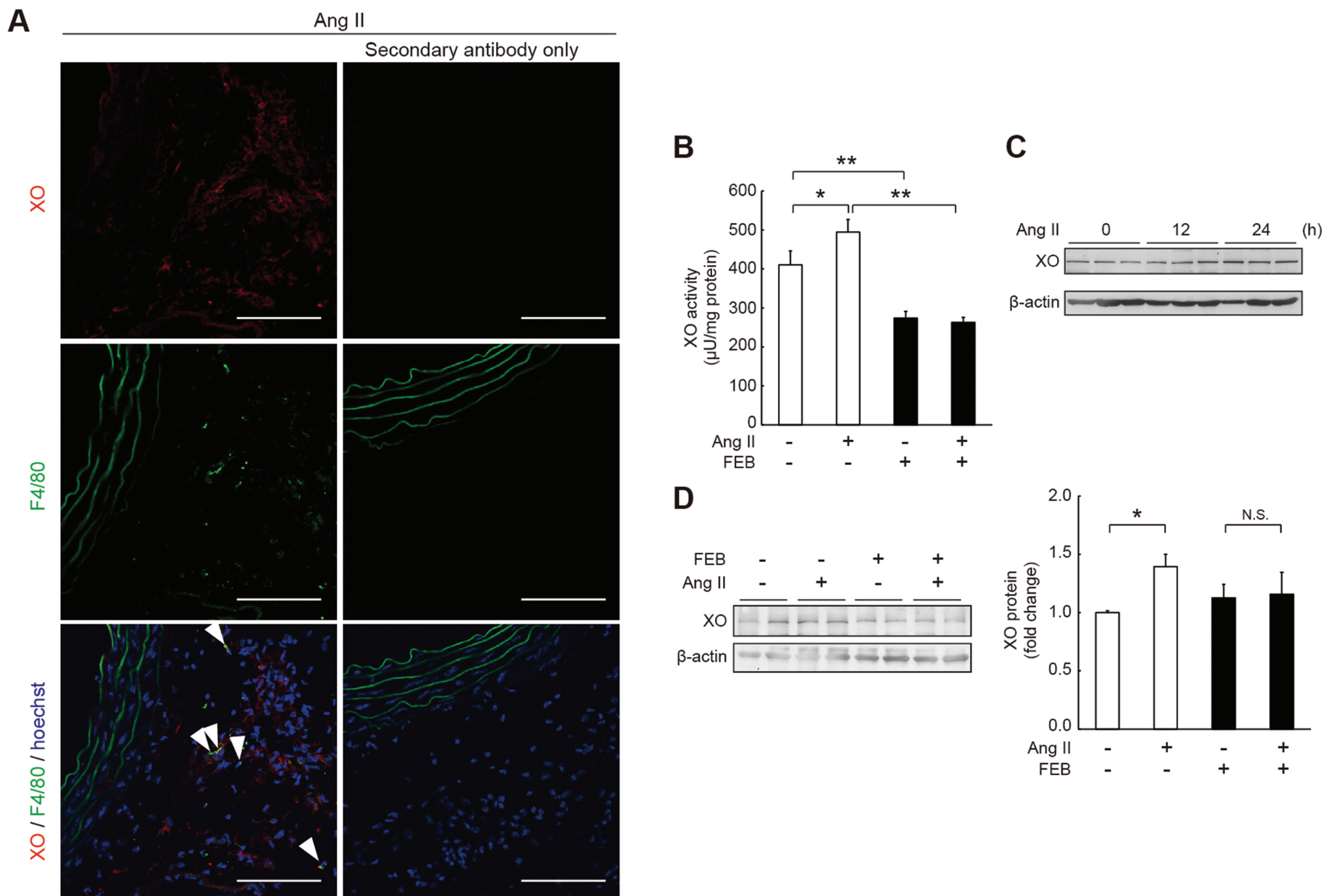

Figure 3. The XO localization in Ang II-administrated aortae and the effects of Ang II on the activity and expression of XO in macrophage cells. (**a**) Some XO high-expressing cells were colocalized with F4/80 positive cells in aortic adventitia (white arrows). Scale bar = 0.1 mm. (**b**) Ang II (1 μM, 24 hours) induced XO activity, and FEB (100 nM) clearly reduced it in macrophage cells, RAW264.7. Statistical significance was assessed by 2-way ANOVA, and multiple comparisons were made by Bonferroni's t-test. Means ± SEM, $*P < 0.05$, $**P < 0.01$. ($n = 3–6$) (**c**) Ang II (1 μM) induced XO expression in RAW264.7. (**d**) Ang II (1 μM, 24 hours) induced XO expression, but not in the presence of FEB (100 nM) in RAW264.7. Statistical significance was assessed by 2-way ANOVA, and multiple comparisons were made by Bonferroni's t-test. Means ± SEM, $*P < 0.05$. ($n = 5–6$). Abbreviations: Ang II, angiotensin II; ANOVA, analysis of variance; FEB, febuxostat; XO, xanthine oxidase.

FEB suppressed Ang II-induced TGF-β1 expression in macrophages

Because our results suggested that FEB mainly affected the accumulated macrophages, we used RAW264.7 macrophage cells in an *in vitro* system. FEB reduced Ang II-induced XO activity in the cells (Figure 3b). We further examined the effect of Ang II and FEB on XO expression. Similar to previous results,[16,17] we found that Ang II induced XO expression (Figure 3c). However, in the presence of FEB, Ang II did not induce XO expression (Figure 3d).

During fibrotic processes, macrophage-derived TGF-β1 plays key roles in fibroblast differentiation into myofibroblasts and extracellular matrix production by fibroblasts and myofibroblasts.[23] FEB significantly suppressed Ang II-induced TGF-β1 mRNA expression in macrophages (Figure 4a). Moreover, in aortic tissue, we found that Ang II administration induced TGF-β1 mRNA expression, but not under FEB administration (Figure 4b).

FEB reduced the number of α-SMA-positive fibroblasts induced by Ang II in the adventitia

Fibroblast differentiation into myofibroblasts expressing α-smooth muscle actin (α-SMA) is a key factor in fibrotic processes.[23] Macrophage-derived TGF-β1 is the primary factor that induces their differentiation.[23] To assess differentiation, we used double immunofluorescence staining of aortae with α-SMA antibody and a fibroblast marker, FSP1 antibody. α-SMA is usually expressed in the medial area; however, in the Ang II alone group, α-SMA-positive cells were also scattered around the outer side of the medial area (Figure 4c). FSP1-expressing cells were found in the outer side of the medial area in the Ang II group and Ang II + FEB group (Figure 4c). In the Ang II alone group, several FSP1-expressing cells, fibroblasts, were α-SMA positive, but almost of the FSP1-expressing cells were α-SMA negative in the Ang II + FEB group (Figure 4c). Thus, FEB reduced the number of α-SMA-positive fibroblasts induced by Ang II in the outer side of the medial area. These results agreed well with

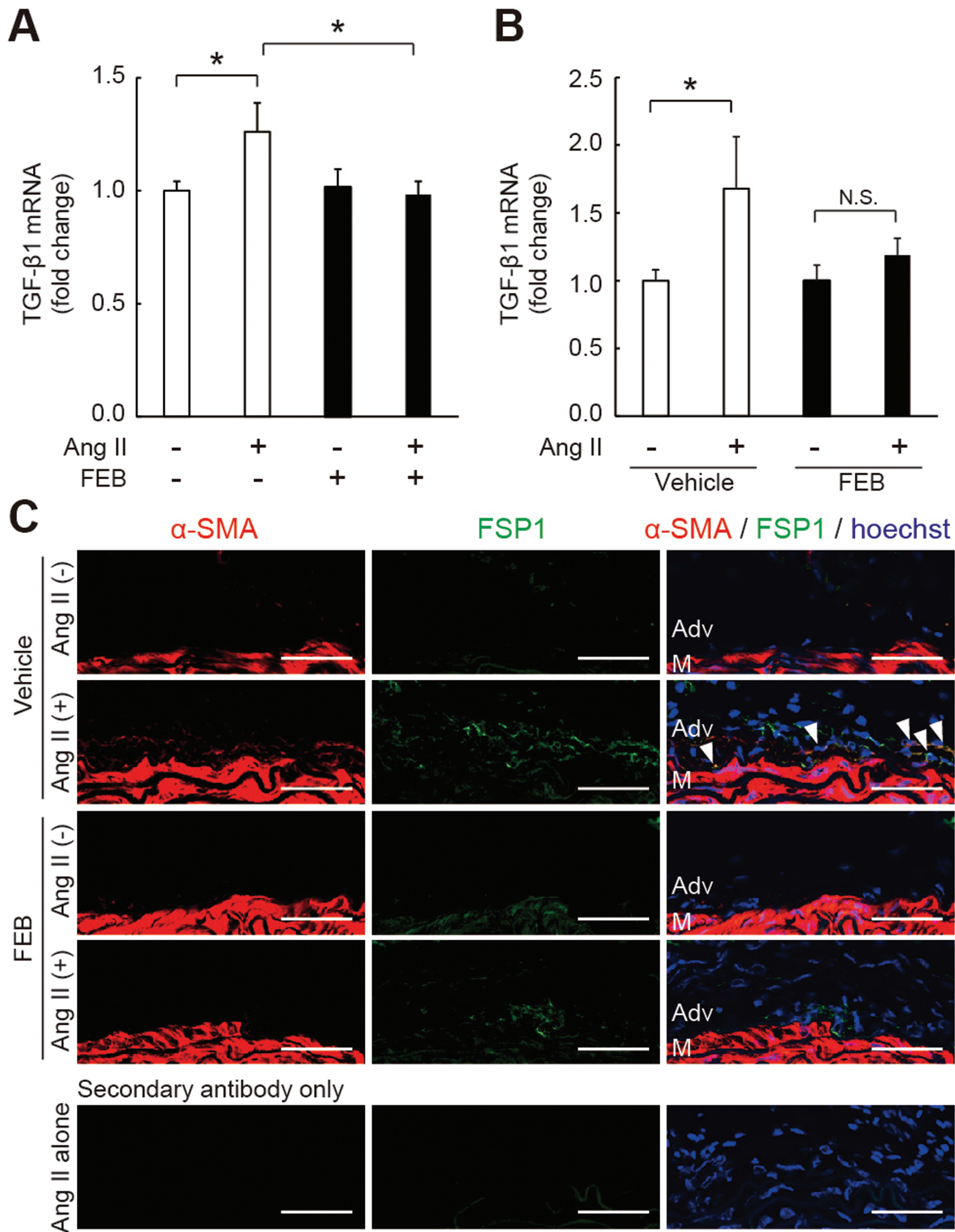

Figure 4. The effects of FEB on Ang II-induced TGF-β1 expression in macrophages. (**a**) Ang II (1 μM, 24 hours) induced TGF-β1 mRNA expression, but not in the presence of FEB (100 nM) in RAW264.7. Statistical significance was assessed by 2-way ANOVA, and multiple comparisons were made by Bonferroni's t-test. Means ± SEM, $^{*}P < 0.05$. (n = 9–12) (**b**) TGF-β1 mRNA expression was induced by Ang II, but not under the FEB administration in the aortae. Statistical significance was assessed by 2-way ANOVA, and multiple comparisons were made by Bonferroni's t-test. Means ± SEM, $^{*}P < 0.05$. (n = 8–11) (**c**) By Ang II administration, FSP1-expressing cells, fibroblasts, were located in the fibrotic area. In Ang II alone group, several fibroblasts were α-SMA positive (white arrowheads), but almost of fibroblasts were α-SMA negative in Ang II + FEB group. Scale bar = 50 μm. Abbreviations: α-SMA, α-smooth muscle actin; Adv, adventitia; Ang II, angiotensin II; ANOVA, analysis of variance; FEB, febuxostat; M, media; TGF, transforming growth factor.

the results indicating the suppressive effects of FEB on Ang II-induced macrophage-derived TGF-β1 mRNA expression.

We also examined the effects of FEB on fibroblasts using mouse embryonic fibroblast in an *in vitro* system. We found that Ang II did not induce TGF-β1 mRNA expression in the fibroblasts and that FEB did not alter this effect (Supplementary Figure 4A). We also found that Ang II slightly induced fibroblast proliferation but that FEB did not affect it (Supplementary Figure 4B). These results suggest that macrophage-derived TGF-β1 expression is a key factor in fibrotic processes and that FEB suppresses Ang II-induced macrophage-derived TGF-β1 expression.

DISCUSSION

The major finding of this study was that a XO inhibitor, FEB, suppressed Ang II-induced vascular fibrosis by affecting mainly the accumulated macrophages in the adventitia. We also found that inhibition of XO by FEB in macrophages suppressed Ang II-induced TGF-β1 expression, which is linked to promoting fibroblast differentiation into myofibroblasts during the fibrotic process. Because we did not find serum uric acid changes in the mouse model of Ang II-induced vascular remodeling we used in this study, these effects of FEB are likely independent of uric acid.

In the present study, FEB suppressed Ang II-induced hypertension, although it did not affect Ang II-induced heart weight elevation. A previous study reported that LysM-positive monocytes mediate Ang II-induced hypertension.[24] Our LysM mRNA expression results in aortae agreed well with this report. Several studies have suggested the beneficial effects of FEB or XO inhibitors on endothelial function and hypertension.[25] Landmesser et al.[16] have demonstrated that Ang II induces reactive oxygen species (ROS) production in part *via* XO activation in endothelial cells. Therefore, FEB might have protective effects in part on endothelial cells in addition to macrophages, which appeared to be the major target of FEB in the present study. The discrepancy between the effects of FEB on hypertension and heart weight suggests that FEB does not affect Ang II signal transduction leading to cardiomyocyte hypertrophy.

Our data suggest that FEB had little effect on SMCs, which is in line with low expression of XO in the aortic media regardless of Ang II infusion. To clarify the mechanisms by which FEB suppressed Ang II-induced aortic fibrosis, we evaluated macrophage accumulation. Both F4/80 immunofluorescence staining and mRNA expression in aortae showed that under FEB administration, Ang II did not induce much macrophage accumulation and the mRNA expression levels of inflammatory cytokines, MCP-1 and IL-1β. These results may partly explain the suppressive effects of FEB on fibrosis.

During the tissue fibrotic processes, macrophage-derived TGF-β1 production plays key roles in extracellular matrix production by fibroblasts or myofibroblasts and fibroblast differentiation into myofibroblasts, which produce more extracellular matrix than normal fibroblasts.[23] Myofibroblasts are active fibroblasts and express α-SMA, whereas normal ones do not. We elucidated that the target of FEB, XO, was highly expressed in accumulated macrophages in aortae and that FEB significantly suppressed Ang II-induced TGF-β1 expression in macrophages. Some reports have suggested that ROS induces TGF-β1 expression,[26] and our preliminary data suggest that FEB can inhibit Ang II-induced ROS production in aortae (Supplementary Figure 5). Thus, FEB might suppress Ang II-induced TGF-β1 expression in part *via* suppressing ROS production. In addition to reduce XO activity, FEB suppressed XO expression induced by Ang II in macrophage cells. Landmesser et al.[16] have demonstrated that Ang II induces XO expression *via* redox-sensitive pathway involving the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Yisireyili et al.[27] have also demonstrated that FEB suppressed the expression levels of NADPH oxidase subunits. Thus, FEB might also suppress expression of XO in part *via* affecting NADPH oxidase.

In the present study, we concluded that FEB suppressed Ang II-induced vascular fibrosis, *via* mainly inhibiting the TGF-β1 expression in the accumulated macrophages in the adventitia, as its additive effects outside of ameliorating hyperuricemia. Our results suggest the possibilities that Ang II/XO/TGF-β1 signaling axis existed in macrophages. In addition, Ives et al.[15] have demonstrated that XO induces IL-1β production *via* mitochondrial ROS generation in macrophages. We showed that under the FEB administration, Ang II did not induce IL-1β expression in aortae. Thus, ROS might be involved in mediating XO effects. FEB is frequently used in patients with hyperuricemia accompanied with metabolic syndrome. These additive effects of FEB can be useful for suppressing the cardiovascular events in patients with hyperuricemia.

SUPPLEMENTARY DATA

Supplementary data are available at *American Journal of Hypertension* online.

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M.I., Y.I.-I., T.T., and K.I. conceived and designed the experiments. M.K., M.I., R.I., Y.M., and Y.H. performed the experiments. M.I., K.F., Y.Z., K.T., M.C., Y.I., H.F. and K.T. analysed the data. This work was supported by JSPS KAKENHI grant number 15K07967 (to K.I.), JSPS KAKENHI grant numbers 16K18884 and 18K14920 (to M.I.), the grant from the Japan Heart Foundation Dr Hiroshi Irisawa & Dr Aya Irisawa Memorial Research Grant (to M.I.), and the grant from Takeda Science Foundation (to M.I.). We appreciate the excellent technical support of the Support Center for Advanced Medical Sciences, Tokushima University Graduate School of Biomedical Sciences.

DISCLOSURE

The authors declared no conflict of interest.

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