Transcriptional regulation of the HO-1 gene in cultured macrophages exposed to model airborne particulate matter

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Chin, Beek Yoke, Michael A. Trush, Augustine M. K. Choi, and Terence H. Risby. Transcriptional regulation of the HO-1 gene in cultured macrophages exposed to model airborne particulate matter. Am J Physiol Lung Cell Mol Physiol 284: L473–L480, 2003. First published November 27, 2002; 10.1152/ajplung.00297.2002.—Respirable particulate matter generated during incomplete combustion of fossil fuels may principally target the cells found in the distal region of the lung. This study characterizes some of the effects that a model particulate matter has on the induction of heme oxygenase-1 (HO-1) in macrophages. HO-1 is a highly inducible stress response gene that has been demonstrated to modulate chemical, physical, and environmental stimuli. Cultured macrophages (RAW 264.7 cells) exposed continuously to a well-defined model of particulate matter (benzo[a]pyrene adsorbed onto carbon black) induced HO-1 gene expression in a time-dependent manner. Likewise, the addition of benzo[a]pyrene-1,6-quinone, a redox cycling metabolite of benzo[a]pyrene, to RAW cells also induced HO-1. This particle-induced gene expression of HO-1 was found to correlate with a corresponding increase in protein levels. Gene regulation studies were performed to delineate the transcriptional regulation of HO-1 after exposure to model particulate matter. Deletional analysis of the HO-1 gene and mutational analysis of activator protein (AP)-1 regulatory element on both distal enhancers demonstrated the importance of this transcriptional factor in mediating HO-1 gene transcription in response to model particulate matter. These results were supported by gel shift analysis demonstrating increased AP-1 binding activity after exposure to particulate matter. In summary, this study demonstrates that model particulate matter enhanced the expression of HO-1. This inductive process may be mediated by AP-1 activation of the regulatory elements on both the 5′-distal enhancers.

heme oxygenase-1; benzo[a]pyrene; benzo[a]pyrene-1,6-quinone

Recent epidemiological studies have found correlations between increases in morbidity and mortality and exposure to fine and ultrafine particulate matter in humans with chronic respiratory ailments (22, 34). These correlations have highlighted the need for research in the area of molecular effects of particles with aerodynamic diameters ranging from 0.1 to <1.0 μm. Particulate matter generated during the combustion of fossil fuels represents a major contributor to this respira-
tory-sized airborne pollutant. This pollutant consists of a carbonaceous particle adsorbed with multiple layers of adsorbed pollutant molecules. When respirable-sized particles are inhaled and deposited in the distal lung, most of the adsorbed pollutants are released into the pulmonary surfactant, and then the underlying epithelial cells are exposed to the released pollutant molecules (3, 4, 9, 29, 35, 36). After this initial rapid release, the particles with their residual burden of adsorbed pollutants remain on the surface of the pulmonary surfactant until either cleared by the mucociliary escalator or phagocytosed by the resident macrophages (10, 15). The residue of particle-associated adsorbed pollutants may be released eventually and metabolized by oxidants generated by cellular processes that accompany phagocytosis.

Heme oxygenase, a cellular antioxidant, is one of the key enzymes catalyzing the degradation of heme-containing molecules to biliverdin (14, 40) and bilirubin (39). The process of degradation also releases iron, which is immediately sequestered into ferritin (41, 43). Heme degradation also produces carbon monoxide, which has been shown to protect against TNF-α-mediated apoptosis and to have anti-inflammatory properties (5, 26). There are three isofoms of heme oxygenase: HO-1 (32 kDa) is an inducible form found ubiquitously in all organs. HO-1 is induced by heme, metals, UV irradiation, lipopolysaccharide, cytokines (for reviews, see Refs. 6, 13, 23, 33), and most recently diesel particulate matter (19). The other isofoms, HO-2 (36 kDa) and HO-3 (33 kDa), are constitutively expressed, primarily in the brain/testes and liver/prostate/kidneys, respectively. Induction of HO-1 or over-expression of HO-1 has also been shown to protect against hyperoxic injury (16, 17, 25, 26). The impor-

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tance of the cytoprotective effects of HO-1 was demonstrated convincingly in HO-1 knockout studies whereby the mice aged prematurely and eventually died within 2 mo postern from oxidant-related complications (27).

In this study, we used a well-defined model particulate matter (8, 28–31, 35, 36) to examine the molecular regulation of HO-1 gene transcription and expression in peritoneal and alveolar macropahges or type II alveolar epithelial cells.

MATERIALS AND METHODS

Cell culture. A murine peritoneal macrophage cell line (RAW 264.7), a murine alveolar macrophage cell line (MHS), and a human type II alveolar epithelial cell line (A549) were purchased from American Type Culture Collection (Rockville, MD). These cell lines have been well characterized and used extensively for in vitro studies. Primary human alveolar macrophages were obtained by bronchoalveolar lavage by a protocol that had been reviewed and approved by the Joint Committee on Clinical Investigation of the Johns Hopkins Medical Institutions. All cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 10% FBS (Hyclone Laboratory, Logan, UT) and gentamicin (50 μg/ml) in a humidified atmosphere of 5% CO2 and 95% air at 37°C.

Model particulate matter. The carbon black (CB, N339) used in this study was manufactured (Cabot, Boston, MA) according to the specifications of the American Society for Testing Materials (ASTM). This CB was selected because it has been shown to have similar physical and chemical properties to the carbonaceous particles produced during the combustion of petroleum-based fuels (28, 30, 31, 46). N339 is an amorphous furnace CB produced from the thermal decomposition of oil feed stock, it has imperfect graphitic structure, and it is devoid of long-range order (28). The major advantage of using an ASTM CB is that its properties do not vary from particle to particle (28). N339 has a measured nitrogen surface area of 109 m2/g, and the intrinsic particle is aciniform with a diameter in the range 2–20 nm. The empirical formula of N339 is C34H27O14N (28). The active sites on N339, a type I adsorbent, arise from surface defects in the short-range graphitic structure and from quinone-like functional groups. Adsorption of molecules onto N339 has been characterized from ultra-low surface coverages to supramolecular interactions (28). Airborne particles undergo multiple aggregation for at least 48 h. The cells were exposed to known concentrations of this suspension. Suspensions of deaggregated untreated CB or a saturated suspension of BaP were used as controls for this study. Cells were continuously exposed to the treatment for varying times. Benzo[a]pyrene (BP; 1,6-quinone (BP-1,6-Q)) was obtained from the NCI Chemical Carcinogen Repository Midwest Research Institute (Kansas City, MO).

RNA extraction and Northern blot analyses. We isolated total RNA from cells by the TRIzol method using direct lysis of the cells in TRIzol buffer followed by chloroform extraction (Life Technologies, Gaithersburg, MD). Northern blot analyses were performed as previously described (8). Samples of total RNA (10 μg) were separated by electrophoresis in a 1% denaturing agarose gel, transferred to Gene Screen Plus nylon membranes (DuPont, Boston, MA) by capillary action, and cross-linked with a UV Stratalinker (Stratagene, La Jolla, CA). The membranes were prehybridized in hybridization buffer [1% BSA, 7% sodium dodecyl sulfate (SDS), 0.5 M phosphate-buffered saline (PBS), pH 7.0, and 1.0 mM EDTA] at 65°C for 2 h, followed by incubation in hybridization buffer containing 10 μg/ml of random-primed [32P]CTP-labeled rat HO-1 cDNA for 12 h. Nylon membranes were washed twice in buffer A (0.5% BSA, 5% SDS, 40 mM phosphate buffer, pH 7.0, and 1.0 mM EDTA) for 15 min at 55°C. Ethidium bromide staining of the gels was used to confirm both RNA integrity and normalization. To control for equal loading, we hybridized blots with an oligonucleotide probe that was complementary to 18s rRNA after removal of the HO-1 probe.

dNA and oligonucleotide probes. A full-length rat cDNA, generously provided by Dr. S. Shibahara of Tohoku University, Japan (34), was subcloned into pBluescript vector, and HindIII/EcoRI digest was performed to isolate a 3.9-kb HO-1 cDNA subfragment. We synthesized a 24-base pair oligonucleotide (5′-ACG-GTA-TCT-GAT-CGT-CTT-CGA-ACC-3′) complementary to 18s rRNA using a DNA synthesizer (Applied Biosystems; Foster City, CA). HO-1 cDNA was labeled with [α-32P]CTP using a random primer kit (Boehringer Mannheim, Mannheim, Germany). The 18s rRNA oligonucleotide was labeled with [α-32P]ATP at the 3′-end with terminal deoxynucleotidyl transferase (Life Technologies).

Western blot analysis. Total cellular protein extracts were obtained for the Western analyses as previously described (8). Briefly, cells were lysed in buffer containing 1% Nonidet P-40, 20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml aprotinin. Protein concentrations of the cell lysates were determined by Coomassie blue dye binding assay (Bio-Rad Laboratories, Hercules, CA). An equal volume of 2× SDS loading buffer (0.125 mM Tris·HCl, pH 7.4, 4% SDS, and 20% glycerol) was added, and the samples were boiled for 5 min. Protein samples (100 μg) were separated by 12% SDS-PAGE, then electroblotted onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). The membranes were incubated with HO-1 antibody (1:100; StressGen Biotech, Vancouver, Canada) for 1.5 h, followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibody for 1.5 h. Signal development was carried out with an enhanced chemiluminescence detection kit (Amersham).

Cellular nuclear protein extract. The RAW 264.7 cells were scraped in cold PBS and centrifuged at 14,000 g at 4°C for 10 min. After removal of the supernatant, the pellet was resuspended in lysis buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, 1 mM dithiothreitol (DTT), 0.5% Nonidet P-40 (Igepal), and 1 mM PMSF). The lysate was maintained on ice and subsequently spun at 1,500 g to separate the cellular nuclei. After a brief wash in lysis buffer without Igepal, the
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briefly, DNA binding activity was determined after incubation of 1.0 μg of RAW 264.7 cell nuclear protein extracts with 20 fmol (20,000–50,000 counts/min) of a 32P-labeled 22-mer oligonucleotide containing the AP-1 consensus binding site (5’-CTA-GTG-AGT-CAG-CG-G-ACT-C-3’) (Stratagene) in a buffer containing 10 mM HEPES, pH 7.9, 1 mM DTT, 1 mM EDTA, 80 mM KCl, 1 mg poly[dI-dC], and 4% Ficoll. The reaction mixture was separated by electrophoresis on a 6% polyacrylamide gel after 20 min of incubation, transferred to DE81 ion exchange chromatography paper (Whatman, Maidstone, England), and dried down before exposure to autoradiographic film.

RESULTS

Dose-dependent induction of HO-1 expression after exposure to model particulate matter for 2 h. Northern blot analyses were used to examine the dose-dependent increases in HO-1 mRNA after exposure to different concentrations of model particulate matter (Fig. 1, CB + BaP). RNA was extracted from RAW 264.7 cells after exposure to model particulate matter: untreated (lane 1), 0.1 (lane 2), 1.0 (lane 3), 2.0 (lane 4), and 4.0 μg/ml (lane 5). As seen in Fig. 1, marked increases in the levels of HO-1 mRNA were observed after exposure to concentration of 2.0 (lane 4) and 4.0 μg/ml (lane 5) for 2 h. The species 18s rRNA was used as a loading control. On the basis of these results, we performed all subsequent studies using model particulate matter at a concentration of 2.0 μg/ml.

Temporal induction of HO-1 expression after exposure to model particulate matter. Northern blot analyses were used to examine the temporal induction of HO-1 mRNA in RAW 264.7 cells after exposure to model particulate matter. As shown in Fig. 2A, there is a marked increase in steady-state levels of HO-1 mRNA after exposure to model particulate matter for 2 h. This increase was maintained for up to 8 h of exposure and declined at 24 h. The species 18s rRNA was used as a loading control. We also observed increased HO-1 protein expression after 4–8 h of exposure to model particulate matter.
sure to model particulate matter. RAW 264.7 cells exposed to untreated CB displayed different kinetics of HO-1 expression. In Fig. 2B, one can see a slight increase in steady-state HO-1 mRNA after 4 h of exposure, a phenomenon that was heightened only after 24 h of continuous exposure. Ethidium bromide was used as the normalization control. There were no detectable increases in the HO-1 mRNA extracted from cells exposed to a saturated solution of BaP (2 µg/ml, data not shown). However, there was a striking increase in HO-1 mRNA after exposure for 1 h to a BaP metabolite, BP-1,6-Q (1 µM). This increase was maintained for up to 8 h of exposure (Fig. 3). Ethidium bromide was used as the normalization control.

Model particulate matter induces HO-1 gene expression in alveolar cells. We also performed preliminary studies in other cell lines to ensure that the induction of HO-1 mRNA by this model particulate matter extended to pulmonary cell types. A type II alveolar epithelial cell line (A549) and an alveolar macrophage cell line (MHS) were exposed to model particulate matter for up to 24 h, and HO-1 expression was monitored by Northern blot analyses. There were measurable increases in the steady-state levels of HO-1 mRNA in type II alveolar epithelial cells (Fig. 4A) and the macrophage cell line (Fig. 4B) after exposure for 1 h to...
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Fig. 4. Northern blot analysis of HO-1 mRNA expression in alveolar cell lines A549, type II alveolar epithelial cells (A); MHS, alveolar macrophage cells (B); and primary alveolar human macrophages (C) after exposure to 2 μg/ml CB + BaP. Total RNA was isolated from the cell lines after times indicated and analyzed for HO-1 mRNA. Et Br and 18s rRNA were used as loading controls. Lane 1, Ctl; lane 2, 1-h CB + BaP; lane 3, 2-h CB + BaP; lane 4, 4-h CB + BaP; lane 5, 8-h CB + BaP. There is a time-dependent increase in HO-1 expression in A549 (A) and MHS (B) cells after exposure to CB + BaP. In C, alveolar macrophages were isolated from human bronchiolar alveolar lavage fluid and exposed to 4 h of CB + BaP.

Deletional analysis of HO-1 gene after exposure to model particulate matter. Within the time frame examined, the highest levels of HO-1 mRNA were observed at 8 h in the A549 cells and at 4 h in the MHS cells. Ethidium bromide and 18s rRNA were used as normalization controls, respectively. Primary human alveolar macrophages (obtained by bronchoalveolar lavage) exposed to model particulate matter for 4 h also demonstrated marked induction of HO-1 expression (Fig. 4C).

5.7-fold induction after exposure to model particulate matter (Fig. 5B). Additional deletions of the 5′-flanking region of the HO-1 gene demonstrated a reduction of CAT activity in the minimal promoter alone (pMHO1CAT), 2.5-fold, and in the pMHO4CAT containing only the SX2 enhancer (3.8-fold).

Mutational analysis of the AB1 enhancer. Because full activation of the HO-1 gene required the AB1 enhancer fragment, additional stable cell transfectants possessing modifications of the AB1 enhancer sites were generated (Fig. 6). The AB1 fragment contains three putative AP-1 sites. Mutation of the first AP-1 site reduced CAT activity fourfold (AB1M16). Mutation of a site other than AP-1 did not produce an effect (AB1M17).

Transcriptional factor AP-1 is activated. We performed electromobility shift assays to confirm the role of AP-1 activation using a synthetic, double-stranded DNA probe specific for the AP-1 binding sites. Increases in AP-1 binding activity in RAW 264.7 cells after exposure to model particulate matter for 2 h are shown in Fig. 7. The specificity on AP-1 binding was exhibited by the ability of the "cold" (unlabeled) 10- and 100-fold (Fig. 7, lanes 5 and 6) AP-1 to compete for the labeled AP-1 counterparts. Further confirmation of AP-1 binding was enhanced by the inability of another transcriptional factor, SP-1, to compete with labeled AP-1. A potential protein involved in AP-1 dimerization was confirmed by addition of c-fos antibody to the nuclear protein extract, demonstrated by supershifted bands to AP-1 in lane 7.
Airborne particulate matter is a public health problem that has been related to increased morbidity and mortality. We have demonstrated that a model airborne particle, CB + BaP, markedly induced the expression of a stress response gene, HO-1, after 2 h of exposure in macrophages. Macrophages exposed to untreated CB also exhibited increased levels of HO-1. However, the HO-1 mRNA levels were much less than those observed with CB + BaP and were only detectable after 4 h of exposure to CB. Interestingly, exposure of macrophages to a saturated solution of BaP without carbon particles did not induce HO-1. Treatment of macrophages with one of its metabolites, BP-1,6-Q, increased HO-1 mRNA levels dramatically after exposure for 1 h. BP-1,6-Q is a redox cycling metabolite of BaP (42).

Little is known about the exact molecular mechanisms governing HO-1 gene expression. Different inducers of HO-1 have been demonstrated to utilize either the AB1 or SX25/distal enhancer elements of the HO-1 gene (7, 17, 18), and it has been shown that the promoter alone is inadequate. Herein, we demonstrate that exposure of cells containing the minimal promoter (pMHO1CAT) to CB + BaP is sufficient to generate basal activity. We further show that mutations of the AP-1 binding sites at either enhancer would abrogate gene activity. Together, these results, although not conclusive, demonstrate that exposure to CB + BaP induced HO-1 gene activity irrespective of the participation of either enhancer. Maximal gene activity, however, requires activation of AP-1 binding activity on both enhancers. The selective differences in gene activation could also be explained by recent reports that the overlapping consensus sequences shared by both the antioxidant response elements (GTGAC-NNNGC) and AP-1 (TGAC-NNNTCA) may function either cooperatively or independently (13, 31, 44). Additional studies will be required to explain this gene activation.

Our model particulate matter, CB + BaP, has allowed us to study how gene expression is related to the...
bioavailability of the prototypical adsorbed pollutant molecule, BaP. This model, CP + BaP, was proposed, since it is difficult to predict the bioavailabilities of molecules adsorbed on the surface of actual airborne particulate matter. The enthalpy of adsorption is dependent on the identity of the adsorbing molecule, surface properties of the particle, surface coverage, and history of when the molecule was adsorbed on the surface (29,35). On the basis of these studies, free BaP can be eliminated as an inducer of HO-1 expression in these cells. Rather, the phagocytic process acts as the stimulus for carbon particle-induced HO-1 expression as a result of the oxidative environment of the phagolysosome contributing to the oxidation of BaP to a redox-reactive metabolite capable of eliciting reactive oxygen species (ROS)-mediated signal transduction. Adsorption of BaP onto the carbon particle will decrease the number of degrees of freedom and may increase the lifetime of the reaction complex. Moreover, the activation energy for the oxidation of BaP may be decreased as a result of being adsorbed on the surface of a carbon particle.

These results suggest that there is a mechanistic relationship between particle phagocytosis by macrophages, particle-adsorbed BaP, metabolic activation of BaP, and the induction of HO-1. It is well known that phagocytosis is accompanied by a respiratory burst resulting in the generation of ROS. The ROS can contribute to the metabolic activation of many xenobiotics (21). On the basis of the results of studies with free BaP vs. BP-1,6-Q, it is reasonable to propose that carbonaceous particle-adsorbed BaP is oxidized by phagocytic burst of ROS to a more active metabolite. The following sequence of events are proposed as an explanation of the induction of HO-1 expression: 1) carbonaceous particles are phagocytized by the macrophages and are subjected to the phagolysosomal milieu that includes NADPH oxidase-derived ROS, 2) BaP adsorbed on a carbon particle is oxidized by ROS to produce 6-hydroxybenzo[a]pyrene (based on T. H. Risby’s unpublished observations with model ROS-generating systems), and 3) 6-hydroxybenzo[a]pyrene diffuses out of the phagolysosome and is further oxidized to BP-1,6-Q. This BaP metabolite can redox cycle in cells leading to increased cellular production of ROS (42). This activity has been shown to result in increased gene expression (45). The role of ROS in HO-1 induction is supported by a study by Li et al. (19). These researchers have shown that pretreatment with the antioxidant N-acetyl cysteine before exposure to diesel exhaust particles prevented the expression of HO-1.

The use of HO-1 as a biologically relevant indicator of particulate matter-induced stress was exemplified in a study whereby the polynuclear aromatic hydrocarbon content derived from airborne particulate matter positively correlated with an increase in HO-1 expression (20). The physiological significance of induction of HO-1 as a result of exposure to carbonaceous airborne particulate matter is currently unknown. However, the induction of heme oxygenase and its catalytic by-products has been shown to be protective in chronic renal inflammation (24), cardiac ischemia/reperfusion (11), and hyperbaric oxygen treatment (38). Moreover, there were also indications that HO-1 may be used as a therapeutic target (12).

REFERENCES


