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Profiles of Antioxidant/Electrophile Response Element (ARE/EpRE) Nuclear Protein Binding and *c-Ha-ras* Transactivation in Vascular Smooth Muscle Cells Treated with Oxidative Metabolites of Benzo[*a*]pyrene

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ABSTRACT. Activation of nuclear protein binding to the antioxidant/electrophile response element (ARE/EpRE) by benzo[*a*]pyrene (BaP) in vascular smooth muscle cells (vSMCs) is associated with transcriptional deregulation of *c-Ha-ras*. This response may be mediated by oxidative intermediates of BaP generated during the course of cellular metabolism. To test this hypothesis, the profile of ARE/EpRE protein binding and transactivation elicited by BaP was compared with that of 3-hydroxy BaP (3-OH BaP) (0.03 to 3.0 μM), BaP 7,8-dihydrodiol (BaP 7,8-diol) (0.03 to 3.0 μM), BaP 3,6-quinone (BaP 3,6-Q) (0.0003 to 3.0 μM), and H_2O_2 (25 to 100 μM). Specific protein binding to the consensus *c-Ha-ras* ARE/EpRE was observed in vSMCs treated with all BaP metabolites at concentrations considerably lower than those required for the parent compound. H_2O_2, a by-product of BaP 3,6-Q redox cycling, also increased binding to the ARE/EpRE. Treatment of vSMCs with oxidative BaP metabolites or H_2O_2 transactivated the *c-Ha-ras* promoter in all instances, but the response was consistently half of the maximal induction elicited by BaP. Similar proteins cross-linked specifically to the consensus *c-Ha-ras* ARE/EpRE sequence in cells treated with BaP or its oxidative intermediates. The protein binding profile in the *c-Ha-ras* promoter was similar to that in the NADPH:quinone reductase gene (NQO_1) and the glutathione *S*-transferase Ya gene (GSTYa) promoters, but the relative abundance of individual complexes was promoter-specific. We conclude that oxidative intermediates of BaP mediate activation of nuclear protein binding to ARE/EpRE and contribute to transcriptional de-regulation of *c-Ha-ras* in vSMCs. BIOCHEM PHARMACOL **60**;9:1285–1296, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. aromatic hydrocarbons; atherogenesis; antioxidant/electrophile response element; gene expression; oxidative stress; vascular injury

The atherogenic potential of BaP‡ and related hydrocarbons has been evaluated in some detail [1–5]. BaP alters vSMC functions by several mechanisms, including inhibition of protein kinase C signal transduction [6], modulation of transcription factor activity [7, 8], and/or DNA adduction [9, 10]. These biological effects are mediated partly by oxidative intermediates generated by cytochrome P450-mediated metabolism. For instance, 3-OH BaP, a stable oxidative metabolite of BaP in vSMCs [11], inhibits protein kinase C [12], whereas BaP 3,6-Q and H_2O_2 modulate *c-Ha-ras* gene expression in vSMCs [13].

Oxidative metabolites of BaP can be produced in the form of free radicals, peroxides, quinones, or other reactive intermediates [14]. Quinones account for a significant fraction of the metabolic yield from BaP in a variety of tissues [15], and participate in one-electron redox cycles between their corresponding hydroquinones (BaP diols) and semiquinone radicals to generate large amounts of H_2O_2 and ROS [16]. Such redox cycles operate under physiological conditions and can be aided by cellular respiratory enzymes. Prior to the formation of BaP quinones, phenolic intermediates such as 6-OH and 3-OH BaP

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‡ *Abbreviations*: 3-OH BaP, 3-hydroxybenzo[*a*]pyrene; AhR, aryl hydrocarbon receptor; AhRE, aryl hydrocarbon response element; AP-1, activator protein 1 gene; ARE/EpRE, antioxidant/electrophile response element; ARE/EpRE-BPs, ARE/EpRE binding proteins; ARNT, aryl hydrocarbon nuclear translocator; BaP, benzo[*a*]pyrene; BaP 3,6-Q, benzo[*a*]pyrene 3,6-quinone; BaP 7,8-diol, benzo[*a*]pyrene 7,8-dihydrodiol; CAT, chloramphenicol acetyltransferase; C1, complex 1; C2, complex 2; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; GSTYa, glutathione *S*-transferase Ya gene; hARE, human antioxidant response element; hHa-ras, human *c-Ha-ras* gene; $hNQO_1$, human NADPH: quinone reductase gene; NAC, *N*-acetylcysteine; NFκB, nuclear factor κB gene; NQO_1, NADPH:quinone reductase gene; NS, nonspecific; PAH, polycyclic aromatic hydrocarbons; rGSTYa, rat glutathione *S*-transferase Ya gene; $rNQO_1$, rat NADPH:quinone reductase gene; ROS, reactive oxygen species; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; TRE, TPA-like response element; and vSMCs, vascular smooth muscle cells.

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TABLE 1. DNA sequences for various ARE/EpRE-containing promoters and oligonucleotide competitors used

hHa-ras:	5′-AGCTCCTGGGTGACAGAGCGAGAAGCT-3′
$rNQO_1$:	5′-TCTAGAGTCACAGTGACTTGGCAGATATCGTAC-3′
$hNQO_1$:	5′-GATGCAGTCACAGTGACTCAGCAGATATCGTAC-3′
rGSTYa:	5′-GAGCTTGGAAATGGCATTGCTAATGGTGACAAAGCAACTTT-3′
NFκB:	5′-AGTTGAGGGGACTTTCCCAGGC-3′
AP-1	5′-ATAAGCTATGACTCATCCGGGGG-3′
AhRE:	5′-TCAGGCATGTTGCGTGCATCCCTGAGGCCAGCC-3′
ARE/EpRE Wo:	5′-CCTGGGGAGA**AGAAAGAGAG**GTAC-3′

ARE/EpRE sequences for the various promoters are underlined. The random mutations of ARE/EpRE in the hHa-ras promoter (ARE/EpRE Wo) are bolded for comparison.

are formed. For instance, 3-OH BaP leads to quinone formation upon air oxidation [17], and conversion to BaP 3,6-Q upon incubation with heat-inactivated microsomes [18].

We have hypothesized that BaP modulates redox signaling in vSMCs during the course of the atherogenic response in part through activation of ARE/EpREs located in the 5′ region of critical target genes. The ARE/EpRE is a *cis*-acting regulatory sequence involved in basal and inducible expression of several genes, including Phase II enzymes responsible for the metabolism and detoxification of planar aromatic hydrocarbons and phenolic antioxidants [19, 20]. The minimal core sequence for inducible expression of the ARE/EpRE in rGSTYa and $rNQO_1$ is 5′-GTGAC NNNGC-3′, where N represents any nucleotide [21]. The same core sequence is also required for inducible expression of hHa-ras [8]. The 3′ GC dinucleotide is required for the element to act as the ARE/EpRE [22], and mutations in TGA completely eliminate basal and inducible activity in the $rNQO_1$ [23]. However, depending upon the binding proteins and promoter examined, some regions within the DNA sequence are more essential than others [8, 23–25]. Favreau and Pickett [23] examined the ARE/EpRE in the $rNQO_1$ promoter with the palindromic sequence 5′-TCTA GAGTCACAGTGACTTGGC-3′, where both half-sites act synergistically to induce high basal level gene expression. They also studied the ARE/EpRE in the rGSTYa promoter, which contains a consensus ARE/EpRE site that is sufficient for transcriptional activation, and a distal ARE/EpRE-like site [22]. Jaiswal and coworkers [26] reported that the basal and induced expression in the $hNQO_1$ promoter requires an ARE/EpRE containing two TREs (5′-GCAGTCACAGTGACTCAGCAGAATC-3′). To date, several ARE/EpRE-BPs have been described in human hepatoma cells and vSMCs [8, 24, 27–29]. Although these proteins may be part of the ARE/EpRE-BP complex, their cellular specificity and mechanism of activation are not fully understood.

In the present study, we examined the ability of oxidative metabolites of BaP and H_2O_2, a by-product of BaP 3,6-Q redox cycling, to activate ARE/EpRE protein binding and transactivate the *c-Ha-ras* promoter. We also evaluated the protein binding profile of the hHa-ras ARE/EpRE by UV cross-linking, relative to the better characterized NQO_1 and GSTYa promoters. Our results suggest that oxidative metabolites of BaP may contribute to transcriptional deregulation of *c-Ha-ras* in vSMCs by modulation of specific protein binding to hHa-ras regulatory sequences.

MATERIALS AND METHODS

Reagents

BaP (98% purity) was obtained from the Aldrich Chemical Co., and BaP 3,6-Q (≥98% purity by HPLC), 3-hydroxy BaP (≥99% purity by HPLC), and BaP 7,8-diol (≥98% purity by HPLC) were obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repositories (c/o Midwest Research Institute). All other chemicals were purchased from the Sigma Chemical Co. unless otherwise noted. BaP, 3-OH BaP, BaP 7,8-diol, and BaP 3,6-Q stock solutions were prepared in DMSO, and H_2O_2 stock solutions were prepared in ddH_2O.

Oligonucleotides

Oligonucleotide sequences used in this study are shown in Table 1. hHa-ras, $rNQO_1$, $hNQO_1$, and rGSTYa oligonucleotides were used to assess protein binding to ARE/EpRE motifs (underlined) located in each promoter. NFκB (Promega), AP-1, and AhRE were used in competition cross-linking assays to assess specificity of protein binding. ARE/EpRE Wo, an oligonucleotide containing several mutations in the ARE/EpRE sequence (underlined), was prepared to evaluate binding specificity in gel shift assays (Genosys). Poly(dI-dC) was also used in cross-linking studies to assess binding specificity. Double-stranded oligonucleotides containing the ARE/EpRE motif of the *c-Ha-ras* promoter were prepared as described previously [8], filled in using the Klenow fragment of DNA polymerase I, and end-labeled using [γ-^{32}P]ATP (New England Nuclear Research Products) and T4 polynucleotide kinase (Promega). Oligonucleotide sequences containing the ARE/EpRE motifs of the $rNQO_1$ and $hNQO_1$ promoters were synthesized (Genosys) to contain *Kpn*I restriction sites and an *Eco*RV region for use in future cloning experiments, then trimmed with mung bean nuclease (New England BioLabs), and end-labeled as described above. AP-1, AhRE, and rGSTYa oligonucleotides were synthesized to contain blunted ends upon annealing (Oligonucleotide Synthesis Services, Department of Biochemistry and Biophysics, Texas A&M

University), and end-labeled as described. The hHa-ras, $rNQO_1$, $hNQO_1$, and rGSTYa cross-linking probes were synthesized by annealing the top strand of each with their respective primers (hHa-ras: 5′-TCTCGCTC-3′) ($rNQO_1$ and $hNQO_1$: 5′-GTACGATA-3′) (rGSTYa: 5′-AAAGT-TGC-3′) and synthesizing the opposite strand in a Klenow reaction with 0.1 mM dATP, 0.1 mM dGTP, 0.1 mM BrdUTP, 1 mM DTT, and 100 μCi [α-^{32}P]dCTP for 1 hr at 37°. Labeled oligonucleotides were purified using Sephadex G-25 spin columns (Boehringer-Mannheim Biochemicals).

Cell Culture

Primary cultures of vSMCs were isolated from female C57BL/6 mouse aortae and maintained under standard conditions as described [30]. Cells were grown in Medium 199 (Gibco) containing 10% fetal bovine serum (Atlanta Biologicals), 2 mM glutamine, 100 U/mL of penicillin, 0.1 mg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Gibco). Subcultures were prepared by trypsinization (Gibco) of subconfluent primary cultures. Cells were seeded at 75 cells/mm^2 onto 150-mm plates, allowed to acclimate for 48 hr, then challenged with BaP, 3-OH BaP, BaP 7,8-diol, BaP 3,6-Q, or H_2O_2 for 3 hr as defined in the figure legends. DMSO concentrations in all experiments were kept below 0.1% at all times, as required to solubilize BaP or its oxidative metabolites in aqueous media.

Nuclear Extract Preparation and EMSA

Nuclear extracts were prepared as previously described [31]. In brief, cells were washed twice with ice-cold HEGD (25 mM HEPES–Cl, pH 7.6, 1 mM DTT, 1.5 mM EDTA, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) and scraped from plates. Cells then were transferred to a Dounce homogenizer and lysed with 30 strokes. Nuclei were pelleted at ~10,000 *g* at 4° in a variable speed microfuge, and the supernatant was discarded. Nuclei were resuspended in 80 μL HEGDK (HEGD plus 0.5 M KCl) and incubated on ice for 1 hr to extract nuclear proteins. Nuclear ghosts were removed by centrifugation at 10,000 *g*, 4°, in a microfuge, and the supernatant was quick-frozen in liquid nitrogen and stored at −80°. Protein concentration was determined by the method of Bradford [32]. For EMSA reactions, 5 μg of nuclear protein was incubated with 10 fmol of double-stranded [γ-^{32}P]-labeled oligonucleotide at room temperature for 20 min to 1 hr. Binding reactions were performed in 0.25x HEGDK supplemented with 1 mM DTT, 20 μg BSA, and 50 ng poly(dI-dC) in a total volume of 25 μL. Loading dyes were added [15% Ficoll (Type 400; Pharmacia Biotech), 0.25% bromophenol blue, 0.25% xylene cyanol FF], and reactions were loaded immediately on a 7% non-denaturing polyacrylamide gel with 0.5x TBE buffer (50 mM Tris, 45 mM boric acid, 1 mM Na_2EDTA, pH 8.3) and electrophoresed at 25 mA. For competition experiments where unlabeled ARE/EpRE Wo was compared with hHa-ras, 1 ng ARE/EpRE Wo was added to the reaction mixture. Gels were dried and exposed to Kodak X-OMAT film for autoradiography at room temperature without an intensifying screen for 12–16 hr or viewed using a STORM PhosphorImager (Molecular Dynamics). The data shown are representative of at least three independent experiments. ImageQuant software was used for quantification of band intensities by densitometry.

Transient Transfections

Cells were seeded at 150/mm^2 onto 60-mm plates and allowed to recover for 24 hr before transfection. Cultures were transfected using a cationic liposome formulation available from InVitrogen (pFx-6). For each plate, 11 μg DNA (9 μg pARERC2A, a CAT vector containing a minimal *c-Ha-ras* promoter with an ARE/EpRE site directly upstream, and 2 μg β-galactosidase control) and 33 μL pFx-6 were added to individual tubes containing 1 mL OptiMEM-I (Gibco). Cells were washed twice with Opti-MEM-I, and DNA and lipid were mixed gently and overlaid onto the cells for 5 hr at 37° (5% CO_2). After this incubation, the transfection mixture was replaced with standard growth medium. Cells were allowed to grow for an additional 36 hr and then challenged with chemicals added directly to the growth medium or by replacing the standard growth medium with treated growth medium. Transfected cells were harvested 24 hr after challenge, and cellular extracts were prepared by five freeze/thaw cycles (liquid nitrogen/37°). Total cellular protein concentration was determined according to the method of Bradford [32]. CAT activity was determined according to the method of Gorman *et al.* [33], using 5–30 μg protein in an overnight incubation at 37°. β-Galactosidase concentrations were determined using a commercially available enzyme-linked immunosorbent assay kit (Boehringer-Mannheim Biochemicals) according to the manufacturer's instructions. CAT activity was normalized to β-galactosidase protein concentration driven from the pcDNA/HIS/lacZ vector (InVitrogen) to control for variation in transfection efficiency. Normalized CAT activities were then expressed as a percentage of vehicle-treated control expression for three individual experiments and averaged.

UV Cross-linking

Equilibrium binding reactions were prepared as described above using 5 μg of nuclear protein and 10^5 cpm of cross-linking probe. The reaction was allowed to attain equilibrium for 20 min at room temperature in non-competition experiments, and 1 hr in competition experiments, followed by UV irradiation at 254 nm for 30 min on ice. After loading dyes were added, samples were boiled for 5 min and subsequently run on denaturing SDS gels (10% resolving) along with molecular weight markers. Gels were dried, and markers were labeled manually with [γ-^{32}P]ATP, followed by exposure to Kodak X-OMAT film for autoradiography at room temperature for 55–72 hr.

RESULTS

Activation of Protein Binding to c-Ha-ras ARE/EpRE by BaP Metabolites

Previous work in this laboratory has shown that BaP induces specific protein binding to hHa-ras ARE/EpRE [8]. To assess the possible role of BaP metabolites in the activation of protein binding, the effects of 3-OH BaP, BaP 7,8-diol, and BaP 3,6-Q in vSMCs were examined. Cells were challenged for 3 hr with individual metabolites at concentrations comparable to or lower than those required to activate ARE/EpRE protein binding by the parent compound. The range of concentrations examined was based on the ratio of metabolite formation in liver microsomes *in vitro*, where approximately 1.81% 3-OH BaP, 1.5% BaP 7,8-diol, and 0.69% BaP 3,6-Q are found [18]. Protein binding to *c-Ha-ras* ARE/EpRE appeared as a doublet of high specificity (denoted C1 and C2), with the lower band of the doublet showing enhanced sensitivity to chemical effects in most instances. BaP increased protein binding to hHa-ras ARE/EpRE in a concentration-dependent manner, resulting in a 2.4-fold induction over control at 3 μM and remaining elevated at the highest concentration examined (Fig. 1A). 3-OH BaP and BaP 7,8-diol also increased hHa-ras ARE/EpRE protein binding in a concentration-dependent manner up to a 2.4- and 2.2-fold induction, respectively, over DMSO controls (Fig. 1B). The threshold and concentration-dependent profiles of protein binding elicited by 3-OH BaP and BaP-7,8-diol were comparable to those of the parent compound, suggesting that similar mechanisms mediate activation of ARE/EpRE protein binding by BaP and its oxidative metabolites.

Challenge of vSMCs with BaP 3,6-Q also increased protein binding, but maximal elevation of signal intensity occurred at concentrations 1,000–10,000 times lower than those required for the parent hydrocarbon (Fig. 2A). Such differences in potency and reactivity implicate redox-active BaP intermediates in the activation of ARE/EpRE protein binding in vSMCs. A gradual reduction in signal intensity below control levels was observed as the BaP 3,6-Q concentration increased to 3.0 μM, a pattern consistent with the high reactivity and cytotoxicity of quinone intermediates. Competition assays with unlabeled specific (hHa-ras) and nonspecific (ARE/EpRE Wo) oligonucleotides in vSMCs treated with 0.0003 μM BaP 3,6-Q showed binding to be specific for C1 and C2 (Fig. 2B). C2 was competed less effectively than C1, but the stringency of this interaction awaits identification of the proteins that constitute the ARE/EpRE doublet. The NS complex in Fig. 2A showed an induction pattern similar to C1 and C2, but was competed by both specific and nonspecific unlabeled oligonucleotides, and thus was regarded as nonspecific. We next examined the effects of H_2O_2, a stable intermediate of quinone redox cycling in vSMCs [13]. Challenge of cells with 25–100 μM H_2O_2 markedly increased protein binding to hHa-ras ARE/EpRE, reaching 1.9-fold over controls at

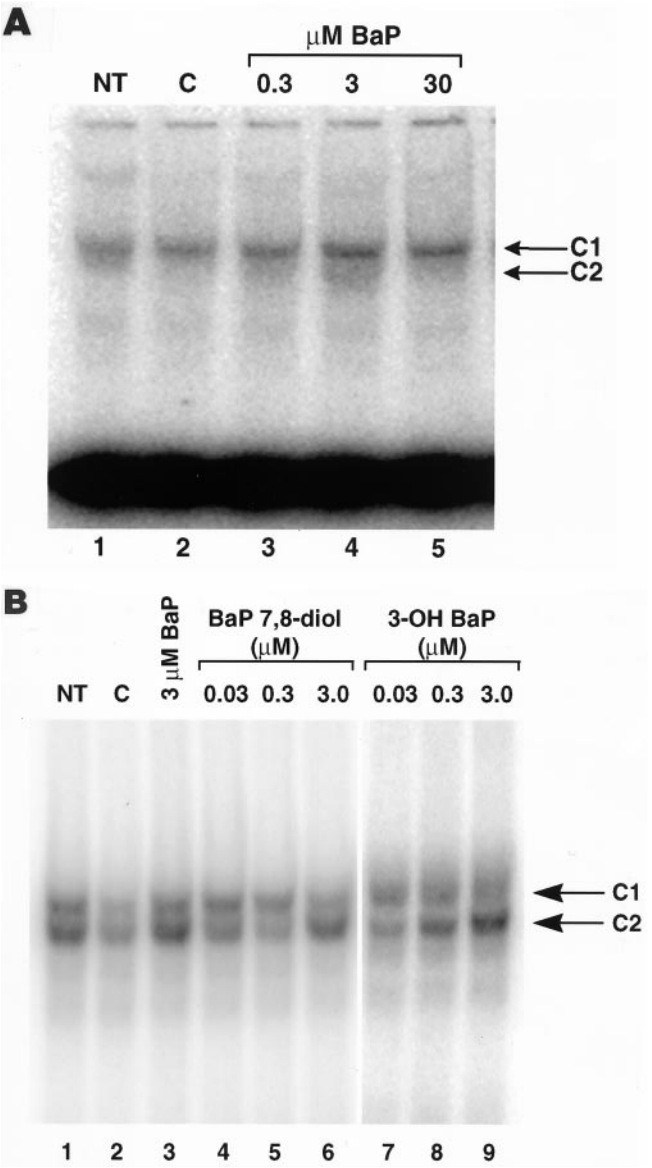

FIG. 1. Protein binding to *c-Ha-ras* ARE/EpRE induced by BaP, BaP 7,8-diol, and 3-OH-BaP. (A) Concentration-dependent profiles of ARE/EpRE protein binding induced by 0.3, 3, and 30 μM BaP. Nuclear extracts of vSMCs (5 μg) were incubated at room temperature with 10 fmol of end-labeled *c-Ha-ras* ARE/EpRE for 20 min. Protein/DNA complexes were resolved on a 7% non-denaturing polyacrylamide gel. Lane 1, nuclear extract of non-treated cells (NT). Lane 2, extract of cells treated with DMSO (C = control). Lanes 3–5, extracts of cells treated with increasing concentrations of BaP. (B) Concentration-dependent profiles of ARE/EpRE protein binding induced by 0.03, 0.3, and 3.0 μM BaP 7,8-diol or 3-OH BaP. Nuclear extracts of vSMCs (5 μg) were incubated at room temperature with 10 fmol of end-labeled *c-Ha-ras* ARE/EpRE for 20 min. Protein/DNA complexes were resolved on a 5% non-denaturing polyacrylamide gel. Lane 1, nuclear extract of non-treated cells (NT). Lane 2, extract of cells treated with DMSO (C = control). Lane 3, extract of cells treated with 3 μM BaP. Lanes 4–6, extracts of cells treated with increasing concentrations of BaP 7,8-diol. Lanes 7–9, extracts of cells treated with increasing concentrations of 3-OH BaP. Arrows indicate specific complexes (C1 and C2). Results are representative of three different experiments.

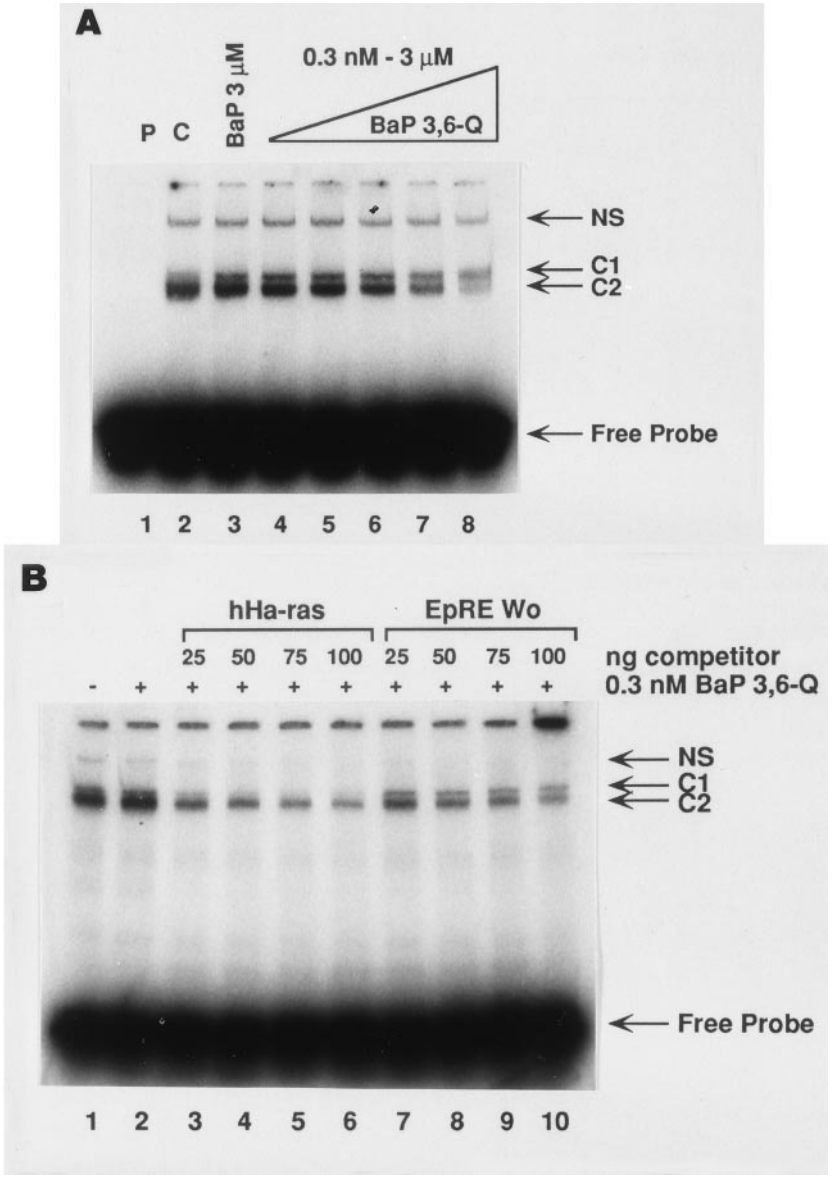

FIG. 2. Protein binding to *c-Ha-ras* ARE/EpRE induced by BaP 3,6-Q. (A) Nuclear extracts of vSMCs (5 μg) were incubated at room temperature with 10 fmol of end-labeled *c-Ha-ras* ARE/EpRE for 20 min. Protein/DNA complexes were resolved on a 7% non-denaturing polyacrylamide gel. Sonicated herring testes DNA (40 ng) was used instead of poly(dI-dC) for nonspecific reaction binding, and a 1x TGE (10 mM Tris–Cl, 76 mM glycine, 0.4 mM Na_2EDTA, pH 8.4) buffer was used instead of 0.5x TBE. Lane 1, reaction run without nuclear extract (P = probe). Lane 2, extracts of cells treated with DMSO. Lane 3, extracts of cells treated with 3 μM BaP. Lanes 4–8, extracts of cells treated with increasing concentrations of BaP 3,6-Q. Arrows indicate specific complexes (C1 and C2), a nonspecific complex (NS), as well as free probe. (B) Nuclear extracts of vSMCs (5 μg) were incubated at room temperature with 10 fmol of end-labeled *c-Ha-ras* ARE/EpRE for 1 hr. Protein/DNA complexes were resolved on a 7% non-denaturing polyacrylamide gel. Specific complexes were identified by competition with unlabeled specific (hHa-ras) and nonspecific (ARE/EpRE Wo) oligonucleotides. Lane 1, extracts of cells treated with DMSO. Lanes 2–10, extracts of cells treated with 0.3 nM BaP 3,6-Q, and subsequently competed as shown. Arrows indicate specific complexes (C1 and C2), a nonspecific complex (NS), as well as free probe. Results are representative of three different experiments.

100 μM (Fig. 3). However, the concentrations of H_2O_2 required to increase ARE/EpRE protein binding were consistently higher than those required to elicit a comparable response by BaP and oxidative intermediates.

Inducibility of protein binding to ARE/EpREs may exhibit promoter-specific characteristics. Therefore, binding to the $rNQO_1$ promoter region was examined in nuclear extracts isolated from vSMCs treated with BaP 3,6-Q to determine if the pattern of ARE/EpRE protein interactions was comparable to that seen for the human *c-Ha-ras* promoter. The NQO_1 gene contains a proximal consensus ARE/EpRE similar to that within the *c-Ha-ras* promoter, along with a distal palindromic consensus sequence (Table 1). As shown for *c-Ha-ras*, induction of C1 and C2 was seen in the $rNQO_1$ promoter (Fig. 4). However, BaP 3,6-Q increased protein binding in a concentration-dependent manner, suggesting that chemical sensitivity of this promoter sequence differs from that of *c-Ha-ras*.

Transcriptional Activation of c-Ha-ras *via ARE/EpRE by BaP Metabolites*

BaP-induced transcriptional activation of *c-Ha-ras* is mediated by ARE/EpRE sequences within the 5′ regulatory region [8]. To assess the functionality of the protein/DNA interactions induced by BaP metabolites and oxidative by-products, transient transfection assays were performed using pARERC2A, a plasmid containing the ARE/EpRE sequence directly upstream of well-characterized regulatory sequences [8]. The maximal induction of the pARERC2A construct in vSMCs is 2-fold, a pattern consistent with mitogen- or BaP-inducible profiles at the mRNA and protein level [8, 13]. Transiently transfected cells were challenged individually with BaP (0.3 to 30 μM), 3-OH BaP (0.03 to 3 μM), BaP 7,8-diol (0.03 to 3 μM), BaP 3,6-Q (0.003 to 0.3 μM), or H_2O_2 (12.5 to 50 μM) for 24 hr. Marked increases in transcriptional activity were seen at

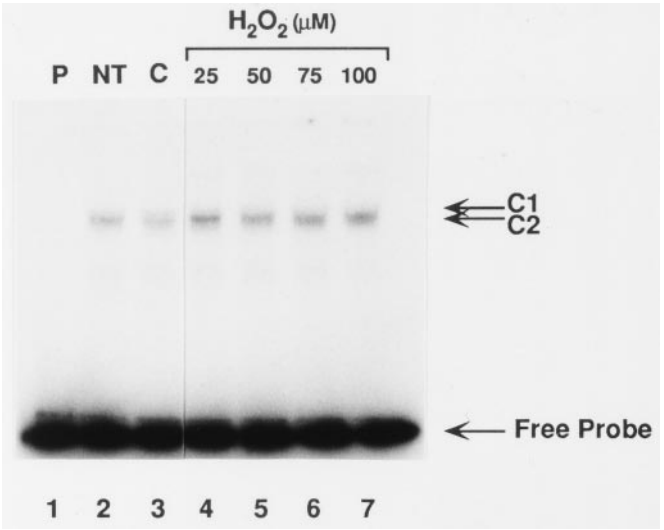

FIG. 3. Protein binding to *c-Ha-ras* ARE/EpRE induced by H_2O_2. Nuclear extracts of vSMCs (5 μg) were incubated at room temperature with 10 fmol of end-labeled *c-Ha-ras* ARE/EpRE for 20 min. Protein/DNA complexes were resolved on a 7% non-denaturing polyacrylamide gel. Lane 1, reaction run without nuclear extract. Lane 2, extracts from non-treated cells. Lane 3, extracts from cells treated with H_2O. Lanes 4–7, extracts from cells treated with increasing concentrations of H_2O_2. Arrows indicate specific complexes (C1 and C2), as well as free probe. Results are representative of three different experiments.

all concentrations in BaP-treated cells (Fig. 5A). Increased transcription also was seen in vSMCs treated with 3-OH BaP (Fig. 5B), BaP 7,8-diol (Fig. 5C), BaP 3,6-Q (Fig. 5D), and H_2O_2 (Fig. 5E). In the case of 3-OH BaP and BaP 3,6-Q, threshold concentrations for increased transcription were considerably lower than those of the parent compound. A biphasic response was observed in vSMCs treated with BaP 7,8-diol, where transcription increased at 0.3 μM, but diminished at 3 μM. H_2O_2 increased transcriptional activity, but only at concentrations considerably higher than those required to modulate transcription by BaP or its oxidative metabolites. In all instances, transcriptional activation by BaP metabolites and H_2O_2 was only half of the maximal induction potential of the gene in response to the parent compound.

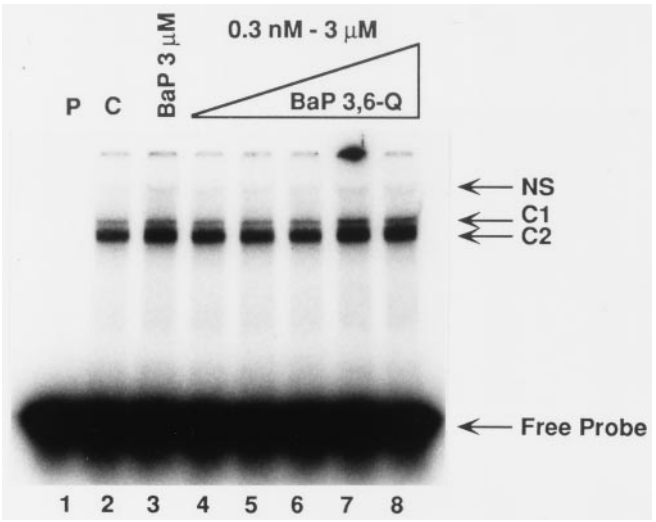

FIG. 4. BaP 3,6-Q induction of binding to an ARE/EpRE homologue. Nuclear extracts of vSMCs (5 μg) were incubated at room temperature with 10 fmol of end-labeled $rNQO_1$ ARE/EpRE for 1 hr. Protein/DNA complexes were resolved on a 7% non-denaturing polyacrylamide gel. Lane 1, reaction run without nuclear extract. Lane 2, extracts of cells treated with DMSO. Lane 3, extracts of cells treated with 3 μM BaP. Lanes 4–8, extracts of cells treated with increasing concentrations of BaP 3,6-Q. Arrows indicate specific complexes (C1 and C2), a nonspecific complex (NS), as well as free probe. Results are representative of three different experiments.

Protein Binding Profile to ARE/EpREs in Different Promoters

We used UV cross-linking EMSA to compare the patterns of protein binding to the *c-Ha-ras* ARE/EpRE elicited by BaP and its oxidative intermediates in vSMCs (Fig. 6A). Significant levels of protein binding to the ARE/EpRE were observed in nuclear extracts isolated from vSMCs under constitutive conditions. The protein binding profiles induced by BaP (3 μM), 3-OH-BaP (0.3 μM), BaP 7,8-diol (0.3 μM), BaP 3,6-Q (3 μM), or H_2O_2 (75 μM) were identical, and comparable to those observed in extracts from vehicle-treated cells. Proteins of 110, 80, 65, 60, 55, 25, and 23 kDa were most prominent in all treatment groups. Next, nuclear extracts from BaP-treated cells were used to compare the cross-linking profiles of hHa-ras, $rNQO_1$, $hNQO_1$, and rGSTYa ARE/EpREs. DNA:protein complexes were resolved on a denaturing gel, alongside samples treated with specific and nonspecific oligonucleotide competitors (Fig. 6B). Similar binding patterns for most of the prominent complexes identified were seen between hHa-ras and rGSTYa, and between $rNQO_1$ and $hNQO_1$. Bands homologous for all promoters were located in the 80-, 65-, and 55-kDa region; however, relative intensities were promoter-specific. The intensity of the 80-kDa protein was most prominent in $hNQO_1$, while the 65- and 55-kDa bands showed prominence in rGSTYa and $rNQO_1$/$hNQO_1$, respectively. Although UV cross-linking experiments did not allow comparison of binding inducibility for different chemical treatments, low molecular weight proteins at 25 and 23 kDa predominated in hHa-ras and rGSTYa. Higher molecular weight proteins were found primarily in the $rNQO_1$, $hNQO_1$, and GSTYa promoters. The 80-kDa protein was specific for all promoters, whereas the 65- and 55-kDa proteins were specific for $rNQO_1$, $hNQO_1$, and rGSTYa, but not hHa-ras (Fig. 6B). Bands at 25 and 23 kDa were specific for both *c-Ha-ras* and rGSTYa. To further define binding specificity profiles, the degree of competition of hHa-ras and $rNQO_1$ ARE/EpREs by $hNQO_1$, rGSTYa, NFκB, AP-1, and AhRE was examined (Fig. 6C). For *c-Ha-ras*, the 80-kDa protein was competed to varying degrees by all oligonucleotides, except NFκB and

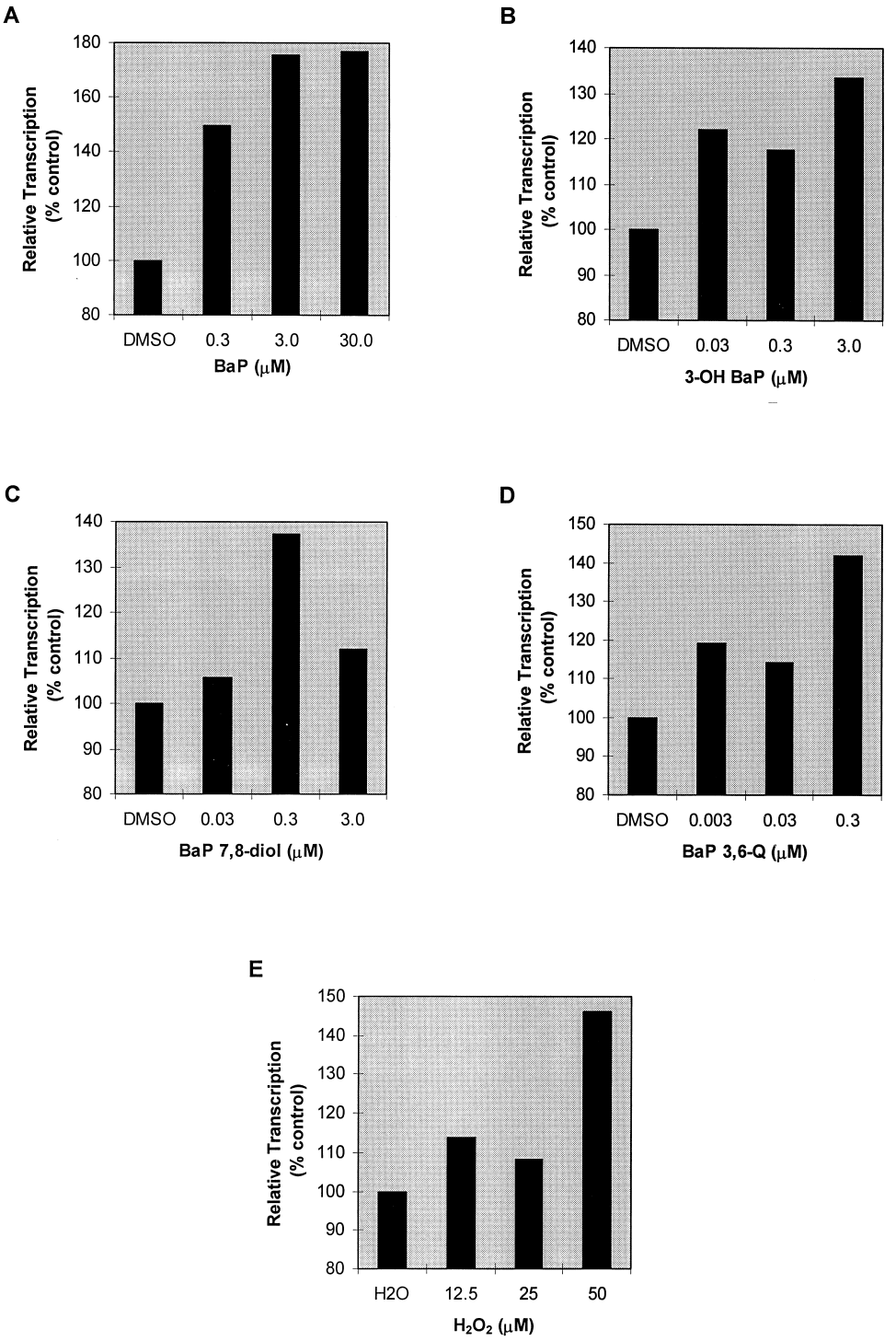

FIG. 5. Induction of pARERC2A by BaP and related oxidative intermediates. Mouse vSMCs were transiently co-transfected with a CAT plasmid construct containing an ARE/EpRE element directly upstream of the minimal *c-Ha-ras* promoter and a β-galactosidase plasmid pcDNA3.1/HIS/LacZ. (A) Cells were treated with DMSO as a control or 0.3, 3.0, or 30.0 μM BaP. Values were normalized to β-galactosidase activity and expressed as a percent of control. (B) Cells were treated with DMSO as a control or 0.03, 0.3, or 3.0 μM 3-OH BaP. Values were normalized to β-galactosidase activity and expressed as a percent of control. (C) Cells were treated with DMSO as a control or 0.03, 0.3, or 3.0 μM BaP 7,8-diol. Values were normalized to β-galactosidase activity and expressed as a percent of control. (D) Cells were treated with DMSO as a control or 0.003, 0.03, or 0.3 μM BaP 3,6-Q. Values were normalized to β-galactosidase activity and expressed as a percent of control. (E) Cells were treated with H_2O as a control or 12.5, 25, or 50 μM H_2O_2. Values were normalized to β-galactosidase activity and expressed as a percent of control. Results are representative of at least three different experiments.

poly(dI-dC). The proteins at 65 and 55 kDa were competed to a small extent by all oligonucleotides. Protein bands at 25 and 23 kDa were ablated by hHa-ras and NFκB oligos, with AP-1 competing to a lesser extent. The specificity of the 80-kDa protein appeared questionable for protein complexes probed with $rNQO_1$. Proteins at 65 and 55 kDa were competed mostly by $hNQO_1$ and AP-1, while the 55-kDa protein also exhibited slight competition by $rNQO_1$ and rGSTYa.

DISCUSSION

In this report we showed that oxidative intermediates of BaP activate protein binding to the ARE/EpRE and upregulate transcription of reporter plasmids containing the ARE/EpRE sequence directly upstream of *c-Ha-ras* regulatory sequences. The threshold and concentration-dependent profiles of protein binding and transactivation in response to 3-OH BaP, BaP 7,8-diol, and BaP 3,6-Q suggest

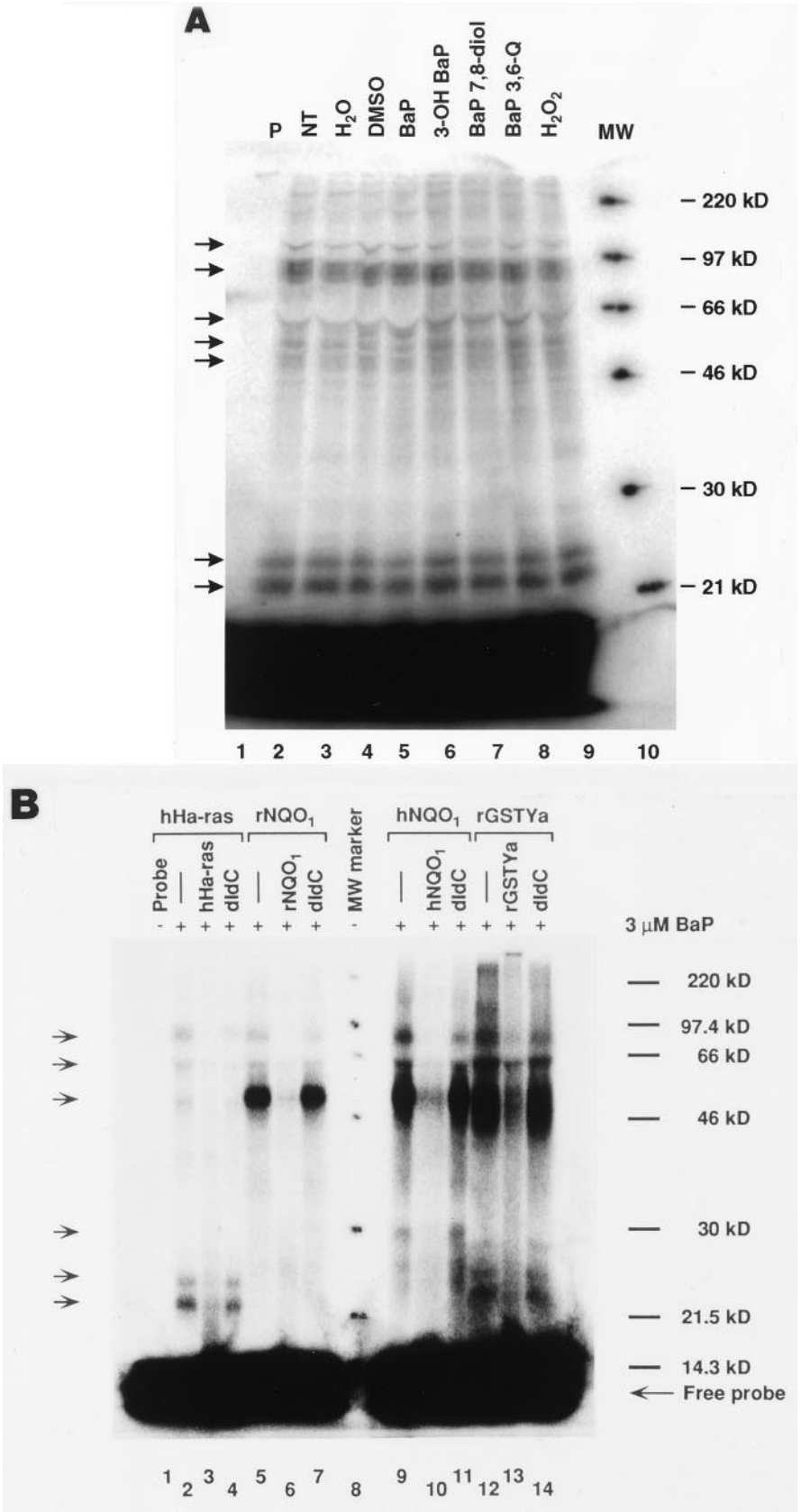

FIG. 6. Cross-linking profiles of ARE/EpRE binding proteins. (A) Nuclear extracts of non-treated cells and cells challenged with H_2O, DMSO, BaP, or metabolites were incubated at room temperature with 10^5 cpm of hHa-ras ARE/EpRE cross-linking probe. Lane 1, reaction run without nuclear extract. Lane 2, extract of non-treated cells. Lane 3, extract of H_2O-treated cells. Lane 4, extract of DMSO-treated cells. Lane 5, extract of cells treated with 3 μM BaP. Lane 6, extract of cells treated with 0.3 μM 3-OH BaP. Lane 7, extract of cells treated with 0.3 μM BaP 7,8-diol. Lane 8, extract of cells treated with 3 μM BaP 3,6-Q. Lane 9, extract of cells treated with 75 μM H_2O_2. Lane 10, molecular weight markers. Arrows indicate predominant proteins. (B) Nuclear extracts of cells challenged with 3 μM BaP were probed with 10^5 cpm hHa-ras, $rNQO_1$, $hNQO_1$, and rGSTYa. Specific complexes were identified by competition with indicated oligonucleotides. Lane 1, reaction run without nuclear extract. Lanes 2–4, extracts cross-linked to hHa-ras. Lanes 5–7, extracts cross-linked to $rNQO_1$. Lane 8, MW markers. Lanes 9–11, extracts cross-linked to $hNQO_1$. Lanes 12–14, extracts cross-linked to rGSTYa. Arrows indicate specific complexes, as well as free probe. (C) Nuclear extracts of cells challenged with 3 μM BaP were probed with 10^5 cpm of either hHa-ras or $rNQO_1$. Specific complexes were identified by competition with unlabeled [hHa-ras, $rNQO_1$, $hNQO_1$, rGSTYa, NFκB, AP-1, AhRE, or poly(dI-dC)] oligonucleotides. Lane 1, reaction run without nuclear extract. Lanes 2–10, extracts competed by the indicated oligonucleotides and cross-linked to hHa-ras. Lane 11, MW markers. Lanes 12–20, extracts competed by the indicated oligonucleotides and cross-linked to $rNQO_1$. Arrows indicate specific complexes, as well as free probe. Results are representative of at least three different experiments.

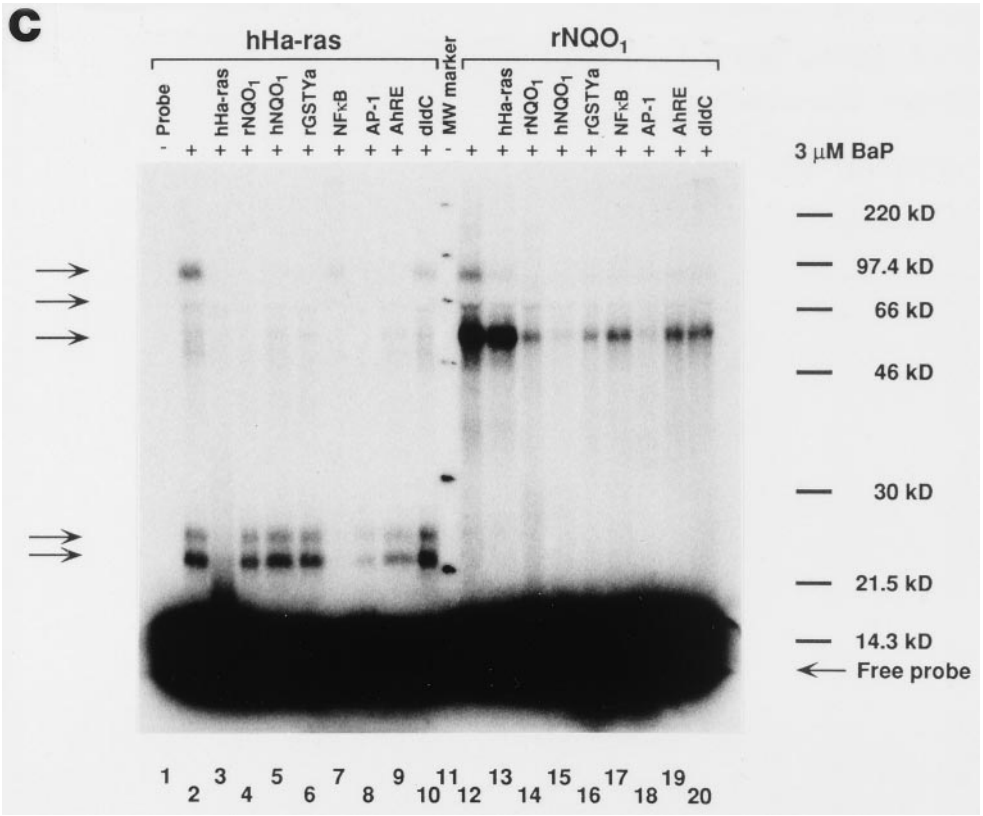

FIG. 6. Continued.

that oxidative intermediates participate in the deregulation of *c-Ha-ras* gene expression by BaP in vSMCs. Modulation of ARE/EpRE-regulated functions by these intermediates is consistent with the role of redox mechanisms in the regulation of *c-Ha-ras* expression in vSMCs [13].

vSMCs express constitutive and inducible aryl hydrocarbon hydroxylase activities encoded by CYPIB1 and CYPIA1 [13, 34]. P450 metabolism of BaP gives rise to several oxidative intermediates capable of interacting with cellular macromolecules and inducing oxidative stress [35]. 3-OH BaP, a precursor to quinone formation and generator of ROS, is a predominant BaP metabolite detected in vSMCs [11]. BaP 3,6-Q accounts for 44% of the oxidation of 6-OH BaP [36], and binding of BaP to DNA at the 1, 3, and 6 positions is associated with a free-radical mechanism of toxic action [37]. Although formation of BaP 7,8-diol in vSMCs has not been reported, DNA adduction by the BaP 7,8-diol-9,10-epoxide intermediate in vSMCs has been documented by ^{32}P-postlabeling in preliminary studies.* The ARE/EpRE protein binding profiles of 3-OH BaP and 7,8-diol were comparable to those of the parent compound, where maximal activation was observed at the 3 μM concentration. In contrast, BaP 3,6-Q was considerably more potent than either 3-OH BaP or BaP 7,8-diol, giving rise to maximal protein binding at concentrations 1,000–10,000 times lower than those required for the parent compound. These findings suggest that BaP 3,6-Q is the proximal BaP intermediate involved in activation of ARE/EpRE protein binding in vSMCs.

The reduction in nuclear protein binding to hHa-ras ARE/EpRE observed when vSMCs were treated with BaP 3,6-Q suggests that cytotoxicity may occur as the quinone concentration increases. However, the inverse pattern of protein binding to hHa-ras likely involves unique differences in the profile of activation of ARE/EpRE-BPs, since concentration-dependent increases in protein binding were observed when nuclear extracts were shifted against a rNQO$_1$ oligonucleotide. In contrast to hHa-ras, rNQO$_1$ contains a proximal consensus site and a distal palindromic site, separated by one base pair. Because only the proximal consensus site is similar in both promoters, we infer that similarities in protein binding profiles are representative of common proteins binding to the consensus ARE/EpRE sequence within both oligonucleotides. Consequently, differences in the concentration-dependent patterns of ARE/EpRE protein recognition between the two promoters may involve differential protein/DNA interactions afforded by the distal palindromic site. Precise characterization of the molecular determinants of protein:ARE/EpRE interaction await identification of ARE/EpRE-BPs and the post-translational events that regulate protein binding kinetics and stability. In this context, it should be noted that the appearance of a doublet that specifically binds to ARE/EpREs in vSMCs may reflect differences in phosphorylation, degree of oxidative modification, or composition in

* Miller KP et al., unpublished observations.

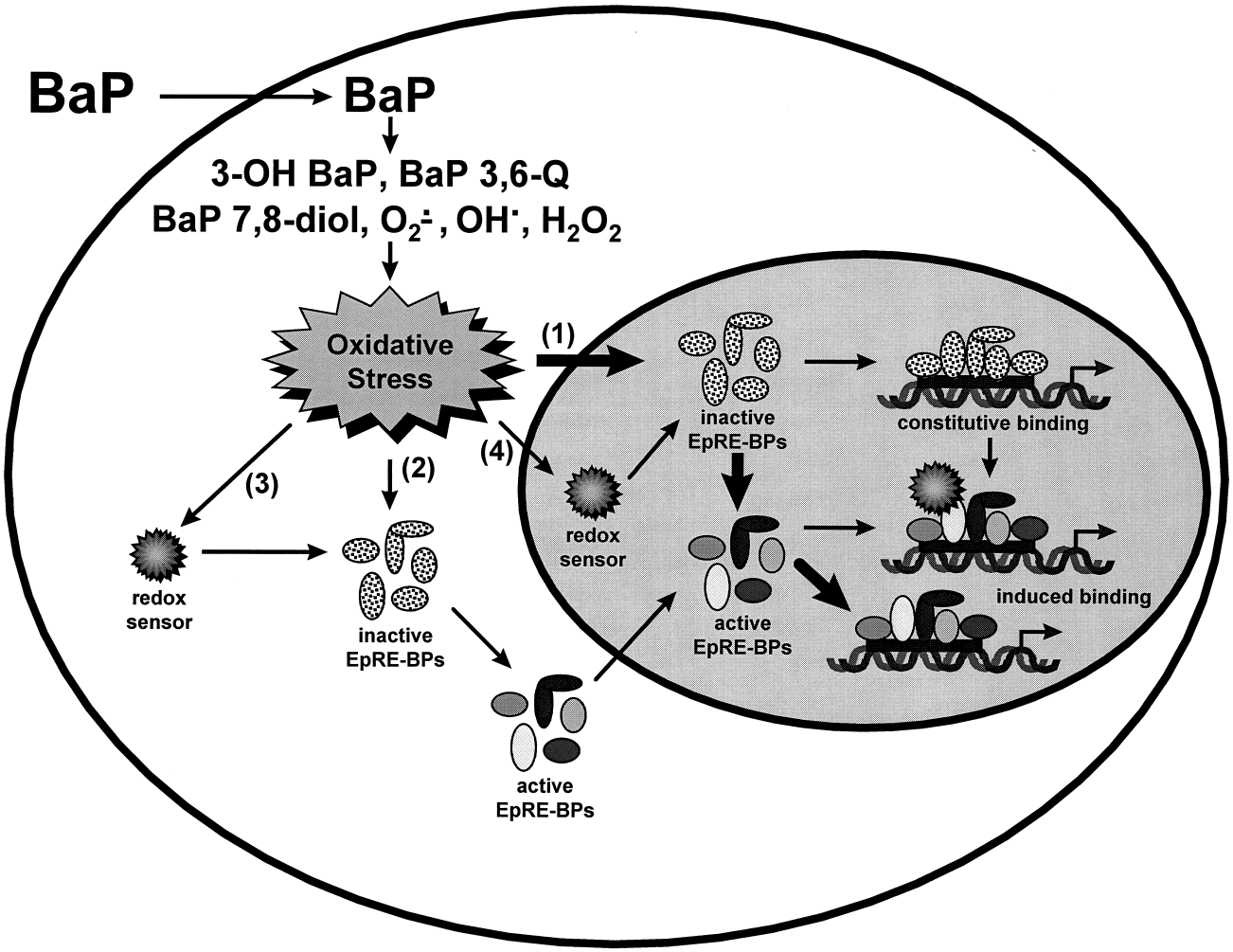

FIG. 7. Proposed mechanisms of protein binding to ARE/EpRE in *c-Ha-ras*. Treatment of vSMCs with BaP is followed by metabolism within the cell to reactive metabolites and by-products including 3-OH BaP, BaP 3,6-Q, BaP 7,8-diol, $O_2^{\cdot-}$, $OH^{\cdot}$, and H_2O_2. Accumulation of these ROS induces oxidative stress, leading to a number of different possible outcomes including activation of nuclear proteins to bind ARE/EpRE [1], activation of cytosolic proteins that translocate and bind ARE/EpRE [2], and finally activation by oxidative stress of a cytosolic [3] or nuclear [4] redox sensor that interacts with cellular proteins to activate binding to ARE/EpRE.

response to oxidant treatment. This hypothesis remains to be examined.

A role for oxidative metabolism of BaP in the activation of protein binding to ARE/EpRE is consistent with the ability of H_2O_2, an intermediate in the metabolism of BaP in vSMCs [13], to activate protein binding to the ARE/EpRE. Thus, redox status in vSMCs may be altered through the generation of ROS following oxidative metabolism of BaP to hydroxylated intermediates, leading to activation of signals that regulate ARE/EpRE-BPs. To our surprise, however, the concentrations of peroxide required to increase ARE/EpRE protein binding were 100 times higher than those of BaP. In our cell system, the effects of exogenous H_2O_2 can be neutralized by antioxidants present in serum [38]. It is also possible that signals responsible for activation of ARE/EpRE protein binding by BaP and its oxidative intermediates in vSMCs are not solely dependent on redox stress and involve mechanisms unaffected by H_2O_2. This possibility is, in fact, consistent with the role of AhR in ARE/EpRE protein binding in hepatoma cells [39] and its involvement in the regulation of ARE/EpRE-regulated transcription of *c-Ha-ras* [13].

BaP and its oxidative intermediates regulated transcription of reporter plasmids containing *c-Ha-ras* regulatory sequences. A role for oxidative mechanisms in the regulation of *c-Ha-ras* is consistent with the ability of buthionine sulfoximine to induce transcriptional activation of pARERC2A in vSMCs (data not shown). We have also obtained evidence that catalase inhibits both constitutive and inducible expression of *c-Ha-ras* in vSMCs.* The activation of *c-Ha-ras* in BaP-treated cells is consistent with the finding of elevated Ras protein levels in vSMCs from BaP-treated animals [40] and the demonstration that increased free-radical load initiates a signaling cascade leading to growth in vSMCs [41]. It is interesting to note that BaP metabolites were only capable of eliciting half-maxi-

* Kerzee JK and Ramos KS, unpublished observations.

mal induction of the gene relative to the parent compound. This finding suggests that the balance between activating gene regulatory signals and cytotoxicity may directly influence ARE/EpRE-regulated transcription.

Nguyen and Pickett [42] have isolated a nuclear protein (YABP) that specifically binds to the ARE/EpRE in the GSTYa promoter. YABP is a heterodimer of two proteins with molecular weights of 28 and 45 kDa, which binds with high affinity to the core ARE/EpRE sequence in control and *tert*-butylhydroquinone-treated nuclear extracts of HepG2 cells [42], but is unable to transactivate the GSTYa promoter [19]. Venugopal and Jaiswal [25, 27] have identified Nrf1 and Nrf2 (66 kDa) and Jun-D, CNC-bZip nuclear transcription factors, as proteins that bind to the ARE in the $hNQO_1$ promoter. Nrf2 has also been associated with regulation of transcription of the γ-glutamylcysteine synthetase gene, a process that may involve heterodimerization with small Maf proteins and/or Jun-D [43]. Jun and Fos proteins bind to ARE/EpRE via the AP-1 binding site; however, they do not bind to the $rNQO_1$ ARE/EpRE, although both elements have highly conserved sequences [23]. Antiestrogen induction of $hNQO_1$ activity is mediated by estrogen receptor binding to proximal and distal ARE/EpRE sites, indicating another pathway potentially involved in the regulation of ARE/EpRE binding [44].

The identity of ARE/EpRE binding proteins in vSMCs has not been defined firmly. We have shown previously that vSMCs express an 80-kDa protein (ARE/EpRE-BP80) that binds specifically to the ARE/EpRE in *c-Ha-ras* [8]. ARE/EpRE-BP80 is distinct from AhR and ARNT [8], and dissimilar from most of the ARE/EpRE-BPs recognized to date. To characterize the proteins that bind to hHa-ras ARE/EpRE, we performed cross-linking studies to define apparent molecular weights and specificities of these proteins. Multiple proteins were identified that bind the consensus ARE/EpRE site in the *c-Ha-ras* promoter, as well as $rNQO_1$, $hNQO_1$, and rGSTYa. ARE/EpRE-BP80 was found to interact specifically with all the promoters examined. Proteins in the 65- and 55-kDa region in the NQO_1 promoter may share homology with Nrf or AP-1 proteins, since they are competed by either AP-1 or $hNQO_1$, elements with TRE or TRE-like binding sites [45]. These two proteins are not predominant in hHa-ras binding, suggesting that AP-1-like factors may not be major contributors to ARE/EpRE regulation in *c-Ha-ras*. It should be noted that proteins in the 23- and 25-kDa region were specific for hHa-ras and rGSTYa ARE/EpRE binding, and, therefore, identification of these proteins is a priority.

In summary, oxidative intermediates of BaP participate in transcriptional deregulation of *c-Ha-ras* gene expression in vSMCs (Fig. 7). The signals responsible for the direct activation of ARE/EpRE-BPs remain to be characterized fully. The possibility remains that oxidative stress induced by oxidative intermediates of BaP also activates redox-sensing mechanisms that, in turn, modulate nuclear protein binding to ARE/EpREs in target genes. Finally, cell- and promoter-specific characteristics appear to influence the nature of proteins binding to redox-regulated DNA elements involved in the cellular adaptation to chemical stress.

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