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Nrf2-Dependent Induction of NQO1 in Mouse Aortic Endothelial Cells Overexpressing Catalase

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Abstract

Overexpression of catalase has been shown to accelerate benzo(a)pyrene (BaP) detoxification in mouse aortic endothelial cells (MAECs). NAD(P)H:quinone oxidoreductase1 (NQO1) is an enzyme that catalyzes BaP-quinone detoxification. Aryl hydrocarbon receptor (AhR) and nuclear factor erythroid 2-related factor-2 (Nrf2) are transcription factors that control NQO1 expression. Here, we investigated the effect of catalase overexpression on NQO1, Nrf2 and AhR expressions. The levels of NQO1 mRNA and protein were comparable in MAECs isolated from wild-type and transgenic mice that overexpress human catalase (*hCatTg*). BaP treatment increased NQO1 mRNA and protein levels in both groups, with a significantly greater induction in *hCatTg* MAECs than in wild-type cells. BaP-induced NQO1 promoter activity was dramatically higher in *hCatTg* MAECs than in wild-type cells. Our data also showed that the basal level of AhR and the BaP-induced level of Nrf2 were significantly higher in *hCatTg* MAECs than in wild-type cells. Inhibition of specificity protein-1 (Sp1) binding to the AhR promoter region by mithramycin A reversed the enhanced effect of catalase overexpression on AhR expression. Knockdown of AhR by RNA interference diminished BaP-induced expression of Nrf2 and NQO1. Knockdown of Nrf2 significantly decreased NQO1 mRNA and protein levels in cells with or without BaP treatment. NQO1 promoter activity was abrogated by mutation of the Nrf2-binding site in this promoter. In contrast, mutation of the AhR-binding site in NQO1 promoter did not affect the promoter activity. These results suggest that catalase overexpression upregulates BaP-induced NQO1 expression via enhancing the Sp1-AhR-Nrf2 signaling cascade.

Keywords

endothelial cell; catalase; NAD(P)H:quinone oxidoreductase1; aryl hydrocarbon receptor; nuclear factor erythroid 2-related factor-2

INTRODUCTION

NAD(P)H:quinone oxidoreductase1 (NQO1) is a phase II enzyme catalyzing the two-electron reduction of quinones [1], thereby diminishing the toxicity of quinone-structured compounds [2]. The expression of NQO1 is highly inducible by several xenobiotics and oxidants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), benzo(a)pyrene (BaP), and

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tert-butylhydroquinone [3-5]. Xenobiotics such as TCDD and BaP are well-established inducers of gene expression mediated by activation of the aryl hydrocarbon receptor (AhR) [6,7]. In the absence of ligand binding, AhR exists predominantly in the cytoplasm. Upon binding ligands such as BaP and TCDD, AhR translocates into the nucleus and heterodimerizes with AhR nuclear translocator (ARNT) [8]. To induce transcription of target genes, this heterodimer binds to consensus regulatory sequences called xenobiotic response elements (XREs) in promoters of target genes [9]. XRE enhancers have been identified in the NQO1 promoter in the rat, human, and mouse [10-12]. Studies in rats show that xenobiotic-induced NQO1 expression occurs via binding of AhR to the NQO1-XRE enhancer [10]. However, recent studies in mice indicate that NQO1 expression depends on both AhR and nuclear factor-E2-related factor-2 (Nrf2) [4,13].

The promoter region in the mouse NQO1 gene contains a Nrf2-binding consensus regulatory sequence known as the antioxidant response element (ARE), which is essential for both constitutive and xenobiotics-induced NQO1 promoter activity [14]. It has been shown that basal NQO1 expression is nearly abolished in cells or tissues obtained from Nrf2-knockout mice [4,13,15], and that treatment of Nrf2-deficient mice or cells with BaP [15] or TCDD [4,13] failed to induce NQO1 expression. These findings suggest that Nrf2 is involved in regulating the expression of NQO1 under both basal and stressed conditions.

Xenobiotics increase Nrf2 protein levels by upregulating transcription of its mRNA [13,16]. Treatment of wild-type animals or cells with TCDD [13,16] or BaP [17] led to a significant increase in the Nrf2 mRNA level. In contrast, these xenobiotics failed to induce Nrf2 mRNA following silencing of AhR by siRNA/shRNA [16,17] or in a AhR null knockout [13,16]. These observations suggest that AhR is involved in regulating Nrf2 transcription induced by xenobiotics. It has been suggested that AhR expression is regulated by the transcription factor specificity protein-1 (Sp1) [18,19]. Inhibition of Sp1 DNA-binding has been shown to attenuate the enhancing effect of catalase overexpression on AhR promoter activity [20].

In this report, we studied the effect of catalase overexpression on NQO1, Nrf2 and AhR expression, and Sp1 DNA-binding activity in MAECs. Our data demonstrated that overexpression of catalase enhanced BaP-induced NQO1 expression, which might explain the increased BaP detoxification observed in cells that overexpress catalase [7]. In addition, we demonstrated that enhancing the Sp1-AhR-Nrf2 signaling cascade is a mechanism by which overexpression of catalase upregulates BaP-induced NQO1 expression in MAECs.

MATERIALS AND METHODS

Isolating and culturing mouse aortic endothelial cells

Transgenic mice overexpressing human catalase (*hCatTg*) were generated as previously described [21]. In this study, mouse aorta endothelial cells (MAECs) were isolated from the aortas of *hCatTg* mice and their wild-type littermates using an outgrowth technique. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in 95% humidity and 5% CO₂. These cells grew as a cobblestone-like monolayer and expressed von Willebrand factor and platelet-endothelial cell molecule-1 (CD31), which are characteristic of freshly isolated endothelial cells [22]. *hCatTg* MAECs exhibited an approximately 2.5-fold higher catalase activity than cells from wild-type littermates [22,23]. Cells that underwent eight or nine passages were used for experiments, unless otherwise indicated.

Measurement of peroxides

MAECs grown in a black-wall bottomed, -clear 96-well plate were incubated for 1 h with 10 µg/ml 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (CDC-H2F diacetate). After

three washes in Hank's buffer, 100 μ l serum-free DMEM containing BaP (final concentration of 1 μ M) or an equal volume of the diluent dimethyl sulfoxide (DMSO) as a control was added to each well and incubated for 1 h. The fluorescence of samples was then read using a Fluoskan Ascent AL fluorometer with excitation/emission wavelengths of 480/540 nm for CDC-H2F to monitor peroxide generation. Protein levels in the lysate were determined using a BCA protein assay kit (Thermo Scientific, Rockford, IL). The level of peroxides was expressed FI (fluorescence intensity) per μ g protein.

Measurement of F₂-isoprostanes

MAECs in serum-free DMEM were treated with or without 1 μ M BaP for 6 h, and then sonicated in ice-cold HPLC-grade water containing 100 μ M butylhydroxytoluene and 1 mM EDTA. Esterified F₂-isoprostanes (F₂-IsoPs) were extracted using C18 followed by silica Sep-Paks, derivatized to pentafluorobenzyl esters, further purified by TLC, and finally 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.

Quantitative real-time RT-PCR assay

MAECs grown to confluence in six-well plates were made quiescent in serum-free DMEM for 12 h and then treated with 1 μ M BaP or an equal volume of dimethyl sulfoxide (DMSO) as control for 4 h. In the experiments for inhibition of catalase activity, 10 μ M 3-amino-1,2,4-triazole (AT, Sigma-Aldrich, St. Louis, MO) was added into the culture medium. In a previous study, we demonstrated that AT at this concentration was able to reduce catalase activity by about 44% in *hCatTg* MAECs [24]. In those particular experiments involving H₂O₂, MAECs were first incubated with 100 μ M H₂O₂ or culture medium alone for 1 h, and then 1 μ M BaP or DMSO was added for additional 4 h as above. Total RNA was extracted using RNeasy plus mini kits (Qiagen, Valencia, CA) and subjected to quantitative real-time PCR using the iCycler system (Bio-Rad, Hercules, CA) as described previously [20]. These specific primers were used for amplification: catalase forward: 5'-AAATGCTTCAGGGCCGCTT-3', reverse: 5'-GTAGGGACAGTTCACAGGTA-3'; NQO1 forward: 5'-TTCTGTGGCTTCCAGGTCTT-3', reverse: 5'-AGGCTGCTTGGAGCAAAATA-3'; Nrf2 forward: 5'-CCTCGCTGGAAAAAGAAGTG-3', reverse: 5'-GGAGAGGATGCTGCTGAAAG-3'; AhR forward: 5'-ACTTCACACCTATTGGTTGT-3', reverse: 5'-ATGCCACTTTCTCCAGTCTT-3'; and GAPDH forward: 5'-GAGCCAAAAGGGTCATCATC-3', reverse: 5'-TAAGCAGTTGGTGGTGCAGG-3'. The expression levels of target mRNAs were normalized to GAPDH mRNA.

Western blot analysis

Quiescent MAECs in serum-free DMEM were treated with or without 1 μ M BaP for 6 h. In experiments where AT and N-acetylcysteine (NAC) were used, MAECs were incubated with 10 μ M AT (Sigma) or 10 mM NAC (Sigma) for 12 h prior to BaP treatment. For whole cell protein extraction, cells were lysed in M-PER mammalian protein extraction reagent (Thermo Scientific). For nuclear extracts, nuclei were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's protocol (Thermo Scientific). Samples containing 5 μ g (for β -actin detection) or 40 μ g of protein (for detection of protein of interest) were resolved on 10% SDS-PAGE gels and then transferred to a PVDF membrane (Millipore, Billerica, MA). Antibodies against catalase, β -actin, AhR, or NQO1 (Santa Cruz Biotechnology, Santa Cruz, CA), Nrf2 (R&D Systems, Minneapolis, MN) were added, followed by detection with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology), as previously described [20]. Immunoreactive bands

were visualized using ECL-plus chemiluminescence reagent (GE Healthcare Healthcare–Amersham, Piscataway, NJ) and analyzed with a GS-700 Imaging Densitometer (Bio-Rad).

Chromatin immunoprecipitation (ChIP)

Quiescent MAECs were treated with 1 μ M BaP or diluent for 4 h. Nrf2 binding to the NQO1 promoter region and Sp1 binding to the AhR promoter region were determined by ChIP as previously described [20]. Briefly, cells were crosslinked with 1% formaldehyde at room temperature, and then lysed in 500 μ l of cell lysis buffer (5 mM PIPES, 85 mM KCl, 0.5% Nonidet P-40, 1 mM PMSF, and protease inhibitor cocktail, pH 8.0). Nuclei were isolated and homogenized in 300 μ l of nuclear lysis buffer (50 mM Tris–HCl, 1% SDS, 10 mM EDTA, and protease inhibitor cocktail, pH 8.1). The resulting nuclear lysate was sonicated until crosslinked chromatin was sheared to an average length of 0.3–1.0 kb. Supernatant (5 μ l) was used as an input control. The remaining lysate 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 samples were incubated with Nrf2 or Sp1 antibody, followed by salmon sperm DNA/protein A-agarose. Bound protein-DNA complexes were eluted with a solution containing 0.1 M NaHCO₃ and 1% SDS. Following reversion of the protein-DNA crosslinks, DNA fragments in the eluate and input controls were purified using a QIAquick PCR purification kit (Qiagen), and then subjected to quantitative real-time PCR using an iCycler system (Bio-Rad). A 201-bp DNA fragment containing the ARE sequence of the NQO1 promoter region was amplified with primers: forward 5'-TCTAAGAGCAGAACGCAGCA-3' and reverse 5'-TTCGTGGGACCTGCCTACAT-3'. A 192-bp DNA fragment containing Sp1-binding sites in the AhR promoter region was amplified with a pair of primers as follows: forward 5'-AACAGGTACTGGGCGAACAC-3' and reverse 5'-CTCCGCAGCCGCGACCCCGC-3'. The amount of DNA-bound Nrf2 or Sp1 was expressed as a ratio of the PCR product amplified from the immunoprecipitated samples versus the input controls.

Small interfering RNA (siRNA) knockdown of AhR and Nrf2

MAECs grown in six-well plates to approximately 60–70% confluence were transfected with control or specific siRNA against AhR or Nrf2 (Santa Cruz Biotechnology) using the GenMute siRNA & DNA Transfection Reagent (SignaGen Laboratories, Ijamsville, MD) according to the manufacturer's instructions. At 24 h post-transfection, the cells were cultured in serum-free DMEM for 4 h. For quantitative real-time RT-PCR, the cells were then treated with or without 1 μ M BaP for 4 h. For Western blot analysis, the cells were treated with or without 1 μ M BaP for 6 h.

Recombinant plasmid construction and cell transfection

A 1101-bp NQO1 promoter fragment was amplified by PCR from wild-type C57BL/6J mouse genomic DNA using Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA). The forward primer starts at position 988 upstream of the NQO1 transcription start site (5'-ATCCGCTCGAGTGTCTACCCTACTTTGGGAG-3'). The reverse primer starts at position 113 downstream of the NQO1 transcription start site (5'-ATCCCAAGCTTATCGTAATACCGAACGCTGA-3'). The PCR products were cloned into the *Xho*I/*Hind*III sites of the pGL2 luciferase vector (Promega, Madison, WI). Mutations in the binding sites for Nrf2 (ARE) or AhR (XRE) within the NQO1 promoter were generated by site-directed mutagenesis of the wild-type pGL2-NQO1-promoter plasmid using the following primers (mutation sites are underlined): ARE forward: 5'-TCAGTCTAGAGTCACAGCTGCAGGTGAAAATTTGAGCCCATC-3', and reverse 5'-GATGGGCTCAAATTTTCACCTGCAGCTGTGACTCTAGACTGA-3'. The XRE mutation primers were: forward 5'-

GCCCCACCCTTCCCCTGCAGCACAAAGGTGACTTCCCA-3', and reverse 5'-TGGGAAGTCACCTTTGTGCTGCAGGGAAGGGTGGGGC-3'. The mutants were selected by analysis of *DpnI* digestion [25].

The recombinant plasmid constructs and a β -galactosidase expression plasmid, pCMV-SPORT- β gal (Promega) were co-transfected into MAECs using the Lipofectamine 2000 kit (Invitrogen) according to the manufacturer's protocol. Briefly, MAECs grown in six-well plates to approximately 60-70% confluence were incubated with 2 ml/well serum-free DMEM containing 10 μ l of Lipofectamine 2000, 0.5 μ g of β -galactosidase expression plasmid, and 4 μ g of pGL2 plasmid encoding either the wild-type or mutated NQO1 promoter fragments. At 6 h post-transfection, the cells were replenished with fresh medium containing 10% FBS and then cultured for an additional 24 h. The cells were grown in serum-free DMEM for 4 h and treated with or without 1 μ M BaP for 6 h prior to reporter gene analysis.

Luciferase and β -galactosidase assays

For the luciferase assays, transfected cells were lysed with 100 μ l of reporter lysis buffer provided by the luciferase assay kit (Luciferase Assay System, Promega). Lysate (10 μ l) was incubated in a 96-well plate at room temperature for 2 min with 100 μ l of luciferase assay reagent (Promega). For the β -galactosidase assays, cells were lysed with 100 μ l of lysis buffer provided by a galactosidase assay kit (Galacto-Star™ β -Galactosidase Reporter Gene Assay System for Mammalian Cells, Applied Biosystems, Foster City, CA). Lysate (10 μ l) was incubated in a 96-well plate at room temperature for 60 min with 100 μ l of reaction buffer (Applied Biosystems). Luminescence was measured using the BL10000 LumiCount (Packard BioScience, Meriden, CT). Luciferase activity was expressed as the luminosity ratio of the luciferase assay to the β -galactosidase assay.

Statistical analysis

For experiments using the microplate reader, the mean value for each experiment was averaged from triplicate wells in the same plate. Data are reported as the mean $\pm$ the standard error of the mean of at least five independent experiments. Differences between wild-type and *hCatTg* MAECs with or without treatment were analyzed by one-way or multiple factor analysis of variance and the Student's unpaired *t*-test. Statistical significance was considered when *P* was less than 0.05. Statistix software (Statistix, Tallahassee, FL) was used for statistical analysis.

RESULTS

Overexpression of catalase diminishes oxidative stress in MAECs

Previous studies from our laboratory demonstrated that endothelial cells and vascular smooth muscle cells (VSMCs) obtained from *hCatTg* mice had approximately a 2.5-fold increase in their catalase activity, and no significant change in the activities of other antioxidant scavengers, including Mn-SOD, extracellular-SOD, and glutathione peroxidase-1, when compared with properties of cells obtained from wild-type littermates [22,23]. Data from the present study show that the catalase mRNA and protein levels are about 2.5-fold higher in *hCatTg* MAECs than in wild-type cells (Fig. 1 A and B). Data in Fig. 1C and D also show that, under control conditions, the level of peroxides and isoprostanes is slightly lower in *hCatTg* MAECs than in wild-type control cells, but the differences were not statistically significant. In contrast, the *hCatTg* and wild-type MAECs show fundamentally different responses to BaP with regard to peroxides and isoprostane generation. Thus, addition of 1 μ M BaP to the culture medium elevated the levels of peroxides and isoprostane by 2.3- and 2.6-fold in wild-type MAECs, respectively. However,

the same dose of BaP did not significantly alter the peroxides level in *hCatTg* cells. In addition, BaP-induced isoprostanes were significantly decreased in *hCatTg* MAECs as compared to wild-type cells. These results are consistent with our previous observations that overexpression of catalase diminishes cellular oxidative stress, reflected by a decrease in peroxides and oxidized lipids [7,26].

Overexpression of catalase enhances BaP-induced NQO1 expression in MAECs

We reported previously that catalase overexpression led to an accelerated detoxification of BaP in MAECs [7]. To elucidate the mechanism underlying this phenomenon in *hCatTg* MAECs, we examined the effect of catalase overexpression on NQO1 expression. Our data demonstrated that NQO1 mRNA and protein levels were comparable in wild-type and *hCatTg* MAECs in the absence of BaP (Fig. 2). However, NQO1 induction in response to BaP was significantly greater in *hCatTg* MAECs than in wild-type cells. Addition of aminotriazole (AT) into the culture attenuated the upregulatory effect of catalase overexpression on BaP-induced NQO1 expression. Specifically, treatment of wild-type MAECs with 1 μ M BaP increased NQO1 mRNA and protein by 5.4- and 1.8-fold, respectively. In BaP-treated *hCatTg* MAECs, NQO1 mRNA and protein levels were increased by 12- and 2.9-fold, respectively. In the presence of 10 μ M aminotriazole, BaP treatment increased NQO1 mRNA and protein by 8.2- and 2.1-fold, respectively, in *hCatTg* MAECs. These data indicate that overexpression of catalase enhances BaP-induced NQO1 expression.

Overexpression of catalase enhances BaP-induced Nrf2 expression and DNA binding in MAECs

Nrf2 has been reported to be essential for constitutive and xenobiotic-induced expression of NQO1 [13,14]. Therefore, we proceeded to study the effect of catalase overexpression on Nrf2 expression and DNA binding. The expression pattern of Nrf2 in MAECs (Fig. 3) was similar to that of NQO1 in Fig. 2. The levels of Nrf2 mRNA and protein in untreated wild-type and *hCatTg* MAECs were comparable (Fig. 3). BaP treatment increased Nrf2 mRNA and protein levels in wild-type and *hCatTg* MAECs. In fact, this induction was significantly greater in *hCatTg* MAECs than in wild-type cells. These data indicate that, while overexpression of catalase did not affect basal Nrf2 expression, it enhanced BaP-induced Nrf2 expression in MAECs.

Consistent with this, catalase overexpression led to increased BaP-induced accumulation of Nrf2 in the nucleus and binding to the NQO1 promoter in MAECs without altering basal levels. For example, exposure of wild-type MAECs to 1 μ M BaP increased the level of nuclear Nrf2 and the amount of Nrf2 bound to the ARE in the NQO1 promoter by 60% and 40%, respectively (Fig. 4). However, same BaP treatment of *hCatTg* MAECs increased the level of Nrf2 in the nucleus and bound to the NQO1-ARE by 180% and 80%, respectively (Fig. 4). These results suggest that the increase in BaP-induced Nrf2 expression in *hCatTg* MAECs is coupled with augmented Nrf2 binding to the NQO1 promoter.

Nrf2 knockdown reduces NQO1 expression in MAECs

To validate the involvement of Nrf2 in regulating NQO1 expression in *hCatTg* MAECs, we examined the effect of Nrf2 knockdown on the expression of NQO1 in wild-type and *hCatTg* MAECs. The efficiency of siRNA knockdown of Nrf2 was confirmed by Western blot analysis and quantitative real-time RT-PCR (Fig. 5A and B). Wild-type and *hCatTg* MAECs transfected with control siRNA showed comparable NQO1 protein and mRNA levels in the absence of BaP (Fig. 5C and D). Furthermore, treatment of MAECs with BaP induced NQO1 expression, indicating that the presence of siRNA itself did not affect the expression of the gene. In fact, *hCatTg* MAECs exhibited significantly higher NQO1

expression than wild-type cells as expected. In contrast, transfection with Nrf2-specific siRNA reduced the basal and BaP-induced levels of NQO1 expression in wild-type and *hCatTg* MAECs. NQO1 protein and mRNA levels in the Nrf2 siRNA-transfected cells were significantly lower than in cells transfected with the control siRNA (Fig. 5C and D). These data indicate a causal role for Nrf2 in enhancing NQO1 expression in *hCatTg* MAECs following BaP treatment.

AhR knockdown diminishes BaP-induced Nrf2 and NQO1 expression in MAECs

Consistent with our previous observations that overexpression of catalase upregulates AhR expression [7], *hCatTg* MAECs exhibited higher AhR mRNA and protein levels (Fig. 6A-C) compared to wild-type cells. To explore the regulatory role of AhR in BaP-induced Nrf2 and NQO1 expression in wild-type and *hCatTg* MAECs, we investigated the effects of siRNA-mediated knockdown of AhR. In the absence of BaP, AhR siRNA reduced AhR protein level by about 53% and 54% in wild-type and *hCatTg* MAECs, respectively (Fig. 6A and B). In the presence of BaP, AhR siRNA reduced AhR protein level by about 50% and 56% in wild-type and *hCatTg* cells, respectively. A similar inhibitory effect of AhR siRNA on AhR mRNA expression was observed in these cells (Fig. C). AhR knockdown in the absence of BaP did not significantly alter Nrf2 and NQO1 mRNA levels (data not shown). In contrast, knockdown of AhR inhibited BaP-induced transcription of Nrf2 (Fig. 6D) and NQO1 (Fig. 6E) significantly in wild-type and *hCatTg* MAECs. Specifically, AhR siRNA reduced BaP-induced Nrf2 and NQO1 mRNA levels by about 35% and 24%, respectively, in both wild-type and *hCatTg* MAECs. The data in Fig. 6 also show that BaP-induced Nrf2 and NQO1 mRNA levels were significantly higher in *hCatTg* MAECs than in wild-type cells under the same treatment conditions, which was consistent with the AhR levels in these cells. These results suggest that BaP-induced Nrf2 and NQO1 expression may be regulated by AhR.

Overexpression of catalase increases NQO1 promoter activity

Analysis of a 1-kb region upstream of the mouse NQO1 transcription start site (TSS) reveals that the promoter of this gene contains an XRE and an ARE [14,27]. The ARE is located ~426-441 nucleotides upstream of the TSS, while the XRE is located ~379-386 nucleotides upstream of the TSS [14,27]. We examined the transcriptional activity of these regions by luciferase assay. Mouse endothelial cells were transfected with promoter-luciferase constructs encoding wild-type (P1) or a mutated ARE (P2) or XRE (P3) (Fig. 7A). Wild-type NQO1 promoter activity was comparable in wild-type and *hCatTg* MAECs in the absence of BaP. Treatment of these cells with 1 μ M BaP increased NQO1 promoter activity significantly (Fig. 7B). This enhancement was significantly higher in *hCatTg* MAECs than in wild-type cells (2.1 ± 0.1 vs. 1.5 ± 0.1 -fold, $P < 0.05$). These results are consistent with those shown in Fig. 1. Interestingly, mutation of the ARE did not significantly alter NQO1 promoter activity. Under control conditions, the XRE-mutated NQO1 promoter was equally as active as the wild-type promoter in wild-type and *hCatTg* MAECs regardless of BaP treatment. These observations suggest that AhR does not activate NQO1 promoter directly. In contrast, mutation of the ARE almost completely abolished NQO1 promoter activity in wild-type and *hCatTg* MAECs in the absence of BaP. Furthermore, BaP treatment did not induce transcriptional activity (Fig. 7B). Collectively, these results indicate that Nrf2 binding to the NQO1-ARE is essential for basal and BaP-induced expression of NQO1 in mouse endothelial cells.

The effect of mithramycin A on Sp1 and AhR expression, and Sp1 binding to the AhR promoter

Sp1 is a transcription factor that controls AhR expression [18,19]. Mithramycin A (mmA) is a chemotherapeutic drug that blocks the binding of Sp1 to DNA [28]. We previously

reported that mutation of Sp1 binding sites in the AhR promoter region or inhibition of Sp1 binding with 500 nM mithramycin A abolished the enhancive effect of catalase overexpression on AhR promoter activity [20]. Data from the present study show that the Sp1 protein level in wild-type and *hCatTg* MAECs is comparable (Fig. 8B), however, the amount of Sp1 bound to the AhR promoter region is about 2.2-fold more in *hCatTg* MAECs than in wild-type cells (Fig. 8A). Despite no effect on Sp1 protein level, mmA significantly diminished the amount of Sp1 bound to the *AhR* promoter region and the level of AhR mRNA (Fig. 8A and 8C) in both *hCatTg* and wild-type MAECs, with a greater extent in *hCatTg* cells. Thus, wild-type and *hCatTg* MAECs treated with mmA manifest comparable levels of Sp1 DNA-binding and AhR mRNA. These results suggest that increase in Sp1 DNA-binding activity is a mechanism by which overexpression of catalase upregulates AhR expression.

The effect of H₂O₂ on AhR and NQO1 expression, and Sp1 binding to the AhR promoter

We previously reported that H₂O₂ diminished AhR promoter activity in both wild-type and *hCatTg* MAECs in a concentration-dependent manner, and that 100 μ M H₂O₂ reduced the AhR promoter activity in *hCatTg* MAECs to the level seen in wild-type cells without H₂O₂ treatment [20]. Data in the present study show that 100 μ M H₂O₂ reversed the enhanced effect of catalase overexpression on Sp1 DNA-binding activity and AhR mRNA expression. Specifically, the levels of Sp1 DNA-binding activity and AhR mRNA expression in *hCatTg* MAECs treated with H₂O₂ are comparable to those in wild-type cells without H₂O₂ treatment (Fig. 9A and B). In addition, H₂O₂ also reduces Sp1 binding activity and AhR mRNA level in wild-type MAECs. The data in Fig. 9C-D show that H₂O₂ does not alter the basal levels of NQO1 mRNA and proteins, but significantly reduces BaP-induced NQO1 expression in both wild-type and *hCatTg* MAECs. It appears that the DNA-binding activity of Sp1 and the expression level of AhR and NQO1 are reversely correlated to the cellular H₂O₂ level.

The effect of N-acetylcysteine on AhR and NQO1 expression

To study whether other antioxidants also up-regulate AhR and NQO1 expression, we examined the effect of N-acetylcysteine (NAC), a peroxyl radical scavenger, on AhR and NQO1 protein levels in wild-type MAECs. We previously reported that treatment of wild-type MAECs with 10 mM NAC abolished oxidized lipid-induced peroxides [29]. Data in Fig. 10 show that treatment of wild-type MAECs with 10 mM NAC for 12 h reduced the basal level of cellular peroxides about 15%, and BaP-induced peroxides about 77% (Fig. 10D). The effect of NAC on AhR and NQO1 expression was similar to the effects induced by catalase overexpression (Figs. 2 and 6). Specifically, NAC significantly increased the basal level of AhR and the BaP-induced level of NQO1 in wild-type MAECs (Fig. 10B and D). These data suggest that up-regulation of AhR and NQO1 expression is not a specific effect of catalase overexpression, but a shared function of antioxidants. This view is supported by our previous observations that overexpression of Cu/Zn-SOD up-regulates AhR and its target gene expression [7].

DISCUSSION

In this report, we demonstrated that overexpression of catalase significantly enhanced BaP-induced expression of NQO1 in MAECs, and that inhibition of catalase activity by aminotriazole or addition of H₂O₂ to the culture medium suppressed the upregulatory effect of catalase overexpression on BaP-induced NQO1 expression. Our data also demonstrated that the increase in BaP-induced NQO1 expression in *hCatTg* MAECs was associated with an increase in AhR and Nrf2 protein levels, and that knockdown of either AhR or Nrf2 diminished BaP-induced NQO1 expression significantly. These findings clearly indicate that

upregulation of AhR and Nrf2 is a mechanism whereby overexpression of catalase enhances BaP-induced NQO1 expression.

Mouse NQO1 promoter contains one XRE and one ARE located 379- and 426-bp upstream of the transcription start site, respectively [14,27]. Our data demonstrated that mutation of the XRE, the consensus regulatory sequence for AhR binding, did not alter NQO1 promoter activity, suggesting that AhR regulates NQO1 expression indirectly through other transcription factor(s), not via activation of the NQO1 promoter. In contrast, mutation of the ARE, the consensus regulatory sequence for Nrf2 binding, suppressed the basal and BaP-induced activities of the NQO1 promoter in both wild-type and *hCatTg* MAECs. These data are consistent with previous findings that the ARE is involved in basal and xenobiotic-induced activities of the NQO1 promoter [14]. More importantly, we observed that overexpression of catalase led to an increase in the amount of Nrf2 bound to the NQO1 promoter region, with enhanced NQO1 promoter activity upon BaP treatment. Taken together, it is highly likely that overexpression of catalase enhances Nrf2 expression in response to BaP exposure, thus increasing the amount of Nrf2 bound to the NQO1-ARE, which in turn increases the transcription of NQO1.

Studies have suggested that increasing oxidative stress is a mechanism by which BaP increases Nrf2 expression. BaP has been shown to increase reactive oxygen species (ROS) and oxidative damage in cells [7,30,31]. It has been well established that ROS, such as superoxide and H₂O₂, and other oxidants, such as oxidized lipids, can increase Nrf2 protein level by inhibiting its degradation [32,33] and upregulating Nrf2 mRNA transcription [17]. However, data from our previous studies demonstrated that overexpression of catalase reduced BaP-induced oxidative stress, as measured by H₂O₂ and lipid peroxidation levels in MAECs [7] and mouse aortas [26]. Thus, the increased Nrf2 protein level in *hCatTg* MAECs in response to BaP treatment is not only due to BaP-induced oxidative stress.

Nrf2 is a target gene of AhR [16]. Our results here established that overexpression of catalase increased AhR expression, and that knockdown of AhR attenuated the up-regulatory effect of catalase overexpression on BaP-induced Nrf2 and NQO1 expression. These data suggest that increasing AhR expression is critical for the increased Nrf2 and NQO1 induction by BaP in *hCatTg* MAECs. Sp1 is transcription factor that controls AhR expression. We previously reported that overexpression of catalase increased the AhR promoter activity, and that mutation of the Sp1 binding sites or inhibition of Sp1 binding to the AhR promoter reversed the enhanced effect of catalase overexpression on AhR promoter activity [20]. Data from the present study demonstrated that overexpression of catalase increased Sp1 binding to the AhR promoter region. Inhibition of Sp1 DNA-binding activity inhibited AhR expression. These observations suggest that enhancing the binding activity of Sp1 to the AhR promoter region is a mechanism underlying the increased AhR expression in MAECs overexpressing catalase.

Another important finding from this report is that exogenous H₂O₂ is able to reverse the enhanced DNA-binding activity of Sp1 and the increased AhR expression in MAECs overexpressing catalase. These findings suggest that reduction in endogenous H₂O₂ due to catalase overexpression protects Sp1 protein from oxidation, and therefore increases its ability to bind the AhR promoter and upregulate its expression. This postulation is supported by the evidence that H₂O₂ and other oxidants increase the oxidation of the cysteine sulfhydryl group of Sp1 and inhibit its DNA-binding activity [34,35].

Based on data from current and previous work [20], we propose the following model (Fig. 11) to describe how catalase overexpression regulates NQO1 expression. Reduction in oxidative stress by overexpression of catalase increases the DNA-binding activity of Sp1 to

the AhR promoter, which increases AhR expression and availability. BaP exposure causes AhR nuclear translocation, leading to induction of Nrf2 expression, which in turn increases NQO1 expression. Data from our previous [7] and current studies also show that other antioxidants, such as N-acetylcysteine and Cu/Zn-superoxide dismutase, are able to up-regulate the expression of AhR and xenobiotic-metabolizing enzymes, suggesting that up-regulation of AhR and its downstream genes might be a common activity of various antioxidants.

Several studies have established that the AhR-Nrf2 cascade regulates the expression of other phase II xenobiotic-metabolizing enzymes, such as GSTs and UGTs [7,13,36]. Overexpression of catalase may also upregulate the expression of these enzymes. Indeed, overexpression of catalase has been shown to reduce the accumulation of BaP reactive intermediates, some of which are substrates of these phase II enzymes [7]. These findings contribute to the larger goal of understanding the molecular mechanisms underlying the protective role of catalase overexpression against BaP-induced toxicity, such as atherosclerosis [26].

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Fig. 1.

Overexpression of catalase reduces BaP-induced peroxides and lipid peroxidation. MAECs were obtained from wild-type and *hCatTg* mice. The catalase protein level was determined in whole cell extracts by Western blot analysis (A) and quantitated relative to β -actin (B). The catalase mRNA level was determined by quantitative real-time RT-PCR and normalized to GAPDH mRNA (C). The peroxide level (D) was measured using CDC-H2F diacetate as a probe. The F₂-isoprostane level (E) was measured using GC/MS. Values represent the mean $\pm$ SEM of three separate experiments; MAECs were pooled from 4 mice. * $P < 0.05$ vs. wild-type cells without BaP treatment, $^{\dagger} P < 0.05$ vs. wild-type cells with BaP treatment; and $^{\#} P < 0.05$ vs. *hCatTg* cells without BaP treatment.

Fig. 2.

Overexpression of catalase elevates BaP-induced NQO1 expression. Wild-type and *hCatTg* MAECs at the 3rd or 8th-9th passages were treated with or without 1 μ M BaP in the presence or absence of 10 μ M aminotriazole (AT) as indicated. The level of NQO1 protein was determined in whole cell extracts by Western blot analysis (A) and quantitated relative to β -actin (B). The level of NQO1 mRNA was determined by quantitative real-time RT-PCR and normalized to GAPDH mRNA (C). Values represent the mean $\pm$ SEM of five independent experiments. * $P < 0.05$ vs. the same genotype cells without BaP treatment; † $P < 0.05$ vs. BaP-treated wild-type cells; # $P < 0.05$ vs. *hCatTg* cells without aminotriazole treatment.

Fig. 3.

Overexpression of catalase elevates BaP-induced Nrf2 expression. Wild-type and *hCatTg* MAECs at the 3rd or 8th-9th passages were treated with or without 1 μ M BaP as described in the Materials and Methods. The Nrf2 protein level in the whole cell was determined by Western blot analysis (A) and expressed relative to β -actin (B). The Nrf2 mRNA level was determined by quantitative real-time RT-PCR and expressed relative to GAPDH mRNA (C). Values represent the mean $\pm$ SEM of five independent experiments. * $P < 0.05$ vs. the same genotype cells without BaP treatment; † $P < 0.05$ vs. BaP-treated wild-type cells.

Fig. 4.

Overexpression of catalase increases BaP-induced Nrf2 accumulation in the nucleus and binding to the NQO1 promoter. Wild-type and *hCatTg* MAECs were treated with or without 1 μ M BaP. The level of nuclear Nrf2 was determined by Western blot analysis (A) and expressed relative to β -actin (B). Nrf2 binding to the NQO1 promoter was assessed by ChIP analysis (C). Genomic DNA bound to Nrf2 was recovered and quantified by quantitative real-time PCR using primer pairs specific for the NQO1-ARE region. Data are expressed relative to the quantity of input. Values represent the mean $\pm$ SEM of five independent experiments. * $P < 0.05$ vs. the same genotype cells without BaP treatment; † $P < 0.05$ vs. BaP-treated wild-type cells.

Fig. 5.

Knockdown of Nrf2 reduces NQO1 expression. Wild-type and *hCatTg* MAECs were transfected with Nrf2 siRNA or control siRNA, and then treated with or without 1 μ M BaP as described in Materials and Methods. The level of Nrf2 (A) and NQO1 (C) proteins were determined by Western blot analysis and expressed relative to β -actin. The mRNA levels of Nrf2 (B) and NQO1 (D) were determined by quantitative real-time RT-PCR and expressed relative to GAPDH mRNA. Values represent the mean $\pm$ SEM of five independent experiments. * $P < 0.05$ vs. the same genotype cells with same siRNA transfection and without BaP treatment; # $P < 0.05$ vs. the same genotype cells transfected with control siRNA and the same treatment; † $P < 0.05$ vs. BaP-treated wild-type cells with same siRNA transfection.

Fig. 6.

Knockdown of AhR reduces BaP-induced Nrf2 and NQO1 expression. Wild-type and *hCatTg* MAECs were transfected with AhR siRNA or control siRNA, and then treated with or without 1 μ M BaP as described in Materials and Methods. The level of AhR protein (A) was determined by Western blot analysis and expressed relative to β -actin (B). The mRNA level of AhR was determined by quantitative real-time RT-PCR and expressed relative to GAPDH mRNA (C). The mRNA levels of Nrf2 (D) and NQO1 (E) were determined by quantitative real-time RT-PCR; BaP-induced changes were expressed as a % of those observed in cells without BaP treatment. Values represent the mean $\pm$ SEM of five independent experiments. * $P < 0.05$ vs. the same genotype cells with same siRNA transfection and without BaP treatment; # $P < 0.05$ vs. the same genotype cells transfected with control siRNA and the same treatment; and $^{\dagger} P < 0.05$ vs. BaP-treated wild-type cells with the same siRNA transfection.

Fig. 7.

Functional analysis of the NQO1 promoter. NQO1 promoter-luciferase reporter plasmids were constructed and cotransfected into wild-type and *hCatTg* MAECs with a β -galactosidase expression plasmid as described in Materials and Methods. (A) Schematic diagram depicting wild-type (P1), ARE-mutated (P2), and XRE-mutated (P3) NQO1 promoter-luciferase constructs. Luciferase activity was measured using a luminescence assay and expressed relative to the luminosity of β -galactosidase assay (B). Values represent the mean $\pm$ SEM of five independent experiments. * $P < 0.05$ vs. the same genotype cells with same plasmid transfection and without BaP treatment; # $P < 0.05$ vs. the same genotype cells transfected with wild-type promoter (P1) and the same treatment; † $P < 0.05$ vs. wild-type cells transfected with same plasmid and treated with BaP.

Fig. 8.

The effect of mithramycin A on Sp1 and AhR expression, and Sp1 binding to the AhR promoter. Wild-type and *hCatTg* MAECs were treated with 500 nM mithramycin A (mma) or medium alone as a control for 24 h. (A) The amount of Sp1 bound to the AhR promoter was determined using ChIP analysis, and expressed as the ratio of input controls. (B) The Sp1 protein level was determined by Western blot analysis and expressed relative to the immunoblot intensity of β-actin. (C) The AhR mRNA level was determined by quantitative real-time RT-PCR and expressed relative to GAPDH mRNA level. Values represent means ± SEM of five separate experiments; in each, MAECs were pooled from four mice. * $P < 0.05$ vs. wild-type cells without mma treatment; and † $P < 0.05$ vs. *hCatTg* cells without mma treatment.

Fig. 9.

The effect of H_2O_2 on AhR and NQO1 expression, and Sp1 binding to the AhR promoter. Wild-type and *hCatTg* MAECs were treated with or without 100 μ M H_2O_2 for 2 h, and then treated with or without 1 μ M BaP for 4 h. (A) The amount of Sp1 bound to the AhR promoter was determined using ChIP analysis, and expressed as the ratio of input controls. AhR (B) and NQO1 (C) mRNA levels were determined by quantitative real-time RT-PCR and expressed relative to GAPDH mRNA. (D) The level of NQO1 protein was determined in whole cell extracts by Western blot analysis and quantitated relative to β -actin. Values represent the mean $\pm$ SEM of three independent experiments. * $P < 0.05$ vs. the same genotype cells without H_2O_2 or BaP treatment (control); $^\dagger P < 0.05$ vs. the same genotype cells with BaP treatment alone; and $^\# P < 0.05$ vs. wild-type cells with the same treatment.

Fig. 10.

The effect of N-acetylcysteine on peroxides, and AhR and NQO1 expression. After pre-incubation with or without 10 nM N-acetylcysteine (NAC) for 1 hr, wild-type MAECs were treated with 1 μ M BaP or culture medium alone (control) for 6 h (Western blot analysis) or for 1 h (peroxide measurement). The AhR and NQO1 protein levels were determined by Western blot analysis and quantitated (B, C respectively) relative to β -actin. Cellular peroxyl radicals were measured in terms of fluorescence intensity (FI) per μ g protein using CDC-H2F diacetate as a probe. Values represent the mean $\pm$ SEM of five independent experiments. * $P < 0.05$ vs. cells without NAC and BaP treatment; $^{\dagger} P < 0.05$ vs. cells with BaP treatment but without NAC treatment; and $^{\#} P < 0.05$ vs. cells with NAC treatment but without BaP treatment.

Fig. 11.
Proposed molecular mechanism for the role of catalase overexpression on BaP-induced NQO1 expression.