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Overexpression of Cu/Zn-superoxide dismutase and/or catalase accelerates benzo(α) pyrene detoxification by upregulation of the aryl hydrocarbon receptor in mouse endothelial cells

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Abstract

A reduction in endogenously generated reactive oxygen species in vivo delays benzo(α)pyrene (BaP)-accelerated atherosclerosis, as revealed in hypercholesterolemic mice overexpressing Cu/Zn-superoxide dismutase (SOD) and/or catalase. To understand the molecular events involved in this protective action, we studied the effects of Cu/Zn-SOD and/or catalase overexpression on BaP detoxification and on aryl hydrocarbon receptor (AhR) expression and its target gene expression in mouse aortic endothelial cells (MAECs). Our data demonstrate that overexpression of Cu/Zn-SOD and/or catalase leads to an 18- to 20-fold increase in the expression of AhR protein in MAECs. After BaP exposure, the amount of AhR binding to the cytochrome P450 (CYP) 1A1 promoter was significantly greater, and the concentrations of BaP reactive intermediates were significantly less in MAECs overexpressing Cu/Zn-SOD and/or catalase than in wild-type cells. In addition, the BaP-induced CYP1A1 and 1B1 protein levels and BaP-elevated glutathione *S*-transferase (GST) activity were significantly higher in these transgenic cells, in parallel with elevated GSTp1, CYP1A1, and CYP1B1 mRNA levels, compared to wild-type MAECs. Moreover, knockdown of AhR with RNA interference diminished the Cu/Zn-SOD and catalase enhancement of CYP1A1 expression, GST activity, and BaP detoxification. These data demonstrate that overexpression of Cu/Zn-SOD and/or catalase is associated with upregulation of AhR and its target genes, such as xenobiotic-metabolizing enzymes.

Keywords

Benzo(α)pyrene; Aryl hydrocarbon receptor; Endothelial cells; Cu/Zn-superoxide dismutase; Catalase; Free radicals

Polycyclic aromatic hydrocarbons (PAHs) are a class of chemical carcinogens found in cigarette smoke, automobile exhaust, and foods cooked at high temperature (reviewed in Refs. [17,33]). Benzo(α) pyrene (BaP), a representative PAH compound, has been shown to target vascular cells and accelerate the development of atherosclerosis [4]. A recent study from our laboratory demonstrated that over-expression of Cu/Zn-superoxide dismutase (SOD) and/or catalase inhibited BaP-accelerated atherosclerosis in hypercholesterolemic mice [42].

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However, the mechanism underlying the inhibitory action of these antioxidant enzymes on BaP-induced atherosclerosis has not been defined.

It is generally accepted that the pathologic action of BaP results primarily from its reactive intermediates, which could be generated by multiple simultaneous or sequential metabolic transformations [13]. Initially, BaP is metabolized by cytochrome P450 (CYP) enzymes to epoxides, which can be hydrated to various dihydrodiols by epoxide hydrolase. The *trans*-7,8-dihydroxy-7,8-dihydro-BaP can be further oxidized to BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) by CYPs [9]. BPDE is capable of binding covalently to DNA to form BPDE-DNA adducts, which is a crucial step leading to DNA mutations [10]. BaP-induced bulky DNA adducts and the consequent DNA mutations in vascular cells are considered to be involved in atherogenesis [2,4]. In addition to generation of BPDE, BaP can form phenols via multiple pathways, such as spontaneous rearrangement from epoxides or metabolic transformation from BaP by CYPs. BaP phenols can be further oxidized spontaneously or metabolically to quinones, which undergo redox cycling and generate reactive oxygen species (ROS), such as superoxide anions ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals [3,9,29]. An increase in vascular ROS is believed to contribute to the pathogenesis of atherosclerosis [22].

Mammalian cells have evolved a number of phase II xenobiotic-metabolizing enzymes, such as glutathione *S*-transferase (GST), sulfotransferase (SULT), and UDP glucuronosyl-transferase (UGT) [36]. These enzymes are engaged in detoxification of xenobiotic chemicals usually by adding a conjugate, such as glutathione, to the functional group, resulting in the conversion of relatively hydrophobic drugs and other chemicals to hydrophilic products that are more readily excreted [13]. It has been shown that BaP epoxides are substrates for GSTs [18], whereas phenols, quinones, and dihydrodiols are substrates for UGT and SULT [27]. Thus, the relative expression of CYPs and phase II enzymes determines the relative levels of bioactivated versus detoxified BaP metabolites in cells and tissues.

The expression of CYP genes is regulated by aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor of the basic helix-loop-helix/Per-Arnt-Sim family [11]. In the absence of ligands, AhR exists predominantly in the cytosolic compartment in association with a chaperone complex (Hsp90/XAP/p23). Upon binding ligands, such as BaP, AhR translocates into the nucleus and forms a heterodimer with AhR nuclear translocator already present in the nucleus [16]. This heterodimer binds to consensus regulatory sequences (xenobiotic response element) located upstream in the promoter of CYP genes, inducing transcription. Increasing evidence indicates that the AhR gene battery also includes a number of phase II xenobiotic-metabolizing enzymes, such as GST, UGT, and NADPH: quinone oxidoreductase 1 [25,26], and activation of AhR by toxic substances like BaP or other PAHs has been shown to induce expression of these genes [25,26]. This report studied the effect of overexpressing Cu/Zn-SOD and/or catalase on BaP detoxification and on AhR and its target gene expression. Our findings provide insights into the molecular mechanism underlying the protective action of Cu/Zn-SOD and catalase against BaP-induced injury, such as atherosclerosis.

Materials and methods

Isolating and culturing mouse aortic endothelial cells (MAECs)

Transgenic mice overexpressing human Cu/Zn-superoxide dismutase (*hsod1*Tg), human catalase (*hcat*Tg), or both Cu/Zn-SOD and catalase (*Sod/Cat*Tg) were generated as described previously [15]. MAECs were obtained from mouse aortas using an outgrowth technique and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a 95% air and 5% CO₂ atmosphere [41]. These cells displayed a cobblestone-like monolayer and expressed von Willebrand factor and platelet-endothelial cell molecule-1 (CD31), characteristic of freshly

isolated endothelial cells [41]. The eighth and ninth passages of the cells were used for experiments.

Measurement of reactive oxygen species

For measurement of $O_2^{\bullet-}$, MAECs grown to confluence in a 96-well plate were incubated with 25 μ M lucigenin (Sigma Chemical Co., St. Louis, MO, USA) in the presence or absence of 1 mM BaP (Sigma) for 30 min at 37°C. Luminescence was read using a luminometer (BL10000 Lumicount; Packard BioScience). The cumulative $O_2^{\bullet-}$ concentration was calculated based on a standard curve generated by incubation of lucigenin with xanthine/xanthine oxidase [14].

For measurement of H_2O_2 , MAECs grown to confluence in a 96-well plate were incubated with 150 μ l Amplex red reagent (Invitrogen, Carlsbad, CA, USA) in the presence or absence of 1 mM BaP for 30 min at 37°C. Fluorescence was read using a fluorometer (Fluoroskan Ascent FL; ThermoLabsystems) with the excitation wavelength at 540 nm and the emission wavelength at 590. The cumulative H_2O_2 concentration was determined based on the standard curve obtained by incubation of the Amplex red reagent with H_2O_2 . Because this measurement reflects the ability of MAECs to release H_2O_2 , we refer to it as H_2O_2 release. At the end of the experiments, MAECs were lysed in M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL, USA). Protein levels in the lysate were determined using a BCA protein assay kit (Thermo Scientific). The levels of $O_2^{\bullet-}$ and H_2O_2 were expressed relative to the protein levels.

Western blot analysis

MAECs grown in 100-mm culture dishes were treated with 1 μ M BaP or culture medium alone for 4 h. For whole-cell protein extraction, cells were lysed in M-PER mammalian protein extraction reagent (Thermo Scientific). For cytoplasmic and nuclear extracts, the cells were lysed in 500 μ l of ice-cold buffer A (10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 0.05% Nonidet P-40, 0.5 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF)). Cell lysates were centrifuged for 10 min at 3000g at 4°C. The supernatant was collected as cytoplasmic extract. The pellet containing nuclei was homogenized in 100 μ l of buffer B (5 mM Hepes, 20% glycerol, 1.5 mM $MgCl_2$, 300 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, and 1 mM PMSF). Samples containing 5 μ g (for detection of β -actin) or 35 μ g protein (for detection of AhR, CYP1A1, and CYP1B1) were separated on a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were incubated with antibodies against mouse β -actin, AhR, CYP1A1, or CYP1B1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Immunoreactive bands were visualized using ECL-Plus chemiluminescence reagent (Amersham Biosciences, Piscataway NJ, USA) and analyzed with a Bio-Rad Model GS-700 imaging densitometer (Bio-Rad, Hercules, CA, USA). The uneven sample loading was normalized using the intensity ratio of the immunoreactive bands of the tested proteins relative to β -actin.

Chromatin immunoprecipitation (ChIP) analysis

MAECs grown in 100-mm tissue culture dishes at confluence were treated for 2 h with 1 μ M BaP or culture medium alone. After being cross-linked with 1% formaldehyde, the cells were lysed in buffer A as described for Western analysis. The nuclei were isolated and resuspended in 300 μ l of lysis buffer (50 mM Tris–HCl, 1% SDS, 10 mM EDTA, and protease inhibitor cocktail, pH 8.1). The lysate was sonicated to break genomic DNA to fragments at 0.3–0.6 kb and then centrifuged at 20,000g for 10 min. Five microliters of supernatant was used as an input control. The rest of the supernatant was diluted 10-fold with ChIP dilution buffer (16.7 mM Tris–HCl, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, and protease

inhibitor cocktail, pH 8.1) and precleaned with salmon sperm DNA/protein A agarose (Santa Cruz Biotechnology). The precleaned sample was incubated overnight at 4°C with AhR antibody and then with salmon sperm DNA/protein A agarose for another 3 h. The bound protein–DNA complex was eluted with a solution containing 0.1 M NaHCO₃ and 1% SDS. After reversion of the protein–DNA cross-links, DNA fragments in the eluates and the input controls were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). An AhR binding site in the CYP1A1 promoter region was amplified using polymerase chain reaction (PCR) with a pair of primers: forward, 5'-AGAGCACTCCCTAAGGCTGT-3'; backward 5'-AGCACTCACCTTGGGCTGTA-3'. The DNA-binding activity of AhR was expressed as the ratio of the PCR product derived from the immunoprecipitated eluates versus that from the input controls.

High-performance liquid chromatography (HPLC) analysis for BaP metabolites

Cells were harvested and homogenized in 0.25 M Tris–sucrose–EDTA (pH 7.4) and 0.1% SDS. The homogenate was extracted with a solution containing water, methanol, and chloroform at a ratio of 1:1.5:2 (v/v). The organic phase was dried under N₂ and resuspended in 0.5 ml of methanol. Particulates in the extracts were removed by passing them through Acrodisc filters (0.45 µm, 25-mm diameter; Gelman Sciences, Ann Arbor, MI, USA). The final extracts were stored at 4°C in amber-colored screwtop vials to prevent photodegradation until analysis. The metabolites from the extracts were identified and measured by a reverse-phase HPLC equipped with a UV and a fluorescence detector as described previously [32].

Glutathione S-transferase activity assay

Total GST activity was measured with an assay kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). Briefly, MAECs were sonicated in 100 mM potassium phosphate containing 2 mM EDTA and centrifuged at 10,000g for 15 min at 4°C. The supernatant was used to measure GST activity with an MRX spectrophotometer (ThermoLabsystems, Chantilly, VA, USA) at 340 nm with 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione as substrates. GST activity was expressed as the amount of CDBN conjugated with reduced glutathione per minute.

Measurement of F₂-isoprostanes and isofurans

MAECs were sonicated in ice-cold HPLC-grade water containing 100 µM butylhydroxytoluene and 1 mM EDTA. Esterified F₂-isoprostanes (F₂-IsoP's) and isofurans (IsoF's) were extracted using C18 followed by silica Sep-Paks, derivatized to pentafluorobenzyl esters, further purified by thin layer chromatography, and derivatized to trimethylsilyl ethers. The derivatives were analyzed and quantified by gas chromatography/negative ion chemical ionization mass spectrometry using [²H₄]15-F_{2t}-IsoP as an internal standard [7].

RNA interference to knock down AhR

DNA oligonucleotides encoding AhR small interfering RNA (siRNA) (5'-AAGACTGGAGAAAGTGGCA-3') were synthesized by Invitrogen (San Diego, CA, USA). Negative control siRNA was purchased from Clontech (Mountain View, CA, USA). The double-strand oligonucleotides were cloned into the RNAi-Ready pSIREN-RetroQ-ZsGreen vector (Clontech). The recombinant retroviral constructs were then transfected into MAECs using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Briefly, MAECs grown in six-well plates at about 60–70% confluence were incubated in 2 ml/well serum-free DMEM containing 10 µl Lipofectamine 2000 and 4 µg retroviral DNA. After 6 h of transfection, the cells were replenished with fresh medium containing 10% FBS and cultured for an additional 24 h. These transfection procedures were repeated one more time. To

determine transfection efficiency, the cells were trypsinized. The total cells and the green fluorescent protein-positive cells were counted on a hema-cytometer under a fluorescence microscope (Eclipse TE 2000; Nikon Instruments, Melville, NY, USA). The two-time liposome transfection protocol gave 30–40% transfection efficiency. No difference among wild-type cells and transgenic cells overexpressing Cu/Zn-SOD and/or catalase was noticed. The transfected cells were treated with BaP for the indicated times and harvested for measurement of protein, mRNA, GST activity, and BaP metabolites.

Quantitative real-time RT-PCR assay

Total RNA was isolated using RNAeasy Plus mini kits (Qiagen) and reverse transcribed to cDNA using the SuperScript first-strand synthesis system (Invitrogen). The resulting cDNAs were subjected to quantitative real-time PCR with an iCycler system (Bio-Rad) using the indicated primers synthesized by Qiagen. Reactions were performed in a final volume of 20 μ l with reagents provided by an Absolute Blue QPCR SYBR Green fluorescein kit (ABgene, Rochester, NY, USA). A standard curve of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was generated by 10-fold serial dilutions of total RNA. The expression levels of target mRNAs were normalized to the GAPDH mRNA level.

Statistical analysis

Data are reported as means $\pm$ standard error of the mean. The differences among wild-type and transgenic MAECs treated with or without BaP were analyzed by multiple-factor analysis of variance and Student's unpaired *t* test. Differences were considered significant at a *P* value less than 0.05. Statistics software (Statistix, Tallahassee, FL, USA) was used for statistical analysis of data.

Results

Overexpression of Cu/Zn-SOD and/or catalase reduces BaP-induced ROS in MAECs

Previous studies from our laboratory demonstrated that endothelial cells obtained from *hSod1*Tg, *hCat*Tg, and *Sod/Cat*Tg mice had approximately 2.5-fold increase in the activities of Cu/Zn-SOD and/or catalase, and no significant changes in the activities of other antioxidant scavengers, including Mn-SOD, extracellular SOD, and glutathione peroxidase-1, compared with the cells obtained from their wild-type littermates [41]. Cu/Zn-SOD is a protein that converts $O_2^{\bullet-}$ to H_2O_2 , whereas catalase destroys H_2O_2 by converting it to molecular oxygen and water. Fig. 1 shows that, under control conditions, the levels of $O_2^{\bullet-}$ and H_2O_2 in the transgenic MAECs were similar to those in wild-type control cells. In contrast, transgenic and wild-type MAECs showed fundamentally different responses to BaP with regard to $O_2^{\bullet-}$ production and H_2O_2 release. Thus, BaP increased the level of $O_2^{\bullet-}$ threefold in *hCat*Tg and in wild-type MAECs, but did not alter the $O_2^{\bullet-}$ level in *hSod1*Tg and *Sod/Cat*Tg cells, whereas BaP significantly increased H_2O_2 release in *hSod1*Tg and wild-type MAECs, but not in *hCat*Tg and *Sod/Cat*Tg cells (Fig. 1). In a recent report, we also observed that BaP induced lower levels of intracellular $O_2^{\bullet-}$, as measured by hydroethidine (a superoxide-sensitive dye), in *hSod1*Tg and *Sod/Cat*Tg MAECs and induced lower levels of hydroperoxides, as measured by 6-carboxy-2,7-dichlorodihydrofluorescein diacetate (a peroxide-sensitive dye), in *hCat*Tg and *Sod/Cat*Tg MAECs compared with wild-type cells [42].

Overexpression of Cu/Zn-SOD and/or catalase upregulates AhR in MAECs

This study examined the effects of Cu/Zn-SOD and/or catalase overexpression on the expression of AhR. As shown in Fig. 2, the AhR protein levels in the whole-cell lysate of the *hCat*Tg, *Sod/Cat*Tg, and *hSod1*Tg MAECs were about 19-, 20-, and 18-fold higher, respectively, than that in wild-type cells (Figs. 2B and C). The increased AhR proteins in the

cells overexpressing Cu/Zn-SOD and/or catalase were proportionally distributed in both the cytosolic and the nuclear fractions (Figs. 2B–E). Treatment of MAECs with BaP resulted in AhR translocation from the cytosol to the nucleus. Fig. 2A shows time-related changes in AhR protein after the wild-type MAECs were treated with BaP. Addition of 1 μ M BaP to the culture medium induced a rapidly increased AhR protein level in the nucleus, reaching a maximal level at 1 h and declining thereafter, with reciprocal changes in the relative amount of the AhR protein in the cytosol (Fig. 2A). As shown in Fig. 2A, BaP treatment also significantly reduced the AhR protein level in the whole-cell lysate. These observations suggest that BaP exposure not only induces AhR translocation to the nucleus but also downregulates AhR expression in MAECs. BaP treatment induced similar changes in the MAECs overexpressing Cu/Zn-SOD and/or catalase compared to wild-type cells, i.e., elevating nuclear AhR but lowering cytosolic AhR protein levels. However, the AhR protein level was always significantly higher in *hSod1*Tg, *hCat*Tg, and *Sod/Cat*Tg MAECs than in wild-type cells, regardless of the presence or absence of BaP (Figs. 2B–E).

BaP enhances the DNA binding activity of AhR

Having demonstrated the BaP induced AhR nuclear translocation, we then examined the binding of AhR to the promoter region of CYP1A1, a target gene of AhR. The DNA-binding activity of AhR was analyzed using ChIP analysis with a pair of primers that amplify the DNA containing one AhR binding site adjacent to the transcription starting site of the CYP1A1 promoter. Under the control conditions, the DNA-binding activity of AhR was barely detectable; there were no significant differences among *hSod1*Tg, *hCat*Tg, *Sod/Cat*Tg, and wild-type MAECs (Fig. 3). After cells were treated with BaP, the binding of AhR to the CYP1A1 promoter was significantly increased, to a greater extent in the transgenic cells than in the wild-type cells. As shown in Fig. 3, the amount of DNA precipitated by AhR antibody from the *hCat*Tg, *Sod/Cat*Tg, and *hSod1*Tg MAECs was 80, 157, and 91% greater, respectively, than that from wild-type cells treated with BaP. These data suggest that the amount of AhR proteins bound to the CYP1A1 promoter region is greater in MAECs overexpressing Cu/Zn-SOD and/or catalase than in wild-type cells.

Overexpression of Cu/Zn-SOD and/or catalase enhances BaP-induced CYP1A1 and 1B1 expression

This study examined the effects of overexpression of Cu/Zn-SOD and/or catalase on the expression of CYP1A1 and 1B1 in MAECs. Fig. 4 shows that the mRNA levels of either CYP1A1 or CYP1B1 were comparable among *hSod1*Tg, *hCat*Tg, *Sod/Cat*Tg, and wild-type MAECs under the control conditions. BaP strikingly upregulated CYP1A1 mRNA expression in *hSod1*Tg, *hCat*Tg, *Sod/Cat*Tg, and wild-type MAECs, with a greater magnitude in the transgenic cells. Thus, BaP (1 μ M) exposure for 2 h elevated the CYP1A1 mRNA level in *hSod1*Tg, *hCat*Tg, and *Sod/Cat*Tg MAECs by 1000- to 2000-fold, but only about 60-fold in wild-type cells (Fig. 4). Fig. 4 also shows that BaP exposure elevated the CYP1B1 mRNA levels about 1.6- to 2.4-fold in MAECs, with no significant difference in CYP1B1 mRNA among *hSod1*Tg, *hCat*Tg, *Sod/Cat*Tg, and wild-type cells.

This study also determined the protein levels of CYP1A1 and 1B1 in MAECs with or without overexpression of Cu/Zn-SOD and/or catalase. Under control conditions, the protein expression of CYP1B1 was higher than that of CYP1A1, which was barely detectable. The protein levels of both CYP1A1 and CYP1B1 were comparable in *hSod1*Tg, *hCat*Tg, *Sod/Cat*Tg, and wild-type cells (Fig. 4). Similar to the observations for BaP-induced changes in mRNA, BaP exposure elevated both CYP1A1 and CYP1B1 protein levels in *hSod1*Tg, *hCat*Tg, *Sod/Cat*Tg, and wild-type MAECs, with a greater magnitude in the transgenic cells. These findings provide evidence that over-expression of Cu/Zn-SOD and/or catalase enhances BaP-induced CYP1A1 and 1B1 expression, without altering the constitutive level of these genes.

Overexpression of Cu/Zn-SOD and/or catalase enhances GSTp1 expression and GST activity

GSTs are a multigene family involved in the detoxification of xenobiotics, including BaP reactive intermediates. As Fig. 5 shows, the total GST activity was comparable in BaP-untreated MAECs with or without overexpression of Cu/Zn-SOD and/or catalase, suggesting that overexpression of these antioxidant enzymes does not alter the basal activity of GSTs. BaP exposure for 4 h enhanced the total GST activity about 42, 38, and 37%, respectively, in *hCatTg*, *Sod/CatTg*, and *hSod1Tg* MAECs, but only about 17% in wild-type cells, leading to a significantly higher GST activity in the transgenic MAECs than in wild-type cells after BaP treatment (Fig. 5).

Fig. 5 also shows that overexpression of Cu/Zn-SOD and/or catalase did not significantly affect the mRNA level of GSTp1, a member of GST family, under the control conditions. Exposure of wild-type MAECs to 1 μ M BaP for 2 h did not significantly alter the GSTp1 mRNA level. However, the same dose of BaP increased GSTp1 mRNA in *hCatTg*, *Sod/CatTg*, and *hSod1Tg* cells by about 32, 43, and 34%, respectively. These findings indicate that overexpression of Cu/Zn-SOD and/or catalase upregulates not only phase I xenobiotic-metabolizing enzymes, such as CYP1A1 and 1B1, but also phase II enzymes, such as GSTs, in MAECs in response to BaP exposure.

Overexpression of Cu/Zn-SOD and/or catalase increases BaP detoxification

Having demonstrated the upregulatory effect of Cu/Zn-SOD and/or catalase overexpression on phase I and phase II enzymes, we next studied whether upregulated expression of xenobiotic-metabolizing enzymes coincides with an elevated BaP detoxification. As shown in Fig. 6A, treatment of MAECs with 1 μ M BaP for 8 h induced a detectable accumulation of BaP reactive intermediates, including 9-hydroxybenzo (α)pyrene (9(OH)BaP), 3-hydroxy-BaP (3(OH)BaP), 9,10-dihydroxy-BaP (9,10-diol), 7,8-dihydro-BaP (7,8-diol), 4,5-dihydroxy-BaP (4,5-diol), and BaP-3,6-dione BaP (3,6-dione). The accumulated level was significantly reduced in *hCatTg*, *Sod/CatTg*, and *hSod1Tg* MAECs compared to wild-type cells. These observations provide evidence that overexpression of Cu/Zn-SOD and/or catalase enhances the capability of MAECs to detoxify BaP.

Overexpression of Cu/Zn-SOD and/or catalase reduces BaP-induced lipid peroxidation

Both F₂-IsoP's and IsoF's are oxidized lipids that are produced nonenzymatically by free radical-catalyzed peroxidation of arachidonic acids and believed to be good indicators of oxidative lesions in vivo [7]. Figs. 6B and C show that BaP exposure significantly elevated the levels of F₂-IsoP's and IsoF's in wild-type, *hCatTg*, and *hSod1Tg* MAECs, with a greater magnitude in wild-type cells. In contrast, BaP did not significantly elevate the level of these oxidized lipids in *Sod/CatTg* MAECs. These findings suggest that overexpression of Cu/Zn-SOD and/or catalase inhibits BaP-induced lipid peroxidation and that the inhibitory role induced by combinational overexpression of Cu/Zn-SOD and catalase is greater than overexpression of either Cu/Zn-SOD or catalase alone.

Knockdown of AhR diminishes the enhancing effect of Cu/Zn-SOD and/or catalase overexpression on BaP-induced gene expression and BaP detoxification

To explore the regulatory role of AhR in BaP-induced gene expression, AhR transcription was suppressed by transient transfection with a retroviral construct expressing AhR siRNA. The knockdown efficiency induced by siRNA was confirmed by detection of AhR mRNA (Fig. 7C) and protein (Figs. 7A and B). We observed that transfection of MAECs with the AhR siRNA significantly inhibited the BaP-induced expression of CYP1A1 and GSTp1 mRNAs. For example, BaP exposure increased the CYP1A1 mRNA levels by 1240-, 1385-, 1299-, and 72-fold, respectively, in the *hCatTg*, *Sod/CatTg*, *hSod1Tg*, and wild-type MAECs transfected

with the control siRNA, but only about 110-, 180-, 90-, and 25-fold in these lines of cells transfected with the AhR siRNA (Fig. 7F). In addition, BaP-induced GST activity (7H) and BaP-induced GSTp1 mRNA (7G) were significantly reduced in *hSod1*Tg, *hCat*Tg, and *Sod/Cat*Tg MAECs transfected with AhR siRNA, compared to the same genotype cells transfected with the control siRNA. In contrast, the BaP-induced increase in CYP1B1 mRNA in wild-type and in transgenic MAECs was not diminished by transfection with AhR siRNA, and there was no difference between AhR siRNA- and control siRNA-transfected cells.

Fig. 7D shows that the levels of BaP metabolites were significantly lower in MAECs overexpressing Cu/Zn-SOD and/or catalase than in wild-type cells, which is consistent with the data derived from MAECs without siRNA transfection (Fig. 6A). Fig. 7D also shows that the *hCat*Tg, *Sod/Cat*Tg, and *hSod1*Tg MAECs transfected with AhR siRNA manifested significantly higher levels of BaP metabolites compared to the same genotype cells transfected with the control siRNA (Fig. 7D). In contrast, the level of BaP metabolites was comparable in wild-type cells transfected with AhR or control siRNA (Fig. 7D). These findings suggest that upregulation of AhR is a mechanism by which over-expression of Cu/Zn-SOD and/or catalase accelerates BaP metabolism.

Discussion

This report, for the first time, brings to light that overexpression of Cu/Zn-SOD and/or catalase significantly increases AhR protein and mRNA levels in MAECs under an untreated control condition. To date, the mechanism by which overexpression of Cu/Zn-SOD and/or catalase upregulates AhR expression has not been defined. This study and previous reports from our laboratory consistently show that the basal levels of $O_2^{\bullet-}$ and H_2O_2 are 15–25% lower in the vascular smooth muscle cells [35] and endothelial cells [28,41,42] obtained from mice overexpressing Cu/Zn-SOD and catalase than in those obtained from wild-type mice. These observations, together with the fact that Cu/Zn-SOD and catalase are major antioxidant enzymes that remove $O_2^{\bullet-}$ and H_2O_2 , suggest that a constant, even slight, reduction in cellular ROS might upregulate AhR expression. In addition, a number of physiological and pathological conditions that regulate AhR transcription have been described. Specifically, DNA hypermethylation [23] and histone deacetylation [12] have been shown to downregulate AhR expression, whereas activation of transcription factors Sp1 [8] and Nrf2 [37] has been reported to increase AhR transcription. In addition, sequence analysis of the murine AhR promoter reveals consensus DNA recognition sites for transcription factors, including AP-1 and NF- κ B [23]. Further studies are warranted to determine whether Cu/Zn-SOD and catalase overexpression upregulates AhR expression via the aforementioned mechanisms.

This report also demonstrates that BaP exposure induces a time-related decline in AhR protein levels in *hSod1*Tg, *hCat*Tg, *Sod/Cat*Tg, and wild-type MAECs overexpressing Cu/Zn-SOD and/or catalase. The ligand-induced downregulation of AhR protein has been observed in a variety of cultured cell lines, including Hepa-1 (mouse hepatocytes), NIH-3T3 (mouse embryo fibroblasts), A7 (rat smooth muscle cells), and C2C12 (mouse skeletal muscle myoblasts) [30], as well as in rat liver, spleen, thymus, and lung [31]. Ligand-initiated degradation is widely accepted as an important mechanism underlying the regulation of AhR [5,21]. It is believed that nuclear AhR, after execution of its transcriptional activity, is exported back to the cytosol and undergoes proteasome-dependent degradation [5,21]. In addition to a reduced AhR protein level, we observed a significant decrease in AhR mRNA levels in MAECs after BaP exposure. Similar results have been obtained in the craniofacial tissue of mice exposed gestationally to 2,3,7,8-tetrachlorodibenzo-*p*-dione [1]. These findings indicate that ligand-induced downregulation of AhR can occur at the transcriptional level, in addition to the proteasome-dependent protein degradation.

AhR activation leads to enhanced transcription of a number of genes, including the CYP family [11]. Members of the CYP family that are most active in the metabolism of PAHs include CYP1A1 and CYP1B1 [11]. CYP1A1 is expressed ubiquitously at low levels but is markedly induced by ligand-activated AhR [25]. In contrast, CYP1B1 often shows substantial constitutive levels in a number of cells/tissues, including endothelial cells, breast, prostate, uterus, ovary, gastrointestinal tract, and immune cells [36]. It is interesting to note that, in the absence of BaP, either the amount of AhR bound to CYP1A1 promoter or the level of CYP1A1 mRNA is comparable in *hSod1*Tg, *hCat*Tg, *Sod/Cat*Tg, and wild-type MAECs, even though the nuclear AhR protein level in wild-type cells is markedly lower. These data suggest that AhR proteins entering the nucleus in the absence of ligands may fail to bind to DNA and therefore cannot enhance transcription. In contrast, BaP-induced AhR nuclear translocation coincides with increased DNA binding of AhR and elevated expression of CYP1A1 and CYP1B1 mRNAs in *hSod1*Tg, *hCat*Tg, *Sod/Cat*Tg, and wild-type MAECs, with a greater magnitude in the transgenic cells. In addition, the upregulatory effect of BaP seems to be more profound for CYP1A1, as we observed a greater increase in CYP1A1 mRNA than in CYP1B1 mRNA. Moreover, knockdown of AhR inhibits BaP-induced expression of CYP1A1 but does not significantly affect CYP1B1. Taken together, our findings suggest that AhR predominantly regulates CYP1A1 expression in MAECs in response to BaP exposure, whereas an AhR-independent mechanism is responsible for BaP-induced CYP1B1 expression. Indeed, there are reports showing AhR-independent regulation of CYP gene expression. For example, dibenzo (α)pyrene has been shown to induce CYP1A1 mRNA expression in the skin of AhR null knockout mice [24]; BaP has been shown to induce CYP1B1 mRNA in aortic smooth muscle cells of AhR null knockout mice [19]; and 3-methylcholanthrene has been reported to increase CYP1A1, 1A2, and 1B1 protein levels in the liver of AhR null knockout mice [20]. In addition, Sakuma et al. [34] reported that phenobarbital, a typical p450-inducing drug, induced CYP1A2 mRNA and protein expression in the liver of AhR-nonresponsive mice. Taken together, AhR-independent pathway(s) for PAHs appear to be cell-, CYP isoform-, and PAH type-specific. Data from this report provide evidence that there exists an AhR-independent pathway in MAECs responsible for BaP-induced CYP1B1 expression.

It is generally accepted that CYP proteins potentiate the toxicity of BaP by converting it to reactive intermediates. However, increasing evidence suggests that CYP isoforms vary in terms of the BaP reactive intermediates they generate. For example, knockout of CYP1A1 increases BaP–DNA adducts in mouse livers after BaP exposure [38,40]. Compared to CYP1A1 knockout mice, the CYP1A1/1B1 double-knockout mice show fewer BaP–DNA adducts [39]. Conversely, overexpression of CYP1A1 and CYP1A2, but not CYP1B1, reduces BaP–DNA adduct formation in BaP-treated HepG2 cells [6]. However, cells overexpressing CYP1A1 metabolize BaP to hydroxyl, quinone, and diol metabolites more efficiently than do those overexpressing CYP1B1 [6]. These results imply that CYP1A1 is more important in the conversion of BaP to hydroxyl, quinone, and diol metabolites, whereas CYP1B1 is more important in the formation of more complicated intermediates, such as BPDE, which induce bulky lesions in DNA.

Because our data show a greater induction of CYP1A1 than of CYP1B1 in MAECs after BaP exposure, we measured the levels of BaP hydroxyl and diol metabolites accumulated in MAECs after BaP exposure. It is paradoxical that MAECs overexpressing Cu/Zn-SOD and/or catalase displayed a significant reduction in BaP intermediates, in parallel with a significant increase in CYP expression. We postulated that upregulation of phase II proteins by AhR could account for this apparent paradox. Indeed, we observed that BaP exposure significantly increased the activity of total GSTs and the mRNA level of GSTp1 in cells overexpressing Cu/Zn-SOD and/or catalase but not in wild-type cells, suggesting that upregulation of phase II enzymes, such as GSTs, could be a mechanism whereby Cu/Zn-SOD and catalase reduce BaP reactive intermediates in MAECs. In this report, we also observed that knockdown of AhR diminished

BaP-induced GSTp1 expression and total GST activity and elevated the accumulation of BaP reactive intermediates in MAECs overexpressing Cu/Zn-SOD and/or catalase. These observations suggest that over-expression of Cu/Zn-SOD and/or catalase upregulates phase II enzymes, such as GSTs, accelerating BaP detoxification via a mechanism involving AhR. Overexpression of Cu/Zn-SOD and/or catalase might also induce other phase II enzymes in addition to GSTs, as we observed a significantly reduced level of dihydrodiols, which are the substrates for UGT and SULT.

We also observed that BaP increased oxidative stress in MAECs, as reflected by increased levels of ROS and lipid peroxidation, and that overexpression of Cu/Zn-SOD and/or catalase diminished BaP-induced oxidative stress. These observations are not surprising because BaP quinones are known to undergo redox cycling to generate ROS, such as $O_2^{\bullet-}$ and H_2O_2 [3,9, 29], and because ROS are known to mediate lipid peroxidation [15]. Thus, Cu/Zn-SOD and catalase can reduce BaP-induced oxidative stress via two mechanisms. First, Cu/Zn-SOD and catalase scavenge BaP-induced $O_2^{\bullet-}$ and H_2O_2 , respectively, and therefore reduce ROS-mediated lipid peroxidation. Second, overexpression of Cu/Zn-SOD and catalase inhibits BaP reactive metabolite generation and therefore reduces BaP-induced ROS and lipid peroxidation.

In summary, this report presents three novel findings. First, MAECs overexpressing Cu/Zn-SOD and/or catalase show elevated AhR mRNA and protein levels compared to wild-type cells. Second, MAECs overexpressing Cu/Zn-SOD and/or catalase show increased CYP1A1, CYP1B1, and GSTp1 expression; increased GST activity; reduced BaP reactive intermediates; and reduced cellular oxidative stress in response to BaP exposure. The aforementioned effects induced by combinational overexpression of Cu/Zn-SOD and catalase seem to be greater than those induced by overexpression of either Cu/Zn-SOD or catalase alone. Third, knockdown of AhR attenuates the upregulatory effects of Cu/Zn-SOD and/or catalase on xenobiotic-metabolizing enzyme expression and BaP detoxification. It is highly likely that overexpression of Cu/Zn-SOD and catalase upregulates AhR, which in turn increases the expression of phase I and II enzymes in response to BaP exposure and therefore accelerates BaP detoxification. These findings could lead to the development of appropriate therapeutic strategies, such as the synthesis of antioxidant candidates/cocktails, to reduce/prevent BaP-induced deleterious effects, such as atherogenesis and carcinogenesis. However, further studies are required to determine the mechanism underlying the upregulatory roles of Cu/Zn-SOD and catalase in AhR expression.

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Fig. 1.

Overexpression of Cu/Zn-SOD and/or catalase reduces BaP-induced ROS. MAECs obtained from wild-type (WT) and transgenic mice overexpressing Cu/Zn-SOD, catalase (CAT), or both Cu/Zn-SOD and catalase (S/C) were incubated with 1 μ M BaP for 30 min or culture medium alone as a control. The level of O₂^{•-} and the release of H₂O₂ in MAECs were measured as described under Materials and methods. Values represent the means $\pm$ SEM of four separate experiments in which MAECs were pooled from four mice. * P <0.01 vs the same genotype cells without BaP treatment, and † P <0.01 vs BaP-treated wild-type cells.

Fig. 2.

Overexpression of Cu/Zn-SOD and/or catalase upregulates AhR expression. The AhR protein levels were measured by Western blot analysis and were expressed as a ratio of their immunoblot intensity relative to β -actin. (A) MAECs obtained from wild-type (WT) mice were incubated with 1 μ M BaP for the indicated hours. (B–E) MAECs obtained from WT mice and transgenic mice overexpressing Cu/Zn-SOD, catalase (CAT), or both Cu/Zn-SOD and catalase (S/C) were treated with 1 μ M BaP for 4 h. (B) Representative images of Western blots. (C, D, and E) AhR protein levels in the whole-cell lysate and cytosolic and nuclear fractions, respectively. Values represent the means $\pm$ SEM of five separate experiments in which MAECs were pooled from four mice. * P < 0.05 vs the same genotype cells without BaP treatment, $^{\dagger}P$ < 0.01 vs WT cells without BaP treatment, and $^{\#}P$ < 0.01 vs BaP-treated WT cells.

Fig. 3.

BaP increases the DNA-binding activity of AhR. MAECs obtained from wild-type (WT) mice and transgenic mice overexpressing Cu/Zn-SOD (SOD), catalase (CAT), or both Cu/Zn-SOD and catalase (S/C) were treated with 1 μ M BaP for 4 h. The binding activity of AhR to the CYP1A1 promoter region was measured with ChIP analysis using two pairs of PCR primers: (a) a primer pair probing the two AhR binding sites in the enhancer region of the CYP1A1 promoter and (b) a primer pair probing the AhR binding site adjacent to the transcription starting site. Values represent the means $\pm$ SEM of four separate experiments in which MAECs were pooled from four mice. * P < 0.01 vs the same genotype cells without BaP treatment, † P < 0.05 vs BaP-treated WT cells.

Fig. 4.

Overexpression of Cu/Zn-SOD and/or catalase enhances BaP-induced CYP1A1 and CYP1B1 expression. MAECs were obtained from wild-type (WT) mice and transgenic mice overexpressing Cu/Zn-SOD, catalase (CAT), or both Cu/Zn-SOD and catalase (S/C). The cells were incubated with 1 μ M BaP for 4 h to determine protein levels and for 2 h to measure mRNA levels. The CYP1A1 and 1B1 protein levels in the whole-cell lysate were measured by Western blot analysis and were expressed as a ratio of their immunoblot intensity relative to β -actin. The CYP1A1 and 1B1 mRNA levels were determined by quantitative real-time RT-PCR and expressed relative to the GAPDH mRNA level. Values represent the means $\pm$ SEM of five separate experiments in which MAECs were pooled from four mice. * $P < 0.01$ vs the same genotype cells without BaP treatment, $^{\dagger}P < 0.01$ vs BaP-treated WT cells.

Fig. 5.

Overexpression of Cu/Zn-SOD and/or catalase enhances BaP-induced GSTp1 mRNA and total GST activity. MAECs were obtained from wild-type (WT) mice and transgenic mice overexpressing Cu/Zn-SOD, catalase (CAT), or both Cu/Zn-SOD and catalase (S/C). The cells were incubated with 1 μ M BaP for 4 h to determine total GST activity and for 2 h to measure GSTp1 mRNA level. Total GST activity in the whole-cell lysate was determined as described under Materials and methods and expressed as the amount of CDNB conjugated with reduced glutathione. The mRNA level of GSTp1 was determined by quantitative real-time RT-PCR and expressed relative to the GAPDH mRNA level. Values represent the means $\pm$ SEM of five separate experiments in which MAECs were pooled from four mice. * P < 0.05 vs the same genotype control cells without BaP treatment, $^{\dagger}P$ < 0.05 vs BaP-treated WT cells.

Fig. 6.

Overexpression of Cu/Zn-SOD and/or catalase reduces BaP reactive intermediates and lipid peroxidation. MAECs obtained from wild-type (WT) mice and transgenic mice overexpressing Cu/Zn-SOD, catalase (CAT), or both Cu/Zn-SOD and catalase (S/C) were incubated with 1 μ M BaP for 8 h. (A) BaP reactive metabolites were determined with a reverse-phase HPLC system with fluorescence detection. The concentrations of (B) F₂-isoprostanes and (C) isofurans in the MAECs were measured using GC/MS. Values represent the means $\pm$ SEM of five separate experiments in which MAECs were pooled from four mice. * P < 0.05 vs BaP-treated WT cells, [†] P < 0.05 vs the same genotype cells without BaP treatment.

Fig. 7.

Knockdown of AhR inhibits BaP detoxification and diminishes BaP-induced gene expression in MAECs overexpressing Cu/Zn-SOD and/or catalase. MAECs obtained from wild-type (WT) mice and transgenic mice overexpressing Cu/Zn-SOD, catalase (CAT), or both Cu/Zn-SOD and catalase (S/C) were transfected with AhR siRNA or control siRNA. The cells were incubated with 1 μ M BaP or culture medium alone for 4 h for determining protein levels, 2 h for determining mRNA levels, and 8 h for determining BaP metabolites. (A and B) The AhR protein level in the whole-cell lysate was measured by Western blot analysis and expressed as a percentage of their immunoblot intensity relative to β -actin. The mRNA levels of (C) AhR, (E) CYP1B1, (F) CYP1A1, and (G) GSTp1 were determined by quantitative real-time RT-PCR and expressed relative to GAPDH mRNA level. (H) The total GST activity was determined using a Cayman GST activity assay kit. (D) BaP reactive metabolites, including 9-

hydroxy-BaP, 3-hydroxy-BaP, 9,10-dihydroxy-BaP, 7,8-dihydro-BaP, 4,5-dihydroxy-BaP, and 3,6-dihydroxy-BaP, were determined with an HPLC system and expressed in picomoles of total BaP reactive metabolites per milligram of cellular proteins. Values represent the means $\pm$ SEM of five separate experiments in which MAECs were pooled from four mice. * $P < 0.05$ vs the same genotype cells transfected with control siRNA, $^{\dagger}P < 0.05$ vs WT cells transfected with control siRNA, and $^{\#}P < 0.05$ vs WT cells transfected with AhR siRNA.