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Overexpression of Antioxidant Enzymes in ApoE-Deficient Mice Suppresses Benzo(a)pyrene-Accelerated Atherosclerosis

Hong Yang^{1,*}, LiChun Zhou^{1,*}, Zefen Wang¹, L. Jackson Roberts II², Xinghua Lin¹, Yanfeng Zhao¹, and ZhongMao Guo¹

¹Department of Cardiovascular Biology, Meharry Medical College, Nashville, TN 37208

²Department of Pharmacology, Vanderbilt University, Nashville, TN 37232

Abstract

The carcinogenic polycyclic aromatic hydrocarbon, benzo(a)pyrene (BaP), has been shown to generate reactive oxygen species (ROS) and accelerate the development of atherosclerosis. To assess the causal role of BaP-generated ROS in this process, we evaluated atherosclerotic metrics in apolipoprotein E-deficient (*ApoE*^{-/-}) mice with or without overexpression of Cu/Zn-superoxide dismutase (Cu/Zn-SOD) and/or catalase. Without BaP, aortic atherosclerotic lesions were smaller in *ApoE*^{-/-} mice overexpressing catalase or both Cu/Zn-SOD and catalase than in those overexpressing neither or Cu/Zn-SOD only. After treating with BaP or vehicle for 24 weeks, mean lesion sizes in the aortic tree and aortic root of *ApoE*^{-/-} mice were increased by approximately 60% and 40%, respectively. BaP also increased the levels of oxidized lipids in the aortic tree of *ApoE*^{-/-} mice and increased the frequency of advanced lesions. In contrast, BaP did not significantly alter lipid peroxidation levels or atherosclerotic lesions in the aortas of *ApoE*^{-/-} mice overexpressing Cu/Zn-SOD and/or catalase. Overexpression of Cu/Zn-SOD and/or catalase also inhibited BaP-induced expression of cell adhesion molecules in aortas and endothelial cells, and reduced BaP-induced monocyte adhesion to endothelial cells. These observations, together with the functions of catalase and Cu/Zn-SOD to scavenge hydrogen peroxide and superoxide anions, implicate a causal role of ROS in the pathogenesis of BaP-induced atherosclerosis.

Keywords

Benzo(a)pyrene; atherosclerosis; apolipoprotein E-deficient mice; Cu/Zn-superoxide dismutase; catalase

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants found in cigarette smoke, automobile exhaust and foods cooked at high temperature. Benzo[a]pyrene (BaP) is a representative PAH that has been suggested to cause human diseases, including cancers, cardiovascular, pulmonary and neurodegenerative diseases, and has been implicated

All correspondence should be addressed to: Dr. Hong Yang or Dr. ZhongMao Guo, Department of Cardiovascular Biology, Meharry Medical College, Nashville, TN 37208, Phone: (615) 327-6868, Fax: (615) 327-6655, E-mail: or Hyang@mmc.edu or Zguo@mmc.edu.
*H.Y. and L.Z. contribute equally to this work.

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in infertility and retarded child growth [1]. The atherogenic effect of BaP was studied as early as the 1970s when Albert and colleagues demonstrated that chronic exposure to BaP resulted in large, focal, fibromuscular lesions in the abdominal aorta of chickens [2]. Their studies showed that BaP not only induced lesions, but also increased their size. Later studies confirmed the dose-dependent increase in plaque size in BaP-treated chickens [3], White Carneau pigeons [4], fat-fed mice [5] and apolipoprotein E-deficient (*ApoE*^{-/-}) mice [6].

In mammalian cells, BaP is metabolized to a number of reactive intermediates, including BaP-dihydrodiol, BaP-quinones and BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) [7]. Among these metabolites, BPDE is capable of binding covalently to DNA to form BPDE-DNA adducts, considered as a crucial initial step in the sequence leading from the generation of mutations to uncontrolled cell growth and tumor formation [8]. Animal studies have demonstrated that cells in the arterial wall are targets for BaP-induced bulky DNA adducts [6]. Bulky DNA adducts also have been found in atherosclerotic lesions in cigarette-smoking humans [9] and in animals exposed to BaP and other PAHs [10]. Taken together, these observations have led to the postulate that induction of DNA damage/mutations in vascular cells is a mechanism by which BaP and other PAHs initiate atherogenesis. However, evidence from recent studies indicates that induction of DNA damage/mutations cannot fully explain the atherogenic effect of BaP because there is a poor correlation between plaque formation and mutagenicity in BaP-exposed animals.

Currently, atherosclerosis is viewed as an inflammatory process driven, at least in part, by reactive oxygen species (ROS) [11]. BaP is known to generate superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) via one-electron redox cycling of its metabolite, BaP-quinone [12]. The goal of the current report is to study the role of ROS in BaP-induced atherosclerosis, using *ApoE*^{-/-} mice overexpressing Cu/Zn-superoxide dismutase (Cu/Zn-SOD) or catalase alone, and those that doubly overexpress Cu/Zn-SOD and catalase. Cu/Zn-SOD is a protein that converts $O_2^{\cdot-}$ to H_2O_2 , while catalase destroys H_2O_2 by converting it to water. Our data demonstrate that BaP treatment significantly accelerates the development of atherosclerosis in *ApoE*^{-/-} mice that do not overexpress Cu/Zn-SOD and/or catalase, but not in *ApoE*^{-/-} mice overexpressing these antioxidant enzymes. These findings suggest that both $O_2^{\cdot-}$ and H_2O_2 contribute to BaP-induced atherogenesis.

2. Materials and Methods

2.1. Animals

Transgenic mice overexpressing Cu/Zn-SOD (*hSod1*Tg) or catalase (*hCat*Tg) were respectively generated by injection of fertilized 57BL/6 embryos with a fragment of human genomic DNA containing either the entire *Sod1* gene or the entire catalase gene as described previously [13]. *ApoE*^{-/-} mice were obtained from the Jackson laboratory (Bar Harbor, ME). These mice were generated by Piedrahita et al. [14] and were backcrossed to C57BL/6 for over 10 generations. In a previous study [15], we used the following breeding strategies to generate *ApoE*^{-/-} mice with overexpression of Cu/Zn-SOD and/or catalase. First, we crossbred *ApoE*^{-/-} mice with *hSod1*Tg or *hCat*Tg mice, which yielded mice heterozygous for knockout of *ApoE* gene and heterozygous for overexpression of human *Sod1* (*hSod1*Tg/*ApoE*^{+/-}) or catalase transgene (*hCat*Tg/*ApoE*^{+/-}). We then bred *ApoE*^{-/-} mice with *hSod1*Tg/*ApoE*^{+/-} or *hCat*Tg/*ApoE*^{+/-} mice to obtain mice homozygous for knockout of *ApoE* gene and heterozygous for overexpression of human *Sod1* (*hSod1*Tg/*ApoE*^{-/-}) or catalase transgene (*hCat*Tg/*ApoE*^{-/-}). Finally, we bred *hSod1*Tg/*ApoE*^{-/-} mice with *hCat*Tg/*ApoE*^{-/-} mice and obtained four lines of mice: 1) mice with a homozygous deletion of the *ApoE* gene (*ApoE*^{-/-}); 2) *ApoE*^{-/-} mice that heterozygously overexpress human *Sod1* (*hSod1*Tg/*ApoE*^{-/-}); 3) *ApoE*^{-/-} mice that heterozygously overexpress human catalase (*hCat*Tg/*ApoE*^{-/-}); and 4) *ApoE*^{-/-} mice that heterozygously overexpress both human *Sod1* and catalase transgenes (*Sod1*+*Cat*Tg/

ApoE^{-/-}). In this report, only male mice were used because data with respect to the effect of sex on atherosclerosis in *ApoE*^{-/-} mice were inconsistent. For example, female *ApoE*^{-/-} mice have variably been shown to have lesser or greater atherosclerotic lesions than their male littermates [16,17]. After weaning, all mice were fed a chow diet containing approximately 5% fat and 19% protein by weight (Harlan Teklad, Madison, WI).

In the present study, BaP (B1760; Sigma, USA) was dissolved in olive oil at a concentration of 1 mg/ml. At 6 weeks of age, overnight-fasted mice were treated with 2.5 mg/kg body weight of BaP or an equal volume of oil (control) by oral gavage. This procedure was repeated once per week for 24 consecutive weeks. The mice were fasted overnight and anesthetized with a ketamine and xylazine cocktail, as described previously [18]. The heart and the aortic tree were removed from the body. All animal-handling procedures were approved by the Institutional Animal Care and Use Committees of Meharry Medical College.

2.2. Quantification of Atherosclerotic Lesions in the Mouse Aorta

The aorta was cut at a distance of 2 mm from the heart. The distal aorta (2 mm from the heart to the iliac bifurcation) was opened longitudinally using microscissors and pinned flat on a black wax surface in a dissecting pan under a dissecting microscope (SMZ1000, Nikon Instruments Inc., Melville, NY). This *en face* preparation was fixed overnight and stained with Oil-Red-O. The photo-image of the aorta was captured with a CoolSnaps digital camera mounted on the SMZ1000 dissecting microscope. The atherosclerotic lesion area and the total aortic area were measured using a MetaMorph imaging system (Nikon Instruments Inc.).

The proximal aorta attached to the heart was used to prepare cross-sections, as described previously [18]. Briefly, the heart was sectioned transversely immediately below and parallel to a plane formed by the line between atrial leaflets. The lower portion of the heart was discarded. The portion of the heart with the attached aorta was placed on a metal stub using OCT and sectioned from the attached aorta towards the root of the aorta where the aorta valves were attached. Sections (8 μ m) were collected onto two sets of microscope slides, i.e., every other section was collected to one set of the slides. One set of the sections (16 sections for each mouse) was stained with Oil-Red-O to measure the average area (μ m²) of the atherosclerotic lesions, as described previously [18]. The second set of sections was stained with a Masson's trichrome staining kit (Sigma-Aldrich, St. Louis, MO) to visualize the presence of lipid cores and fibrous caps. In the trichrome-stained sections, collagen fibers are stained blue, nuclei are stained black and the cytoplasm is stained red. Lipid core is shown as an acellular area in the atherosclerotic plaque, and fibrous cap is shown as a layer of blue-stained tissue that covers the lipid core [19].

2.3. Measurement of F₂-Isoprostanes and Isofurans in the Mouse Aorta

Aortas were homogenized in ice-cold HPLC-grade water containing 100 μ mol/L butylhydroxytoluene and 1 mmol/L ethylenediamine-tetraacetic acid (EDTA). Total lipids were extracted using the Folch method (chloroform: methanol, 2:1 v/v). The concentrations of F₂-isoprostanes (F₂-IsoPs) and isofurans (IsoFs) in the mouse aorta were quantified by stable isotope dilution gas chromatography/negative ion chemical ionization mass spectrometry (GC/NICI/MS), as described [20].

2.4. Plasma Lipid Analysis

The levels of plasma cholesterol and triglycerides were measured by spectrophotometric quantification using reagents obtained from Sigma Chemical Co. (St. Louis, MO). For measuring cholesterol, the mixture of plasma and cholesterol-reaction reagent was incubated at 37°C for 30 min, and the absorbance was read at 530 nm with a Dynex microplate reader (Thermo Labsystems, Franklin, MA). For measuring triglycerides, the mixture of plasma and

triglyceride-reaction reagent was incubated at 37°C for 10 min, and the absorbance was read at 530 nm. Plasma concentrations of cholesterol and triglycerides were determined based on the absorbance obtained by incubation of the cholesterol and triglyceride standards provided by Sigma.

2.5. Western Blot Analysis

Expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and β -actin protein in mouse aortas was determined by western blotting using specific antibodies obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Aortas were obtained from 10 mice per treatment group. Two aortas were pooled and homogenized in 20 mM Tris-Cl, yielding five samples per group. The homogenates were centrifuged at 14,000 rpm for 10 min at 4°C, and the resulting supernatants containing 15 μ g protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels and transferred to polyvinylidene difluoride membranes. Detection of VCAM-1, ICAM-1 and β -actin was performed according to standard western blotting protocols, using the ECL Western Blotting Detection System (Amersham Biosciences Inc., Piscataway, NJ).

2.6. Adhesion of Mononuclear Cells to Endothelial Cells

Mouse aortic endothelial cells (MAECs) were isolated from *ApoE*^{-/-}, *hSod1Tg/ApoE*^{-/-}, *hCafTg/ApoE*^{-/-} and *Sod1+CafTg/ApoE*^{-/-} mice by an outgrowth technique, as detailed previously [21]. Briefly, mouse mononuclear cells (MNCs) were isolated from the blood of wild-type mice by Histopaque gradient separation and labeled with calcein acetoxymethyl ester provided in the Vybrant Cell Adhesion Assay Kit (Molecular Probes, Eugene, OR) [21]. MAECs were grown to confluence in 96-well plates and treated for 4 h with 1 μ M BaP or culture medium alone (control). The medium was then replaced with serum-free Dulbecco's modified Eagle's medium (DMEM). Fluorescently labeled MNCs (2×10^5 cells/well) were added to wells covered by endothelial cells or wells containing only culture medium. After a 2-min centrifugation at 500 rpm and a 1-h incubation, nonadherent MNCs were removed by two washes with phosphate-buffered saline, and firmly adherent MNCs were lysed with 0.5 N NaOH. Fluorescence was determined with the Fluoroskan Ascent AL fluorometer (Thermo Labsystems, Franklin, MA) with excitation and emission wavelengths of 485 nm and 510 nm, respectively. The number of adherent MNCs to the well was determined by reference to a standard curve generated with known numbers of fluorescently labeled MNCs [21]. The number of MNCs attached to endothelial cells, compared to MNCs attached to the plate alone, was determined by the difference of fluorescence absorption between the wells with and without endothelial cells.

2.7. Endothelial Cell-Surface Expression of VCAM-1 and ICAM-1

Expression of VCAM-1 and ICAM-1 on the surface of MAECs was determined using an enzyme-linked immunosorbent assay (ELISA) [21]. MAECs grown to confluence in 96-well plates were treated for 2 h at 37°C with 1 μ M BaP or culture medium alone (control). After fixing in 1% glutaraldehyde, cells were incubated with a primary antibody against mouse VCAM-1 or ICAM-1, then with a peroxidase-conjugated secondary antibody. The reaction was developed with 100 μ l of a 0.1 mg/ml 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) and 0.003% H₂O₂ solution for approximately 30 min at room temperature. Absorbance at 450 nm was determined with a microplate reader (Dynex Technologies, Chantilly, VA).

2.8. Quantification of ROS Production in MAECs

The levels of peroxy radicals and superoxide anions (O₂^{•-}) were determined using a peroxide-sensitive dye, 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (CDC-H2F diacetate), and a superoxide-sensitive dye, hydroethidine (HE) (Molecular Probes Inc., Eugene, OR). MAECs,

grown to confluence in a black-walled bottom-clear 96-well plate, were incubated for 1 h with 10 $\mu\text{g/ml}$ CDC-H₂F diacetate di(acetoxymethyl) ester or 20 μMHE . After three washes in Hank's buffer, 100 μl serum-free DMEM containing 1 μM BaP was added to each well and incubated for 1 h. The fluorescence was read using a Fluoskan Ascent AL fluorometer with excitation/emission wavelengths of 480/540 nm for CDC-H₂F and 480/590 nm for HE.

2.9. Statistical Analysis

The data are reported as mean $\pm$ standard error of the mean. For the experiments using the 96-well microplate reader, the value for each experiment was averaged from triplicate wells in the same plate (considered as one data point), and five independent experiments were performed per analysis. The differences among *hSod1Tg/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}*, *Sod1+CatTg/ApoE^{-/-}* and *ApoE^{-/-}* mice, and the difference among MAECs obtained from these mice were analyzed by multiple-factor analysis of variance followed by a Student's *t*-test. Differences were considered significant at a *P* value less than 0.05.

3. Results

3.1. Overexpression of Cu/Zn-SOD and/or Catalase Inhibits BaP-Induced Atherosclerosis but Does Not Affect Plasma Lipid Levels

Our previous study showed that the activities of Cu/Zn-SOD and catalase were increased approximately 2.3- and 2.2-fold in aortas obtained from *hSod1Tg/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}* mice, respectively, as compared to *ApoE^{-/-}* (control) mice. The activities of Cu/Zn-SOD and catalase in the aorta of *Sod1+CatTg/ApoE^{-/-}* mice were approximately 2.1-fold higher than *ApoE^{-/-}* mice. In contrast, the activities of other antioxidant enzymes, including Mn-SOD, extracellular-SOD and glutathione peroxidase-1, were not significantly altered in the aorta of *hSod1Tg/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}* and *Sod1+CatTg/ApoE^{-/-}* mice when compared to their *ApoE^{-/-}* littermates. These observations suggest that overexpression of Cu/Zn-SOD and/or catalase does not result in a compensational regulation in other major antioxidant enzymes in the aorta of mice lacking ApoE [15].

Here, we compared the atherosclerotic lesions in these mice with or without BaP treatment. In the absence of BaP treatment, the surface area of the aortic tree covered by atherosclerotic lesions (Fig. 1A and Table 1), and the size of atherosclerotic lesions in the aortic root (Fig. 1B and Table 1) were significantly smaller in *hCatTg/ApoE^{-/-}* and *Sod1+CatTg/ApoE^{-/-}* mice than in *ApoE^{-/-}* mice. In contrast, the size of lesions in *hSod1Tg/ApoE^{-/-}* mice was comparable to that in *ApoE^{-/-}* mice (Fig. 1 and Table 1). Consistent with the observed reduction in lesion size, the overexpression of catalase or combined overexpression of Cu/Zn-SOD and catalase diminished signs of advanced lesions in the proximal aorta of *ApoE^{-/-}* mice, as evidenced by a decrease in the occurrence of fibrous caps and lipid cores (Fig. 1C and Table 1). All aortic root cross-sections obtained from *ApoE^{-/-}* mice showed one or more fibrous caps and lipid cores, whereas only approximately 50% and 20% of the cross-sections obtained from *hCatTg/ApoE^{-/-}* mice showed lipid core and fibrous caps, respectively. Moreover, only ~20% of the sections from *Sod1+CatTg/ApoE^{-/-}* mice showed a lipid core, and none showed fibrous caps (Table 1). These data imply that endogenously produced ROS, especially hydrogen peroxide, contribute to the development of atherosclerosis in *ApoE^{-/-}* mice. These findings are consistent with our previous report [15].

Figure 1 and Table 1 also show that exposure of *ApoE^{-/-}* mice to 2.5 μM BaP for 24 weeks increased the mean lesion size in the aortic tree and the aortic root by about 60% and 40%, respectively. In addition, BaP treatment significantly increased the occurrence of advanced lesions (i.e., the number of fibrous caps and lipid cores) in the aortic roots. These observations are consistent with a report showing that exposure of *ApoE^{-/-}* mice to BaP accelerated the

development of atherosclerosis [6]. In contrast, BaP treatment did not significantly increase the size or severity of atherosclerosis in *hSod1Tg/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}* or *Sod1+CatTg/ApoE^{-/-}* mice (Fig. 1 and Table 1). Thus, the lesion area in the aortic tree and the aortic root, and the number of fibrous caps and lipid cores in the aortic root of the BaP-treated *hSod1Tg/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}* and *Sod1+CatTg/ApoE^{-/-}* mice were significantly reduced compared with those in *ApoE^{-/-}* mice treated with BaP (Fig. 1 and Table 1). Interestingly, the levels of plasma cholesterol and triglycerides were comparable in *hSod1Tg/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}*, *Sod1+CatTg/ApoE^{-/-}* and *ApoE^{-/-}* mice with or without BaP treatment (Table 2). In addition, we observed that 2.5 mg/kg BaP treatment for 24 weeks did not significantly alter the body weight of all the four lines of mice (data not shown).

3.2. Overexpression of Cu/Zn-SOD and/or Catalase Decreases BaP-Induced Lipid Peroxidation in Mouse Aortas

Without BaP treatment, the aortic levels of F₂-IsoPs and IsoFs were significantly lower in *hCatTg/ApoE^{-/-}* and *hSod1+CatTg/ApoE^{-/-}* mice than in *ApoE^{-/-}* mice (Fig. 2). The F₂-IsoPs and IsoFs levels in *hSod1Tg/ApoE^{-/-}* mice trended lower than those in *ApoE^{-/-}* mice; however, the difference was not statistically significant (Fig. 2). BaP treatment significantly elevated the aortic levels of F₂-IsoPs and IsoFs in *ApoE^{-/-}* mice, but not in *hSod1/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}* or *Sod1+CatTg/ApoE^{-/-}* mice (Fig. 2). These results suggest that both O₂^{•-} and H₂O₂ contribute to BaP-induced lipid peroxidation in *ApoE^{-/-}* mouse aortas.

3.3. Overexpression of Cu/Zn-SOD and/or Catalase Reduces BaP-Induced Monocyte Adhesion to Endothelial Cells

Under control conditions, the number of MNCs adhering to MAECs from *hSod1Tg/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}* and *Sod1+CatTg/ApoE^{-/-}* and *ApoE^{-/-}* mice was comparable; in each case, approximately 20–25 MNCs/mm² were firmly adherent to MAECs (Fig. 3). Pretreatment of MAECs with 0.5 μM BaP significantly increased the number of MNCs adhered to MAECs from *ApoE^{-/-}* mice, but did not significantly alter the number of MNCs adhered to MAECs from *hSod1Tg/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}* or *Sod1+CatTg/ApoE^{-/-}* mice. Increasing BaP concentration to 1 μM significantly increased adhesion of MNCs to MAECs obtained from all four lines of mice. However, the magnitude of this increase varied among MAECs. Specifically, the number of MNCs that adhered to *hSod1Tg/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}* and *Sod1+CatTg/ApoE^{-/-}* MAECs was significantly less than that for *ApoE^{-/-}* MAECs. Furthermore, the number of adherent MNCs was less for *Sod1+CatTg/ApoE^{-/-}* MAECs than for *hSod1Tg/ApoE^{-/-}* and *hCatTg/ApoE^{-/-}* MAECs. These results suggest that both O₂^{•-} and H₂O₂ contribute to BaP-induced adhesion of MNCs to MAECs.

3.4. Overexpression of Cu/Zn-SOD and/or Catalase Decreases Expression of Cell Adhesion Molecules

Under control conditions, the expression of VCAM-1 and ICAM-1 proteins on the surface of MAECs was relatively low, and no significant difference was observed among *ApoE^{-/-}*, *hSod1Tg/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}* and *Sod1+CatTg/ApoE^{-/-}* MAECs (Fig. 3). Addition of 1 μM BaP to the culture media increased the expression of VCAM-1 and ICAM-1 on the surface of MAECs obtained from *ApoE^{-/-}* mice by approximately 3-fold (Fig. 3), but did not significantly alter surface expression in MAECs obtained from *hSod1Tg/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}* or *Sod1+CatTg/ApoE^{-/-}* mice. We also compared the expression of VCAM-1 and ICAM-1 in mouse aortas. As shown in Fig. 4, the *hSod1Tg/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}* and *Sod1+CatTg/ApoE^{-/-}* mice had a lower level of VCAM-1 and ICAM-1 proteins in the aorta than did *ApoE^{-/-}* mice in the absence of BaP treatment. Exposure of *ApoE^{-/-}* mice to BaP significantly elevated aortic VCAM-1 and ICAM-1 protein levels; however, BaP treatment did not increase VCAM-1 or ICAM-1 in the aortas of *hSod1Tg/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}* or *Sod1*

+*CatTg/ApoE^{-/-}* mice (Fig. 4). These results provide evidence that overexpression of Cu/Zn-SOD and/or catalase inhibits BaP-induced expression of cell adhesion molecules *in vitro* and *in vivo*.

3.5. Overexpression of Cu/Zn-SOD and/or Catalase Decreases BaP-Induced ROS

Under control conditions, the level of $O_2^{\cdot-}$ and peroxy radicals in MAECs obtained from *hSod1Tg/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}* and *Sod1+CafTg/ApoE^{-/-}* mice was similar to that in MAECs obtained from *ApoE^{-/-}* mice (Fig. 5). However, MAECs obtained from these mice showed different responses to BaP with regards to $O_2^{\cdot-}$ and peroxy radical production. For example, addition of 1 μ M BaP to the culture medium increased the level of $O_2^{\cdot-}$ by about 2-fold in MAECs obtained from *hCatTg/ApoE^{-/-}* and *ApoE^{-/-}*, but did not increase $O_2^{\cdot-}$ in MAECs obtained from *hSod1Tg/ApoE^{-/-}* or *Sod1+CafTg/ApoE^{-/-}* mice (Fig. 5). In contrast, BaP significantly increased peroxy radicals in MAECs from *hSod1Tg/ApoE^{-/-}* and *ApoE^{-/-}* mice, but did not increase peroxy radicals in MAECs obtained from *hCatTg/ApoE^{-/-}* or *Sod1+CafTg/ApoE^{-/-}* mice (Fig. 5). These results suggest that BaP-induced $O_2^{\cdot-}$ and peroxide generation in MAECs are blocked by overexpression of Cu/Zn-SOD and catalase, respectively.

4. Discussion

Although ROS have been suggested to participate in atherogenesis, the relative contribution of different ROS varies in different atherosclerosis models. For example, a previous report from our laboratory demonstrated that overexpression of catalase, but not Cu/Zn-SOD, significantly retarded the development of atherosclerosis in *ApoE^{-/-}* mice [15], suggesting that H_2O_2 , but not $O_2^{\cdot-}$, contributes to atherogenesis in these mice. In addition, Tribble et al. [22] reported that overexpression of Cu/Zn-SOD did not reduce the formation of fatty streaks induced by a high-fat diet. However, in a later study in which mice were first exposed to a single X-ray dose before being placed on a high-fat diet, Tribble et al. [23] observed that atherosclerotic lesions in the proximal aorta of Cu/Zn-SOD transgenic mice were significantly smaller than those in the aorta of wild-type mice. These observations suggest that both H_2O_2 and $O_2^{\cdot-}$ participate in atherogenesis, although their relative contribution varies in different animal models. Thus, it appears that $O_2^{\cdot-}$ contributes to the atherosclerosis induced by insults that directly generate $O_2^{\cdot-}$, such as ionizing radiation. It is known that radiolytic hydrolysis leads to the formation of a number of ROS, with $O_2^{\cdot-}$ being a major product when O_2 is present [24]. However, $O_2^{\cdot-}$ does not appear to participate in the atherosclerosis induced by a high-fat diet or by ApoE knockout. In these models, atherogenic stimuli (e.g., oxidized lipids/lipoproteins) are thought to activate intracellular ROS-generating sources to produce $O_2^{\cdot-}$, which is then converted to H_2O_2 and other radicals [15]. It also has been suggested that these atherogenic stimuli elevate *only* peroxides and not $O_2^{\cdot-}$ [25].

In the present study, we observed that BaP increased both $O_2^{\cdot-}$ and peroxide generation in endothelial cells, and showed that overexpression of Cu/Zn-SOD and/or catalase reduced BaP-induced ROS and inhibited BaP-accelerated atherosclerosis in *ApoE^{-/-}* mice. These observations suggest that both H_2O_2 and $O_2^{\cdot-}$ contribute to the pathogenesis of BaP-induced atherosclerosis. Increasing evidence indicates that BaP increases ROS through multiple mechanisms. For example, BaP-quinones and BaP-diols undergo redox cycling and generate ROS, such as $O_2^{\cdot-}$, H_2O_2 and hydroxyl radicals [12]. In addition, BaP has been shown to reduce the activities of SODs and catalase, and therefore increase $O_2^{\cdot-}$ and H_2O_2 [26]. It is highly likely that overexpression of Cu/Zn-SOD and/or catalase increases the capacity of vascular cells to scavenge BaP-generated ROS, and thereby inhibits BaP-induced atherosclerosis.

One mechanism by which ROS mediate their atherogenic actions is through lipid/lipoprotein oxidation [13]. It has been proposed that once lipids/lipoproteins deposited in the arterial wall become oxidized, they induce damage to endothelial cells (ECs), attracting inflammatory cells

into the arterial wall and inducing migration and proliferation of smooth muscle cells (SMCs) into the intima, leading to the formation of atherosclerotic plaques [27]. BaP and its metabolites have been reported to increase the ability of animals and cells to oxidize lipids. For example, treatment of ApoE-deficient mice with BaP increased lipid peroxidation and oxidized lipid-derived DNA modifications in the aorta [28]. Incubation of mouse peritoneal macrophages with BaP increased their capacity to oxidize LDL [29]. Data from the present study show that reduction in atherosclerotic lesions in *hSod1Tg/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}* and *Sod1+CatTg/ApoE^{-/-}* mice is associated with a reduced level of aortic F₂-IsoPs and IsoFs, *in vivo* markers of lipid peroxidation. These observations suggest that Cu/Zn-SOD and catalase inhibit BaP-accelerated atherosclerosis through suppression of lipid peroxidation in the arterial wall.

Another atherogenic action of ROS is to induce expression of a variety of proteins thought to be involved in the pathogenesis of atherosclerosis [27,30]. Specifically, ROS induce vascular cells to express cell adhesion molecules, which trigger adhesion of leukocytes to the endothelium and promote subsequent migration of leukocytes into the intima, thus initiating the atherogenic process [27,30]. It has been suggested that BaP accumulated in the arterial wall induces expression of cell adherence molecules and triggers adhesion of inflammatory cells to the endothelium [6]. In the present study, we observed that BaP treatment significantly elevated the levels of VCAM-1 and ICAM-1 protein in the aorta of *ApoE^{-/-}* mice, but not in *hSod1Tg/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}* or *Sod1+CatTg/ApoE^{-/-}* mice. Similarly, BaP significantly increased surface expression of VCAM-1 and ICAM-1 in endothelial cells obtained from *ApoE^{-/-}* mice, but not in those obtained from *hSod1Tg/ApoE^{-/-}*, *hCatTg/ApoE^{-/-}* or *Sod1+CatTg/ApoE^{-/-}* mice. In addition, overexpression of Cu/Zn-SOD and catalase significantly reduced BaP-induced monocyte adhesion to MAECs. These observations suggest that the effect of BaP to enhance cell adhesion molecule expression and monocyte adhesion to endothelial cells is associated with its ability to induce ROS.

It is well established that ROS induce cell adhesion molecule expression by activation of redox-sensitive transcription factors such as NF- κ B [31]. Overexpression of Cu/Zn-SOD and catalase in vascular smooth muscle cells has been shown to inhibit VCAM-1 expression through inhibition of NF- κ B [31]. The present study demonstrates that overexpression of Cu/Zn-SOD and catalase efficiently reduces BaP-induced ROS in MAECs. Thus, the inhibitory effect of Cu/Zn-SOD and catalase on BaP-induced cell adhesion molecule expression can most easily be explained by its ability to decrease O₂⁻ and H₂O₂ production in endothelial cells. Suppression of BaP-induced expression of cell adhesion molecules in vascular cells is responsible, at least in part, for the anti-atherogenic function of Cu/Zn-SOD and catalase. However, it is important to note that ROS may not be responsible for all elevated expression of monocyte-attracting proteins in endothelial cells, since a recent report showed that antioxidants could not attenuate BaP-induced monocyte chemotactic protein-1 expression in *ApoE^{-/-}* mouse vasculature tissue and human endothelial cells [32].

In summary, this report demonstrates that exposure of *ApoE^{-/-}* mice to BaP accelerates the development of atherosclerosis. Overexpression of Cu/Zn-SOD and/or catalase in *ApoE^{-/-}* mice inhibits BaP-accelerated atherosclerosis, in association with a reduction in the levels of oxidized lipids and cell adhesion molecules in the aorta. In addition, overexpression of Cu/Zn-SOD and/or catalase decreases cellular responses to BaP, including ROS production and cell adhesion molecule expression in MAECs, and monocyte adhesion to MAECs. Further studies will be needed to establish whether ROS contribute to BaP-induced atherogenesis via additional mechanisms, such as modification of endothelial permeability, changes in nitric oxide metabolism, and induction of vascular cell proliferation and/or death. Nevertheless, the present report provides evidence that both O₂⁻ and H₂O₂ contribute to BaP-induced atherogenesis, and that Cu/Zn-SOD and/or catalase inhibit the development of atherosclerosis by reducing lipid peroxidation and decreasing the sensitivity of vascular cells to BaP.

Collectively, our results validate reduction in intracellular ROS levels as a therapeutic strategy for BaP-induced atherosclerosis.

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Figure 1. Representative *en face* preparations and cross-sections of aortas

Aortas were obtained from *ApoE*^{-/-}, *hSod1* Tg/*ApoE*^{-/-}, *hCat*Tg/*ApoE*^{-/-} and *Sod1*+*Cat*Tg/*ApoE*^{-/-} mice treated with BaP or v as a control. Panel A shows distal aorta *en face* preparations stained with Oil-Red-O. Red-stained areas are atherosclerotic lesions. Panel B shows aorta root cross-sections stained with Oil-Red-O. Panel C shows aorta root cross-sections stained with trichrome, in which the acellular areas represent lipid cores, the blue-stained tissues that cover the lipid cores indicate fibrous caps.

Figure 2. Overexpression of Cu/Zn-SOD and/or catalase reduces BaP-induced lipid peroxidation in mouse aortas

Aortas were obtained from *ApoE*^{-/-}, *hSod1*Tg/*ApoE*^{-/-}, *hCat* Tg/*ApoE*^{-/-} and *Sod1*+*Cat*Tg/*ApoE*^{-/-} mice treated with or without BaP. The levels of F₂-IsoPs and IsoFs were measured using a GC/MS assay as described in Materials and Methods. Values represent the mean ± SEM of six mice. **P* < 0.05 compared to *ApoE*^{-/-} mice without BaP treatment; †*P* < 0.05 compared to BaP-treated *ApoE*^{-/-} mice.

Figure 3. Overexpression of Cu/Zn-SOD and/or catalase reduces BaP-induced expression of cell adhesion molecules in mouse aortas

Aortas were obtained from *ApoE*^{-/-}, *hSod1*Tg/*ApoE*^{-/-}, *hCat* Tg/*ApoE*^{-/-} and *Sod1*+*Cat*Tg/*ApoE*^{-/-} mice treated with or without BaP. The levels of VCAM-1 and ICAM-1 protein were measured by western blotting analysis. Values represent the mean ± SEM of five separate experiments. Protein samples were pooled from two aortas in each experiment. **P* < 0.05 compared to *ApoE*^{-/-} mice without BaP treatment; †*P* < 0.05 compared to BaP-treated *ApoE*^{-/-} mice.

Figure 4. Overexpression of Cu/Zn-SOD and/or catalase inhibits BaP-induced monocyte-endothelium interaction and endothelial expression of cell adhesion molecules

MAECs were obtained from ApoE^{-/-}, hSod1Tg/ApoE^{-/-}, hCatTg/ApoE^{-/-} and Sod1+CatTg/ApoE^{-/-} mice. MNCs were obtained from wild-type mice. MAECs were treated with the indicated concentrations of BaP or culture medium alone (control). The number of MNCs adherent to MAECs was determined by a fluorochrome adhesion assay, and the levels of VCAM-1 and ICAM-1 protein expressed on the MAEC surface were measured by ELISA. Values represent the mean $\pm$ SEM of five separate experiments, in which MAECs were pooled from four mice. * $P < 0.05$ compared to ApoE^{-/-} MAECs without BaP treatment; † $P < 0.05$ compared to BaP-treated ApoE^{-/-} MAECs.

Figure 5. Overexpression of Cu/Zn-SOD and/or catalase inhibits BaP-induced reactive oxygen species

MAECs were obtained from *ApoE*^{-/-}, *hSod1*Tg/*ApoE*^{-/-}, *hCat*Tg/*ApoE*^{-/-} and *Sod1*+*Cat*Tg/*ApoE*^{-/-} mice. Intracellular O₂^{•-} and peroxy radicals were measured after incubating MAECs with 1 μM BaP or culture medium alone (control). Values represent the mean ± SEM of five separate experiments, in which MAECs were pooled from four mice. **P* < 0.05 compared to *ApoE*^{-/-} MAECs without BaP treatment; †*P* < 0.05 as compared with BaP-treated *ApoE*^{-/-} MAECs.

Table 1
The Effect of Overexpressing Cu/Zn-SOD and/or Catalase on Atherosclerosis

	Lesion Area ^A (% aortic tree)	Lesion area ^B ($\mu\text{m}^2 \times 10^3$)	Lipid core ^B (# per section)	Fibrous cap ^B (# per section)
Control				
<i>ApoE</i> ^{-/-}	10.2 ± 2.7	34 ± 5	1.6 ± 0.7	1.3 ± 0.4
<i>hSod1Tg/ApoE</i> ^{-/-}	9.3 ± 1.8	32 ± 7	1.2 ± 0.4	0.9 ± 0.1
<i>ShCat Tg/ApoE</i> ^{-/-}	6.2 ± 1.2*	25 ± 4*	0.5 ± 0.1*	0.2 ± 0.1*
<i>Sod1+CatTg/ApoE</i> ^{-/-}	4.6 ± 1.3*	19 ± 4*	0.2 ± 0.1*	0.0 ± 0.0*
BaP treatment				
<i>ApoE</i> ^{-/-}	16.5 ± 2.6*	49 ± 7*	2.2 ± 0.6*	1.3 ± 0.3
<i>hSod1Tg/ApoE</i> ^{-/-}	8.8 ± 2.5 [†]	35 ± 9 [†]	1.2 ± 0.3 [†]	0.9 ± 0.2
<i>hCat Tg/ApoE</i> ^{-/-}	7.6 ± 2.4 [†]	27 ± 6 [†]	0.5 ± 0.1 [†]	0.2 ± 0.1 [†]
<i>Sod1+CatTg/ApoE</i> ^{-/-}	5.1 ± 1.6 [†]	21 ± 5 [†]	0.2 ± 0.1 [†]	0.1 ± 0.0 [†]

Aortas were obtained from *ApoE*^{-/-}, *hSod1Tg/ApoE*^{-/-}, *hCatTg/ApoE*^{-/-} and *Sod1+CatTg/ApoE*^{-/-} mice treated with or without BaP.

^A The lesion area in the surface of the aortic tree was measured using the *en face* staining method; data are expressed as the percent of the aortic tree surface covered by atherosclerotic lesions (n = 10/group).

^B The area (μm^2) of atherosclerotic lesions, and the number of fibrous caps and lipid core were determined in the aorta roots using the cross-section staining methods (n = 20/group). Values represent the mean ± SEM.

* $P < 0.05$ compared to *ApoE*^{-/-} mice without BaP treatment;

[†] $P < 0.05$ compared to BaP-treated *ApoE*^{-/-} mice.

Table 2
Mouse Plasma Lipids (mg/dL)

	Triglycerides		Total Cholesterol	
	Control	BaP-treated	Control	BaP-treated
<i>ApoE</i> ^{-/-}	117 ± 16	103 ± 18	516 ± 61	531 ± 90
<i>hSod1Tg/ApoE</i> ^{-/-}	104 ± 19	107 ± 17	546 ± 84	497 ± 102
<i>hCatTg/ApoE</i> ^{-/-}	106 ± 14	115 ± 10	558 ± 74	523 ± 68
<i>Sod1+CatTg/ApoE</i> ^{-/-}	98 ± 18	104 ± 16	520 ± 51	509 ± 79

Blood samples were obtained from *hSod1Tg/ApoE*^{-/-}, *hCatTg/ApoE*^{-/-}, *Sod1+CatTg/ApoE*^{-/-} and *ApoE*^{-/-} mice treated with or without BaP. Plasma total triglycerides and total cholesterol were determined as described in Materials and Methods. Values represent the mean ± SEM of 10 mice.