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Antitumor promotion by phenolic antioxidants: Inhibition of AP-1 activity through induction of Fra expression

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ABSTRACT **Induction of phase 2 detoxification enzymes by phenolic antioxidants can account for prevention of tumor initiation but cannot explain why these compounds inhibit tumor promotion. Phase 2 genes are induced through an antioxidant response element (ARE). Although the ARE resembles an AP-1 binding site, we show that the major ARE binding and activating protein is not AP-1. Interestingly, AP-1 DNA binding activity was induced by the phenolic antioxidant *tert*-butylhydroquinone (BHQ), but the induction of AP-1 transcriptional activity by the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was inhibited by this compound. BHQ induced expression of c-*jun*, *junB*, *fra-1*, and *fra-2*, which encode AP-1 components, but was a poor inducer of c-*fos* and had no effect on *fosB*. Like c-Fos and FosB, the Fra proteins heterodimerize with Jun proteins to form stable AP-1 complexes. However, Fra-containing AP-1 complexes have low transactivation potential. Furthermore, Fra-1 repressed AP-1 activity induced by either TPA or expression of c-Jun and c-Fos. We therefore conclude that inhibitory AP-1 complexes composed of Jun–Fra heterodimers, induced by BHQ, antagonize the transcriptional effects of the tumor promoter TPA, which are mediated by Jun–Fos heterodimers. Since AP-1 is an important mediator of tumor promoter action, these findings may explain the anti-tumor-promoting activity of phenolic antioxidants.**

Phenolic antioxidants exhibit anti-inflammatory, antiatherosclerotic, and anticarcinogenic activities (1). Although the mechanism of anticarcinogenesis is not well understood, it may include induction of the phase 2 detoxification enzymes glutathione *S*-transferases and quinone reductase (2). Protection may also be provided by inhibition of phase 1 enzymes, which activate precarcinogens (3). While these mechanisms can explain how phenolic antioxidants prevent tumor initiation, they do not explain why they interfere with tumor promotion (4, 5). The mechanisms accounting for the latter activity are largely unknown. Paradoxically, at high doses phenolic antioxidants are tumor promoters (1).

Induction of the glutathione *S*-transferase Ya subunit and quinone reductase genes is mediated through an antioxidant (or electrophile) response element (ARE, or EpRE) (6–11). The similarity between the ARE and the 12-*O*-tetradecanoylphorbol 13-acetate (TPA) response element (TRE) recognized by AP-1, a transcription factor that mediates gene induction by phorbol esters and other tumor promoters (12), suggested that AP-1 also activates phase 2 genes. Indeed, induction of AP-1 binding activity by phenolic antioxidants was reported (13, 14), and it was suggested to bind to the ARE (10, 13, 14).

AP-1 is a dimeric DNA binding protein composed of the products of the *jun* and *fos* protooncogenes (12). The Jun proteins (c-Jun, JunB, and JunD) form homo- and heterodimers, whereas the Fos proteins (c-Fos, FosB, Fra-1, and Fra-2) cannot associate with each other but form very stable heterodimers with any of the Jun proteins (12, 15–17). Despite their high degree of sequence conservation, the Jun and Fos proteins exhibit different abilities to activate target genes (18–23). While c-Fos-containing heterodimers activate AP-1 target genes (17, 19), Fra-containing heterodimers do not (23). The biological functions of individual AP-1 complexes are largely unknown.

We investigated the relationship between AP-1 and the transcription factor that activates phase 2 genes by binding to the ARE. Although AP-1 binds with low affinity to the ARE, the major ARE binding and activating protein is not related to AP-1. In the course of these studies, we found that phenolic antioxidants such as *tert*-butylhydroquinone (BHQ) prevented induction of AP-1 transcriptional activity by TPA. This inhibition involves a change in composition of the AP-1 complex. Treatment with BHQ caused the appearance of AP-1 complexes containing high levels of Fra rather than Fos proteins. In cotransfection experiments, Fra-1 repressed activation of a reporter gene by Fos-containing AP-1 complexes. As a variety of different tumor promoters, including phorbol esters, UV irradiation, and the trace metal ion As^{3+}, stimulate AP-1 transcriptional activity (refs. 12, 24, and 25; M.C., K.Y., and M.K., unpublished results), these findings suggest a mechanism that can explain the anti-tumor-promoting activity of phenolic antioxidants.

MATERIALS AND METHODS

HeLa, HepG2, and F9 cells were grown and transfected as described (18–20, 24, 26). After transfection, cells were incubated in medium containing 0.1% fetal bovine serum for 20–24 h and were exposed to the various compounds when confluent. Extracts were prepared 18–24 h later, and chloramphenicol acetyltransferase (CAT) and luciferase (LUC) activities were measured. Most of the different reporters and expression vectors have been described (18–20, 27, 28). The ARE–LUC reporter was derived from the 2XTRE–LUC reporter (29) by replacing the TREs with the synthetic ARE shown below. The Fra-1 expression vector was constructed by inserting the rat *fra-1* cDNA (30) into the pSRα expression vector.

Cells were serum starved for 24 h and then exposed to BHQ or TPA. Nuclear extracts were prepared as described (29). Binding reactions were carried out as described (20) using 100 μg of poly(dI-dC) per ml and 0.1 ng of ^{32}P-labeled TRE or ARE probe, whose sequences were

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Abbreviations: ARE, antioxidant response element; BHQ, *tert*-butylhydroquinone; CAT, chloramphenicol acetyltransferase; LUC, luciferase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TRE, TPA response element.

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TRE: 5′-AGCTTAAAGCATGAGTCAGACACCT
           ATTTCGTACTCAGTCTGTGGACTTAA-5′

ARE: 5′-GATCCTAGCTTGGAAATGACATTGCTAATGG-
           GATCGAACCTTTACTGTAACGATTACC-

        TGACAAAGCAACTTTC
        ACTGTTTCGTTGAAAGAGCT-5′
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The DNA–protein complexes were resolved on a 5% nondenaturating polyacrylamide gel at room temperature. To examine the presence of Fra-1 and c-Jun in the AP-1 complexes, anti-Fra-1 (a gift from R. Bravo, Bristol–Meyers Squibb, Princeton) or anti-c-Jun (G56-206.6, Pharmingen) antibodies were added to the reaction mixture.

Total cytoplasmic RNAs were prepared and analyzed as described (25). Nuclear extracts were separated on an SDS/10% polyacrylamide gel, transferred to an Immobilon-P membrane (Millipore), and subjected to immunoblot analysis (31) using anti-Fra-1 antiserum generated against a synthetic peptide corresponding to amino acids of 2–14 of human Fra-1.

RESULTS

The Major ARE Binding Protein Is Not AP-1. We examined whether AP-1 binds to the ARE. Nuclear extracts were prepared from HepG2 cells treated with BHQ or TPA. A synthetic 51-bp fragment containing the ARE of the mouse glutathione *S*-transferase Ya gene (13) and a 30-bp fragment containing the collagenase TRE (26) were used as probes. BHQ did not increase the ARE-specific binding activity (Fig. 1*A*). Similar results were obtained with longer exposure to BHQ (up to 24 h) or TPA (data not shown). TPA, however, induced AP-1 DNA binding activity and the appearance of a weak ARE binding activity that comigrated with AP-1 (Fig. 1*B*). The mobilities of the AP-1–TRE and AP-1–ARE complexes were different from the major protein–ARE complex. Unlabeled ARE did not compete for AP-1 binding, and unlabeled TRE did not interfere with formation of the major protein–ARE complex, although it competed for binding of AP-1 to the ARE (Fig. 1*B*). Similar results were obtained with BHQ- and TPA-treated HeLa cells (data not shown). Thus, AP-1 binds to the ARE with very low affinity, and, as suggested by Nguyen and Picket (32), the major ARE binding protein is not AP-1. However, it is still possible that despite weak binding to the ARE, overexpression of AP-1 proteins may lead to modest activation of ARE-containing promoters as reported (13, 14). Interestingly, exposure to BHQ stimulated AP-1 DNA binding activity in both HeLa and HepG2 cells (data not shown).

BHQ Suppresses Induction of AP-1 Transcriptional Activity. To determine whether AP-1 participates in induction of phase 2 genes by phenolic antioxidants, we examined the effect of BHQ on AP-1 transcriptional activity in HeLa cells. We found that the induction of two AP-1-dependent reporters by TPA was repressed in a dose-dependent manner by BHQ (Fig. 2*A* and data not shown). BHQ alone did not affect either reporter (data not shown). Similar suppression of reporter gene induction was also observed in HepG2 cells (data not shown). Neither compound affected the Rous sarcoma virus (RSV)–CAT reporter, which lacks a functional TRE (Fig. 2*A*) or a β-actin–CAT reporter (data not shown). Therefore, the suppressive effect of BHQ is specific for AP-1-dependent promoters activated by TPA and is not due to general inhibition of translation or transcription.

By contrast, BHQ stimulated expression of a LUC reporter driven by a truncated prolactin promoter upstream to which an ARE was placed, upon transfection into HepG2 (Fig. 2*B*) or HeLa cells (data not shown). This reporter, ARE–LUC, was also induced by TPA and synergistically activated by TPA plus

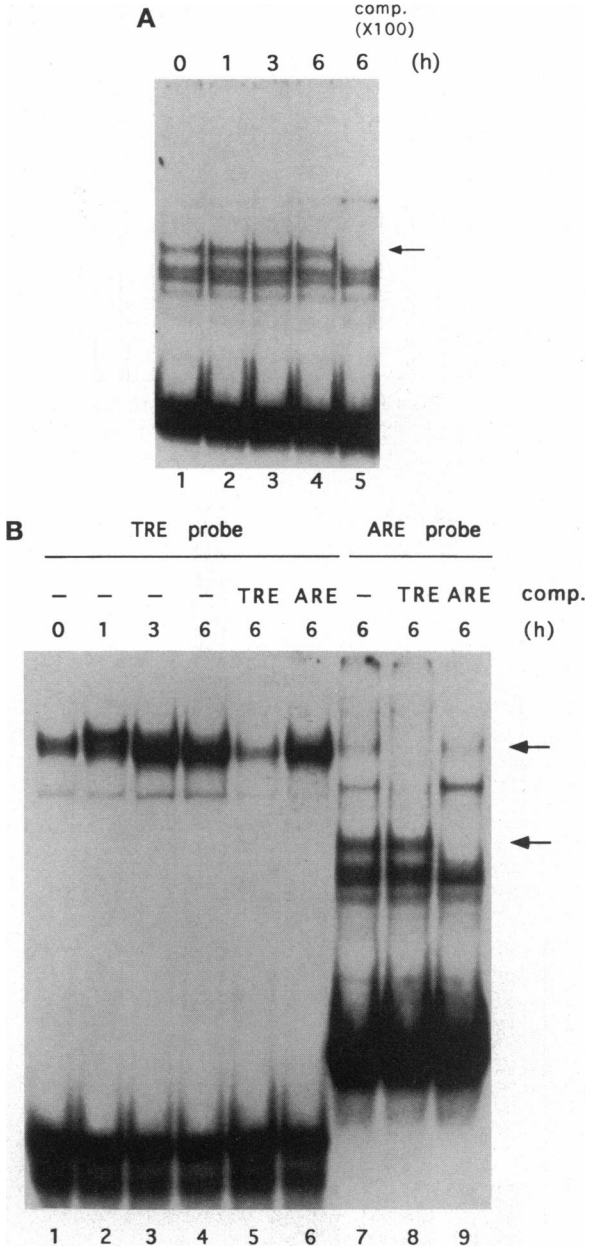

FIG. 1. Effect of BHQ and TPA on ARE and TRE binding activities. HepG2 cells were serum starved for 24 hr, then exposed to BHQ (60 μM) (*A*) or TPA (100 ng/ml) (*B*). At the indicated time points (in h), the cells were harvested and nuclear extracts were prepared. Five-microgram samples were incubated with ^{32}P-labeled ARE (*A*, lanes 1–5; *B*, lanes 7–9) or TRE (*B*, lanes 1–6) probe. To determine the specificity of binding, the 6-h extracts were incubated with the probes in the presence of a 100-fold molar excess of unlabeled ARE or TRE competitor (comp.). The bound and free probes were separated by gel electrophoresis and visualized by autoradiography. The positions of the specific protein–DNA complexes are indicated by the arrows.

BHQ. A similar reporter containing two TREs (2XTRE–LUC), however, was induced only by TPA. Most importantly, the ARE–LUC reporter was inducible by BHQ in F9 teratocarcinoma cells (Fig. 2*C*). These cells do not express endogenous AP-1 activity (19), and congruently the 2XTRE–LUC reporter was not inducible by TPA or BHQ. Thus the major ARE binding activity is not AP-1, its response to BHQ is very different from that of AP-1, and its activation can occur in the absence of AP-1. However, it is still possible that the synergistic activation of the ARE reporter by BHQ plus TPA

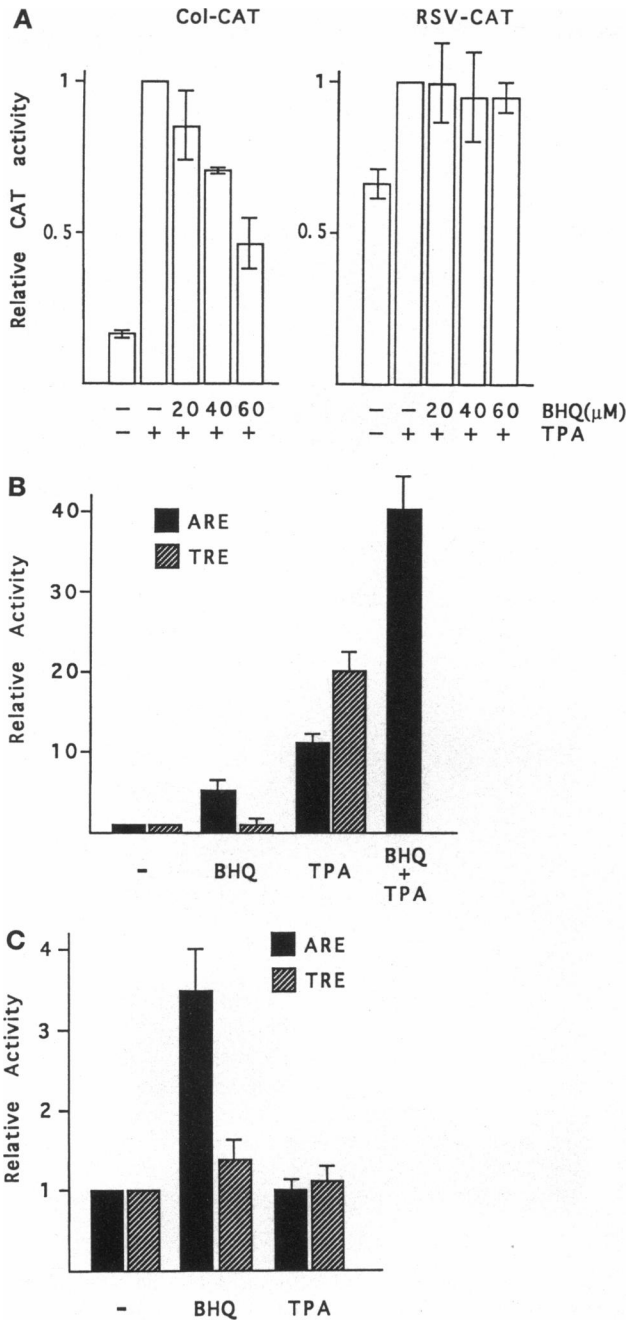

FIG. 2. Effect of BHQ on TRE- and ARE-dependent transcriptional activity. (*A*) HeLa cells were transfected with −73/+63 Col–CAT or RSV–CAT reporters. After serum starvation for 20–24 h the cells were treated with BHQ for 6 h and then cultured with or without TPA (100 ng/ml) for another 18 h. The cells were harvested and CAT activity was determined. CAT activities were calculated relative to the level of CAT activity in TPA-treated cells, which was given an arbitrary value of 1.0. The results shown represent the average of three independent experiments. HepG2 (*B*) or F9 (*C*) cells were transfected with either ARE–LUC (solid bars) or 2XTRE–LUC (hatched bars) reporter. After 24 h of serum starvation, the cells were treated with BHQ (60 μM), TPA (100 ng/ml), or both for 18 h, after which the cells were harvested and LUC activity was determined. The level of LUC expression in nontreated cells was given an arbitrary value of 1.0. Results shown represent averages of three experiments.

involves the interaction of a TPA-induced AP-1 complex with the ARE.

Effects of BHQ on *jun* and *fos* Gene Expression. To understand how BHQ affects AP-1 activity, we examined its effect on *jun* and *fos* gene expression. In the first 3 h after BHQ

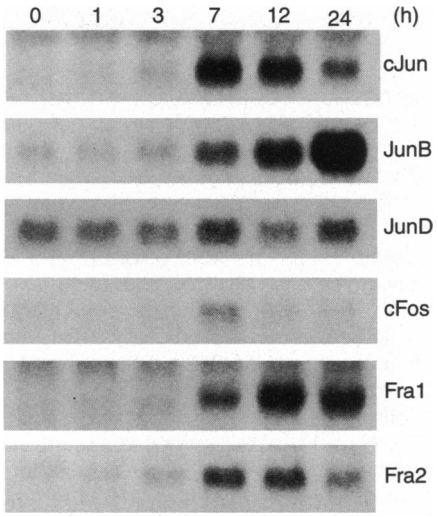

FIG. 3. Effects of BHQ on *jun* and *fos* gene expression. HeLa cells were serum starved for 24 h and treated with BHQ (60 μM) for the indicated times (in h). Total cytoplasmic RNAs were prepared, and 10-μg samples were subjected to Northern analysis using probes specific for c-*jun*, *junB*, *junD*, c-*fos*, *fra-1*, and *fra-2*.

treatment, no significant effect was observed, but after 3 h c-*jun*, *junB*, *fra-1*, and *fra-2* expression was markedly induced (Fig. 3). On the other hand, c-*fos* was very weakly induced, *junD* expression was essentially constitutive, and *fosB* transcripts were undetectable. BHQ treatment does not interfere with the signaling pathway activated by TPA, because induction of c-*fos* by TPA was not affected by a 6-h BHQ pretreatment (data not shown).

BHQ Induces Fra-1-Containing AP-1 Complexes. Analysis of nuclear extracts prepared from cells treated with BHQ revealed marked Fra-1 induction, peaking 12 hr posttreatment

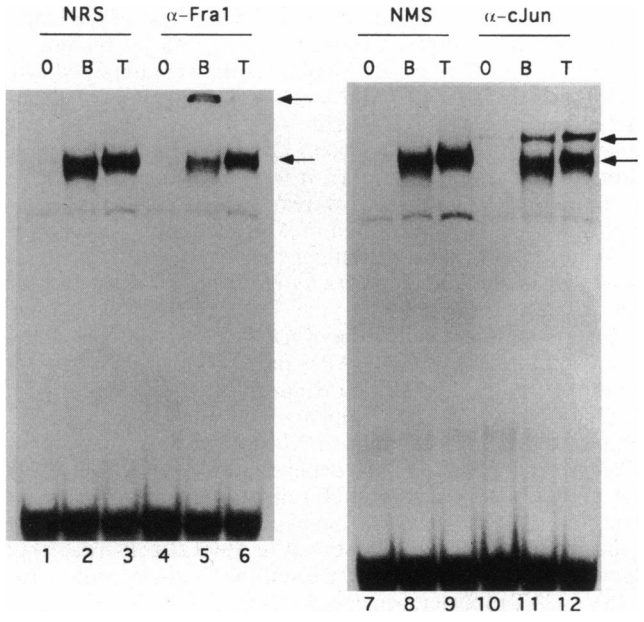

FIG. 4. c-Jun and Fra-1 are part of the BHQ-induced AP-1 complex. Nuclear extracts of untreated (0), BHQ- (B; 16-h posttreatment), or TPA- (T; 1-h posttreatment) treated HeLa cells were incubated with normal rabbit serum (NRS), normal mouse serum (NMS), anti-Fra-1 polyclonal antiserum (α-Fra1), or anti-c-Jun monoclonal antibody (α-cJun) as indicated, prior to incubation with the ^{32}P-labeled TRE probe. Gel mobility shift assays were as described in the legend to Fig. 1*A*. The positions of the specific protein–DNA complexes are indicated by the arrows.

(data not shown). Nuclear extracts were also incubated with anti-c-Jun and anti-Fra-1 antibodies and analyzed by a mobility shift assay (Fig. 4). Incubation with anti-Fra-1 antiserum retarded the mobility of the BHQ-induced, but not the TPA-induced, AP-1–TRE complex. Based on the decrease in intensity of the AP-1–TRE band, it appears that ≈60% of the AP-1 complexes in BHQ-treated cells contain Fra-1. Incubation with an anti-c-Jun antibody retarded the mobilities of both the BHQ- and TPA-induced AP-1–TRE complexes. An anti-cFos antibody supershifted the TPA-induced AP-1–TRE complex but not the BHQ induced complex (data not shown). These data indicate compositional differences between BHQ- and TPA-induced AP-1 complexes. Based on the Northern analysis, we expect that in addition to c-Jun and Fra-1, BHQ-induced AP-1 complexes contain Fra-2 and JunB. As previously shown, JunB is a much weaker transcriptional activator than c-Jun (18–20), even in combination with Fra-1 or Fra-2 (23).

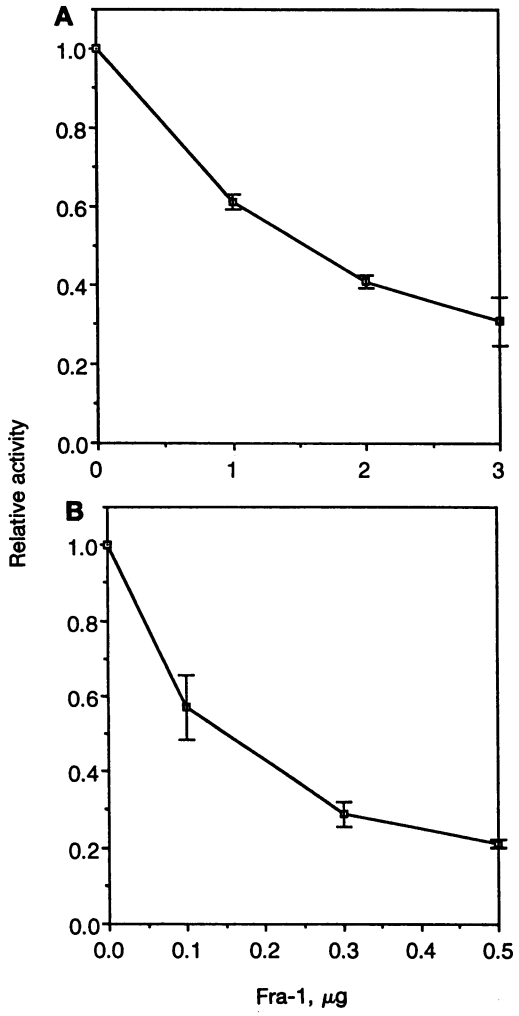

FIG. 5. Fra-1 inhibits AP-1 transcriptional activity. (*A*) HeLa cells were cotransfected with −73/+63 Col–CAT (3 μg) and either a Fra-1 or an empty expression vector (0–3 μg each, as indicated). After 24 h of serum starvation, TPA (100 ng/ml) was added and relative CAT activity was determined 18 h later; the level of CAT expression in cells cotransfected with empty expression vector was given an arbitrary value of 1.0. (*B*) F9 cells were cotransfected with −73/+63 Col-LUC (0.3 μg), c-Jun and c-Fos expression vectors (0.25 μg each), and the indicated amounts of a Fra-1 expression vector. The total amounts of DNA were kept constant using an empty expression vector. After 16 h, the cells were harvested, and the relative LUC activity was determined, giving the level expressed in cells receiving no Fra-1 expression vector an arbitrary value of 1.0.

Fra-1 Inhibits AP-1 Transcriptional Activity. Unlike c-Fos, Fra-1 and Fra-2 proteins lack a transcriptional activation function (23). Therefore, the preferential induction of Fra-containing AP-1 complexes may explain the inhibitory effect of BHQ. To examine this possibility, we cotransfected the −73Col–CAT reporter with increasing amounts of a Fra-1 expression vector. This resulted in a dose-dependent inhibition of collagenase promoter activation by TPA (Fig. 5*A*). As shown in Fig. 5*B*, Fra-1 also repressed AP-1 activity generated by cotransfection of c-Jun and c-Fos expression vectors into F9 cells. Similar results were obtained using the ARE–LUC reporter, which is modestly activated by c-Jun and c-Fos (data not shown). We also examined the transcriptional activity of c-Fos–Fra-1 chimeras. While a chimeric protein containing the N-terminal half of c-Fos and the C-terminal half of Fra-1 had the same low activity as Fra-1, the converse chimera containing the N-terminal half of Fra-1 and the C-terminal half of c-Fos was almost as active as c-Fos (data not shown). A c-Fos deletion mutant lacking the C-terminal activation domain (33) had the same low activity as Fra-1.

Only Redox-Active Phenolic Antioxidants Induce *fra-1*. We also examined the effects of the BHQ analogs 1,2-diphenol (catechol), 1,3-diphenol (resorcinol), and 1,4-diphenol (hydroquinone) on *fra-1* and *c-jun* expression. Only the redox-active BHQ analogs catechol and hydroquinone induced *c-jun* and *fra-1* expression (Fig. 6). No effect was observed with redox-inactive resorcinol. These results reveal that the ability to undergo redox cycling (34) is required for induction of *fra-1* and *c-jun* by phenolic antioxidants. The butyl group of BHQ appears to augment its activity in comparison to the nonbutylated analog hydroquinone. Redox cycling of these diphenols results in conversion into the corresponding quinones, which are likely to be the active principles in the induction process, due to their electrophilic nature (11).

DISCUSSION

As shown above, the ARE-binding protein(s) that mediates induction of phase 2 genes by phenolic antioxidants is not AP-1. Most critically, the ARE confers an effective response to BHQ even in cells lacking AP-1 activity. In addition, AP-1 transcriptional activity is inhibited by BHQ. Our results are therefore consistent with those of Nguyen *et al.* (34). Others, however, suggested that AP-1 may be the activator of the ARE (10, 13, 14). We also find that an AP-1-like entity binds weakly

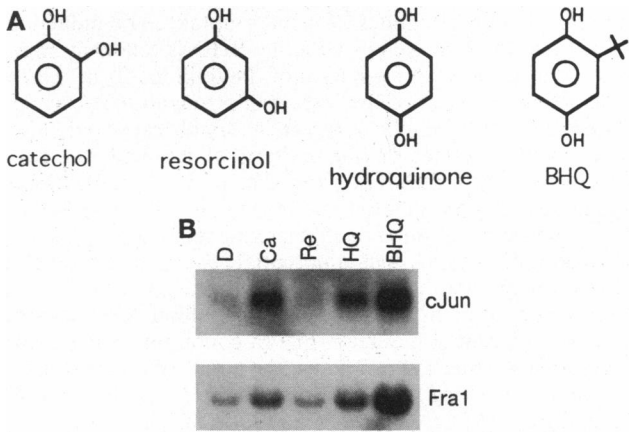

FIG. 6. Effects of various phenolic antioxidants on *c-jun* and *fra-1* expression. (*A*) Chemical structures of the compounds used. (*B*) HeLa cells were serum starved for 24 h and exposed to 60 μM catechol (Ca), resorcinol (Re), hydroquinone (HQ), or BHQ for 12 h. Dimethyl sulfoxide (D) was used as a vehicle control. Total cytoplasmic RNA was extracted and subjected to Northern analysis with *c-jun* and *fra-1* cDNA probes.

to the ARE and that cotransfection with c-Jun and c-Fos expression vectors results in modest activation of ARE-dependent reporters (K.Y., unpublished results). However, as explained above, it is extremely unlikely that AP-1 mediates the induction response to phenolic antioxidants. On the other hand, the weak AP-1 binding site within the ARE may mediate induction of phase 2 enzymes by TPA (35).

The most important and unexpected finding of the present study is that despite its ability to induce AP-1 binding activity, BHQ suppresses TPA-induced AP-1 transcriptional activity. Although certain phenolic antioxidants inhibit protein kinase C activity (36), this cannot account for their effect on AP-1 activity. At the doses used here, BHQ did not inhibit the activation of protein kinase ERK2 or the induction of c-*fos* expression by TPA (K.Y., unpublished results). The inhibition of AP-1 activity by BHQ appears to involve a change in the composition of the AP-1 complex. Unlike TPA, which effectively induces c-*jun* and c-*fos* but is a delayed and weak inducer of *fra-1* and *fra-2* (18, 37), BHQ effectively induces c-*jun*, *junB*, *fra-1*, and *fra-2*, but not *c-fos*. Thus, TPA-induced AP-1 complexes contain mostly c-Fos, whereas BHQ-induced AP-1 complexes contain mostly Fra-1 and probably also Fra-2. Although capable of heterodimerization with Jun proteins and binding to AP-1 sites, the Fra proteins are devoid of transcriptional activation function (23). In c-Fos this function resides in its C-terminal region (32), which is absent from Fra-1 and -2. Therefore, the difference between the composition of BHQ-induced AP-1 complexes and those induced by phorbol esters or other mitogens can explain how BHQ suppresses AP-1 activity. Indeed, we find that transient expression of Fra-1 suppresses AP-1 activity. Similar inhibitory effects on AP-1 activity of a truncated form of FosB, as well as Fra-1 and Fra-2, were reported (23, 38).

An intriguing question is how phenolic antioxidants induce *fra-1* and *fra-2* expression. Their failure to induce high level of c-*fos* or *fosB* expression indicates that their activity is specific and distinct from that of other inducers, such as TPA, UV, and H_2O_2, which induce c-*fos* to a much higher extent than any of the *fra* genes (25). Only redox-active compounds, which can be converted intracellularly to electrophilic quinones, induce *fra-1*. Among these compounds, BHQ is the most potent inducer. Similar activity profiles were found when the abilities of these compounds to induce quinone reductase expression (33) or an ARE-dependent reporter gene (11) were measured. Since the electron-donating butyl group stabilizes the quinone form, BHQ has a lower reduction potential than hydroquinone (39). Alternatively, the butyl group makes BHQ more lipophilic and thus facilitates its cellular uptake. The induction of *fra-1* and *fra-2*, as well as c-*jun*, by BHQ is relatively slow. This is consistent with formation of the quinone form, which is the actual mediator of the response, through redox cycling. It is unlikely that phenolic antioxidants simply cause oxidative stress, as their effect on the pattern of *jun* and *fos* gene expression is very different from that of H_2O_2 (25). These findings also suggest that the *fra-1* gene may contain an ARE. Indeed, an examination of the 5′-flanking region of the human *fra-1* gene (40) reveals such a potential sequence at positions −104 through −94.

The ability of phenolic antioxidants to inhibit AP-1 activity through preferential induction of Fra-containing AP-1 complexes stands in marked contrast to the ability of diverse tumor promoters, including phorbol esters (26), UV irradiation (24, 25), and As^{3+} (M.C., K.Y., and M.K., unpublished results), to induce AP-1 activity. As expression of dominant-negative *jun* mutants reverts the transformed phenotype (41), it is also possible that highly effective and specific inducers of *fra* expression may be useful both in chemoprevention and in cancer therapy.

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