Original Article

Ablation of the Bach1 Gene Leads to the Suppression of Atherosclerosis in Bach1 and Apolipoprotein E Double Knockout Mice

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This study was designed to determine whether Bach1 gene ablation leads to suppression of atherosclerosis in apolipoprotein E (Apo E)/Bach1 double knockout (DKO) mice. Apo E/Bach1 DKO mice were generated by intercrossing Apo E knockout (KO) and Bach1 KO mice. The animals were fed a high-fat diet for 8 weeks, and the atherosclerotic plaques in the thoracic and abdominal aorta were visualized by oil red O staining. In DKO mice, the total plaque area was reduced by 32% compared with that in Apo E KO mice. In DKO mice, heme oxygenase-1 (HO-1) was upregulated in the endothelium and, to a lesser extent, in vascular smooth muscles. In atherosclerotic plaques in Apo E KO mice and DKO mice, HO-1 was abundantly expressed in the endothelium and macrophages. Urine excretion of 8-iso-prostaglandin (PG) F2 α , a marker for lipid peroxidation, was reduced in DKO mice compared with that in Apo E KO mice. The effects of Bach1 ablation on the plaque area and 8-iso-PG F2 α excretion were almost completely abolished by treating DKO mice with Sn protoporphyrin, an inhibitor of HO activity. Disruption of the Bach1 gene in Apo E KO mice caused inhibition of atherosclerosis through upregulation of HO-1. Inhibition of Bach1, conversely, may be a novel therapeutic strategy to treat atherosclerotic diseases. (*Hypertens Res* 2008; 31: 783–792)

Key Words: Bach1 knockout mice, apolipoprotein E knockout mice, heme oxygenase-1, atherosclerosis

Introduction

Heme oxygenase (HO) is the rate-limiting enzyme catalyzing cleavage of heme into free iron, biliverdin and carbon monoxide (CO). Free iron is subsequently sequestered into ferritin, and biliverdin is rapidly converted to bilirubin (1). HO-1 is the inducible form of the enzyme that is upregulated in response to stimuli such as hypoxia (2) and oxidative stresses (3–5). HO-1 is a cytoprotective enzyme (6) that degrades the

potent prooxidant heme to generate CO and bilirubin. CO is a vasodilatory gas that has anti-proliferative effects on vascular smooth muscle cells (VSMCs) (7) and has anti-inflammatory properties (8). Bilirubin is a scavenger for reactive oxygen species (ROS) (9). In addition, ferrous ion, another product of HO-1, upregulates ferritin, which also has scavenger activity. HO-1 gene-deficient mice display reduced stress defense, a pro-inflammatory tendency (10), and susceptibility to atherosclerotic lesion formation (11). Interestingly, Yachie *et al.* reported a human case of HO-1 deficiency displaying various

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systemic disorders that stemmed from endothelial cell injury (12), suggesting that HO-1 is important for maintaining endothelial function. Supporting a possible association between HO-1 function and atherosclerosis, HO-1 gene delivery into the artery has been reported to ameliorate atherosclerosis (13, 14). Furthermore, in humans, we have recently demonstrated that the capacity to upregulate HO-1 was inversely correlated with the severity of coronary artery disease (15). These observations indicate that the ability to induce HO-1 is a key factor that determines susceptibility to atherosclerosis.

Since HO-1 is an inducible factor, an understanding of the transcriptional mechanism of HO-1 is important. Inducible enhancers of the HO-1 gene carry multiple Maf-recognition elements (MAREs), also known as stress-responsive elements. Heterodimers of the small Maf proteins and NF-E2-related factor 2 (Nrf2), which belongs to the basic leucine zipper (bZip) family of transcriptional factors, activate HO-1 through binding to MAREs (16–19). In contrast, heterodimers of small Maf and Bach1 or Bach2 repress MARE-dependent transcription (20). However, Nrf2 and Bach1 are not equally competitive for binding to MAREs. Under normal conditions, Bach1 dominantly binds to MAREs, almost completely suppressing HO-1 expression. Once cells are subjected to oxidative stress, Bach1 loses its DNA-binding activity, and is exported out of the nuclei, which allows Nrf2 to access MARE to upregulate HO-1 (3, 4, 21). These observations suggest that HO-1 expression is under suppressive control by Bach1 and that inactivation of Bach1 is a key mechanism of HO-1 induction (3, 4, 21). Although the downstream genes controlled by Bach1 have not been fully elucidated, it appears that Bach1 plays a pivotal role in the deployment of cytoprotective programs, including activation of HO-1.

We have recently demonstrated that mice lacking the Bach1 gene showed suppression of neointimal formation after vascular injury *in vivo* (22). Accordingly, proliferative activity of Bach1-deficient vascular smooth muscle cells *in vitro* was suppressed. Deletion of Bach1 in mice causes marked upregulation of HO-1 protein in the cardiovascular system (2, 22), which may in part explain the inhibition of neointimal proliferation. However, the anti-atherosclerotic actions of Bach-deficient macrophages and vascular cells *in vitro* were only partially mediated by HO activity (22). The roles of Bach1 and HO-1 in the mechanism of atherosclerosis remain unclear. In the present study, we investigated the effect of Bach1 gene disruption on the development of atherosclerosis in mice lacking the apolipoprotein E (Apo E) gene, which provides an established model of cholesterol-mediated atherosclerotic lesion formation. The results suggest that the major outcome of Bach1 disruption was upregulation of HO-1, which caused inhibition of atherosclerotic lesion formation.

Methods

Animal Model

All experimental procedures were approved and carried out in accordance with the Guidelines of Hiroshima University Graduate School of Biomedical Sciences. Animals were housed individually under ordinary conditions with a 12-h light/12-h dark cycle. We generated Apo E^{-/-}/Bach1^{-/-} double knockout (DKO) mice by intercrossing Apo E^{-/-}/Bach1^{+/-} (Apo E knockout [KO]) mice and Apo E^{+/-}/Bach1^{-/-} (Bach1 KO) mice. The methods for generating Bach1 KO mice have been reported previously (3). Apo E KO mice were purchased from Jackson Laboratory (Bar Harbor, USA). Both strains of homozygous KO mice were backcrossed onto C57BL/6J (Jackson Laboratory) for at least 10 generations.

Mouse genotypes of Apo E and Bach1 were determined by polymerase chain reaction according to a protocol provided by Jackson Laboratory and a protocol reported previously (23), respectively. After determining the genotypes of all littermates, DKO mice, Apo E KO mice, Bach1 KO mice, and wild-type (WT) mice were chosen and enrolled into the experimental protocol as described below.

Mouse Treatments

Mice were fed a high-fat diet containing 20% fat, 1.25% cholesterol, and 0.5% cholic acid (Oriental Yeast Co., Tokyo, Japan), starting at the age of 5–7 weeks, for 4 or 8 weeks. The mice had ad libitum access to chow and water. The total numbers of mice used for the experiments were 36 DKO mice, 33 Apo E KO mice, 33 Bach1 KO mice and 30 WT mice.

In addition to these 4 groups, the effect of Sn (IV) protoporphyrin IX dichloride (SnPP; Frontier Scientific Inc., Lancashire, UK), an inhibitor of HO activity, was studied in another group of DKO mice on a high-fat diet (*n*=25). SnPP was injected at a dose of 7.5 mg/kg into the peritoneum 3 times per week. SnPP was dissolved in phosphate buffer with 0.1 mol/L NaOH and neutralized with 0.1 mol/L HCl immediately before administration, and the pH was maintained at 7.4 (2).

During the treatment with a high-fat diet, body weight was measured every 4 weeks. Systolic blood pressure and heart rate were measured 8 weeks after starting the high-fat diet by the tail cuff method (2) (BP98A system; Softron, Tokyo, Japan). Serum levels of low-density lipoprotein were determined before and at 4 and 8 weeks after starting the high-fat diet. To analyze the serum lipid level, mice were fasted overnight and blood was collected from the heart.

Atherosclerotic Plaque Area

The extent of atherosclerotic plaque formation in the thoracic and abdominal aorta was examined 8 weeks after starting the

Table 1. Changes in Low-Density Lipoprotein and Body Weight

	Weeks	WT	Bach1 KO	Apo E KO	DKO	SnPP-treated DKO
LDL (mg/dL)		(n=5)	(n=5)	(n=5)	(n=5)	(n=5)
	0	10.8±1.1	10.6±0.6	82.0±11.8*	81.4±8.7*	76.4±8.4*
	4	57.6±8.4	60.0±4.2	600.5±29.5*	672.0±32.5*	577.0±38.9*
	8	45.4±4.0	54.2±7.3	425.3±41.0*	450.2±53.7*	494.0±38.5*
BW (g)		(n=13)	(n=10)	(n=17)	(n=14)	(n=9)
	0	18.7±0.4	18.7±0.4	17.6±0.4	18.1±0.5	18.0±0.9
	4	22.9±0.3	23.0±0.3	19.9±0.5*	19.8±0.6*	19.5±0.9*
	8	26.1±0.3	25.0±0.4	20.6±0.6*	20.1±0.8*	20.2±0.9*

Values are means±SEM. **p*<0.01 vs. WT mice. WT, wild-type; KO, knockout; Apo E, apolipoprotein E; DKO, double knockout; SnPP, Sn (IV) protoporphyrin IX dichloride; LDL, low-density lipoprotein; BW, body weight.

Table 2. Systolic Blood Pressure and Heart Rate after 8 Weeks of High-Fat Diet

	WT	Bach1 KO	Apo E KO	DKO	SnPP-treated DKO
	(n=5)	(n=5)	(n=5)	(n=6)	(n=5)
SBP (mmHg)	119.5±3.2	125.0±3.3	123.7±5.9	119.5±3.2	118.0±2.1
HR (bpm)	556.5±24.9	542.6±18.7	507.1±17.9	514.5±8.2	519.9±33.0

Values are means±SEM. WT, wild-type; KO, knockout; Apo E, apolipoprotein E; DKO, double knockout; SnPP, Sn (IV) protoporphyrin IX dichloride; SBP, systolic blood pressure; HR, heart rate.

high-fat diet. The whole aorta was cleaned, excised, opened longitudinally, and fixed in 4% paraformaldehyde for 2 to 3 d. The vessel was then rinsed, stained with oil red O solution (30 min), rinsed again, and pinned onto a rubber surface. Digital images of the aortic lumen en face under the microscope were captured and analyzed by using SCION image software (<http://www.scioncorp.com>). The total area of red-stained lipid plaques and total surface area of aorta were measured, and [total plaque area/total surface area] × 100 (%) was calculated as an index of atherosclerotic formation (24–26).

Immunohistochemistry

Eight weeks after starting the high-fat diet, mice were perfused first with phosphate-buffered saline (PBS, pH 7.4) and subsequently with freshly prepared 4% paraformaldehyde from the left ventricle. The excised hearts and aortas were post-fixed in the same fixative for 6 h at 4°C. The tissues were then embedded in paraffin, and 4-μm-thick sections were cut. The sections were used for immunohistochemistry to identify the HO-1 with polyclonal rabbit anti-mouse HO-1 antibody (1:200; StressGen Biotechnologies, Victoria, Canada), macrophages with monoclonal rat anti-mouse CD68 antibody (1:400; AbD Serotec, Oxford, UK), and endothelium with monoclonal rat anti-mouse CD31 antibody (1:200; Becton and Dickinson, Sunnyvale, USA). The immunohistochemical signals were detected by the immunoperoxidase method (ABC Elite Kit; Vector Laboratories, Burlingame, USA).

Western Blot Analysis

The amount of HO-1, HO-2 and Bach1 protein in the aorta was analyzed before and at 4 and 8 weeks after starting the high-fat diet. The excised aorta was immediately frozen in liquid nitrogen, homogenized in a buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1 mmol/L PMSF, 0.25 mmol/L sucrose, 0.5% deoxycholate, 2 μg/mL leupeptin and 2 μg/mL aprotinin), and centrifuged for 15 min at 15,000 rpm, and then the supernatant was used for analysis. The detection of HO-1 and HO-2 protein with polyclonal anti-HO-1 and anti-HO-2 antibody (1:1,000; StressGen Biotechnologies) was performed as previously described (2, 27), respectively. The detection of Bach1 protein with polyclonal antiserum against Bach1 antibody (generated by Dr. Kazuhiko Igarashi) was also performed as previously described (4). The amount of HO-1 was semi-quantified in comparison with the amount of β actin (1:5,000; abCAM, Cambridge, UK) as previously described (2).

HO Activity

HO activity in the aorta was determined by measuring bilirubin formation in the entire aorta and expressed as nmol bilirubin formed per h per mg of protein as previously described (2, 28). The bilirubin level was measured by a Beckman DU640 spectrophotometer (450 nm; Beckman Coulter, Fullerton, USA). One sample included 3 aorta tissues from each mouse group.

Fig. 1. *A*: Oil red O staining of the aortas. Beginning at left, representative results are shown for the WT mice, Bach1 KO mice, Apo E KO mice, DKO mice and SnPP-treated DKO mice after 8 weeks of high-fat diet. *B*: The index of atherosclerotic formation was calculated as the ratio of the total plaque area to the total surface area. The index was reduced in DKO mice by 32% ($p < 0.01$) compared with that in Apo E KO mice. Values are the means $\pm$ SEM. * $p < 0.01$ vs. WT mice and Bach1 KO mice; † $p < 0.01$ vs. Apo E KO mice and SnPP-treated DKO mice.

Urinary 8-iso-Prostaglandin F2 α Excretion

The systemic oxidative stress level was estimated from urinary 8-iso-prostaglandin (PG) F2 α excretion. Before and at 4 and 8 weeks after starting the high-fat diet, urine was collected by bladder puncture in overnight-fasted mice. The urinary concentration of 8-iso-PG F2 α was assayed with EIA kits (Assay Designs Inc., Ann Arbor, USA) and standardized by 1 mg creatinine excretion, which correlates well with 24-h excretion level (29–31).

Statistical Analysis

All values are expressed as the means $\pm$ SEM. Comparisons among 4 and 5 groups were made by ANOVA with Fisher's post hoc test (Stat View software program; Abacus Corp., Berkeley, USA). Values of $p < 0.05$ were considered statistically significant.

Results

Changes in the body weight of WT mice, Bach1 KO mice, Apo E KO mice, DKO mice, and SnPP-treated DKO mice

Fig. 2. A: Western blot analysis of Bach1, HO-1 and HO-2 protein expressions in the aorta of each mouse group. Beginning at left, results are shown for the basal level measurement (Week 0) and after 4 and 8 weeks of the high-fat diet. B: Quantifications of HO-1 protein. Values are the means $\pm$ SEM. * p < 0.05 vs. WT mice (Week 0, 4 or 8); † p < 0.05 vs. Bach1 KO (Week 0); § p < 0.05 vs. DKO (Week 0).

during the diet protocol are shown in Table 1. The rates of body weight increase were significantly slower in Apo E KO mice, DKO mice, and SnPP-treated DKO mice than in WT and Bach1 KO mice, indicating a detrimental influence of Apo E gene disruption on mouse growth. However, there was no difference in body weights in the three groups of mice lacking the Apo E gene throughout the study period. Eight weeks after starting the high-fat diet, there was no significant difference in systolic blood pressures and heart rates among the 5 mouse groups (Table 2). In Apo E KO mice, DKO mice, and SnPP-treated DKO mice, the high-fat diet caused marked increases in low-density lipoprotein (LDL) cholesterol levels, reaching approximately 600 mg/dL after 4 weeks, whereas the same diet had no effect on LDL cholesterol level in WT mice and Bach1 KO mice (Table 1). The extents of elevation

in LDL cholesterol level were almost the same in Apo E KO mice, DKO mice, and DKO mice treated with SnPP.

Atherosclerotic Plaque Formation

Oil red O staining of the aorta (Fig. 1A) showed that the high-fat diet for 8 weeks resulted in formation of multiple lipid-rich plaques in Apo E KO mice and, to a lesser extent, in DKO mice, but not at all in WT mice and Bach1 KO mice. The indexes (%) of atherosclerotic formation (Fig. 1B) were 0 ± 0 in WT mice, 0 ± 0 in Bach1 KO mice, 8.6 ± 0.9 in Apo E KO mice, 5.4 ± 0.4 in DKO mice and 9.5 ± 1.0 in SnPP-treated DKO mice. The plaque area was significantly (p < 0.01) reduced by 32% in DKO mice compared with that in Apo E KO mice. These results together with the results of serum

Fig. 3. Heme oxygenase activity in the aorta 4 weeks after starting the high-fat diet. Activity was determined by measuring bilirubin formation in the entire aorta and expressed as nmol bilirubin formed per h per mg of protein. Values are the means $\pm$ SEM. * $p < 0.05$ vs. WT mice; $^{\dagger}p < 0.05$ vs. Bach1 KO mice and DKO mice.

LDL cholesterol level indicate that the deletion of Bach1 significantly inhibited the atherosclerosis resulting from elevated serum LDL cholesterol level.

The total plaque area of DKO mice treated with SnPP was similar to that of Apo E KO mice, suggesting that the inhibition of atherosclerosis by Bach1 disruption was mediated by upregulation of HO-1.

Disruption of Bach1 Caused Upregulation of HO-1 in the Aorta

To confirm that suppression of atherosclerosis in DKO mice was mediated by upregulation of HO-1, the HO-1 protein level and HO activity were evaluated in the aorta. Figure 2 shows the results of Western blot analysis for the protein levels of Bach1, HO-1, and HO-2 in the aorta. Bach1 protein was not expressed in the aorta of Bach1 KO mice and DKO mice. In WT mice and Apo E KO mice, the Bach1 level was constant throughout the experimental protocol. HO-1 protein was upregulated in the aorta of Bach1 KO mice and DKO mice even before starting the diet, indicating that Bach1 disruption caused the elevation of basal HO-1 expression. After initiation of the high-fat diet, HO-1 expression in Bach1 KO mice gradually declined, although the reason for this was unclear. In Apo E KO mice, HO-1 expression tended to increase during the 8 weeks after starting the diet, although the change did not reach the level of statistical significance. In DKO mice, the HO-1 level was as high as that in Bach1 KO mice in the

4th week, and it was further increased in the 8th week after starting the high fat diet. The high fat diet had no effect on HO-1 expression in WT mice. HO-2 levels were not altered by Bach1 deficiency or by the high-fat diet in any mouse group.

Consistent with the upregulation of HO-1 protein in Bach1 KO mice and DKO mice, HO activities (4 weeks after starting the high-fat diet) in the aorta of these two strains were significantly elevated compared with those in WT mice and Apo E KO mice (Fig. 3). Furthermore, the HO activity in SnPP-treated DKO mice was comparable to the level in WT mice, suggesting that SnPP treatment effectively blocked HO activity. The HO activity consists of both HO-1 activity and HO-2 activity. Given that HO-2 expression was constant (Fig. 2), the HO activity was well correlated with the HO-1 protein level (2).

Immunohistochemistry

To further clarify the mechanism of the anti-atherosclerotic action of HO-1 in DKO mice, we investigated the localization of HO-1 within the aorta by immunohistochemistry (Fig. 4). In a plaque-free portion of the aorta either from WT mice or Apo E KO mice, HO-1 protein was expressed in the endothelium but not in the VSMCs. On the other hand, in the aorta of mice lacking Bach1, *i.e.*, Bach1 KO mice and DKO mice, HO-1 protein was expressed both in the endothelium and the VSMC layer (Fig. 4A). In atherosclerotic plaques in Apo E

Fig. 4. *A: HO-1 protein expression in the plaque-free portion of the aorta after administration of a high-fat diet for 8 weeks. Scale bar: 50 μ m. B: HO-1 protein expression in plaque lesions of the aorta after a high-fat diet for 8 weeks in the Apo E KO mice group and DKO mice group at high power magnification. Scale bar: 50 μ m. C: HO-1 protein in plaques and VSMCs of DKO mice (8 weeks after initiation of the high-fat diet). The staining intensity of HO-1 in VSMCs was not homogeneous. Arrows indicate HO-1-positive areas and open arrows indicate the atherosclerotic plaques. Scale bar: 50 μ m. All sections were immunostained using the immunoperoxidase technique and then counterstained with hematoxylin.*

KO mice and DKO mice, HO-1 was localized in the endothelium and in lipid-laden macrophages (Fig. 4B). There were no clear differences in the distribution and intensity of HO-1 staining for HO-1 in the plaques between Apo E KO and DKO mice. The endothelium and macrophages were identified by morphology as well as by staining of CD31 and CD68 (data not shown), respectively, in adjacent sections. In comparing the staining intensity, HO-1 was much stronger in the plaques (open arrow in Fig. 4C) than in the VSMC layer (arrows in Fig. 4C). Furthermore, the HO-1 signals within the VSMCs of DKO mice and Bach1 KO mice (data not shown)

did not show a homogeneous pattern.

Oxidant Stress Level

It has been suggested that increased oxidative stress plays a major role in the development of atherosclerosis in Apo E KO mice (32, 33). In addition, a substantial body of evidence indicates that 8-iso-PG F2 α plays an important role in the progression of arteriosclerosis (34). Thus, we estimated the systemic oxidative stress level in each group of mice by measuring the urinary excretion of 8-iso-PG F2 α (Fig. 5). Before

Fig. 5. Comparison of urinary 8-iso-PG F2 levels before (Week 0) and after 8 weeks (Week 8) of the high-fat diet. Data are corrected by urinary creatinine (mg/dL) levels. Values are the means $\pm$ SEM. * $p < 0.05$ vs. WT mice (Week 0); † $p < 0.05$ vs. DKO mice (Week 8).

initiation of the high-fat diet, urine 8-iso-PG F2 α levels (ng/mg) were 28.3 ± 4.9 in WT mice, 24.5 ± 4.0 in Bach1 KO mice, 26.7 ± 2.8 in Apo E KO mice, 28.6 ± 4.5 in DKO mice and 29.1 ± 5.5 in SnPP-treated DKO mice, with no significant difference among the groups. Eight weeks of a high-fat diet caused significant elevation of urine 8-iso-PG F2 α levels in Apo E KO mice (47.5 ± 6.1) and SnPP-treated DKO mice (46.2 ± 4.1) but not in Bach1 KO mice (26.6 ± 3.9) and DKO mice (34.5 ± 3.9), suggesting that the systemic oxidative stress level was elevated in Apo E KO mice, but deletion of Bach1 inhibited the increase in oxidative stress through a mechanism dependent on HO activation.

Discussion

In the present study we demonstrated that Bach1 gene ablation in Apo E KO mice resulted in inhibition of atherosclerotic plaque formation. Bach1 disruption, either in Bach1 KO mice or DKO mice, caused marked upregulation of HO-1 protein and HO activity in the aorta, and treatment of DKO mice with SnPP, an inhibitor of HO, abolished the suppressive effects of Bach1 ablation on plaque formation. These results indicate that Bach1 normally has an inhibitory effect on transcriptional induction of HO-1 in the arteries and that inhibition of Bach1 may activate HO-1, leading to inhibition of atherosclerosis. Such effects of Bach1 ablation were almost completely abolished by SnPP (Fig. 1A), indicating that HO-1, among the downstream target molecules of Bach1, appears

to be the major effector molecule for protection against atherosclerosis in DKO mice.

In DKO mice, HO-1 protein expression in the aorta was continuously increased during the diet protocol (Fig. 2). A similar tendency was also observed in Apo E KO mice. These results may indicate that the oxidative stress associated with the developing atherosclerosis caused upregulation of HO-1 in the aorta. On the other hand, HO-1 expression was gradually decreased during the diet protocol in Bach1 KO mice, although the mechanism of this effect remains unclear.

We demonstrated that Bach1 deficiency causes upregulation of HO-1. However, it remains to be determined whether the function of Bach1 is altered in the presence of disease. In Apo E KO mice, the Bach1 level was not different from that in WT mice, and it was unaffected by the high-fat diet (Fig. 2) despite the significant elevation of systemic oxidative stress (Fig. 5). It is unlikely that the protein abundance of Bach1 is a simple determinant of HO-1 expression.

In DKO mice, 8-iso-PG F2 α excretion was reduced, whereas this effect of Bach1 ablation was reversed by treatment of mice with SnPP, suggesting that Bach1 ablation resulted in a reduction of systemic oxidative stress through upregulation of HO-1. Increased lipid peroxidation is one of the central mechanisms of atherogenesis in Apo E KO mice (35, 36). Several lines of evidence (10, 11) indicate that the presence of HO-1 is directly associated with resistance to oxidative stress. Thus, it is conceivable that increased capacity to diminish oxidative stress through upregulation of HO-1 may

be responsible for the suppression of atherogenesis in DKO mice. HO-1 upregulation in DKO mice may result in exaggerated heme degradation and reduction of intracellular heme availability, leading to inactivation of heme proteins, including NADPH oxidase, which is thought to be the major source of ROS in vascular cells (37). The catalytic products of HO may also have direct antioxidant activity. Biliverdin and its metabolite by biliverdin reductase, bilirubin, are powerful antioxidants capable of inhibiting the oxidation of LDL (9). Supporting the idea that bilirubin plays a role in the mechanism of atherosclerosis, it has been reported that bilirubin alleviated endothelial dysfunction in the thoracic aortic rings of LDL receptor KO mice fed a high-fat diet, and this effect was associated with restoration of attenuated endothelial nitric oxide synthase (eNOS) expression in the endothelium (38). In addition, several lines of evidence suggest that the coordinated upregulation of ferritin following heme degradation also plays an important role in antioxidant activity of HO-1 (39).

In the aorta of DKO mice, HO-1 expression as evaluated by Western blot analysis was markedly elevated compared with that in the aorta of Apo E KO mice (Fig. 2). To better understand what cell types are responsible for the inhibition of atherosclerosis in DKO mice, we performed immunohistochemical analysis. Immunohistochemical analysis of the aortic portion without plaques revealed that HO-1 was expressed both in the endothelium and VSMCs in DKO mice and only in the endothelium in Apo E KO mice. In the plaques, HO-1 was expressed in the endothelium and macrophages, with the expression level being much greater than that in the normal arterial wall, but there were no differences between the two strains. These observations suggest that upregulation of HO-1 in either or all of the endothelium, macrophages, and VSMCs contributed to the inhibition of atherosclerosis in DKO mice, although the precise mechanisms remain unclear. Interestingly, the HO-1 staining pattern in VSMCs of DKO mice was not homogeneous (Fig 4C), raising a possibility that the plaques occur preferentially in portions of the artery where HO-1 expression is reduced.

It should be noted that Bach1 deletion results in upregulation of HO-1 in various tissues, but the Bach1 KO mouse is not a simple HO-1 overexpression model. In Bach1-deleted animals, HO-1 expression was just de-suppressed, while transcriptional activators such as Nrf2 were functional. Therefore, in DKO mice, the differential regulation of HO-1 expression in response to stress in the endothelium, macrophages, and smooth muscle in the atherosclerotic lesion may be preserved. Bach1 ablation provides a suitable model to study the pure effects of HO-1 activation without administering toxic pro-oxidants such as hemin. Bach1 ablation may also be superior to direct HO-1 gene transfer because the cell type-specific differential expression pattern may be preserved.

In summary, we have demonstrated that the transcriptional factor Bach1 normally suppresses HO-1 expression, thereby

limiting the threshold level of deployment of the protective mechanism against atherogenesis. Although the physiological significance of this function of Bach1 remains unclear, our data suggest that inhibition of Bach1 provides a promising therapeutic option for enhancing HO-1 activity and attenuating atherosclerosis without any prominent disadvantages.

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