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Activation of *c-Ha-ras* by Benzo(*a*)pyrene in Vascular Smooth Muscle Cells Involves Redox Stress and Aryl Hydrocarbon Receptor

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ABSTRACT

Repeated cycles of vascular injury by benzo(*a*)pyrene (BaP) increase the onset and progression of atherosclerotic lesions in laboratory animals. This atherogenic response is partly mediated by activation of cis-acting antioxidant/electrophile response elements that enhance *c-Ha-ras* transcription in vascular smooth muscle cells (vSMCs). Activation of antioxidant/electrophile responsive cis-acting elements may depend on metabolism of BaP by cytochrome P450s to intermediates that induce oxidative stress and modulate gene expression. To test this hypothesis, we evaluated mitogen-activated *c-Ha-ras* expression in vSMCs treated with BaP or its metabolic intermediates alone, and in combination with agents that modulate cellular redox status. BaP (0.3 and 3 μM), BaP-3,6-quinone (0.3 μM), or hydrogen peroxide (50 μM) enhanced serum-activated *c-Ha-ras*. Ellipticine (0.01 nM), a known inhibitor of cytochrome P450 metabolism and aryl hydrocarbon receptor (AhR) antagonist, inhibited *c-Ha-ras* induction by BaP (3 μM). Serum challenge of G_0 synchronized cultures of vSMCs with DL-buthionine-(*S*,*R*)-sulfoximine (0.1 mM), a depletor of cellular glutathione, increased *c-Ha-ras* mRNA levels during the early phase of the mitogenic response. Combined BaP/DL-buthionine-(*S*,*R*)-sulfoximine challenge was cytotoxic to the cells and inhibited *c-Ha-ras* expression, whereas up-regulation of antioxidant capacity by *N*-acetylcysteine (0.5 mM) precluded BaP-induced *ras* expression. BaP increased formation of reactive oxygen species and depleted cellular glutathione, but these changes did not correlate with the kinetics of *c-Ha-ras* induction. BaP did not enhance *c-Ha-ras* expression in vSMCs from AhR knockout mice, although aryl hydrocarbon hydroxylase activity was constitutively expressed in these cells. These results suggest that *c-Ha-ras* activation in vSMCs by BaP involves a redox-sensitive mechanism that is coupled to AhR receptor-dependent functions.

Aberrant proliferation and migration of vascular smooth muscle cells (vSMCs) from the tunica media into the lumen of the artery are key early events in atherosclerosis (Ross, 1993). During initial stages of atherosclerotic plaque formation, oxidized low-density lipoproteins circulating in blood are engulfed by macrophages in the subendothelial space, giving rise to foam cells that become "fatty streaks." Oxidized low-density lipoproteins also injure cells within the vessel wall (Ross, 1993) and modulate mitogenic signaling in vSMCs (Kusuhara et al., 1997). A correlation exists between levels of homocysteine, an intermediate in cellular methionine metabolism and inducer of oxidative stress, and the formation of oxidized low-density lipoproteins and H_2O_2 (McCully, 1996). Homocysteine also is linked to myointimal cellular proliferation in baboons (Harker et al., 1983), and vSMC proliferation in vitro after up-regulation of cyclins D1 and A (Tsai et al., 1994). In a similar fashion, oxidants and pro-oxidants present in tobacco smoke increase the formation of vascular atherosclerotic lesions in laboratory animals (Ramos et al., 1994). For benzo(α)pyrene (BaP), the atherogenic response involves reprogramming of mitogenic signal transduction pathways and induction of proliferative phenotypes (Ramos et al., 1996).

The responses elicited by BaP in vSMCs are reminiscent of those in epithelial cells where BaP acts as a complete carcinogen. This homology has led to the suggestion that common molecular links exist between atherogenesis and carcinogenesis. BaP carcinogenicity is mediated by oxidative metabolism of the parent compound to BaP-7,8-diol-9,10-epoxide after repeated cycles of cytochrome P450 (CYP)-mediated metabolism (Pelkonen and Nebert, 1982). BaP-7,8-diol-9,10-epoxide covalently binds to cellular macromolecules, leading

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ABBREVIATIONS: vSMC, vascular smooth muscle cell; BaP, benzo(*a*)pyrene; CYP, cytochrome P450; ROS, reactive oxygen species; ARE/EpRE, antioxidant/electrophile-responsive cis-acting elements; AhR, aryl hydrocarbon receptor; BaPQ, BaP-3,6-quinone; FBS, fetal bovine serum; NaC, *N*-acetylcysteine; BSO, DL-buthionine-(*S*,*R*)-sulfoximine; AHH, aryl hydrocarbon hydroxylase; GSSG, glutathione disulfide; DCFDA, dichlorofluoroscin diacetate; ellip, ellipticine.

to formation of mutagenic DNA adducts (Pelkonen and Nebert, 1982). BaP also is oxidized to 3-hydroxy- and 6-hydroxy-BaP, which further oxidize to form BaP quinones that can undergo redox cycling and generate reactive oxygen species (ROS; Lesko et al., 1975). Although both of these pathways are operative in vSMCs (Bond et al., 1979, 1980), their contributions to BaP atherogenesis are not yet fully understood.

The occurrence of oxidized BaP metabolites in vSMCs implicates oxidative stress as a mechanism in the modulation of cellular phenotypes and mitogenic signaling. This hypothesis is consistent with the demonstration that H_2O_2 and O_2^- induce *c-myc* and *c-fos* expression and enhance DNA synthesis (Rao and Berk, 1992), and differentially activate protein kinases (Baas and Berk, 1994) in vSMCs. As shown by Baas and Berk (1994), O_2^- enhances mitogen-activated protein kinase activity, whereas H_2O_2 increases the activity of mitogen-activated protein kinase phosphatase, an important regulator of growth in *ras*-transformed cells (Sun et al., 1994), and H_2O_2 stimulates tyrosine phosphorylation of epidermal growth factor receptor to activate $p21^{ras}$ in vSMCs (Rao, 1996). Thus, interactions probably exist between growth regulatory genes and redox status in vSMCs.

The *c-Ha-ras* proto-oncogene encodes for $p21^{ras}$, a membrane-bound GTP-binding protein that acts as an upstream regulator of mitogen-activated protein kinase signaling. A role for this gene in atherogenesis was first described by this laboratory in studies showing that overexpression of mutant $Ha\text{-}ras^{EJ}$ in vSMCs induces proliferative phenotypes and loss of differentiation (Sadhu et al., 1994). Interestingly, angioplasty-induced restenosis has been associated with $p21^{ras}$ activity within the artery wall (Ueno et al., 1997). A central role for *c-Ha-ras* in the regulation of vSMC phenotypes and atherogenesis is consistent with the ability of chemical atherogens, such as BaP, to disrupt the kinetics of gene induction and growth factor-dependent *ras* signaling (Sadhu et al., 1993; Ramos et al., 1996).

Given that activation of *c-Ha-ras* transcription by BaP in vSMCs is mediated by antioxidant/electrophile-responsive cis-acting elements (ARE/EpREs) within the regulatory region of the gene (Bral and Ramos, 1997), this study was conducted to evaluate the role of redox mechanisms in the regulation of *c-Ha-ras*. We present evidence that modulation of mitogen-stimulated *c-Ha-ras* expression in vSMCs by BaP involves a redox-sensitive mechanism that is coupled to aryl hydrocarbon receptor (AhR)-dependent functions.

Materials and Methods

Chemicals. BaP (98% purity) was obtained from Aldrich Chemical Co. (Milwaukee, WI). BaP-3,6-quinone (BaPQ) and 3-OH BaP were obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repositories (c/o Midwest Research Institute, KS City, MO). Medium 199, Dulbecco's modified Eagle's medium with F12 salts, antibiotic, and trypsin were purchased from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Norcross, GA). Collagenase was purchased from Worthington (Freehold, NJ). Nylon membranes were purchased from Amersham (Chicago, IL). X-ray film (XAR 5 and MR) for autoradiography was from Kodak (Rochester, NY). [α-^{32}P]dCTP (3000 Ci/mmol) was from New England Nuclear (Boston, MA). High Prime random-primed labeling kit was purchased from Boehringer Mannheim (Indianapolis, IN). Tri-reagent was purchased from Molecular Research Center, Inc. (Cincinnati, OH). Restriction enzymes were purchased from Promega (Madison, WI). Tris/glycine/SDS buffer, Tris/glycine buffer, and polyvinylidene difluoride membranes were purchased from Bio-Rad (Hercules, CA). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture Procedure. vSMCs were isolated by successive enzymatic digestion of the thoracic aorta from $AhR^{+/+}$, $AhR^{+/-}$, and $AhR^{-/-}$ female C57/Bl6J mice (20–30 g). Cells were grown in Medium 199 supplemented with 10% FBS, 2 mM glutamine, and antibiotics (10,000 U/ml penicillin, 10 mg/ml streptomycin, and 25 mg/ml amphotericin) at 37°C in 5% CO_2, 95% air. Subcultures were prepared by trypsinization of subconfluent primary cultures and used between passages 12 to 26.

Chemical Treatments. Stock solutions of BaP (40 mM) and BaPQ (8.33 mM) were prepared in dimethyl sulfoxide and kept at −20°C in the dark. Stock solutions of *N*-acetylcysteine (NaC; 100 mM) and DL-buthionine-(*S*,*R*)-sulfoximine (BSO; 100 mM) were prepared in sterile PBS and stored at 4°C. For glutathione (GSH) measurements, vSMCs were seeded in 6-well culture plates at a density of 150 cells/mm^2. Cells were allowed to attach for 24 h and then serum-deprived in Medium 199 containing 0.1% FBS for 72 h to synchronize cells in G_0 (Sadhu and Ramos, 1993). The BaP concentrations tested were chosen based on established gene inducibility profiles (Bral and Ramos, 1997). BaPQ was tested at 0.3 μM because this concentration falls within the BaP-responsive concentration range and is a noncytotoxic quinone concentration that can be readily solubilized in aqueous media. H_2O_2 (25, 50, and 100 μM) was tested at concentrations previously reported to enhance cell signaling and proto-oncogene expression (Rao and Berk, 1992). vSMCs were pretreated with NaC (0.5 mM) or BSO (0.1 mM) in the absence of serum for 8 h. vSMCs were then incubated with BaP (0.3 or 3 μM) alone, or in combination with NaC (0.5 mM) or BSO (0.1 mM), in the presence of serum for various times. For measurements of ROS, vSMCs were seeded in 100-mm culture dishes at a density of 150 cells/mm^2 and allowed to attach for 24 h. Cells were G_0 synchronized in Medium 199/0.1% FBS for 72 h, transferred to Lab-Tek (Naperville, IL) glass slide wells in Medium 199/10% FBS, and then challenged with BaP for various times. For Northern analysis and aryl hydrocarbon hydroxylase (AHH) measurements, vSMCs were seeded in 100-mm culture dishes at a density of 150 cells/mm^2. Final dimethyl sulfoxide concentrations in the cultures never exceeded 0.075%.

GSH Measurements. Cells were rinsed with PBS to remove excess media and freeze/thawed 3× in 5% metaphosphoric acid. Cells were scraped and transferred to microfuge tubes and centrifuged at 12,000*g*. Aliquots of the supernatant were taken from each sample for GSH measurements. GSH was measured by the 5,5′-dithio-bis-(2-nitrobenzoic acid)-glutathione disulfide (GSSG) reductase recycling assay as described by Anderson (1985). Briefly, 20 μl of sample was aliquoted into a microfuge tube and warmed to 37°C for 15 min. Each sample was then combined with 700 μl of daily buffer (NADPH; 0.3 mM) in stock buffer [Na_2HPO_4 (143 mM) and Na_4EDTA (6.3 mM), final pH = 7.5)], 10 μl of dithiobisnitrobenzoic acid (6 mM stock), and 10 μl of GSH reductase (2.86 U). The absorbance at 412 nm was measured every 30 s for 5 min in a kinetic mode. The ΔA/min was measured and compared with GSH and GSSG standards to calculate actual concentrations. Measurements were normalized to cellular protein content in each dish by a microbiuret assay.

Measurement of ROS. Serum-deprived vSMCs were seeded in 2-well slides at a density of 150 cells/mm^2 in Medium 199/10% FBS. Kinetic measurements of H_2O_2 levels were conducted with dichlorofluoroscin diacetate (DCFDA) dissolved in Dulbecco's modified Eagle's medium with F12 salts. At the appropriate times the Medium 199/10% FBS was removed from the slides and rinsed with PBS. Dulbecco's modified Eagle's medium with F12 salts with DCFDA was applied to the cells and fluorescence measurements were conducted at 488 nm by argon-ion laser cytometry with the Meridian ACAS Ultima.

RNA Extraction and Analysis. Total RNA was extracted with Tri-reagent according to manufacturer's specifications as described by Chomczynski and Sacchi (1987). Briefly, cells were scraped in 0.8 ml of Tri-reagent and allowed to sit at room temperature for 5 min. Samples were then combined with 0.2 ml of chloroform, vortexed, and allowed to sit at room temperature for 2 min. After centrifugation at 12,000*g* (4°C) for 15 min, the aqueous layer was mixed with an equal volume of isopropanol and stored at −20°C overnight. This solution was centrifuged for 15 min at 12,000*g* (4°C) and the pellet washed with 70% ethanol, dried, and resuspended in 50% formamide. RNA concentration was determined spectrophotometrically at 260 nm.

Northern Analysis. Ten micrograms of total RNA was dissolved in 50% formamide, mixed with 10 μl of 2× buffer (63.5% formamide, 7.6% formaldehyde, 1 M Na_2HPO_4, and 15% 6× gel loading buffer), and denatured by heating at 55°C for 10 min. Total RNA was separated by electrophoresis on a formaldehyde denaturing gel [1.2% agarose, 1 M formaldehyde, and 1× SPC (20 mM Na_2HPO_4, 2 mM *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′*N*′-tetraacetic acid, final pH = 6.8)] in 1× SPC buffer and transferred onto a nylon membrane by capillary action. Membranes were dried at room temperature and cross-linked with a Stratagene (La Jolla, CA) UV crosslinker (4 min at 254 nm). The membrane was prehybridized at 45°C for *c-Ha-ras* and 60°C for β-tubulin for 18 to 24 h with hybridization buffer containing 45% formamide, 6× SSPE (0.75 mol/l NaCl, 0.05 mol/l NaH_2PO_4, and 5 mM EDTA, pH 7.4), 1% SDS, 10% dextran sulfate, and 100 μg/ml sheared herring testes DNA and then hybridized with ^{32}P-labeled probe in the same buffer for 18 to 24 h. β-Tubulin cDNA (1.6 kb) was excised from a pBluescript plasmid with *Eco*RI and *c-Ha-ras* cDNA (0.8 kb) was from Oncor (Gaithersburg, MD). Probes were radiolabeled with a High Prime random-primed labeling kit. After hybridization the blots were subjected to stringent washes in 0.1× SSPE/0.2% SDS at 55°C for *c-Ha-ras* and 65°C for β-tubulin, air dried at room temperature, and exposed to X-ray film at −80°C for 4 to 24 h. mRNA levels were quantified with a Betagen beta scanner. Target mRNAs were standardized against β-tubulin mRNA.

AHH Activity. AHH activity was measured as described by Nebert and Gelboin (1968). Briefly, vSMCs were harvested in ice-cold Tris-sucrose buffer (50 mM Tris and 200 mM sucrose, pH 8.0) and centrifuged at 1100 rpm for 5 min at 4°C. The supernatant was decanted and the pellet resuspended in ice-cold buffer. An aliquot (100 μl) of sample was combined with 850 μl of 0.1 M HEPES (pH 8.0), 10 μl of 0.4 mM NADPH (in 1% Na bicarbonate), and incubated at 37°C for 2 min before addition of 40 μl of 80 μM BaP (dissolved in MeOH) for an additional 15 min. One milliliter of ice-cold acetone and 3.25 ml of hexane were added before the organic layer was removed and combined with 5 ml of 1 N NaOH. The aqueous layer was then transferred to a new tube and monitored on a spectrofluorimeter at an excitation spectrum of 396 nm and emission spectrum of 522 nm. Protein was measured by the method of Bradford (1976). Authentic 3-OH BaP was used as a standard. AHH activity was expressed as picomoles of 3-OH BaP formed/15 min/milligram protein.

Statistical Analysis. ANOVA was used to assess significance followed by Fisher's least-significant difference post hoc test for ROS measurements and AHH activity. Wilcoxon's rank sum test was used to assess significance for GSH and gene expression measurements. The .05 level of probability was accepted as significant. Values represent mean ± S.E.

Results

Identification of functional BaP-responsive ARE/EpREs in the *c-Ha-ras* promoter suggests that a redox-sensitive mechanism is involved in the regulation of *c-Ha-ras* (Bral and Ramos, 1997). The transactivation response may be mediated by oxidative metabolites of BaP that activate redox signaling in vSMCs. To test this hypothesis, G_0-synchronized vSMCs were treated with BaP (0.3 and 3 μM), BaPQ (0.3 μM), or H_2O_2 (50 μM). BaPQ and H_2O_2 were studied because they are recognized intermediates of BaP metabolism in mammalian cells (Sullivan, 1985). BaP and related oxidants increased *c-Ha-ras* mRNA levels relative to controls at all time points examined (Fig. 1, A and B). Although induction by all agents was most pronounced at 1 h relative to controls, time-related increases in *c-Ha-ras* signal were observed at 3 and 5 h. BaP was a more effective inducer of *c-Ha-ras* than BaPQ or H_2O_2 at the concentrations tested. Higher BaPQ concentrations were cytotoxic and inhibited ARE/EpRE signaling in vSMCs (Miller et al., 2000). *c-Ha-ras* activation was observed in vSMCs treated with 25 μM H_2O_2, but at 100 μM we observed a decrease in *c-Ha-ras* signal due to cytotoxicity and cell death (data not shown). These data indicate that oxidative intermediates of BaP significantly enhance *c-Ha-ras* expression at noncytotoxic concentrations, but exhibit induction profiles that are different from the parent compound. Pretreatment of cells for 24 h with ellipticine (ellip; 0.01 nM) inhibited induction of *c-Ha-ras* by BaP (3 μM) at 1 and 3 h (Fig. 2), indicating that either the AhR or CYP-mediated metabolism is required for gene activation.

The activation of *c-Ha-ras* by BaP and its oxidative intermediates implicate a redox-sensitive mechanism in the regulation of the gene. Therefore, subsequent experiments were conducted to examine the profile of *c-Ha-ras* gene expression after chemical modulation of cellular redox balance. vSMCs were synchronized in G_0 by serum deprivation and challenged with BSO (0.1 mM) or NaC (0.5 mM) for 8 h before serum-stimulated release into the cell cycle. BSO inhibits GSH synthesis by specifically blocking the binding site of glutamate on γ-glutamylcysteine synthetase, the rate limiting enzyme in GSH synthesis, whereas NaC provides free cysteine for GSH synthesis de novo or directly detoxifies quinones by acting as a free electron donor. The concentrations of BSO and NaC examined were defined in dose-range finding studies showing a 68 ± 0.3% depletion and 124 ± 16% induction of GSH, respectively (n = 3).

After synchronized vSMC entry into the cell cycle by serum stimulation, cells were challenged with BaP (3 μM) alone, or in combination with BSO (0.1 mM) or NaC (0.5 mM). BaP (3 μM) enhanced *c-Ha-ras* mRNA levels by 1 h with maximal induction at 3 and 5 h (Fig. 3). BSO (0.1 mM) enhanced steady-state *c-Ha-ras* mRNA levels during the early phase of the mitogenic response (Fig. 3). The induction of *c-Ha-ras* was immediate with a >5.5-fold increase at 1 h in BaP-treated cells compared with a 4-fold induction in BSO-treated cells. Combined challenge of vSMCs with BaP and BSO was cytotoxic and caused cellular shrinking and blebbing (data not shown), and loss of *c-Ha-ras* signal (Fig. 3). NaC (0.5 mM) alone did not influence *c-Ha-ras* mRNA levels at 1 or 3 h, but up-regulated *c-Ha-ras* expression by 5 h. The induction of *c-Ha-ras* by BaP (3 μM) was prevented by NaC (0.5 mM; Fig. 3), suggesting that a redox mechanism mediates the gene activation response.

With DCFDA, the formation of H_2O_2 was monitored as an indicator of ROS formation in synchronized vSMCs challenged with 10% FBS in the presence of BaP (3 μM). ROS levels were not increased within the first 60 min after BaP challenge relative to controls (Fig. 4) but were enhanced at 2 (125%) and 4 h (120%). To determine whether ROS formation

by BaP-compromised redox balance in vSMCs, we measured cellular GHS levels in vSMCs after challenge with BaP (3 μM) alone, or in combination with BSO (0.1 mM) or NaC (0.5 mM). BaP (3 μM) depleted GSH levels by 11 $\pm$ 4.2% within 1 h, and 32 $\pm$ 3.8% within 2 h compared with controls (Fig. 5). In contrast, BSO alone induced a quick and sustained decrease in cellular GSH levels. The return of cellular GSH levels to control values in BaP-treated cells probably involves transcriptional activation of γ-glutamylcysteine synthetase via redox cycling (Shi et al., 1994; Moinova and Mulcahy, 1999). BaP (3 μM) in combination with BSO (0.1 mM) enhanced GSH depletion at 3 and 4 h (Fig. 5). NaC (0.5 mM) enhanced GSH levels in a time-dependent manner reaching up to 256 $\pm$ 33% by 4 h, and completely protected cells from BaP (3 μM)-induced GSH depletion (Fig. 5). Collectively, these data indicate that BaP promotes ROS formation and causes depletion of cellular GSH in vSMCs, but that a clear disassociation exists in vSMCs between the kinetics of *c-Ha-ras* activation and the modulation of redox status by BaP.

To further evaluate mechanisms of BaP-induced activation of *c-Ha-ras*, gene expression was examined in vSMCs from $AhR^{+/+}$, $AhR^{+/-}$, and $AhR^{-/-}$ mice challenged with BaP. Our focus on the AhR was based on the finding that ellip, an inhibitor of AhR-dependent functions, effectively antagonized *c-Ha-ras* activation by BaP. BaP enhanced *c-Ha-ras* expression in $AhR^{+/+}$ and $AhR^{+/-}$ vSMCs compared with controls, but not in $AhR^{-/-}$ vSMCs (Fig. 6). Similar results were seen in wild-type vSMCs pretreated with AhR antisense oligonucleotide before BaP challenge (data not shown). Next, we examined AHH activity in vSMCs challenged with 10% serum in the absence or presence of BaP (0.3 and 3 μM) for 5 and 24 h to determine whether loss of *c-Ha-ras* inducibility was due to loss of metabolic activation potential in AhR null vSMCs (Fig. 7). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (1 nM) was included in this experiment as a positive control for AhR-mediated inducibility. Constitutive AHH expression was observed in G_0-synchronized and randomly cycling vSMCs independent of the AhR phenotype. The level of AHH activity in synchronized vSMCs was markedly reduced compared with randomly cycling counterparts, confirming that expression of CYPs is influenced by growth status (Ou and Ramos, 1995). Induction of CYP1B1-encoded AHH activity by BaP increased as a function of dose and time, but was independent of AhR status.

Discussion

Previous studies in this laboratory have established a link between vSMC proliferation and *c-Ha-ras* (Ramos et al., 1996). Mitogenic stimulation of G_0-synchronized vSMCs was associated with induction of *c-Ha-ras* before progression into S-phase, whereas pharmacological interference with gene induction precluded continued cell cycle progression (Sadhu et al., 1993). Overexpression of oncogenic *Ha-ras* disrupts mitogenic signaling and induces vSMC dedifferentiation (Sadhu et al., 1994). These responses are reminiscent of those in vSMCs isolated from animals challenged with atherogenic doses of BaP (Ramos et al., 1996). Because of the central role of *c-Ha-ras* in the regulation of vSMC functions, we are interested in defining the molecular basis of *c-Ha-ras* induction by atherogenic stimuli. For BaP, activation of *c-Ha-ras*

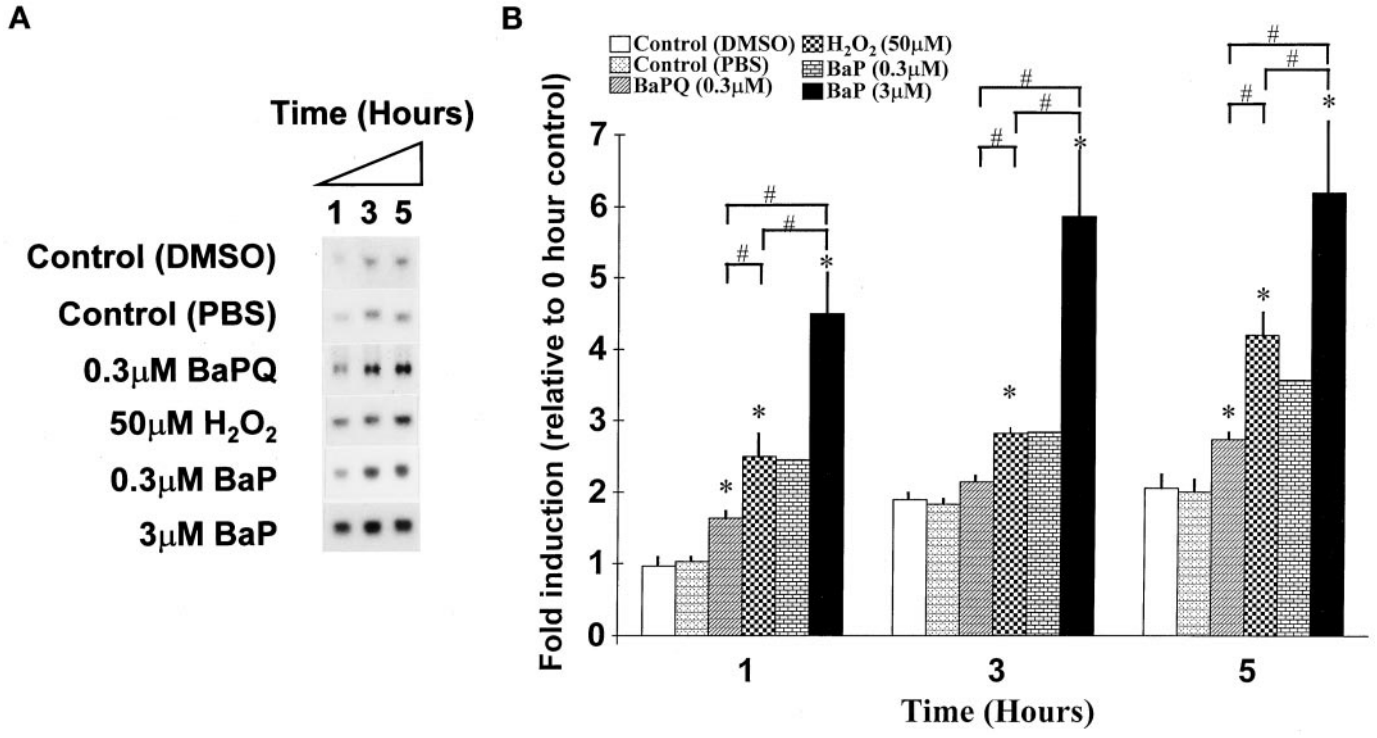

Fig. 1. c-Ha-ras mRNA induction profiles in vSMCs challenged with BaP, BaPQ, or H_2O_2. vSMCs were synchronized in G_0 and subsequently challenged with H_2O_2 (50 μM), BaPQ (0.3 μM), or BaP (0.3 and 3 μM) in the presence of serum (10% FBS) for 1, 3, and 5 h. RNA extraction and analysis were performed as described in *Materials and Methods*. A, representative Northern of c-Ha-ras induction profiles in vSMCs challenged with H_2O_2, BaPQ, or BaP. B, data was normalized to β-tubulin mRNA and expressed as fold induction relative to the 0 h control. mRNA levels were quantified with Zero Dscan Image Analysis version 1.0. Data for H_2O_2, BaPQ, and 3 μM BaP are representative of three individual experiments.. Values for 0.3 μM BaP ($n = 1$) are included for comparison. *, significance ($P < .05$) compared with control values for each respective time point. #, significance ($P < .05$) between chemical treatments at each respective time point. Filled columns, $AhR^{+/+}$; hatched columns, $AhR^{+/-}$; open columns, $AhR^{-/-}$.

involves a transcriptional mechanism mediated in part by activation of ARE/EpREs within the *c-Ha-ras* regulatory region (Bral and Ramos, 1997).

Because BaP is metabolized by vascular CYPs to intermediates that undergo redox cycling and induce oxidative stress (Bond et al., 1979, 1980), we hypothesized that activation of ARE/EpREs within the *c-Ha-ras* promoter involves modulation of redox status by oxidative intermediates of BaP. In support of this hypothesis, we report herein that both BaPQ and H_2O_2 enhanced serum-stimulated *c-Ha-ras* gene expression in vSMCs and that ellip, a CYP inhibitor and AhR antagonist, inhibited early induction of *c-Ha-ras* by BaP. Interestingly, the magnitude of *c-Ha-ras* induction by BaP-derived intermediates was not as pronounced as that of BaP, suggesting that either gene regulation is not entirely dependent on formation of oxidative intermediates, or that differences in the relative balance of oxidative stress and cytotoxicity influence patterns of gene inducibility.

To determine whether modulation of cellular redox potential participates in the regulation of *c-Ha-ras* gene expression, we examined the ability of BSO and NaC alone or in combination with BaP to influence patterns of gene inducibility and GSH status. BSO increased *c-Ha-ras* mRNA levels and depleted cellular GSH, showing that gene activation can be influenced by a redox-sensitive mechanism. In combination with BaP, BSO was cytotoxic and inhibited *c-Ha-ras* induction. Because BaP conjugates with GSH and consumes GSH-reducing equivalents, combined treatment with both agents probably overwhelms antioxidant capacity and enhances vSMC susceptibility to oxidative injury. The ability of nonlethal concentrations of BaP and BSO to promote a prooxidant state and increase *c-Ha-ras* expression suggests that coordinate regulation of redox balance and *c-Ha-ras* is operative in vSMCs. This interpretation is in fact consistent with the delayed increase of *c-Ha-ras* mRNA levels in NaC-treated cultures at 5 h when cellular adaptation to altered redox status can lead to activation of redox signaling (Tsai et al., 1996). NaC is known to induce oxidative stress and to modulate protein kinases involved in functional regulation of ARE/EpRE-binding proteins (Ng et al., 1998). As such, inhibition of BaP-induced *c-Ha-ras* activation at 5 h by NaC was unexpected. The consumption of excess reducing equivalents by oxidative intermediates of BaP in the presence of NaC may offset the gradual loss of redox control associated with extended antioxidant treatment. This interpretation is consistent with differences in cellular GSH levels between cells treated with NaC alone or in combination with BaP.

Differences between the induction profiles of BaP and its oxidative intermediates, or agents that modulate redox sta-

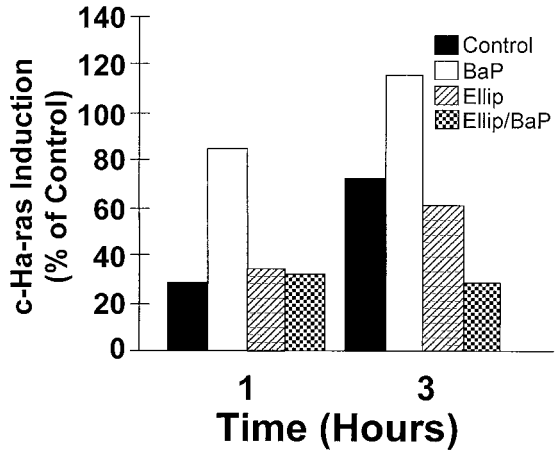

Fig. 2. c-Ha-ras mRNA levels in vSMCs challenged with BaP alone or in combination with ellip. Synchronized vSMCs were pretreated with ellip (0.01 nM) for 24 h and subsequently challenged with BaP (3 μM) for 1 and 3 h. Data was normalized to β-tubulin mRNA and expressed as percentage of the maximal induction response of c-Ha-ras mRNA in controls at 5 h, the time at which maximal induction is observed. RNA extraction and analysis were performed as described in *Materials and Methods*. mRNA levels were quantified with Zero Dscan Image Analysis version 1.0. Data shown are representative of duplicate experiments. The range of values for individual treatment groups were control 1 h (24.7–28.9%), control 3 h (75.2–80.5%), BaP 1 h (85.3–99.1%), BaP 3 h (115.3–132.4%), ellip 1 h (29.8–34.5%), ellip 3 h (55.2–62.3%), ellip/BaP 1 h (31.6–33.1%), and ellip/BaP 3 h (25.4–28.6%). Filled columns $AhR^{+/+}$; hatched columns $AhR^{+/-}$; open columns, $AhR^{-/-}$.

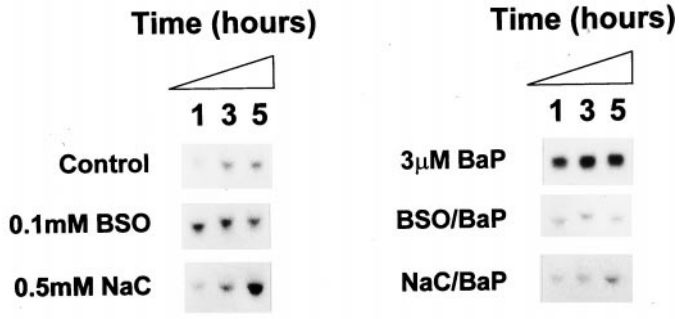

Fig. 3. c-Ha-ras mRNA levels in vSMCs challenged with 3 μM BaP alone or in combination with 0.1 mM BSO or 0.5 mM NaC. G_0-synchronized vSMCs were pretreated with BSO (0.1 mM) or NaC (0.5 mM) for 8 h and subsequently challenged with BaP (3 μM) alone or in combination with BSO (0.1 mM) or NaC (0.5 mM) in the presence of serum for 1, 3, and 5 h. RNA extraction and analysis were performed as described in *Materials and Methods*. β-Tubulin is shown as a control for RNA loading and transfer. Data are representative of three individual experiments.

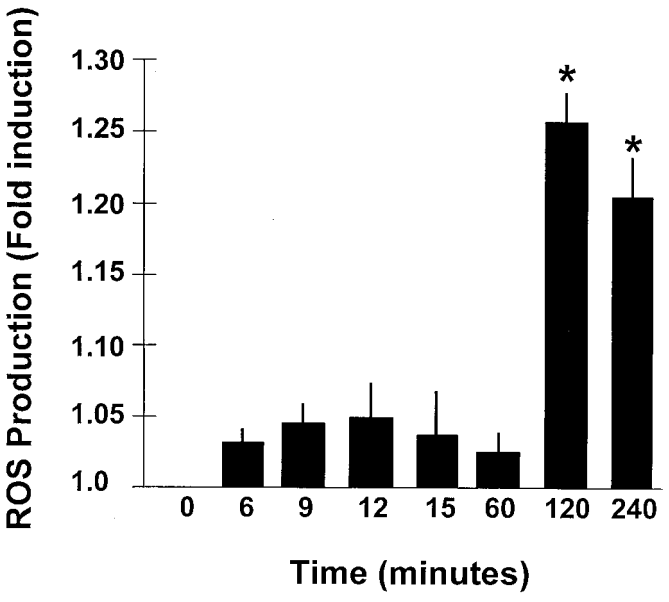

Fig. 4. ROS formation in vSMCs challenged with 3 μM BaP for various times. Synchronized vSMCs were challenged with BaP (3 μM) for up to 4 h and incubated in the presence of DCFDA, which enters the cells and is deacetylated by cellular esterases. Dichlorofluoroscin is oxidized to dichlorofluorosceine in the presence of H_2O_2 and the fluorescence can be measured at 488 nm. Data are expressed as fold induction over control values at each respective time point. Error bars represent S.E.M. Each column is representative of multiple measurements from individual cells (n = 51 to 326). *$P < .05$.

tus, indicate that *c-Ha-ras* gene activation by BaP is not solely dependent on redox status. This interpretation is consistent with the temporal disassociation between *c-Ha-ras* gene expression, ROS production, and GSH depletion in BaP-treated cells. A role for the AhR in gene regulation by BaP is suggested by the finding that activation of *c-Ha-ras* is lost in vSMCs from AhR$^{-/-}$ mice and that down-regulation of AhR protein by antisense oligonucleotides blocks BaP-induced *c-Ha-ras* expression. Within this context, it is important to note that the atherogenic response of mice to polycyclic aromatic hydrocarbons segregates with the high-affinity form of the AhR locus (Paigen et al., 1986). The involvement of the

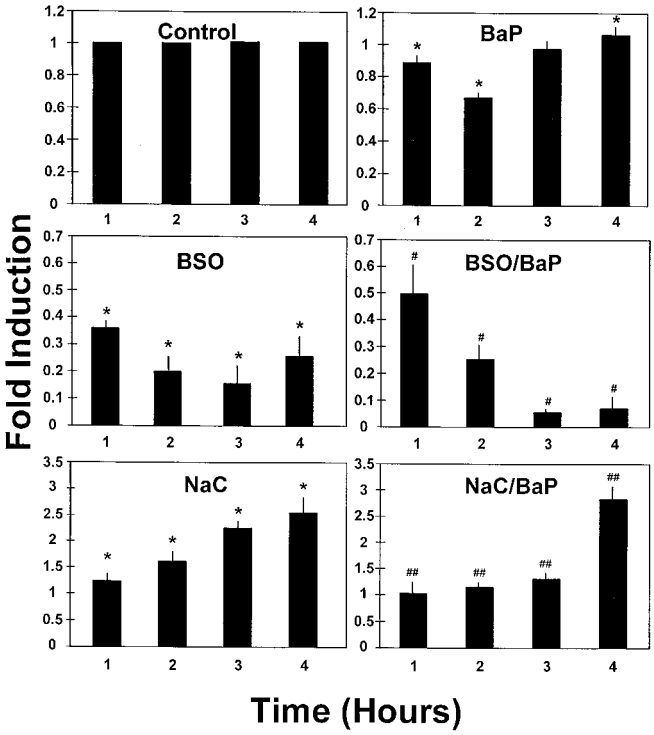

Fig. 5. GSH levels in vSMCs challenged with 3 μM BaP alone or in combination with 0.1 mM BSO or 0.5 mM NaC. G_0-synchronized vSMCs were pretreated with BSO (0.1 mM) or NaC (0.5 mM) for 8 h and subsequently challenged with BaP (3 μM) alone or in combination with BSO (0.1 mM) or NaC (0.5 mM) in the presence of serum for 1, 2, 3, and 4 h. Cellular GSH levels were determined with the 5,5′-dithio-bis-(2-nitrobenzoic acid)-GSH-GSSG reductase recycling assay and protein was determined with a Microbiuret assay as described in *Materials and Methods*. Data are presented as a ratio of control values for each time interval for 3 individual measurements, respectively. *, significance ($P < .05$) compared with control values at each respective time point. #, significance ($P < .05$) compared with BSO alone at each respective time point. ##, significance ($P < .05$) compared with NaC alone at each respective time point. Note the differences in scale for individual treatment groups.

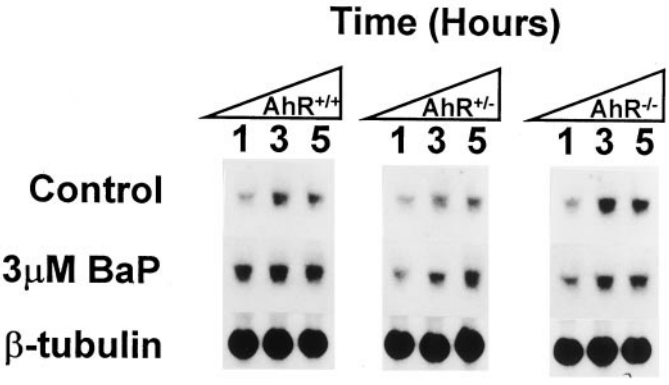

Fig. 6. c-Ha-ras mRNA levels in AhR$^{+/+}$, AhR$^{+/-}$, and AhR$^{-/-}$ vSMCs challenged with BaP. Synchronized vSMCs were challenged with BaP (3 μM) for 1, 3, and 5 h. RNA extraction and analysis were performed as described in *Materials and Methods*. β-Tubulin is shown as a control for RNA loading and transfer. Data are representative of duplicate experiments.

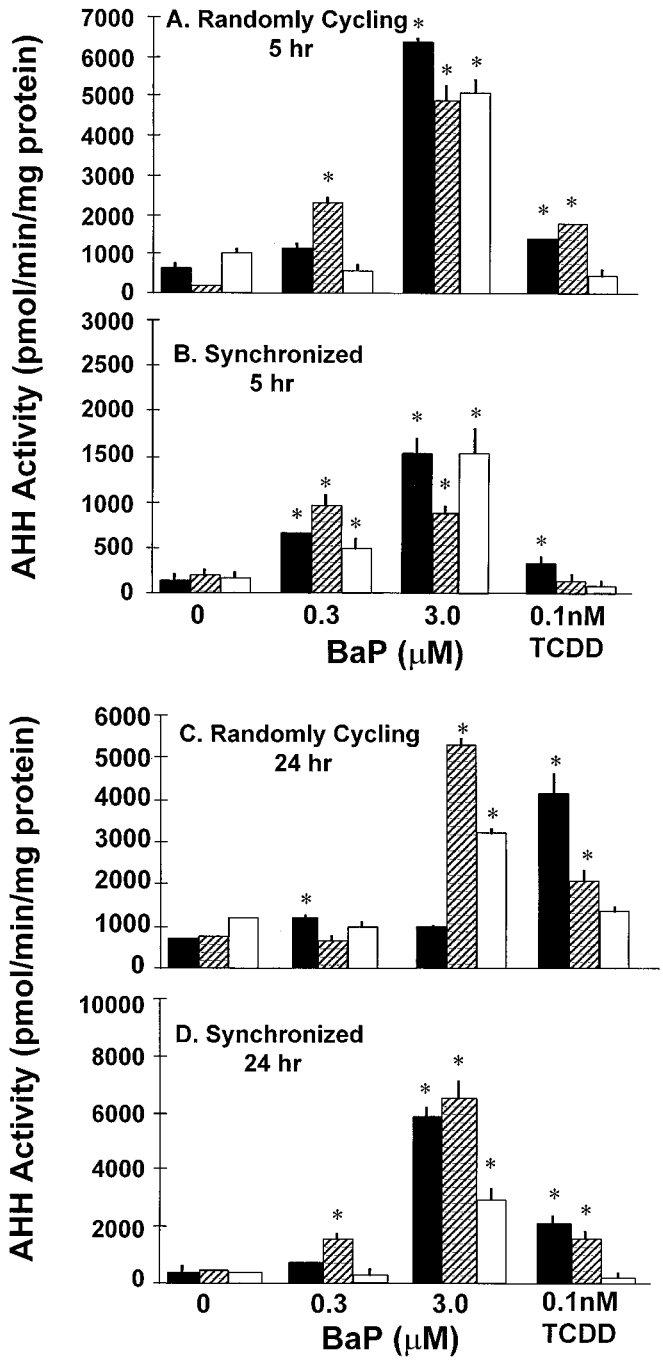

Fig. 7. AHH activity in AhR$^{+/+}$, AhR$^{+/-}$, and AhR$^{-/-}$ vSMCs challenged with BaP. Randomly cycling (A and C) and synchronized (B and D) vSMCs were challenged with BaP (0.3 and 3 μM) or TCDD (1 nM) for 5 (A and B) and 24 h (C and D). AHH analysis was performed as described in *Materials and Methods*. Error bars represent S.E.M. Each column is representative of three measurements. Data are representative of duplicate experiments. *$P < .05$.

AhR is not related to regulation of BaP metabolism because AHH activity was constitutively expressed in $AhR^{-/-}$, as well as $AhR^{+/+}$ vSMCs. Constitutive expression of AHH activity in vSMCs suggests that BaP metabolism occurs immediately upon cell entry and that formation of oxidative intermediates is in fact independent of phase I gene induction. Interestingly, the patterns of AHH activity in $AhR^{+/+}$, $AhR^{+/-}$, and $AhR^{-/-}$ vSMCs implicate multiple mechanisms in the regulation of hydroxylase activity. Previous studies have demonstrated that AHH activity is regulated at the transcriptional level via AhR-dependent and -independent mechanisms (Alexander et al., 1997; Larsen et al., 1998), as well as via a protein stabilization mechanism (Savas and Jefcoate, 1994).

The involvement of AhR in the regulation of *c-Ha-ras* inducibility by BaP and oxidative intermediates may involve functional interactions between AhR and transcription factors that bind ARE/EpREs in the *c-Ha-ras* promoter. This suggestion is consistent with preliminary studies showing that induction of *c-Ha-ras* by both BaPQ and H_2O_2 is AhR-dependent (J. K. Kerzee and K. S. Ramos, unpublished data). Interactions between ARE/EpRE-binding proteins and the AhR may occur, as suggested by Vasiliou et al. (1995) who first established the presence of AhR in protein complexes binding to the ARE/EpRE, and our recent finding that AhR plays a key role in negative regulation of GST-Ya promoter in vSMCs (Chen and Ramos, 1999). The presence of functional ARE/EpREs in the promoter region of several aryl hydrocarbon responsive element-regulated genes suggests that signaling cross talk is part of the adaptive response to chemical stress (Rushmore et al., 1991; Li and Jaiswal, 1993). In this manner, interactions between two distinct signaling pathways involved in the regulation of xenobiotic responsive genes may account for *c-Ha-ras* gene activation by BaP and related oxidants.

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