Role of Oxidants and Antioxidants in the Induction of AP-1, NF-κB, and Glutathione S-Transferase Gene Expression*

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Transcription factors AP-1 and NF-κB have been implicated in the inducible expression of a variety of genes in response to oxidative stress. Recently, based on the observation that butylated hydroxyanisole (BHA) and pyrrolidine dithiocarbamate (PDTC) induce AP-1 binding activity and AP-1-dependent gene expression and assuming that these compounds exert an antioxidant effect, it was claimed that AP-1 is an antioxidant-responsive factor. To determine whether AP-1 can be responsive to both oxidant and antioxidant, we examined the nature of BHA and PDTC inducing activity. Using EPR spectroscopy to detect semiquinone radicals, we demonstrate the autoxidation of BHA metabolite tert-butylhydroquinone (TBHQ) to tert-butylquinone. The kinetics of TBHQ-mediated generation of OH radicals were monitored in intact hepatoma HepG2 cells by EPR spin trapping technique. Exogenous catalase inhibited the rate of OH radical formation and the induction of AP-1-mediated glutathione S-transferase (GST) Ya gene expression by BHA and TBHQ, thus indicating the intermediate formation of H2O2 in the metabolism of these chemicals. Furthermore, we show that the induction of AP-1 and NF-κB activities and GST Ya gene expression by BHA and TBHQ is due to a pro-oxidant activity, since this induction was inhibited by thiol compounds N-acetyl cysteine and GSH. Similarly, induction of AP-1 and GST Ya gene expression by PDTC was inhibited by N-acetyl cysteine and GSH. The present findings do not support the notion that the induction of AP-1 by BHA, TBHQ, or PDTC is an antioxidant response and demonstrate that both AP-1 and NF-κB activities are induced by oxygen radicals.

Reactive oxygen species, such as superoxide anion O2−, H2O2, hydroxyl radical -OH, organic peroxides, and radicals, are generated endogenously by all aerobic cells as byproducts of a number of metabolic reactions (1). Oxidative stress, which is an excess production of reactive oxygen species, can damage cells by lipid peroxidation and alteration of protein and nucleic acid structure. To prevent oxidative damage and allow survival in an environment, mammalian cells have developed an elaborate antioxidant defense system that includes nonenzymatic antioxidants (e.g. glutathione and vitamins C and E) as well as enzymatic activities such as superoxide dismutases, glutathione peroxidase, glutathione reductase, catalase, and other hemoprotein peroxidases (2). In addition, drug-metabolizing enzymes such as glutathione S-transferases, glucuronosyl transferases, and NAD(P)H:quinone reductase, by removing compounds capable of generating reactive oxygen species, decrease the level of oxidative stress and are also part of the antioxidant defense (2). Recent findings indicate that oxidative stress conditions enhance the expression of genes encoding antioxidant enzyme activities such as glutathione S-transferase (3, 4), γ-glutamyl cysteine synthetase (5), and heme oxygenase (6). Reactive oxygen species were also shown to be responsible for the inducible expression of genes involved in inflammatory and immune responses (7–9).

In eukaryotic cells enhanced production of reactive oxygen species and oxidative stress conditions are induced by a variety of stimuli, which include ionizing radiation, exposure to drugs and xenobiotics, or binding of cytokines to cell surface receptors (10). Current evidence indicates that the different stimuli use reactive oxygen species as signaling messengers to activate transcription factors and induce gene expression. Recently it was shown that the activation of transcription factor NF-κB by different stimuli, such as cytokines TNFα and interleukin-1, phorbol ester, cycloheximide, lipopolysaccharide, and in some cell types H2O2, is inhibited by antioxidant compound NAC and metal chelators (7, 8, 11, 12). Based on these observations it was concluded that the activation of NF-κB, which occurs by a posttranslational mechanism involving dissociation of inhibitory protein IκB (13), is controlled by reactive oxygen species and the intracellular redox state. Furthermore, using cell lines stably overexpressing H2O2-degrading enzyme, catalase, or cytoplasmic copper/zinc-dependent superoxide dismutase, which enhances H2O2 production from superoxide, Schmidt et al. (14) show that among reactive oxygen species H2O2 acts as a messenger for TNF-α and okadaic acid-induced activation of NF-κB.

Transcription factor AP-1 is an ubiquitous regulatory protein complex that interacts with AP-1 binding sites of target genes to regulate transcription in response to environmental stimuli (15). The AP-1 factor is composed of protein products of members of fos and jun proto-oncogene families, which form homodimeric (junosjun) or heterodimeric (Fos-Jun) complexes. The expression of fos and jun genes was shown to be induced by a variety of extracellular stimulatory agents (e.g. serum, growth factors, phorbol esters, calcium ionophore, neurotrans-

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1 The abbreviations used are: TNF, tumor necrosis factor; BHA, 3(2)-tert-butyl-4-hydroxyanisole; BHT, butylated hydroxytoluene; DDTT, diethylidithiocarbamate; DMPO, 5,5′-dimethyl-1-pyrroline N-oxide; GST, glutathione S-transferase; NAC, N-acetyl cysteine; PDTC, pyrrolidine dithiocarbamate; Q̄O2, 2-phenyl-4-(butylamino)naphtho[2,3-h]quinoline-7,12-dione; O2−, 2-phenyl-5-nitronaphtho[2,3-g]-indole-6,11-dione; TBHQ, tert-butylhydroquinone; TBO, tert-butylquinone; TEMPO, 2,2,6,6-tetramethylpiperidin-1-oxyl-4-one.
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mitters, and ionizing radiation) that promote cell proliferation and transformation and neuronal excitation (15-18). Recent studies on the induction of xenobiotic-metabolizing enzyme glutathione S-transferase Ya gene expression by a variety of structurally unrelated chemical agents have indicated the involvement of AP-1 in the cellular response to chemical stress. It was shown that the induction of a mouse glutathione S-transferase (GST Ya) gene expression, is mediated by a regulatory element, EpRE, composed of two adjacent AP-1-like binding sites that binds and is transactivated by the AP-1 complex (19, 20). Regulatory elements with similar structure were observed to mediate the inducible expression of several other genes encoding drug-metabolizing enzymes (21).

Exposure of hepatoma cells to chemical inducers of GST Ya gene expression such as β-naphthoflavone, 3-methylcholanthrene, tert-butylhydroquinone, trans-4-phenyl-3-butyne-2-one, phorbol 12-myristate 13-acetate, dioxin, phenobarbital, hydrogen peroxide, arsenite, arsenenate, and heavy metals was found to induce an increase in AP-1 binding activity (21-24). The finding that chemical agents of diverse structure all activate GST Ya gene expression through the induction of AP-1 transcription factor that interacts with the EpRE enhancer, led to the assumption that the various chemicals may produce a common transduction signal responsible for AP-1 induction. It was observed that chemical inducers of GST Ya gene expression can generate by metabolism reactive oxygen species or can modify thiol compounds, both of which may cause depletion of reduced GSH and a change of intracellular redox equilibrium toward a more oxidizing environment (25). Recent studies support the hypothesis that the cellular redox state plays an important role in the induction of AP-1 and AP-1-mediated activation of GST Ya gene expression and indicate that this induction is associated with an increase in intracellular oxidant level (3, 23). The role of reactive oxygen species in the induction of AP-1 activity and GST Ya gene expression and their effect on intracellular GSH levels were recently studied by exposing hepatoma cells to quinones with different capacities to generate oxygen radicals (4). The findings indicate a correlation between quinone-mediated production of OH radicals, a decrease in GSH levels, and the induction of AP-1 binding activity and GST Ya gene expression. In view of the regulatory role played by the AP-1 transcription factor in the inducible expression of GST Ya and other drug-metabolizing enzymes by oxidants and thiol reagents (3, 21), the induction of AP-1 appears to be part of a general mechanism of regulation of gene expression by oxidative stress.

Recent studies, however, based on the assumption that the inducible effect of 2,3(3) tert-butyl-4 hydroxyanisole (BHA) and pyrrolidine dithiocarbamate (PDTC) on the AP-1 binding activity and AP-1-mediated reporter gene expression is due to their antioxidant activities, have concluded that AP-1 is an antioxidant-responsive factor (26, 27). Thus, AP-1 activity would be induced by antagonistic signals, by both oxidative stress and antioxidants. The answer to this apparently paradoxical situation may be found, in our opinion, in a reconsideration of the chemical and biological features of BHA, its metabolite tert-butylhydroquinone (TBHQ), and PDTC. Phenolic compounds such as BHA and BHT, termed phenolic antioxidants due to their chain-breaking action during autoxidation of lipids, are utilized for food preservation and suppression of lipid peroxidation in biological materials. However, these phenolic antioxidants were found to exhibit also tumor-promoting activity in rodents, an activity that seems to be related to the formation of oxidized metabolites (28). Indeed, BHA is oxidatively demethylated in mammalian tissues to TBHQ, which by autoxidation to tert-butylquinone (TBQ) may produce reactive oxygen species by redox cycling (29). Thus, BHA may have the potential to act both as antioxidant and antioxidant. This raises the question of which of the two activities is involved in the induction of AP-1-mediated gene expression.

In the present study we investigated whether the induction of AP-1 activity and GST Ya gene expression by BHA and its metabolite TBHQ is due to generation of reactive oxygen species or to an antioxidant activity. The formation of semiquinone by autooxidation of TBHQ both in vitro and in intact cells was detected by EPR spectroscopy. The generation of ‘OH radicals by TBHQ metabolism was monitored by DMPO-OH spin trapping and EPR spectra measurements. In this report we present evidence that the induction of AP-1 and NF-κB activities and GST Ya gene expression by phenolic antioxidants BHA and TBHQ is due to oxidation and quinone-mediated generation of oxidant radicals.

**EXPERIMENTAL PROCEDURES**

Materials—Adriamycin hydrochloride, 5,5’-dimethyl-1-pyrone N-oxide (DMPO), N-acetylcyesteine, reduced glutathione, dimethyl sulfoxide, 3,2(3)- tert-butyl-4-hydroxyanisole, pyrrolidine dithiocarbamate, and bovine liver catalase (1540 units/mg) were obtained from Sigma; 2,2,6,6-tetramethylpiperidine-1-oxyl-4-one (TEMPO) and tert-butylhydroquinone were from Aldrich. Quinones 2-phenyl-4-(butylamino)naphtho[2,3-h]quinoline-7,12-dione (QhQ) and 2-phenyl-5-nitronaphtho[2,3-g]indole-6,11-dione (QhN) and R-SR nitrooxide biradical were synthesized by Dikalov et al. (30) and Khramtsov et al. (31), respectively.

Cell Cultures and Plasmids—Human HepG2 and rat H4II (a differentiated cell line that still expresses GST Ya gene) hepatoma cells were grown in F12 and Dulbecco’s modified Eagle’s medium (1:1) with 10% fetal calf serum (32). HepG2 and H4II cells have been previously found to yield similar results concerning AP-1 binding of nuclear extracts, transient expression of GST Ya gene constructs, intracellular measurement of GSH levels, and drug-mediated gene expression (3, 4, 21). The EpRE Ya-cat plasmid construct, containing the EpRE 41-base pair enhancer of GST Ya gene ligated into the –187 site of its promoter driving the expression of CAT gene (32), was transfected for transient expression into HepG2 cells as described (3).

Electrophoretic Mobility Shift Assays—HepG2 cells were grown on 100-mm plates and, after exposure to chemicals, nuclear extracts were prepared (33) and assayed by electrophoretic mobility shift assay for 32P-labeled AP-1 or NF-κB oligonucleotide binding (21). Oligonucleotides 5’-GATCATGACTGAGGACGTA-3’ and 5’-CTAGCGTCTGGGAGCTT-TCCAGCC-3’ containing the AP-1 and NF-κB binding sites, respectively, were prepared by O. Goldberg (Weizmann Institute).

RNA Extraction and RNA Blot Analysis—Total cellular RNA was prepared by the guanidium thiocyanate method as described (34) and was fractionated by electrophoresis on 1% agarose-formaldehyde gels followed by transfer onto nitrocellulose filters. The RNA blot was hybridized with 32P-labeled probes for GST Ya cDNA and rRNA.

Measurement of Cellular Sulfhydryl Groups—Total -SH groups were measured in intact HepG2 cells by the EPR spectroscopy method of Weiner (35) as described previously by Pinkus et al. (4) and in cell extracts by optical methods, as described by Bergelson et al. (3), using the Ellman reagent (36) and by the Griffith (37) procedure.

Detection of Free Radical Intermediates—HepG2 cells grown to log phase were harvested by scraping, washed twice in phosphate-buffered saline (pH 7.2), and suspended in the same buffer at a density of 1 × 106 cells/ml. OH radical formation was followed by EPR of spin adduct DMPO-OH (38). A typical 200-μl incubation mixture for trapping OH radicals contained 2.5 × 105 cells, 100 mM DMPO, and 2 μM to 1 mM TBHQ dissolved in Me3SO. For DMPO-OH radical measurements the Me3SO final concentrations were not higher than 0.005% and were obtained by serial dilutions with phosphate-buffered saline of 1 μM DMPO in Me3SO. After incubation in 50% Me3SO for 10 min at 37°C (pH 7.2) was purified before use by passing it twice through a syringe containing activated charcoal. The EPR spectra of DMPO-OH, semiquinone, and TEMPO radicals were recorded in a Bruker electron spin resonance ER200D-SRC spectrometer in a quartz capillary of 150 μl. The standard instrumental parameters were as follows: microwave frequency, 9.7 GHz; incident microwave power, 20 mW; center of the field, 3480; scan range, 100 G; field modulation, 1 G; receiver gain, 63 × 103; and time constant, 640 ms. The EPR spectrum of DMPO-OH consisted of a quartet (1:2:2:1) with hyperfine splitting constants of
RESULTS

OH Radical Formation in tert-Butylhydroquinone-Treated Cells—In order to quantitate the TBHQ-mediated formation of OH radical in cultured cells, we have used the spin trapping of OH by DMPO and measured the EPR spectrum of the resulting DMPO-OH spin adduct. Fig. 1b shows that incubation of intact HepG2 cells with 2 μM TBHQ in the presence of 100 mM DMPO for 15 min before the EPR spectrum was measured, resulted in the formation of an EPR spectrum consisting of a quartet (2:2:2:1) with hyperfine splitting of 14.9 G, which is characteristic for the DMPO-OH spin adduct (39). An EPR spectrum consisting of six components was observed to be equally produced during incubation of 10 mM TBHQ in phosphate-buffered saline in the presence or absence of HepG2 cells (Fig. 1, c and d). Similar sextet spectra were registered during incubation of TBHQ in phosphate-buffered saline by Asworth (42). It should be noted that the EPR sextet spectra observed in Fig. 1 (c and d), which were ascribed by others to the semiquinone of TBHQ (40), are actually the result of inadequate experimental conditions for ESR spectra registration. The kinetics of OH formation in TBHQ-treated cells were studied by measuring the amplitude of the second component of the quartet in the EPR spectrum.

DMPO for 15 min before the EPR spectrum was measured, resulted in the formation of an EPR spectrum consisting of a quartet (2:2:2:1) with hyperfine splitting of 14.9 G, which is characteristic for the DMPO-OH spin adduct (39). An EPR spectrum consisting of six components was observed to be equally produced during incubation of 10 mM TBHQ in phosphate-buffered saline in the presence or absence of HepG2 cells (Fig. 1, c and d). Similar sextet spectra were registered during incubation of TBHQ in phosphate-buffered saline by Schilderman et al. (40). However, using a flat cell and varying the conditions of spectra registration of 10 mM TBHQ solution and the scan range to 25 G we obtained a well resolved EPR spectrum consisting of eight lines of equal intensity (Fig. 1e). This spectrum corresponds to the known EPR spectrum of the semiquinone of TBHQ (41). Computer simulation of the experimental spectrum (Fig. 1f) yielded the hyperfine splitting constants of \( a_x = 0.08 \text{ G}, a_y = 1.65 \text{ G}, a_z = 2.13 \text{ G} \) and \( a_s = 2.82 \text{ G} \), which are close to those estimated for the semiquinone of TBHQ in aqueous solution by Asworth (42). It should be noted that the EPR sextet spectra observed in Fig. 1 (c and d), which were ascribed by others to the semiquinone of TBHQ (40) are actually the result of inadequate experimental conditions for ESR spectra registration. The kinetics of OH formation in TBHQ-treated cells were studied by measuring the amplitude of the EPR signal of DMPO-OH spin adduct (Fig. 1b) produced by exposure for different time periods to TBHQ concentrations from 2 μM to 1 mM. The results presented in Fig. 2 indicate that...
for each TBHQ concentration used there is an increase in the amount of DMPO-OH production as a function of time of incubation. However, both the kinetics of 'OH radical formation and the maximum amplitude of the spin adduct EPR signal are found to decrease with the increase in TBHQ concentration being almost abolished at 1 mM BHA, under conditions similar to those described for TBHQ in Fig. 2, did not produce any DMPO-OH spin adduct signal (data not shown). We should observe that in order to prevent Me2SO interference and form of DMPO-CH3 radical spin adduct, all EPR measurements of the DMPO-OH signal were carried out in the presence of Me2SO concentrations up to 0.005%.

Effect of Catalase on 'OH Radical Formation in tert-Butylhydroquinone- and Adriamycin-treated Cells—To study the involvement of H2O2 in 'OH formation by TBHQ and adriamycin, a quinone producer of 'OH radical previously studied by us (4), we have measured the effect of catalase on the kinetics of DMPO-OH spin adduct formation by these chemicals. Fig. 3 shows that the addition of catalase to an incubation mixture of intact HepG2 cells containing 2 μM TBHQ or 100 μM adriamycin and 100 μM DMPO-OH reduces both the kinetics and the maximal amplitude of the DMPO-OH signal. The effect seems to depend on catalase concentration. Moreover, the addition of catalase (0.1 or 1.0 mg/ml) after the DMPO-OH production reached steady-state levels is found to cause an immediate drop in DMPO-OH concentrations followed by a continued decrease with kinetics dependent upon catalase concentrations (Fig. 3).

Antioxidant Effects of tert-Butylhydroquinone—Assuming that the decrease in 'OH radical production with the increase in TBHQ concentration observed in Fig. 2 may be due to an antioxidant effect of this chemical, we have studied the effect of TBHQ addition to an active 'OH radical producer such as adriamycin. Fig. 4A indicates that the addition of 1 mM TBHQ to HepG2 cells incubated with 100 μM adriamycin and 100 mM DMPO at the point where DMPO-OH spin adduct formation has reached steady state levels causes an immediate drop in DMPO-OH level. An inhibition of DMPO-OH formation is observed in cells preincubated for 30 min with 1 mM TBHQ before the addition of adriamycin. Suspecting that TBHQ may inhibit DMPO-OH spin adduct signal by reducing the DMPO-OH spin adduct to diamagnetic hydroxylamine, we have studied the effect of TBHQ on a stable nitroxy radical such as TEMPO. Aliquots of 100 μM TEMPO were incubated with increasing concentrations of TBHQ from 1 μM to 70 mM, and the TEMPO radical signal was measured from the amplitude of the middle component of the triplet in EPR spectrum. The results presented in Fig. 4B show a decrease in TEMPO radical signal as a function of TBHQ concentration, which indicates a direct reduction of the nitroxy radical and production of EPR silent hydroxylamine.

Effect of tert-Butylhydroquinone on Intracellular -SH Level—The intracellular -SH levels were measured in intact HepG2 cells exposed to TBHQ by the EPR spectroscopy method of Weiner (35) and in cell extracts by optical methods using Ellman reagent as described previously (3) or the Griffith (37) procedure. It was observed that 3-h exposure of cells to 100 mM TBHQ caused a decrease in total -SH levels of about 18% as measured by the different methods (Table I).

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![Fig. 4. TBHQ as an antioxidant.](image)

**TABLE 1**

| GSH levels (nmol/10^6 cells) | Optical methods | Ellman (36) | Griffith (37) |
|-----------------------------|----------------|------------|--------------|
| Control                     | 8.99 ± 0.36a (100)a 13a | 9.93 ± 0.40 (100) 10 | 8.40 ± 0.40 (100) 3 |
| TBHQ                        | 7.38 ± 0.23 (82.0) 19 | 8.10 ± 0.30 (81.5) 8 | 7.00 ± 0.36 (83.3) 3 |

a Mean ± S.D.
b Percent of GSH level.
c Number of experiments.
probe we observe also an induction of NF-κB binding activity in nuclear extracts of cells exposed to 30 μM TBHQ. This induction is inhibited by NAC or GSH (Fig. 5A). Similarly, both AP-1 and NF-κB binding activities were induced in the nuclear extracts of cells exposed for 3 h to 30 μM BHA, and their induction was prevented by GSH (Fig. 5B). PDTC was claimed to act as an inducer of AP-1 binding activity and AP-1-regulated gene expression and as an inhibitor of activation of NF-κB binding activity by virtue of its antioxidant properties (26, 27). To test whether PDTC acts as an antioxidant, HepG2 cells were exposed for 3 h to 100 μM PDTC, nuclear extracts were prepared, and AP-1 and NF-κB binding activities were measured by electrophoretic mobility shift using the respective oligonucleotide probes. Fig. 5C shows that PDTC induced the AP-1 binding activity and that this induction was inhibited by pre-exposure of cells to antioxidant NAC. GSH was also observed to inhibit PDTC induction of AP-1 activity (data not shown). In contrast, PDTC was not found to induce the NF-κB binding activity (Fig. 5C).

Induction of AP-1 and NF-κB Activities by Quinones—In a previous study we showed that the induction of AP-1 binding activity by Adriamycin and two synthetic quinones, Qb and Qn, with different capacities to generate oxygen radicals, correlates with their oxygen radical production (4). Thus, Adriamycin and Qb, which can chelate Fe(III) ions and are more effective hydroxyl radical producers, both in vitro (30) and in vivo (4), were also stronger inducers of AP-1 binding activity than Qn. The effect of the different quinones on the induction of NF-κB binding activity was presently studied in nuclear extracts of HepG2 cells exposed for 3 h to 2 μM concentrations of Adriamycin, Qb or Qn. Adriamycin, Qb or Qn, added directly or after a 60-min preincubation with 0.1 mg/ml catalase or 30 mM NAC as indicated. Nuclear extracts were prepared and assayed by electrophoretic mobility shift assay for 32P-labeled AP-1 or NF-κB oligonucleotide binding. Arrows indicate AP-1 or NF-κB complex. B, the specificity of NF-κB binding activity was tested by competition with a 100-fold molar excess of unlabeled NF-κB or AP-1 oligonucleotides as indicated.

Antioxidants Inhibit Induction of GST Ya Gene Expression by Adriamycin, PDTC, and tert-Butylhydroquinone—We have previously described the induction of GST Ya gene expression by TBHQ (25), Adriamycin, and two other quinones with different capacities to produce OH radicals and have shown that the induction of GST Ya mRNA by these quinones correlates with their oxygen radical production (4). To further study the effect of oxidants and antioxidants on GST Ya gene expression, hepatoma H4II cells that express this gene were exposed for 3 h to 2 μM Adriamycin, 100 μM TBHQ, or 100 μM PDTC in the absence or presence of NAC or GSH. Fig. 7 shows that the induction of GST Ya mRNA by Adriamycin, TBHQ, or PDTC is considerably inhibited by the presence of the antioxidants. NAC, GSH, and Catalase Inhibit Induction of EpRE Ya-cat by PDTC, tert-Butylhydroquinone, and BHA—To further question whether the induction of AP-1 binding activity and NF-κB binding activity by Adriamycin and two synthetic quinones, Qb and Qn, with different capacities to generate oxygen radicals, correlates with their oxygen radical production (4). Thus, Adriamycin and Qb, which can chelate Fe(III) ions and are more effective hydroxyl radical producers, both in vitro (30) and in vivo (4), were also stronger inducers of AP-1 binding activity than Qn. The
this chemical occurs with the intermediate production of H₂O₂. Previously, it was shown that NAC and GSH inhibit EpRE Ya-cat induction by TBHQ (3). Similarly, induction of EpRE Ya-cat by BHA is found to be inhibited by GSH and catalase but not by PDTC (Fig. 8B).

**DISCUSSION**

In the present study we have addressed the question whether AP-1 can be induced by both oxidant and antioxidant conditions as recently claimed (26, 27). We have examined the chemical properties of AP-1-inducing compounds, BHA and its metabolite TBHQ, and questioned the nature of their action on the activation of gene expression. In this study, using EPR spectroscopy, we have observed that TBHQ in buffered solution or in HepG2 cellular suspensions gives rise to paramagnetic molecular species (Fig. 1, c, d, and e). We ascribed these species to a semiquinone form of TBHQ on the basis of similarity of the experimental EPR spectra with those of TBHQ semiquinones described in the literature (41, 42). It can be assumed that, in solution, the autoxidation of TBHQ to semiquinone (TBQ⁺) takes place according to the reaction,

\[ \text{TBHQ} + \text{O}_2 \rightarrow \text{TBQ}^+ + \text{O}_2^- \]

which occurs at a noticeable rate when hydroquinones are in the anionic form (43, 44). In fact we observed that the concentration of TBQ⁺ increased dramatically with the pH and oxygen pressure (data not shown). The electron transfer from TBQ⁺ into molecular oxygen follows the reaction,

\[ \text{TBQ}^+ + \text{O}_2 \rightarrow \text{TBQ}^0 + \text{O}_2^- \]

The rate of reaction (2) and the equilibrium concentration of TBQ⁺ and O₂⁻ depend on the redox potential of TBQ/TBQ⁺ and O₂/O₂⁻ known as E₁/₂ as \(-520 \text{ mV (in dimethylformamide)}\) and \(-155 \text{ mV (in water)}\), respectively (45, 46). Although E₁/₂ becomes more positive with an increase in the polarity of solvent, this does not seem to be a marked effect for p-benzoquinone (47), and one can assume an E₁/₂ for TBQ/TBQ⁺ in water that would still allow it to participate in O₂⁻ production according to Reaction 2. OH radicals will be further generated from O₂⁻ by Haber-Weiss and Fenton reactions as described by Weiner (48). We have used the spin trapping EPR technique to monitor the generation of OH radicals by TBHQ in intact cells. In the presence of spin trapping agent DMPO the TBHQ-induced OH radical production was detected by EPR spectra of DMPO-OH spin adducts (Fig. 1b). The study of DMPO-OH formation in cells exposed to TBHQ shows a decrease in both kinetics and maximum amplitude of the spin adduct EPR signal with increase in TBHQ concentration (Fig. 2). This may indicate an antioxidant effect since TBHQ, at concentrations above 1 mM, caused an inhibition of DMPO-OH formation by adriamycin and a decrease in TEMPO nitroxy radical signal (Fig. 4). BHA, under conditions similar to those where TBHQ is active in generating oxygen radicals, did not produce any DMPO-OH spin adduct signal (data not shown). This finding may be rationalized by postulating that (i) TBHQ, a metabolite of BHA, is not produced under the experimental conditions described in Fig. 2, which do not allow BHA demethylation, and (ii) BHA itself does not cause radical formation. This interpretation is consistent with the conclusions of Kahl et al. (29) that no oxygen activating properties can be ascribed to BHA itself.

Working with intact HepG2 cells we show that the redox cycling of TBHQ/TBQ leading to the generation of OH radicals, which is probably accelerated in cells by the availability of enzymes that support this process, occurs with intermediate formation of H₂O₂. Thus, the addition of catalase to the culture medium inhibited the OH generation in a catalase concentration-dependent fashion (Fig. 3). This effect is surprising since, due to its molecular mass, catalase is not expected to enter the cells. It may, however, be explained by the difusibility of H₂O₂ through biological membranes, which would allow catalase, without entering the cell, to lower intracellular H₂O₂ levels by decomposition of extracellular H₂O₂ and enhance the efflux gradient across plasma membrane. The addition of exogenous catalase to HepG2 cells is presently found to inhibit also the...
and AP-1-dependent gene expression by this chemical is due to enhanced detoxification of hydrogen peroxide by GSH peroxidase and oxidation of GSH to GSSG (49). Our present data indicate that a short term exposure (3 h) of HepG2 cells to TBHQ causes a modest (−18%) but reproducible decrease in intracellular -SH levels and support the pro-oxidant notion for the activity of this chemical (Table I). Further evidence that TBHQ and BHA function as oxidants to induce AP-1 binding activity is provided by the inhibitory effect of thiol compounds NAC and GSH on this induction (Fig. 5). In previous studies we have shown that depletion of intracellular GSH levels, by the specific inhibition of γ-glutamyl cysteine synthetase with l-buthionine-S,R-sulfoximine (50) or by direct oxidation to GSSG by diamide, causes an increase in AP-1 binding activity in the absence of chemical inducer as well as an enhanced induction of AP-1 binding activity by TBHQ. In addition, the depletion of intracellular GSH levels was found to enhance the activation of AP-1-mediated EpRE Ya-cat expression by a variety of chemical agents (3). The inducible increase in the AP-1 binding activity by chemicals was shown to involve the induction of fos and jun gene expression with accumulation of increased levels of the respective mRNAs (21, 22, 24) and a de novo synthesis of the AP-1 protein components (21, 24).

In contrast to AP-1, the activation of transcription factor NF-κB in response to pro-oxidant conditions produced by various stimuli (7, 9, 12) occurs by a post-translational mechanism, which involves the dissociation of the inhibitory protein IκB. There are, however, similarities between the induction of AP-1 and NF-κB transcription factor activity by reactive oxygen species. Working with adriamycin and two different quinones, Qb and Qm, with different capacities to generate 'OH radicals, we previously showed that the induction of AP-1 binding activity by these quinones correlates with their oxygen radical production (4). Similarly, in the present study we find that the induction of NF-κB binding activity by these quinones is dependent on their capacity to generate 'OH radicals, the two Fe(III) chelating quinones and potent 'OH radical producers, adriamycin and Q5, being stronger NF-κB inducers than Qm (Fig. 6). We may therefore conclude that 'OH radicals probably constitute the reactive oxygen species responsible for the induction of both AP-1 and NF-κB transcription factors.

In view of the present findings, which show that TBHQ is a producer of 'OH radicals and that the induction of AP-1 activity and AP-1-dependent gene expression by this chemical is due to its pro-oxidant properties, we have reconsidered the effect of BHA and its cellular metabolite TBHQ on NF-κB activation. Recently Israel et al. (51) and Schulze-Osthoff et al. (9) have observed that BHA inhibits the phorbol 12-myristate 13-acetate- or TNF-induced activation of NF-κB binding activity and attributed this inhibition to an antioxidant effect of BHA. We find, however, that a direct treatment of HepG2 cells with TBHQ or BHA, at the relatively low concentration of 30 μM, causes an induction of NF-κB binding activity (Fig. 5). Since this induction is inhibited by NAC and GSH, it is evident that TBHQ and BHA act as pro-oxidants to induce NF-κB. It should be noted that pyrogalol, a triphenol antioxidant, was reported to generate reactive oxygen and induce NF-κB binding activity (52). The inhibitory effects of BHA on phorbol 12-myristate 13-acetate or TNF induction of NF-κB binding activity and NF-κB-mediated human immunodeficiency virus enhancer activation described by Israel et al. (51) and Schulze-Osthoff et al. (9) may still be explained by the large amounts of BHA required (200–400 μM), which at these concentrations may have an antioxidant effect in the lymphoblastoid T (J. Jhan) and monocyteic (U937) cell lines studied.

Because the pyrrolidine derivative of dithiocarbamate, PDTC, was shown to suppress activation of NF-κB in response to diverse stimuli involving reactive oxygen production, being more potent than thiol compound NAC, its mode of action was attributed to an antioxidant thiol function (26). However, this conclusion may not be correct in view of the fact that dithiocarbamates may induce a variety of biological effects in mammalian cells that are not consistent with this hypothesis. Thus, dithiocarbamates PDTC, DDT, and disulfiram (which may be reduced by GSH to DDT), due to their metal-chelating properties, inhibit (copper/zinc) superoxide dismutase activity and were also shown to potentiate oxygen toxicity in animal tissues and cause a decrease in glutathione peroxidase activity and thiol levels (53–55). It was demonstrated that dithiocarbamates, like other xenobiotics bearing a thiol function, may cause oxidation of GSH by a nonradical mechanism (56). The thiol functions of these compounds are oxidized by microsomal flavin-containing monooxygenases to reactive intermediates sulfenic acids, which by a nonenzymatic reaction oxidize GSH to GSSG, regenerating the parent xenobiotic (57). In this respect dithiocarbamates seem to act catalytically, relatively small amounts leading to the oxidation of several hundred molar equivalents of GSH (56). In fact, exposure of thymocytes to PDTC was found to cause a fast increase in intracellular GSSG level (58). This, and the observation that nonthiol metal chelators, such as phenanthroline and desferal, also inhibit NF-κB activation (12) suggests that the inhibitory effects of dithiocarbamates on NF-κB activation may be due to their metal chelator properties rather than to an antioxidant effect of their thiol function.

The hypothesis regarding an antioxidant function for PDTC was also invoked to explain the induction of AP-1 activity by this chemical (26, 27). In this report we show, however, that the PDTC induction of AP-1 binding activity is completely inhibited by NAC (Fig. 5C). Furthermore, we show that PDTC induction of EpRE Ya-cat expression is inhibited by NAC and GSH and is stimulated by the pro-oxidant activity of TBHQ (Fig. 8). In addition, the induction of endogenous GST Ya gene expression in H4II cells by PDTC, TBHQ, or adriamycin was also inhibited by NAC or GSH (Fig. 7). These findings clearly indicate that the activation of AP-1 transcription factor and GST Ya gene expression by PDTC is not due to an antioxidant effect of this chemical.

In conclusion, the present study presents evidence that phe nolic antioxidant BHA and its active metabolite TBHQ have dual capacity to act both as antioxidants and as producers of reactive oxygen. Here we show, for the first time, that the induction of AP-1 and NF-κB activities and GST Ya gene expression by BHA and TBHQ is due to the oxidative stress conditions generated by these chemicals. This conclusion is supported by the following findings: (i) exposure of cells to TBHQ causes generation of 'OH radicals and a decrease in intracellular GSH level, (ii) induction of AP-1 and NF-κB by BHA and TBHQ is inhibited by antioxidants NAC or GSH and by exogenous catalase, (iii) induction of endogenous GST Ya gene in hepatoma cells or a transfected EpRE Ya-cat construct by BHA or TBHQ is inhibited by NAC, GSH, and exogenous catalase. In view of these findings the regulatory element responsible for the inducible expression of GST Ya gene by TBHQ...
is actually an oxidative stress response element, and the term “antioxidant response element” (ARE) currently used by a number of laboratories (59, 60) to define this element is misleading. In the present study we have also shown that induction of AP-1 and GST Ya gene expression by PDTC is due to an oxidative effect, since it is inhibited by antioxidants NAC and GSH but not by BHA or TBHQ.

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