Peroxiredoxin 2 Deficiency Exacerbates Atherosclerosis in Apolipoprotein E–Deficient Mice

Jong-Gil Park, Ji-Young Yoo, Se-Jin Jeong, Jae-Hoon Choi, Mi-Ran Lee, Mi-Ni Lee, Jeong Hwa Lee, Hyoung Chin Kim, Hanjoong Jo, Dae-Yeul Yu, Sang Won Kang, Sue Goo Rhee, Mun-Han Lee, Goo Taeg Oh

Rationale: **Peroxiredoxin 2 (Prdx2), a thiol-specific peroxidase, has been reported to regulate proinflammatory responses, vascular remodeling, and global oxidative stress.**

Objective: **Although Prdx2 has been proposed to retard atherosclerosis development, no direct evidence and mechanisms have been reported.**

Methods and Results: **We show that Prdx2 is highly expressed in endothelial and immune cells in atherosclerotic lesions and blocked the increase of endogenous H_2O_2 by atherogenic stimulation. Deficiency of Prdx2 in apolipoprotein E–deficient ($ApoE^{-/-}$) mice accelerated plaque formation with enhanced activation of p65, c-Jun, JNKs, and p38 mitogen-activated protein kinase; and these proatherogenic effects of Prdx2 deficiency were rescued by administration of the antioxidant ebselen. In bone marrow transplantation experiments, we found that Prdx2 has a major role in inhibiting atherogenic responses in both vascular and immune cells. Prdx2 deficiency resulted in increased expression of vascular adhesion molecule-1, intercellular adhesion molecule-1, and monocyte chemotactic protein-1, which led to increased immune cell adhesion and infiltration into the aortic intima. Compared with deficiency of glutathione peroxidase 1 or catalase, Prdx2 deficiency showed a severe predisposition to develop atherosclerosis.**

Conclusions: **Prdx2 is a specific peroxidase that inhibits atherogenic responses in vascular and inflammatory cells, and specific activation of Prdx2 may be an effective means of antiatherogenic therapy. (*Circ Res*. 2011;109: 739-749.)**

Key Words: peroxiredoxin 2 ■ atherosclerosis ■ inflammation ■ VCAM-1 ■ ICAM-1

Oxidative stress by reactive oxygen species (ROS), including superoxide anions and H_2O_2, has been implicated in contributing to the initiation and progression of atherosclerosis.[1] ROS are produced by many endogenous and exogenous contributors, such as hypercholesterolemia, hyperglycemia, hypertension, and shear stress in vascular and inflammatory cells.[2] ROS mediate various signaling pathways that upregulate a number of atherogenic processes, such as monocyte infiltration, platelet activation, smooth muscle cell migration, and cell adhesion.[2] Therefore, increased antioxidant defense against accumulation of oxidative stress has been proposed to retard the development of atherosclerosis.

Peroxiredoxins (Prdxs) are thiol-specific antioxidant proteins found in mammals, yeast, and bacteria and are classified largely on the basis of having either 1 (1-Cys) or 2 (2-Cys) conserved cysteine residues.[3] Among the 6 isoforms of Prdxs, Prdx1 and Prdx2 are the most abundant, constituting a total of 0.2% to 1% of soluble protein in cultured mammalian cells,[3] and exhibit higher affinity toward low concentrations of H_2O_2.[4] Prdx1 is induced by exposing macrophages to oxidized LDL[5] and by laminar shear stress in endothelial cells.[6] In addition, Prdx1 deficiency enhanced the regulated secretion pathway in endothelial cells by promotion of excessive release of several proinflammatory components of Weibel-Palade bodies, such as P-selectin and von Willebrand factor.[7] However, Prdx1 deficiency did not affect transcriptional regulation of receptors such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule-1 (VCAM-1). Prdx2 removes transiently produced H_2O_2 in response to activation of various cell surface receptors.[8]

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From the Division of Life and Pharmaceutical Sciences, Ewha Womans University, Seoul, Republic of Korea (J.G.P., J.Y.Y., S.J.J., M.R.L., M.N.L., J.H.L., S.W.K., S.G.R., G.T.O.); College of Veterinary Medicine, Seoul National University, Seoul, Republic of Korea (J.G.P., M.H.L.); Department of Life Science, College of Natural Sciences, Hanyang University, Seoul, Republic of Korea (J.H.C.); Bio-Evaluation Center, KRIBB, Yangcheongri, Ochangeup, Cheongwongun, Republic of Korea (H.C.K.); Coulter Department of Biomedical Engineering and the Division of Cardiology, Georgia Institute of Technology and Emory University, Atlanta, Georgia (H.J.); and Aging Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea (D.Y.Y.).

Correspondence to Goo Taeg Oh, Division of Life and Pharmaceutical Science, Ewha Womans University, Seoul 120-750, Korea. E-mail gootaeg@ewha.ac.kr

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Non-standard Abbreviations and Acronyms	
ApoE$^{-/-}$	apolipoprotein E deficient
Cys	cysteine residue
GPx	glutathione peroxidase
LC	lesser curvature
MAEC	mouse aortic endothelial cell
Prdx	peroxiredoxin

Prdx2 regulates platelet-derived growth factor (PDGF) signaling, including enhanced activation of the PDGF receptor and phospholipase Cγ1, and vascular remodeling, including PDGF-dependent neointimal thickening of vascular smooth muscle cells.[9] Prdx2 also inhibits general immune cell responsiveness through scavenging low levels of ROS,[10] modulates lipopolysaccharide-induced proinflammatory responses, and protects against endotoxin-induced lethal shock.[11] Consistent with this notion, the roles of Prdx1 and Prdx2 are also apparent in phenotypes of the corresponding knockout mice, including hemolytic anemia and cellular senescence,[12,13] and differences in tissue distribution.[14] A recent report showed that Prdx2 is more susceptible than Prdx1 to hyperoxidation in cells subjected to sustained global oxidative stress,[15] which suggests that Prdx2 deficiency may lead to accelerated atherosclerosis due to failure to eliminate ROS. However, Prdx2 has not yet been reported to be connected to atherosclerosis.

Previous studies showed that dietary antioxidants or antioxidant enzymes can protect against atherosclerosis by reducing oxidative stress.[16–18] However, many antioxidant drugs or agents have shown poor outcomes. Therefore, we focused on identifying the specific antioxidant enzyme responsible for modulating H_2O_2 levels as part of in vivo atherogenic signaling pathways. In the present study, we found that Prdx2 is a specific peroxidase critical for proinflammatory and atherogenic responses in the vasculature.

Methods

Experimental Animals

Animal study protocols were approved by the Animal Care Committee of Ewha Womans University. To generate *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ animals, Prdx2-deficient mice were crossed with apolipoprotein E–deficient (*ApoE*$^{-/-}$) mice. Both strains were *C57BL/6* congenic lines backcrossed more than 10 times with *C57BL/6J* mice. The atherogenic cholate-containing diet contained 0.15% cholesterol, 20% fat, and 0.05% sodium cholate (all wt/wt; Research Diets Inc, New Brunswick, NJ; C12348). Mice were euthanized and hearts and aortas perfused with phosphate-buffered saline (PBS) through the left ventricle. Hearts were embedded in OCT (Sakura, Tokyo, Japan) and frozen on dry ice. Aortas were dissected from the proximal ascending aorta to the bifurcation of the iliac artery, and adventitial fat was removed. For *en face* analysis, aortas were split longitudinally, pinned onto flat black silicone plates, and fixed in 10% (vol/vol) formaldehyde in PBS overnight. Fixed aortas were stained with oil red O for 4 hours, washed with PBS briefly, and digitally photographed at a fixed magnification. Total aortic areas and lesion areas were calculated with AxioVision (Carl Zeiss, Jena, Germany). For analysis of aortic sinus plaque lesions and aortic arch lesions, cryosectioning was performed. Each section was stained with oil red O overnight, and images were digitized. Plasma lipid levels were measured with an automatic blood chemical analyzer (Hitachi, Tokyo, Japan).

Statistical Analysis

Results were analyzed with the Wilcoxon rank sum test for comparison of 2 groups or the Kruskal-Wallis test followed by Wilcoxon rank sum test for multiple comparisons. For matched experiments, the results were analyzed with the Wilcoxon signed rank test.

Supplemental Methodology

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org. For detailed methods related to analysis of atherosclerosis, bone marrow transplantation, infusion protocol for ebselen, cell culture and aortic organ culture, in vitro and ex vivo adhesion assay, in vitro transmigration assay, in vitro transmigration rate measure, en face confocal imaging, quantitative real-time polymerase chain reaction polymerase chain reaction, reagents, and immunostaining, see the online-only Data Supplement.

Results

Prdx2 Is Highly Expressed in Endothelial and Immune Cells in Atherosclerosis-Prone Areas and Regulates Endogenous H_2O_2 Production

To study the expression pattern of Prdx2 in the vasculature, we stained Prdx2 in the aorta from 8-week-old *C57BL/6* and *ApoE*$^{-/-}$ mice (Figures 1A and 1B). Prdx2 was not only expressed in the adventitia region of both mice but was also detected in the endothelial layer of *ApoE*$^{-/-}$ mice predisposed to atherosclerosis (Figure 1A). To focus on specific Prdx2 expression in the endothelial layer in the atherosclerosis-prone condition, we stained for Prdx2 using *en face* aorta preparation. Prdx2 expression in the lesser curvature (LC) was higher than that in the thoracic aorta from *C57BL/6* mice (Figure 1B, upper panels), and Prdx2 expression was upregulated in the LC of atherosclerosis-prone *ApoE*$^{-/-}$ mice compared with normal *C57BL/6* mice (Figure 1B, lower panels). In addition, CD45^{+} cells that infiltrated into the plaque formed in the aortic intima of 60-week-old *ApoE*$^{-/-}$ mice highly expressed Prdx2 (Figure 1C). To identify the effect of endogenous H_2O_2 production associated with Prdx2 deficiency in the vasculature, the aorta segments from 8-week-old *Prdx2*$^{+/+}$ or *Prdx2*$^{-/-}$ mice were incubated with or without tumor necrosis factor-α (TNF-α). As expected, Prdx2 deficiency upregulated the endogenous H_2O_2 level in both basal and inflammation conditions (Figure 1D).

Prdx2 Deficiency Accelerates Atherosclerotic Plaque Formation

To test the effect of Prdx2 deficiency on the development of atherosclerosis, we generated double-knockout mice lacking ApoE and Prdx2 (Online Figure I) and compared plaque formation in *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ control littermates at 2 different stages. The plaque area in the aortic arch was significantly higher in 20-week-old female *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice (16.4%) than in their *ApoE*$^{-/-}$ counterparts (10.4%, $P<0.01$). The same was true of male *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice (9.63% versus 6.41% in *ApoE*$^{-/-}$ males, $P<0.01$; Online Figure II). After a 10-week atherogenic cholate-containing diet, plaque formation was significantly higher in female *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice (19.5%) than in

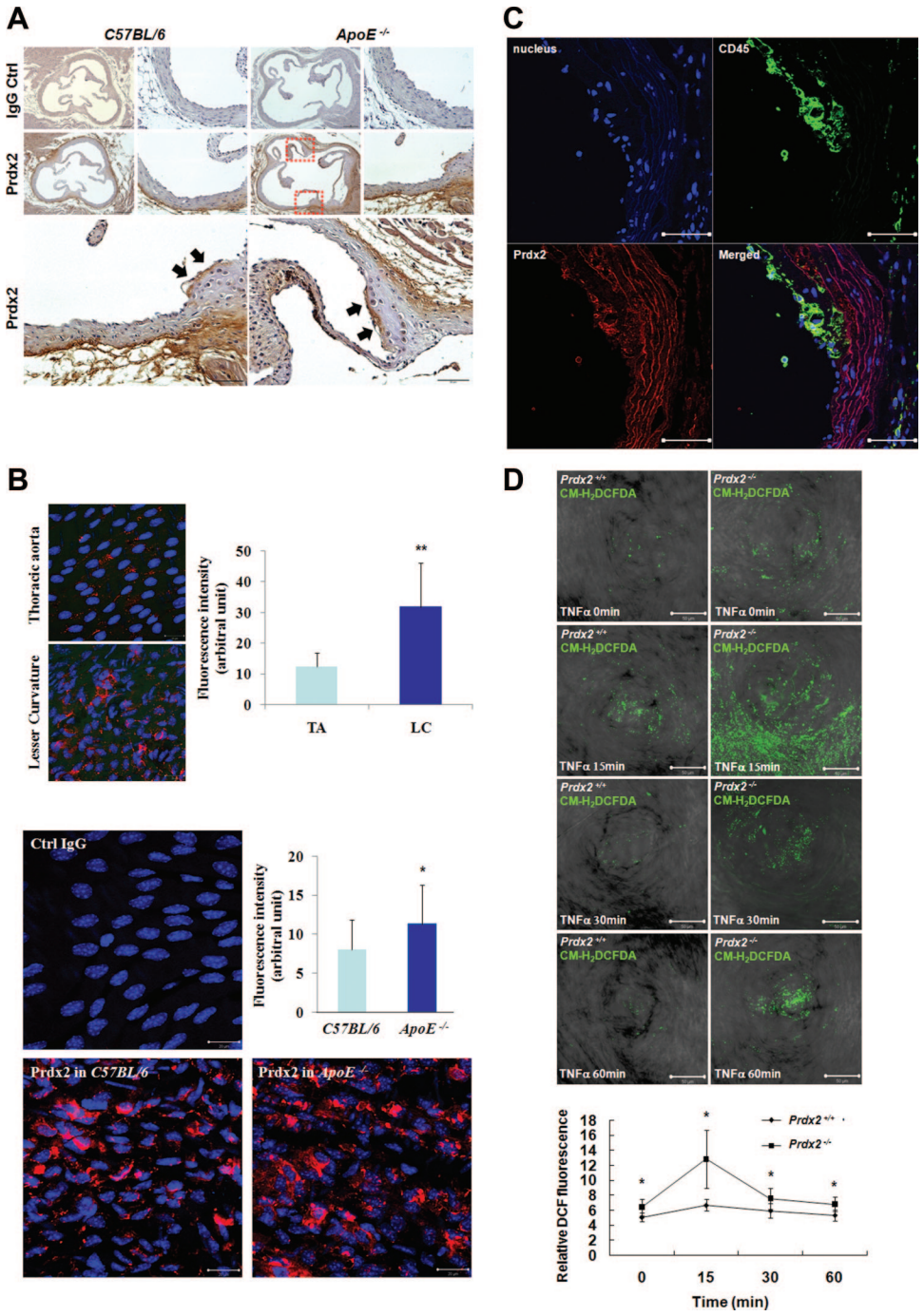

Figure 1. Prdx2 regulates endogenous H_2O_2 production and is highly expressed in endothelial cells in atherosclerosis-prone areas and immune cells. A and **B**, Expression pattern of Prdx2 in the vasculature from 8-week-old *C57BL/6* and *ApoE*$^{-/-}$ mice. **A**, Representative immunostaining of aortic sinus sections. **Scale bars**, 200 μm or 50 μm. Lower panels are higher magnification images of dashed boxes. Prdx2 is expressed in the adventitia region of both mice and the endothelial layer of *ApoE*$^{-/-}$ mice. **Scale bars**, 50 μm. **B**, Representative *en face* immunofluorescence staining of the lesser curvature (LC) of aortic arch and thoracic aorta (TA) from *C57BL/6* mice (n=3) for Prdx2; quantitative graph in upper panel. Representative *en face* immunofluorescence staining of the LC from *C57BL/6* mice (n=5) and *ApoE*$^{-/-}$ mice (n=5); quantitative graph in lower panel. **P*<0.05, ***P*<0.01 compared with counterpart. **Scale bars**, 20 μm. **C**, Representative immunostaining for CD45 (green) and Prdx2 (red) of atherosclerotic plaques in 60-week-old *ApoE*$^{-/-}$ mice. Prdx2 is expressed in CD45^{+} cells on plaques. **Scale bars**, 50 μm. **D**, CM-H_2DCFDA fluorescence image of TNF-α–stimulated aortic tissue. Four isolated pieces of aorta tissue from 8-week-old *Prdx2*$^{+/+}$ (n=6) and *Prdx2*$^{-/-}$ (n=6) mice were incubated with or without TNF-α (10 ng/mL). Quantitative data in the graph represent relative DCF fluorescence intensity. In the endothelial layer of the aortas, intracellular H_2O_2 production was higher in *Prdx2*$^{-/-}$ mice than in *Prdx2*$^{+/+}$ mice. **P*<0.05 compared with counterpart. **Scale bars**, 50 μm.

female *ApoE*$^{-/-}$ mice (8.1%, $P<0.001$; Figures 2A and 2B, left panels). In addition, plaques in the ascending and descending regions of the aortic arch (Figures 2C and 2D) and aortic sinus (Figure 2E) were also significantly larger in *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice than in *ApoE*$^{-/-}$ mice, which expressed Prdx2 abundantly (Online Figure III). Prdx2 deficiency also exacerbated atherosclerosis in male mice, as demonstrated by the significantly larger plaques in *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice than in *ApoE*$^{-/-}$ mice after 10 weeks on the atherogenic cholate-containing diet (Figures 2A and 2B, right panels). However, compared with *ApoE*$^{-/-}$ mice, *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice did not have altered body weight (data not shown), total cholesterol, LDL cholesterol, HDL cholesterol, or triglyceride levels when fed either the normal chow diet or atherogenic cholate-containing diet (Online Table I).

Prdx2 Deficiency Activates Redox-Dependent Signaling

To find mechanisms of accelerated plaque formation induced by Prdx2 deficiency, we tested redox-dependent signaling involving nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and mitogen-activated protein kinases (MAPKs). In association with increased atherosclerotic lesions, 20-week-old *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice displayed enhanced activation of redox-dependent signaling molecules such as p65, c-Jun, c-Jun N-terminal kinases (JNKs), and p38 MAPK, but not extracellu-

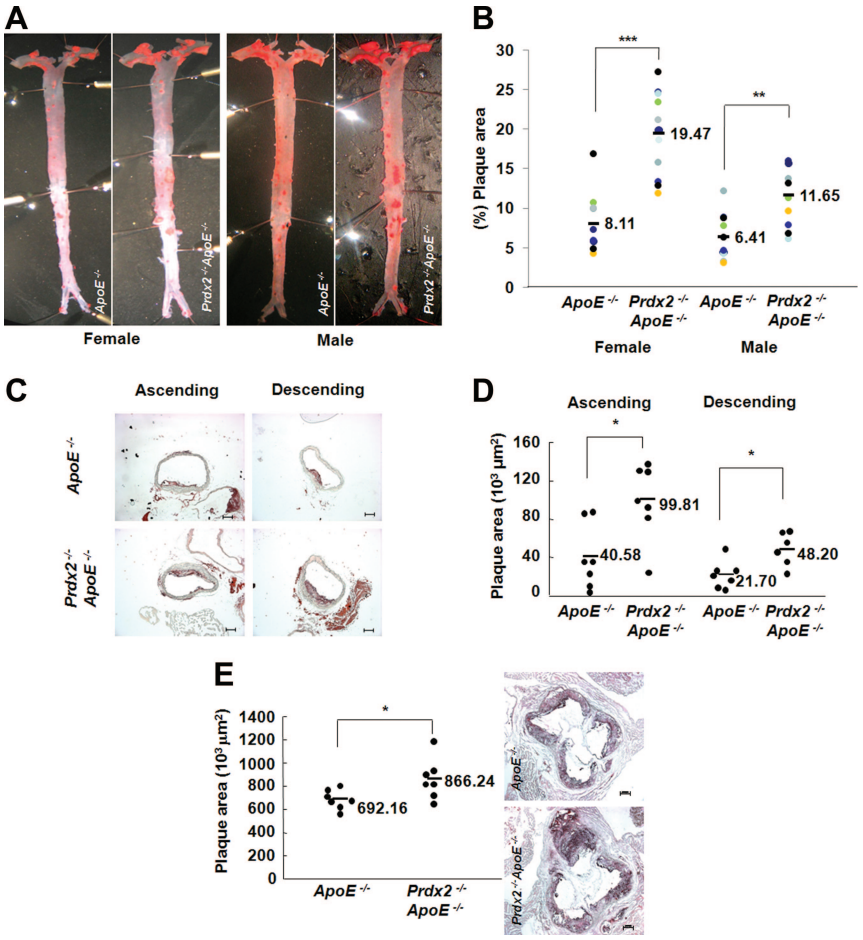

Figure 2. Prdx2 deficiency accelerates atherosclerotic plaque formation in *ApoE*$^{-/-}$ mice. A and **B,** *ApoE*$^{-/-}$and *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice were fed an atherogenic cholate-containing diet for 10 weeks. Representative *en face* images (**A**) showing oil red O–stained plaque areas of aortas and (**B**) percentage of plaque areas (mean±SD; n=10 to 12). ***P*<0.01 and ****P*<0.001. **C,** Frozen sections of ascending and descending aortas of *ApoE*$^{-/-}$ and *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ female mice fed an atherogenic cholate-containing diet for 10 weeks were stained with oil red O; **D**, plaque areas were quantified. Quantitative data (mean±SD; n=6 to 7). **P*<0.05. **Scale bars**, 200 µm. **E,** Atherosclerotic plaques in the aortic sinuses of *ApoE*$^{-/-}$ and *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ female mice. Quantitative data (mean±SD; n=7). **P*<0.05. **Scale bars**, 200 µm.

lar signal-related kinases (ERKs; Figure 3A and B), compared with the control mice, as shown in Online Figure II.

To examine whether the reduction of elevated endogenous H_2O_2 levels associated with Prdx2 deficiency could ameliorate atherosclerotic progression, we administered ebselen[19] to *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice using osmotic pumps. After infusion, the 2 control groups (dimethyl sulfoxide [DMSO]-treated *ApoE*$^{-/-}$ and *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice) and 2 antioxidant-treated groups (ebselen-treated *ApoE*$^{-/-}$ and *Prdx2*$^{-/-}$ *ApoE*$^{-/-}$ mice) were examined and compared after being fed an atherogenic cholate-containing diet. Consistent with Figure 2, the plaque area of the aorta in DMSO-treated *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice was greater than that in DMSO-treated *ApoE*$^{-/-}$ mice. The plaque in *ApoE*$^{-/-}$ mice and *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice was attenuated by treatment with ebselen compared with DMSO-treated *ApoE*$^{-/-}$ and *Prdx2*$^{-/-}$ *ApoE*$^{-/-}$ mice, respectively. Atherosclerotic plaques in ebselen-treated *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice were reduced to a level similar to that in DMSO-treated *ApoE*$^{-/-}$ mice (Online Figure IV).

Exacerbation of Atherosclerosis by Prdx2 Deficiency Is Mediated by Both Vascular and Hematopoietic Cells

To determine whether the increased plaque formation in Prdx2-deficient *ApoE*$^{-/-}$ mice was due to Prdx2 deficiency in either arterial walls or hematopoietic cells, we conducted bone marrow transplantation experiments. Bone marrow from *ApoE*$^{-/-}$ or *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice was transplanted into 4-week-old lethally irradiated *ApoE*$^{-/-}$ or *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice, respectively. After undergoing a 4-week recovery, the mice were fed an atherogenic cholate-containing diet for 10 additional weeks. Successful chimerism of bone marrow cells was confirmed by polymerase chain reaction in blood cells (Online Figure V, A). Comparing recipient mice of identical genotypes, we confirmed that Prdx2-deficient hematopoietic cells accelerated plaque formation compared with Prdx2 wild-type cells. In addition, *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ recipient mice showed significantly more plaque formation than *ApoE*$^{-/-}$ recipient mice when transplanted with identical bone marrow cells (Figures 4A and 4B). To examine the accumulation of immune cells in the plaque of bone marrow transplanted mice, we stained CD45$^+$ cell in aortic sinus of each group of mice. The proportion of the CD45$^+$ area in the plaque was increased by Prdx2-null bone marrow transplantation in both *ApoE*$^{-/-}$ and *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ recipient mice (Online Figure V, B; $P<0.01$, respectively).

Adhesion of Immune Cells on Endothelial Cells Is Enhanced by Prdx2 Deficiency via Increased VCAM-1 and ICAM-1 Expression

Leukocytes attach to endothelial cells that express selective adhesion molecules on their surfaces.[20] In particular, ICAM-1 and VCAM-1 preferentially bind to monocytes and T lym-

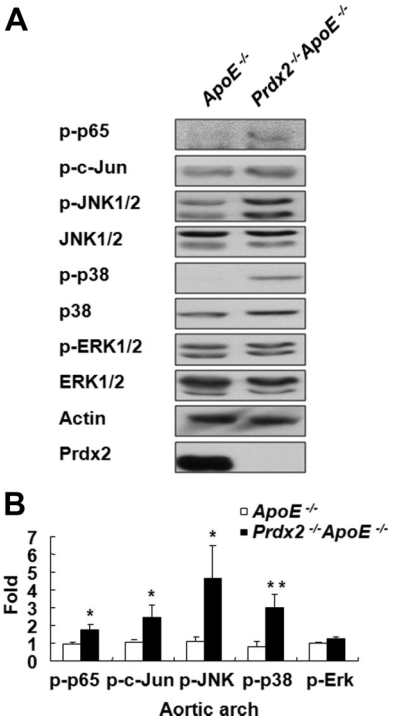

Figure 3. Prdx2 deficiency enhances activation of redox-dependent signaling molecules. A, Western blot analyses of p65, c-Jun, JNK1/2, p38 MAPK, and ERK1/2 in extracts from the aortic arch (pooling at least 3 samples) of 20-week-old $ApoE^{-/-}$ or $Prdx2^{-/-}ApoE^{-/-}$ mice. β-Actin, JNK1/2, p38 MAPK, and ERK1/2 levels were used as loading controls. The blot is representative of 3 independent experiments. **B,** Quantitative data (mean±SD; n=3). *$P<0.05$; **$P<0.01$.

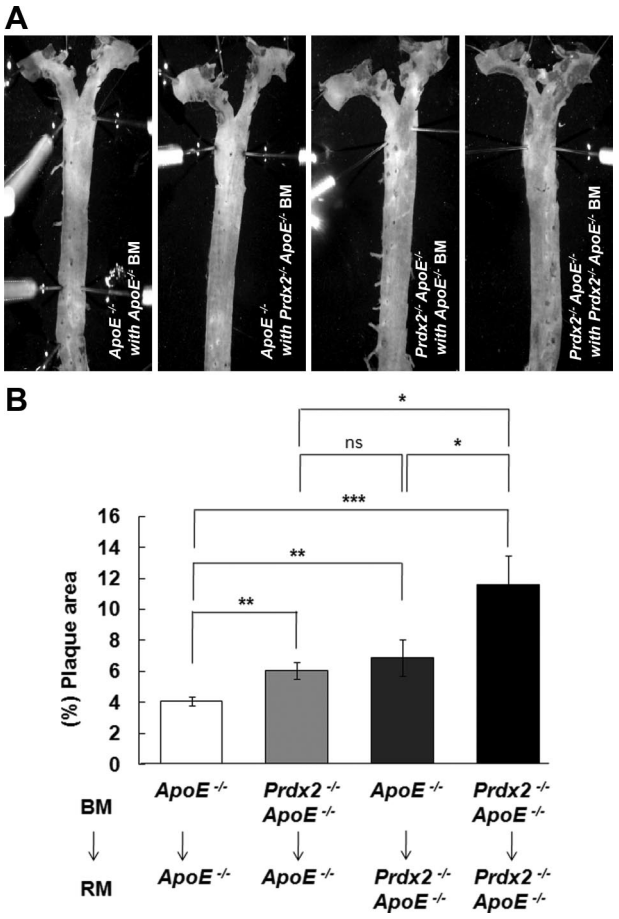

Figure 4. The proatherogenic effects of Prdx2 deficiency are mediated by both vasculature and hematopoietic cells. A, Representative images for oil red O staining of aortas from $ApoE^{-/-}$ or $Prdx2^{-/-}ApoE^{-/-}$ bone marrow (BM)–transplanted $ApoE^{-/-}$ and $Prdx2^{-/-}ApoE^{-/-}$ recipient mice (RM) fed an atherogenic cholate-containing diet for 10 weeks. **B,** Quantitative data (mean±SEM; n=5 to 8). *$P<0.05$, **$P<0.01$, ***$P<0.001$.

phocytes.[21] The aortic arch of 20-week-old $Prdx2^{-/-}$ $ApoE^{-/-}$ mice showed higher expression of VCAM-1 and ICAM-1 than $ApoE^{-/-}$ control littermates (Figures 5A and 5B). Compared with control mice, VCAM-1 expression was elevated in the LC of the aorta, but not in the thoracic aorta, of Prdx2-deficient mice fed an atherogenic cholate-containing diet for 2 weeks (Online Figure VI, upper panels). ICAM-1 expression was higher in both the LC and thoracic aorta of Prdx2-deficient mice than in control mice (Online Figure VI, lower panels). As shown in Figures 5C and 5D, TNF-α–induced expression of VCAM-1 ($P<0.05$) and ICAM-1 ($P<0.01$) was significantly higher in $Prdx2^{-/-}$ aortas than in $Prdx2^{+/+}$ aortas. Importantly, incubation of aortic segments with the antioxidant ebselen[22] for 30 minutes before TNF-α stimulation effectively reduced H_2O_2 levels (unpublished data) and aortic VCAM-1 and ICAM-1 expression in aortas of both $Prdx2^{+/+}$ and $Prdx2^{-/-}$ mice (Figures 5C and 5D, third and tenth lanes), which suggests a role for H_2O_2 in aortic expression of adhesion molecules. Next, we studied signaling pathways that regulate TNF-α–dependent adhesion molecule expression.[22] MAPK inhibitors including the p38 MAPK inhibitor SB203580, JNK inhibitor SP600125, and ERK inhibitor PD98059 only marginally reduced the TNF-α–induced expression of VCAM-1 and ICAM-1 in $Prdx2^{+/+}$ aortas, in contrast to the significant reduction on treatment with the NF-κB inhibitor BAY-11. However, all the inhibitors significantly reduced adhesion molecule expression in $Prdx2^{-/-}$ aortas (Figures 5C and 5D), which suggests their involvement. These results showed that both MAPK activation and NF-κB signaling are important in TNF-α–dependent adhesion molecule expression in $Prdx2^{-/-}$ aortas.

To compare the interaction between monocytes and aortic walls in $ApoE^{-/-}$ or $Prdx2^{-/-}ApoE^{-/-}$ mice, we performed ex vivo adhesion assays with aortas isolated from $ApoE^{-/-}$ and $Prdx2^{-/-}ApoE^{-/-}$ mice.[23] Consistent with the results of the expression of VCAM-1 and ICAM-1, the number of $CD11b^+GFP^+$ monocytes bound to $Prdx2^{-/-}ApoE^{-/-}$ aortas was significantly higher than that bound to $ApoE^{-/-}$ aortas (Figures 5E and 5F). In isolated mouse aortic endothelial cells (MAECs) from $Prdx2^{+/+}$ or $Prdx2^{-/-}$ mice,[23] Prdx2 deficiency increased the expression of adhesion molecules (unpublished data) and binding of $CD11b^+GFP^+$ monocytic cells (Online Figure VII). The blockade of VCAM-1 and ICAM-1 with antibodies reduced monocytic cell binding (Figure 5G).

Prdx2 Deficiency Increases the Accumulation of Immune Cells in Atherosclerotic Lesions

To assess leukocyte transmigration, we examined the effect of Prdx2 deficiency on the expression of monocyte chemot-

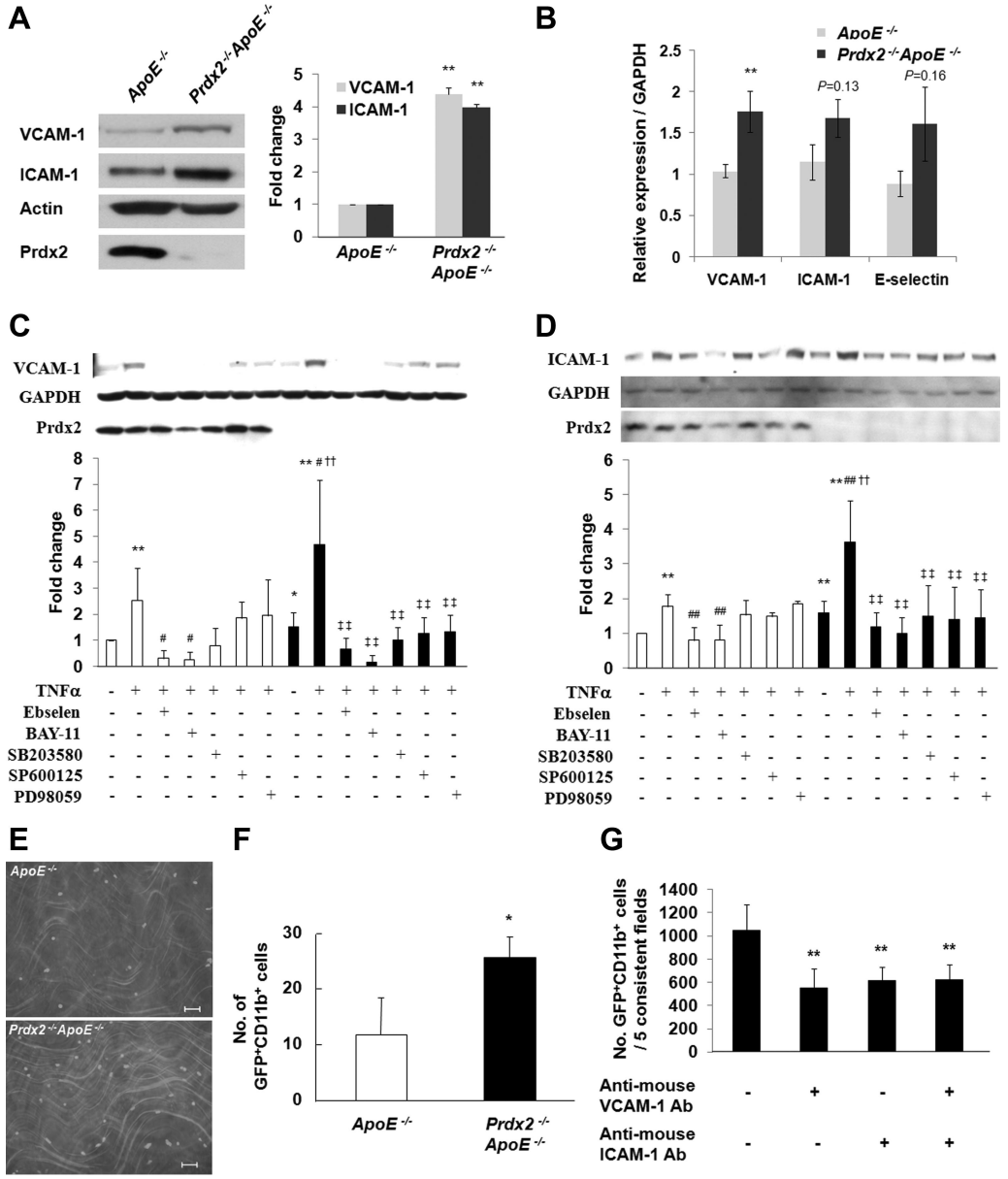

Figure 5. Adhesion of monocytes is enhanced by Prdx2 deficiency via increasing expression of VCAM-1 and ICAM-1. A, VCAM-1 and ICAM-1 expression in aortic arches from $Prdx2^{-/-}ApoE^{-/-}$ mice was higher than in $ApoE^{-/-}$ mice. The blot is representative of 3 experiments. $^{**}P<0.01$ compared with control group. **B,** mRNA levels of VCAM-1, ICAM-1, and E-selectin were increased in the aortic arch of 20-week-old $Prdx2^{-/-}ApoE^{-/-}$ mice compared with $ApoE^{-/-}$ mice (n=3), as determined by quantitative reverse transcription–polymerase chain reaction. $^{**}P<0.01$ compared with control group. **C** and **D,** VCAM-1 (C) and ICAM-1 (D) expression in extracts from the aortas of $Prdx2^{+/+}$ and $Prdx2^{-/-}$ mice. Aortas were treated with or without TNF-α (10 ng/mL) either with or without inhibitors for 12 hours ex vivo. VCAM-1 and ICAM-1 expression in extracts from the aorta of $Prdx2^{-/-}$ mice were increased compared with $Prdx2^{+/+}$ mice by TNF-α stimulation, and inhibitors reduced adhesion molecule expression to basal levels in $Prdx2^{-/-}$ aortas. Quantitative data in **C** and **D** are mean±SD (n=3 to 10) of fold changes. $^{*}P<0.05$ and $^{**}P<0.01$ versus $Prdx2^{+/+}$ control, $\#P<0.05$ and $\#\#P<0.01$ versus $Prdx2^{+/+}$ treated with TNF-α, $\dagger\dagger P<0.01$ versus $Prdx2^{-/-}$ ctrl, and $\ddagger\ddagger P<0.01$ versus $Prdx2^{-/-}$ treated with TNF-α. **E,** Representative ex vivo binding assay of GFP^+CD11b^+ cells to aortas isolated from $ApoE^{-/-}$ or $Prdx2^{-/-}ApoE^{-/-}$ mice (n=4). **Scale bars**, 50 μm. **F,** Number of bound cells in panel E were significantly increased in $Prdx2^{-/-}ApoE^{-/-}$ mice. $^{*}P<0.05$ compared with aortas of $ApoE^{-/-}$ mice. **G,** The number of bound GFP^+CD11b^+ cells on $Prdx2^{-/-}$ MAECs treated with TNF-α for 12 hours with blockade of VCAM-1 and/or ICAM-1. $^{**}P<0.01$ compared with number of GFP^+CD11b^+ cells in MAECs without anti-VCAM-1 and anti-ICAM-1 Ab.

actic protein-1 (MCP-1). MCP-1 was significantly higher in the culture medium of TNF-α–treated aortas from $Prdx2^{-/-}$ mice than in $Prdx2^{+/+}$ controls (Figure 6A, $P<0.05$). The enhanced production of MCP-1 in $Prdx2^{-/-}$ aortas was blocked by ebselen, BAY-11, and MAPK inhibitors (Figure 6B). To test the effect of Prdx2 deficiency on leukocyte transmigration, we performed an in vitro transmigration assay. After MAECs on transwells were stimulated with TNF-α for 6 hours, $CD11b^+$ monocytes were added and incubated for 4 or 18 hours. In Prdx2-deficient MAECs, transmigrated monocytes were increased at both time points (Figures 6C and 6D). Pretreatment with the antioxidant ebselen, SB203580, SP600125, or BAY-11 before TNF-α stimulation on MAECs showed that ebselen and SB203580

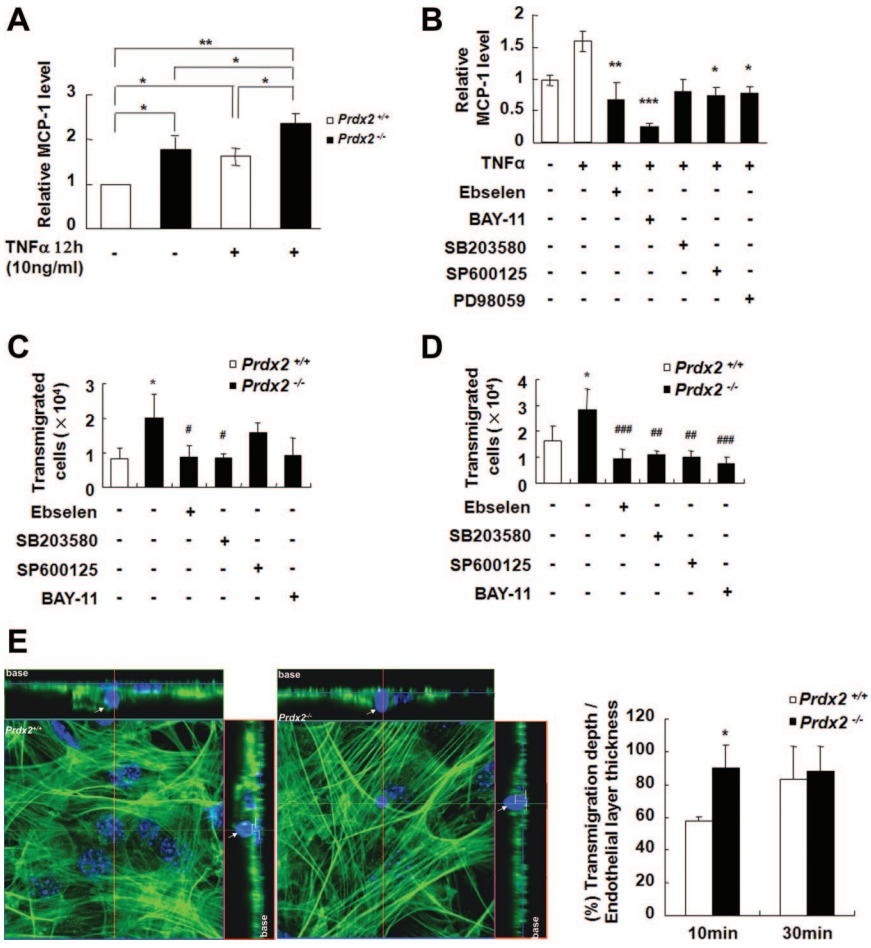

Figure 6. Prdx2 deficiency increases MCP-1 production and leukocyte transmigration. A, TNF-α–induced MCP-1 secretion in isolated aortas from *Prdx2*$^{-/-}$ mice is increased compared with *Prdx2*$^{+/+}$ mice. Isolated aortas were cultured with or without TNF-α (10 ng/mL) for 12 hours and media were analyzed by ELISA (mean±SEM; n=5 to 7). **P*<0.05, ***P*<0.01 compared with control group. **B,** TNF-α–induced MCP-1 secretion by isolated aortas of *Prdx2*$^{-/-}$ mice. Isolated aortas were cultured with or without TNF-α (10 ng/mL) for 12 hours with or without inhibitors. **P*<0.05, ***P*<0.01, ****P*<0.001 relative to *Prdx2*$^{-/-}$ mice treated with TNF-α. **C** and **D,** In vitro transmigration assay. MAECs from *Prdx2*$^{+/+}$ and *Prdx2*$^{-/-}$ mice in transwells were activated by TNF-α (10 ng/mL) for 6 hours. Inhibitors were added to MAECs for 1 hour before TNF-α treatment. After 4 hours (**C**) or 18 hours (**D**), CD11b^{+} monocytes that transmigrated into the low chamber were counted. **P*<0.05 compared with *Prdx2*$^{+/+}$ controls. #*P*<0.05, ##*P*<0.01, ###*P*<0.001 compared with *Prdx2*$^{-/-}$ MAECs without inhibitors. **E,** Representative confocal images for analyzing in vitro transmigration rate after 10 minutes and quantitative graph. Arrow indicates the CD11b^{+} monocyte. Transmigration rate was estimated as percentage of transmigration depth of leukocyte over endothelial layer thickness and was accelerated in Prdx2-deficient MAECs after 10 minutes.**P*<0.05 compared with counterpart.

effectively suppressed the transmigration of CD11b^{+} monocytes at both time points, whereas SP600125 and BAY-11 only inhibited the transmigration of CD11b^{+} monocytes at 18 hours. To investigate whether Prdx2 deletion affects the transmigration rate, we obtained confocal microscopic images and measured transmigration rates by Z stack analysis. TNF-α–stimulated MAECs from *Prdx2*$^{+/+}$ or *Prdx2*$^{-/-}$ mice were incubated with CD11b^{+} monocytes for 10 or 30 minutes, and we confirmed that Prdx2 depletion in MAECs accelerated the transmigration rate of cells after 10 minutes (Figure 6E).

Consistent with the in vitro transmigration studies, plasma levels of MCP-1 were significantly higher in *Prdx2*$^{-/-}$ *ApoE*$^{-/-}$ mice (36.0±1.5 pg/mL) than in *ApoE*$^{-/-}$ mice (32.6±0.9 pg/mL, $P<0.05$), and focal expression of MCP-1 in the plaque was also increased in *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice. After the increased MCP-1 levels, CD45^{+} cells, macrophages, and CD4^{+} cells were more abundant in the plaques of *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice than in *ApoE*$^{-/-}$ mice (Figures 7A and 7B). In addition, TNF-α focal expression in the plaque was more pronounced in *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice than in *ApoE*$^{-/-}$controls (Figure 7A, fifth row, and Figure 7B).

Prdx2 Deficiency Shows More Severe Atherosclerosis Development Than Deficiency of Glutathione Peroxidase 1 or Catalase

To compare the effect of Prdx2 to other major peroxidases such as glutathione peroxidase 1 (GPx1) and catalase on the development of atherosclerosis, we generated double knockout mice lacking ApoE and GPx1 or catalase (Online Figure I). The distributions of Prdx2, GPx1, and catalase in tissues from C57BL/6 mice were confirmed in Online Figure VIII, then we identified that Prdx2 and GPx1 were highly expressed compared with catalase in the aortas. Next, male mice of each group were fed an atherogenic cholate-containing diet for 10 weeks to examine plaque formation at an early stage of atherosclerosis. In spite of Gpx1 deficiency showing increased tendency to develop atherosclerosis, plaque formation in *GPx1*$^{-/-}$ *ApoE*$^{-/-}$ was less than that in *Prdx2*$^{-/-}$ *ApoE*$^{-/-}$ mice (Figures 8A and 8B). Our results from *GPx1*$^{-/-}$ *ApoE*$^{-/-}$ mice are consistent with previous reports that *GPx1*$^{-/-}$ *ApoE*$^{-/-}$ mice developed more atherosclerosis on the Western diet than control *ApoE*$^{-/-}$ mice.[17] *En face* immunofluorescence staining of the corresponding aortic lesion areas was performed. Prdx2-deficient mice showed highly elevated VCAM-1 or ICAM-1 expression in the LC of the aorta compared with GPx1-deficient mice, whereas the expression level in catalase-deficient mice was similar to control mice (Figure 8C).

Discussion

Prdx2 expression is more pronounced in endothelial and immune cells on atherosclerotic-prone regions and plaques. Endothelial and immune cells activated by hyperlipidemia are major contributors to the development of atherosclerosis. We

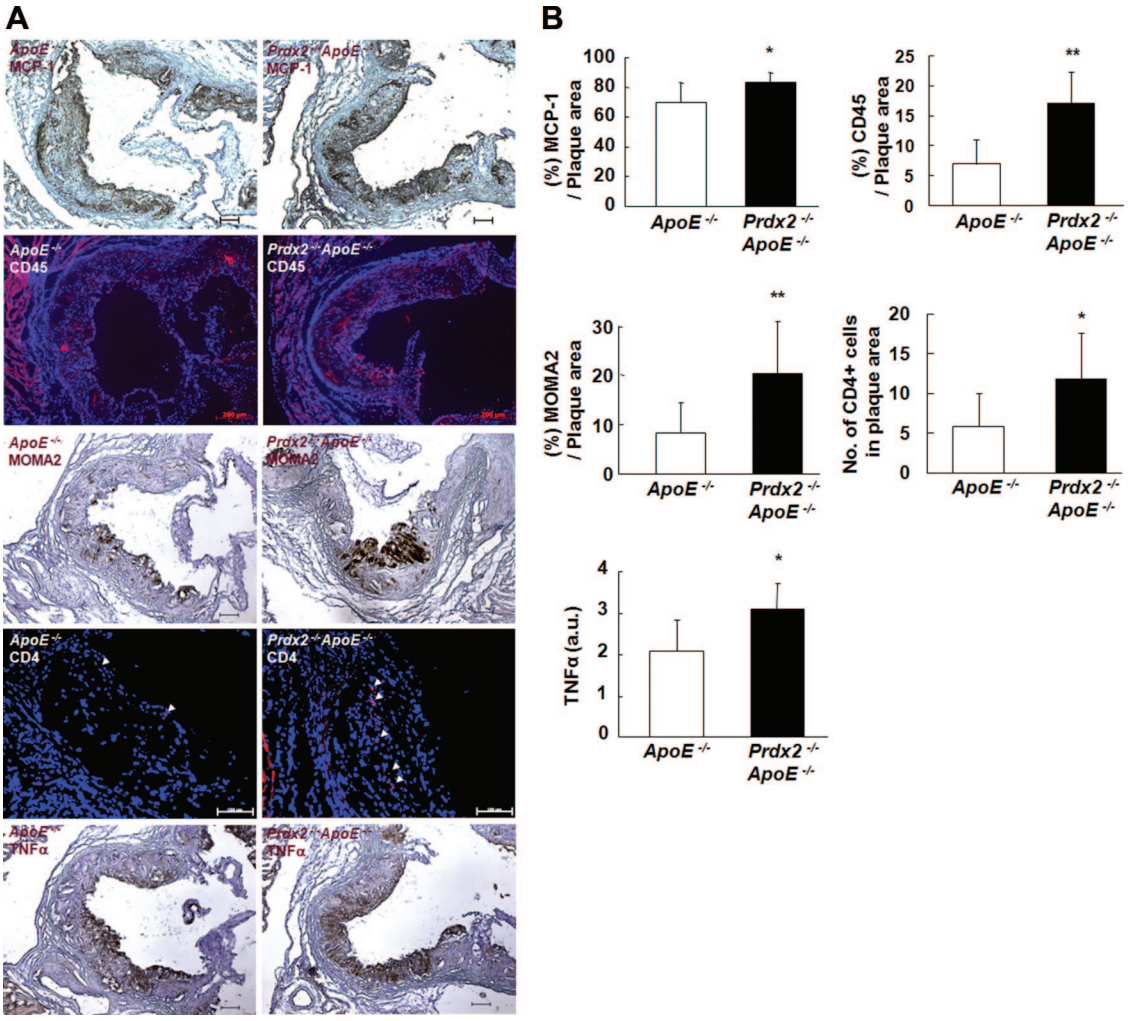

Figure 7. Prdx2 deficiency increases accumulation of inflammatory cells in plaques. A, Representative immunostaining for MCP-1, CD45, MOMA2, CD4, and TNF-α in aortic sinus plaques from $ApoE^{-/-}$ and $Prdx2^{-/-}ApoE^{-/-}$ mice fed an atherogenic cholate-containing diet for 10 weeks. **Arrows** indicate $CD4^{+}$ cells. Nuclei were stained with hematoxylin or DAPI. **Scale bars**, 100 μm. **B,** Quantitative data (mean±SD) in the graph represent the positively stained area percentage of plaque area and show an increased stained area in $Prdx2^{-/-}ApoE^{-/-}$ mice compared with $ApoE^{-/-}$ mice. *$P<0.05$, **$P<0.01$ relative to $ApoE^{-/-}$ mice.

therefore investigated which cells have major roles in accelerating plaque formation in Prdx2-deficient mice. Transplantation of bone marrow into $ApoE^{-/-}$ or $Prdx2^{-/-}ApoE^{-/-}$ mice showed that Prdx2 deficiency in either vascular tissue or blood cells was sufficient to mediate the observed exacerbation of plaque formation. In addition, Prdx2 deficiency in both vascular and blood cells synergistically increased atherosclerotic plaque formation. We also confirmed that accumulation of $CD45^{+}$ cells was significantly increased in the plaques of $ApoE^{-/-}$ and $Prdx2^{-/-}ApoE^{-/-}$ recipient mice transplanted with $Prdx2^{-/-}ApoE^{-/-}$ bone marrow than in those transplanted with $ApoE^{-/-}$ bone marrow. Moreover, the size of the necrotic core in the lesions of $Prdx2^{-/-}ApoE^{-/-}$ recipient mice increased significantly compared with $ApoE^{-/-}$ recipient mice (Online Figure V, B), which probably leads to the increasing CD45 antigen degradation responsible for the reduction in CD45 staining in $Prdx2^{-/-}ApoE^{-/-}$ recipient mice compared with $ApoE^{-/-}$ recipient mice.

Atherosclerosis preferentially develops in branched or curved arterial regions exposed to turbulent flow, including oscillatory shear stress.[24] Turbulent flow induces inflammation via increasing ROS production from NADPH oxidases and accelerates atherosclerosis, including upregulated expression of VCAM-1 and ICAM-1. We showed that Prdx2 is more abundant in the aortic LC exposed to turbulent flow than in the thoracic aorta exposed to undisturbed laminar shear stress, and the expression of VCAM-1 and ICAM-1 was enhanced in the LC of Prdx2-deficient mice. These results suggest that upregulated Prdx2 in the LC has a protective role in inhibiting the expression of VCAM-1 and ICAM-1. Mowbray et al[6] reported that exposure to chronic laminar shear stress upregulated Prdx1 and Prdx5 levels in bovine aortic endothelial cells and suggested that Prdx1 is a novel mechanosensitive antioxidant. On the other hand, our studies showed that Prdx2 levels in the LC were regulated by turbulent flow in mice.

Increased expression of adhesion molecules by activated endothelial cells is a key feature of atherosclerosis.[25] VCAM-1 and ICAM-1 are expressed by endothelial cells in regions predisposed to atherosclerotic lesion formation.[21] We

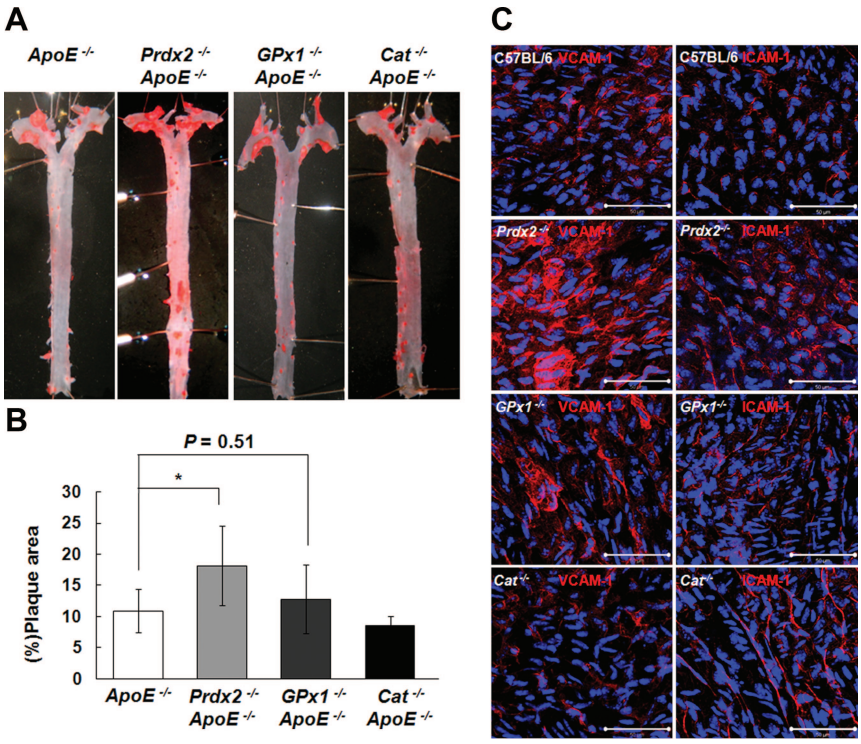

Figure 8. Prdx2 deficiency shows severe predisposition to develop atherosclerosis than deficiency of GPx1 or catalase. A and **B,** *ApoE*$^{-/-}$, *Prdx2*$^{-/-}$ *ApoE*$^{-/-}$, *GPx1*$^{-/-}$*ApoE*$^{-/-}$, and *Cat*$^{-/-}$ *ApoE*$^{-/-}$ male mice were fed an atherogenic cholate-containing diet for 10 weeks. **A**, Representative *en face* images showing oil red O–stained plaque areas of aortas. **B**, Percentage of plaque areas (mean±SD; n=7 to 11). *$P<0.05$. **C,** Prdx2 deficiency increases VCAM-1 and ICAM-1 expression compared with deficiency of GPx1 or catalase in the arterial wall. *En face* immunofluorescence staining of VCAM-1 and ICAM-1 expression in the LC region of aortas from *C57BL/6*, *Prdx2*$^{-/-}$, *GPx1*$^{-/-}$, and *Cat*$^{-/-}$ mice (n=3) fed an atherogenic cholate-containing diet for 2 weeks.

showed that VCAM-1 and ICAM-1 expression is also highly dependent on vessel structure. In endothelial cells, VCAM-1 and ICAM-1 expression is stimulated by ERKs, JNKs, p38MAPK, AP-1, and NF-κB.[26] Consistent with these results, we found that adhesion molecule expression was stimulated by accumulation of H_2O_2 and accompanied by MAPK and NF-κB activation in isolated aortic tissue stimulated with TNF-α. We also showed that MAPKs, NF-κB, and c-Jun were activated by endogenous H_2O_2 in the Prdx2-deficient aorta. In particular, the active, phosphorylated forms of JNK and p38 MAPK were significantly elevated in Prdx2-deficient mice, which suggests that JNKs and p38 MAPK are the major mediators of in vivo atherogenic signaling initiated by H_2O_2.

Leukocyte transmigration has been shown to be regulated by signaling pathways initiated by clustering of ICAM-1 and VCAM-1.[27] ROS participate in these pathways, activating Src and p38 MAPK and inactivating protein tyrosine phosphatases.[27] In Prdx2-deficient endothelial cells, leukocyte transmigration was increased through the upregulation of H_2O_2 and activation of p38 MAPK. To block intracellular H_2O_2 for leukocyte transmigration, Prdx2 null MAECs were treated with ebselen. The present data show that ebselen effectively suppressed the transmigration of $CD11b^+$ monocytes. Others have shown that activation of p38 MAPK was required for ICAM-1–induced cytoskeletal remodeling, such as the generation of actin stress fibers.[27] Here, increasing transmigration in Prdx2-deficient MAECs was inhibited by a p38 MAPK inhibitor, and JNKs and NF-κB inhibitors also reduced leukocyte transmigration at 18 hours. This may be the effect of abrogation on VE-cadherin phosphorylation and junctional separation by the JNK inhibitor[28] and reduced expression of VCAM-1 and ICAM-1 by the NF-κB inhibitor. Taken together, these data suggest that upregulated H_2O_2 stimulates enhanced leukocyte transmigration in Prdx2-deficient endothelial cells via activation of p38 MAPK, JNKs, and NF-κB.

An atherogenic diet that includes cholesterol or cholate components can induce hepatic inflammation, which causes increased complication of atherosclerotic plaque.[29,30] In particular, mice fed an atherogenic diet containing 0.5% sodium cholate increased accumulation of collagen and extracellular matrix components in liver, which induce fibrosis, and expression of genes related to the response to chronic inflammation.[30] Although we used an atherogenic diet that contained low-dose sodium cholate (0.05%), we could not ignore the effects of cholate on an atherogenic cholate-containing diet in *ApoE*$^{-/-}$ mice undergoing hyperlipidemia and inflammatory responses themselves. Therefore, we tested the antiatherogenic effects of Prdx2 in *ApoE*$^{-/-}$ mice fed a normal chow diet and confirmed that deficiency of Prdx2 in *ApoE*$^{-/-}$ mice revealed significantly increased plaque formation compared with control mice (Online Figure II).

An increased antioxidant defense against accumulation of oxidative stress has been proposed to retard the development of atherosclerosis. Ectopic overexpression of catalase, known to remove H_2O_2, has protective effects in cardiovascular disease, such as inhibition of oxidized LDL-induced proliferation of vascular smooth muscle cells[31] and retardation of atherosclerosis in *ApoE*$^{-/-}$ mice.[18] Interestingly, atherosclerosis in *ApoE*$^{-/-}$ mice overexpressing Cu/Zn-superoxide dismutase was comparable to that in *ApoE*$^{-/-}$ control mice. However, catalase deficiency in *ApoE*$^{-/-}$ mice has not been reported to date. In the present study, we found that catalase expression is lower than GPx1 or Prdx2 expression in the vasculature, and catalase deficiency did not affect atherosclerosis

rosis development in $ApoE^{-/-}$ mice. The major isoform of GPx, GPx1, is located in the cytosol and mitochondria. In patients with coronary artery disease, the low activity of red blood cell GPx1 is associated with the risk of cardiovascular events.[32] GPx1 deficiency accelerates the progression of atherosclerosis in $ApoE^{-/-}$ mice.[33] These results showed that $GPx1^{-/-}$ $ApoE^{-/-}$ mice developed significantly more atherosclerosis after 24 weeks, but not at 6 or 12 weeks, on the Western diet than control $ApoE^{-/-}$ mice. Haan et al reported that GPx1 deficiency does not increase atherosclerosis in C57BL/J6 mice fed a high-fat diet[34] or in nondiabetic $ApoE^{-/-}$ mice.[35] The present results showed that deficiency of Gpx1 in $ApoE^{-/-}$ mice accelerated atherosclerosis slightly. Like Prdx2, Prdx6 is located in the cytosol and is expressed in the endothelium and macrophages and regulates oxidative stress and H_2O_2 as a second messenger. Paigen et al[36] reported that Prdx6 deficiency in a B6;129 background showed significantly larger aortic root lesions than in wild-type controls, but there was no difference in other genetic backgrounds, which suggests that Prdx6 does not play a major antiatherogenic role in mice. Furthermore, Prdx6 overexpression in $ApoE^{-/-}$ background did not affect atherosclerosis.[36] However, we still think that the effect of Prdx6 deficiency on atherosclerosis should be conclusively shown in ApoE-deficient mice. Peroxiredoxin 2 function in human atherogenesis has yet to be investigated. In ruptured abdominal aortic aneurysms, the expression of PRDX2 was increased compared with unruptured controls.[37] Although the pathophysiological processes are somewhat different between atherogenesis and aneurysm, the 2 diseases share many common factors, including several inflammatory mediators and oxidative stress. Therefore, research on polymorphisms of the *PRDX2* gene and activity of PRDX2 in human atherosclerosis patients may help to identify a novel biomarker of cardiovascular events.

In summary, Prdx2 was highly expressed in endothelial and immune cells in atherosclerotic lesions and blocked upregulation of endogenous H_2O_2 by proinflammatory cytokines. Deficiency of Prdx2 in $ApoE^{-/-}$ mice accelerated plaque formation after enhanced activation of p65, c-Jun, JNKs, and p38 MAPK. These proatherogenic effects of Prdx2 deficiency were rescued in part by administration of the antioxidant ebselen and mediated not only by vascular cells but also by bone marrow–derived inflammatory cells. Prdx2 deficiency increased the expression of VCAM-1, ICAM-1, and MCP-1. Immune cell infiltration into the plaque and TNF-α production by inflammatory cells were also increased by Prdx2 deficiency. Compared with glutathione peroxidase 1 and catalase, Prdx2 deficiency revealed a severe predisposition to develop atherosclerosis. In conclusion, Prdx2 is a specific peroxidase that inhibits atherogenic responses in vasculature and immune cells, and specific activation of Prdx2 may be an effective means of antiinflammatory and antiatherogenic therapy.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Reactive oxygen species (ROS) has been implicated in the initiation and progression of atherosclerosis.
- Peroxiredoxin 2 (Prdx2), which is an antioxidant enzyme, regulates proinflammatory responses, vascular remodeling, and global oxidative stress; however, little is known about its roles in pathogenesis of atherosclerosis.

What New Information Does This Article Contribute?

- Expression of Prdx2 in endothelial cell is increased in atherosclerosis-prone area and hyperlipidemic mice.
- Prdx2 is a specific antioxidant enzyme that inhibits endogenous ROS level and thereby attenuates the activation of the redox-dependent signaling molecules including p65, c-Jun, c-Jun N-terminal kinases (JNKs), and p38 mitogen-activated protein kinase (MAPK) on atherogenic stimulation.
- Deficiency of Prdx2 in apolipoprotein E–deficient (*ApoE*$^{-/-}$) mice accelerates atherosclerosis by increasing the infiltration of immune cells into plaques after increased expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1).

Although several animal studies show that antioxidants or antioxidant enzymes can protect against atherosclerosis, clinical trials with antioxidant interventions have led to poor therapeutic outcomes Therefore, we focused on identifying the specific antioxidant enzyme responsible for regulating H_2O_2 levels as part of in vivo atherogenic signaling pathways. We show that Prxd2 is highly expressed in vascular endothelial cells in atherosclerosis-prone areas and in immune cells in plaques. Deficiency of Prdx2 in the atherosclerosis-prone *ApoE*$^{-/-}$ mice accelerated atherogenesis. We found that increased activation of redox-dependent signaling molecules enhanced the expression of VCAM-1 and ICAM-1 and increased infiltration of immune cells into plaques. These atherogenic effects by Prdx2 deficiency were rescued by the administering the antioxidant ebselen. The antiatherogenic effects of Prdx2 were found to be important for both vascular and immune cell function, as shown by bone marrow transplantation experiments. Finally, Prdx2 deficiency led to more severe atherosclerosis than the deficiency of glutathione peroxidase 1 or catalase in *ApoE*$^{-/-}$ mice. Our study demonstrates for the first time that Prdx2 is a critical regulator in the pathogenesis of atherosclerosis. Therefore, specific activation of Prdx2 could be an effective approach for developing antiatherogenic therapy.