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Activation of the NRF2 Signaling Pathway by Copper-Mediated Redox Cycling of *Para*- and *Ortho*-Hydroquinones

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SUMMARY

Transcription factor NF-E2 p45-related factor 2 (Nrf2) mediates adaptation to oxidants and electrophiles through up-regulating genes that contain antioxidant response elements (AREs) in their promoters. Using the stably transfected human AREc32 reporter cell line, we found that copper and other transition metals enhanced induction of ARE-driven luciferase by 2-*tert*-butyl-1,4-hydroquinone (tBHQ) as a result of increased oxidation to 2-*tert*-butyl-1,4-benzoquinone (tBQ). Following exposure to tBHQ for 30 min, ARE-luciferase activity measured after 24 hr was dependent on the presence of Cu^{2+}. In contrast, tBQ-induced activity was Cu^{2+}-independent. The metal-catalyzed oxidation of tBHQ to tBQ occured rapidly and stoichiometrically. Compounds that share *para*- or *ortho*-hydroquinone structures, such as catechol estrogens, dopamine, and L-DOPA, also induced ARE-driven luciferase in a Cu^{2+}-dependent manner. Thus, the oxidation of *para*- and *ortho*-hydroquinones to quinones represents the rate-limiting step in the activation of Nrf2.

INTRODUCTION

Nrf2 (NF-E2 p45-related factor 2) is a cap'n'collar (CNC) basic-region leucine zipper (bZIP) transcription factor that allows adaptation to oxidative and electrophilic stress. It regulates a battery of genes for antioxidant enzymes and detoxifying proteins, including glutamate-cysteine ligase (comprising catalytic [GCLC] and modifier [GCLM] subunits), NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione *S*-transferases, and aldo-keto reductases, through antioxidant response elements (AREs) in the regulatory regions of these genes. Under homeostatic conditions, Keap1 (Kelch-like ECH-associated protein 1) suppresses the activity of Nrf2 by acting as an E3 ubiquitin ligase substrate adaptor for Cul3/Rbx1 and targeting the CNC-bZIP transcription factor for proteasomal degradation (Hayes and McMahon, 2009; Kensler et al., 2007; Nguyen et al., 2009). During oxidative or electrophilic stress, Keap1 is inactivated as a consequence of modification of its highly reactive cysteine residues (Eggler et al., 2005; Zhang, 2006). Under such circumstances, the stability of Nrf2 increases and it accumulates in the nucleus, where after dimerization with a small Maf (Musculo-Aponeurotic Fibrosarcoma) protein (Motohashi and Yamamoto, 2004; Nioi et al., 2003), it transactivates genes that contain an ARE in their promoter regions.

Nrf2 has been widely studied from a pharmacology and toxicology perspective. This body of work has revealed that the CNC-bZIP transcription factor is activated by many thiol-reactive electrophilic xenobiotics and can mediate resistance against these compounds, in part because it controls GSH synthesis. Evidence also suggests that Nrf2 is activated by redox-cycling agents and can confer protection against this class of chemicals, but that the resistance, at least in the case of menadione, is independent of GSH (Higgins et al., 2009). Relatively little is known about how quinones and hydroquinones activate Nrf2. This is, however, an important issue because several endogenous hydroquinones exist, such as 4-hydroxyestradiol and dopamine, that could influence Nrf2 activity. Thus, 4-hydroxyestradiol and dopamine could serve as endogenous second messengers to control the expression of antioxidant genes in mammary or neural tissues.

The findings that 1,2-hydroquinones (catechols, *ortho*-hydroquinones) and 1,4-hydroquinones (*para*-hydroquinones), but not 1,3-hydroquinones (resorcinols, *meta*-hydroquinones), induce ARE-driven gene expression suggested that the oxidative lability of these compounds is essential for inducer activity (Prochaska et al., 1985; Rushmore et al., 1991). On the basis of molecular orbital calculations, Bensasson et al. (2008) proposed a two-step mechanism of induction of Nrf2-dependent enzymes by oxidizable diphenols involving (1) oxidation of the diphenols to their quinone derivatives, and (2) oxidation by these quinones of critical cysteine residues in Keap1 that are essential for its ubiquitin ligase substrate adaptor activity, and thus repression of Nrf2.

Copper (Cu) is an essential trace element. It is present in many tissues at micromolar concentrations, and in some tissues, including kidney and liver, its concentrations exceed 100 μmol/l (Linder and Hazegh-Azam, 1996). In the body, copper exists in both oxidation states, oxidized Cu^{2+} and reduced Cu^{+}.

Moreover, it is a redox-active transition metal and can catalyze the oxidative activation of a number of phenolic compounds via a Cu^{2+}/ Cu^{+} redox cycling mechanism that produces reactive oxygen species (ROS) (Li et al., 2002; Li and Trush, 1993).

Although tBHQ is a prototypic inducing agent, the initiating events or species responsible for the activation of Nrf2 by the hydroquinone have not been well defined. Electron spin resonance spectroscopy has revealed that in the presence of O_2, Cu^{2+} mediates the one electron oxidation of tBHQ to a semiquinone anion radical (Li et al., 2002), which, in turn, reacts with a second molecule of O_2 to give $O_2^{\cdot -}$ and tBQ, with the subsequent reoxidation of Cu^{+} by $O_2^{\cdot -}$ to produce H_2O_2 and regenerate Cu^{2+} (Nakamura et al., 2003); 1,4-Hydroquinone can also be oxidized by Cu^{2+} in a similar way to tBHQ (Li and Trush, 1993). In the presence of cytochrome P450-dependent monooxygenases, 2- and 4-hydroxy estrogens are converted into semiquinone radicals and eventually to their corresponding estrogen quinones. Conversion of these catechol estrogens to quinones can also occur through a nonenzymatic mechanism involving Cu^{2+} (Li et al., 1994; Thibodeau and Paquette, 1999).

The finding that Cu^{2+} can promote oxidation of tBHQ has prompted us to question whether this redox-active transition metal might either augment or inhibit induction of ARE-driven gene expression by hydroquinones. Furthermore, we tested whether Cu^{2+} has an effect on induction of gene expression by endogenous hydroquinones formed from estradiol or by dopamine. Using AREc32 cells (Wang et al., 2006), we demonstrate that oxidation of diphenols to quinones is a critical step in the activation of Nrf2. In particular, Cu^{2+} efficiently mediates the redox-cycling of oxidizable diphenols. The concentration of Cu^{2+} in the cell culture medium correlates with the potency of these compounds as inducers of ARE-driven luciferase activity. To our knowledge, we provide the first experimental evidence that the *para*- and *ortho*-hydroquinones themselves are unable to activate Nrf2 directly and that their corresponding quinones react with Keap1 and are the direct activators of Nrf2. In addition, we show that in the presence of Cu^{2+}, the catechol estrogens 2-hydroxyestradiol, 4-hydroxyestradiol, 4-hydroxyestrone, as well as dopamine, are potent Nrf2 activators.

RESULTS

Cu^{2+} and tBHQ Synergistically Enhance ARE-Driven Gene Expression

It has been reported that tBHQ can be oxidized by Cu^{2+} (Li et al., 2002; Nakamura et al., 2003). To test whether copper has any effect on the activation of Nrf2 by tBHQ, $CuCl_2$ was added to the culture medium of AREc32 cells. $CuCl_2$ enhanced the induction of ARE-driven luciferase activity by tBHQ in a dose-dependent manner (Figure 1A). In the absence of $CuCl_2$, treatment for 24 hr with 5 μM tBHQ resulted in ~5-fold induction of luciferase activity. However, in the presence of 10 μM $CuCl_2$, the induction by 5 μM tBHQ was doubled to 10-fold, and in the presence of 100 μM $CuCl_2$, it was increased to ~40-fold. In addition, CuCl also enhanced the induction of ARE-driven luciferase activity by tBHQ in a dose-dependent manner (Figure 1B). In contrast, Cu^{2+} had no effect on the induction by sulforaphane (SFN), an isothiocyanate that activates Nrf2, but is not redox-active.

To confirm that Cu^{2+} can increase induction of endogenous ARE-driven genes by tBHQ, western blotting was performed, and the expression of the endogenous ARE-dependent genes encoding AKR1C, AKR1B10, and heme oxygenase 1 (HO-1) was evaluated (Devling et al., 2005; Lou et al., 2006; Reichard et al., 2007; MacLeod et al., 2009). Induction of AKR1C and AKR1B10 by 5 μM tBHQ was markedly increased by $CuCl_2$ (Figure 1C, lanes 2 and 4), and HO-1 only became detectable in the presence of $CuCl_2$, confirming the potentiating effect of Cu^{2+} on the activation of Nrf2 by tBHQ.

Low Oxygen Inhibits Cu^{2+}-Enhanced Induction of Luciferase Activity by tBHQ

Oxidation of tBHQ by Cu^{2+} requires O_2 (Li et al., 2002; Nakamura et al., 2003). When AREc32 cells were cultured in DMEM under 1% (vol) O_2, without the addition of Cu^{2+}, 5 μM tBHQ induced luciferase activity to a similar degree as it did under 21% O_2 (Figure 2A); this is probably because the hydroquinone is still capable of undergoing slow auto-oxidation to the quinone even under low oxygen conditions. In addition, under low oxygen conditions, $CuCl_2$ was still able to enhance the induction of luciferase activity by tBHQ ~4-fold (from 5-fold to 20-fold). Most importantly however, this potentiation was significantly lower than that observed under aerobic conditions (from 5-fold to 50-fold). The low oxygen conditions did not affect the induction of luciferase activity by SFN significantly (Figure 2B). These results indicate that Cu^{2+}-enhanced activation of Nrf2 by tBHQ is dependent on the oxygen concentration, whereas activation of Nrf2 by SFN is not increased by Cu^{2+} nor is it oxygen-dependent.

1,2- and 1,4-Diphenols Are Not Direct Inducers of ARE-Driven Genes

Quinones are electrophilic compounds that can readily react with thiols. To avoid interference by thiol compounds in the culture medium, we exposed AREc32 cells to 5 μM tBHQ in PBS for only 30 min and then continued to grow the cells for a further 24 hr in the growth medium to which the hydroquinone had not been added. This short-time exposure to tBHQ had no effect on the expression of luciferase, as determined by measuring its activity 24 hr later (Figure 3 and Table 1). However, when the 30 min exposure to 5 μM tBHQ was performed in the presence of 50 μM $CuCl_2$ or 100 μM CuCl, the ARE-driven luciferase activity was induced ~30-fold. Importantly, induction was completely blocked by the metal chelators DFX (100 μmol/l) and EDTA (100 μmol/l) (Figure 3), indicating that Cu^{2+}-mediated oxidation of tBHQ is essential to activate ARE-dependent gene expression. Under identical conditions, the quinone tBQ induced luciferase activity in the absence of Cu^{2+}, and this induction was not affected by DFX or EDTA. These data further demonstrate that (1) Cu^{2+}-mediated oxidation of tBHQ to the quinone tBQ occurs rapidly, (2) oxidation of tBHQ to tBQ is an essential step in inducing ARE-dependent gene expression, and (3) tBQ, and not tBHQ, is the ultimate inducing agent.

The conversion of 1,4-hydroquinones to quinones can be mediated by Cu^{2+} (Li et al., 1994; Thibodeau and Paquette, 1999). We therefore examined whether Cu^{2+} can potentiate the ability of other oxidizable diphenols to activate ARE-driven reporter gene activity. As shown in Table 1, 1,4-hydroquinone

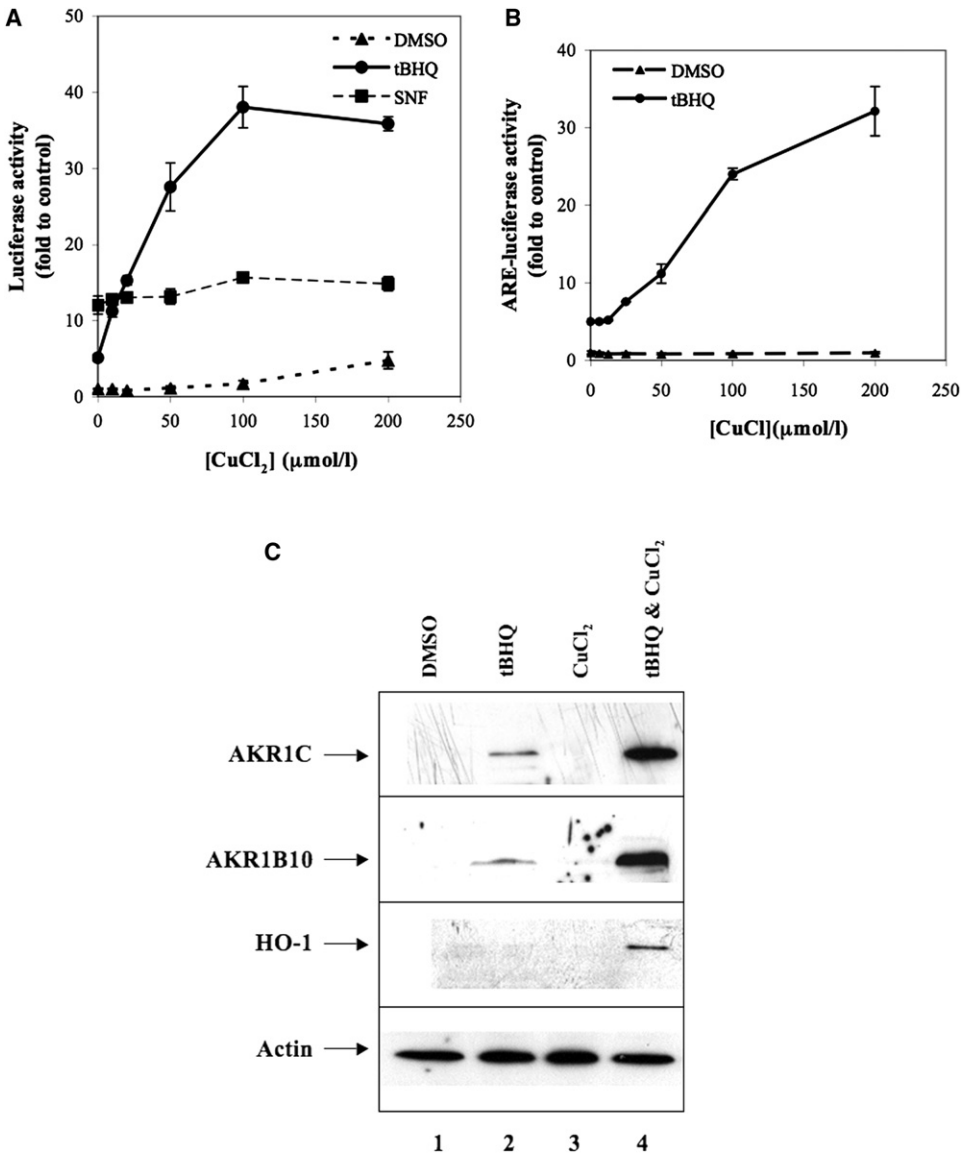


Figure 1. Cu^{2+} and Cu^{+} Enhance tBHQ-Induced ARE-Driven Gene Expression

(A and B) AREc32 cells were exposed to (A) $CuCl_2$ or (B) CuCl (10 to 200 μmol/l) in DMEM for 24 hr in the presence of 0.1% (v/v) DMSO, 5 μM tBHQ or 5 μM SFN. The luciferase activity of cells treated with DMSO (control) was arbitrarily set at 1.

(C) Induction of AKR1C, AKR1B10, and HO-1 by tBHQ is enhanced by $CuCl_2$. Cells were cultured in DMEM containing 5 μM tBHQ in the presence or absence of 50 μM $CuCl_2$ at 21% (v/v) O_2 for 24 hr. Whole cell extracts (30 μg of protein) were separated by 15% SDS-PAGE and the levels of AKR1C, AKR1B10, and HO-1 analyzed by western blotting. Immunoblotting for actin was used as the loading control. Results are representative of three separate experiments.

and catechol were only able to induce ARE-luciferase in the presence of $CuCl_2$. In contrast, after treatment with 20 μM 1,4-benzoquinone (1,4-BQ) for 30 min, luciferase activity was induced 16-fold 24 hr later. 1,3-Diphenol (resorcinol), which is unable to undergo oxidation to a quinone, was inactive both in the presence and in the absence of $CuCl_2$; 1,3-diphenol was inactive as an inducer in both murine Hepalclc7 cells (Prochaska et al., 1985) and in human HepG2 cells (Rushmore et al., 1991). These data indicate that the formation of quinones is the essential step in the activation of Nrf2 by 1,2- and 1,4-diphenols.

Surprisingly, Cu^{2+} further enhanced induction of ARE-luciferase activity by quinones (Figure 3 and Table 1). To characterize this further, the effect of metal chelators on the Cu^{2+}-dependent potentiation of induction by quinones of ARE-driven gene expression was examined. In contrast to the complete suppression of the inducer activity of hydroquinones by DFX and EDTA, the metal chelators only partially blocked the induction of luciferase activity by the cotreatment with tBQ and $CuCl_2$ (Figure 3), to yield levels of induction similar to those observed for tBQ alone. This finding suggests that the potentiation by Cu^{2+} of tBQ-induced luciferase activity could be due to the activity of

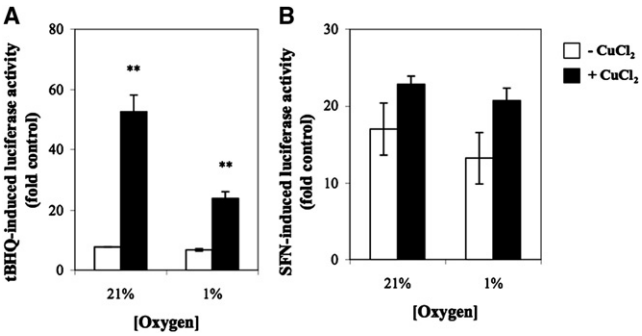


Figure 2. Hypoxia Inhibits the Potentiation by $CuCl_2$ of tBHQ-Induced Luciferase Activity

AREc32 cells were cultured in DMEM containing 5 μM tBHQ (A) or 2 μM SFN (B) in the presence or absence of 50 μM $CuCl_2$ at 21% (v/v) O_2 or 1% (v/v) O_2 for 24 hr. The luciferase activity of cells treated with 0.1% DMSO (control) and cultured at 21% (v/v) O_2 was set at 1.0. Values are mean ± SD. Results are representative of three separate experiments. $^{**}p < 0.001$.

NQO1, which can catalyze the reduction of a portion of the tBQ to yield tBHQ, thereby increasing the effective concentration of the hydroquinone. According to this hypothesis, the newly formed tBHQ will be susceptible to Cu^{2+}-dependent oxidation and thus capable of activating Nrf2. Indeed, high levels of NQO1 activity were detected in AREc32 cells (1004.8 ± 135 nmol/min/mg protein), consistent with the proposal that NQO1 catalyzes the obligatory 2 electron-reduction of tBQ to tBHQ (Talalay and Dinkova-Kostova, 2004). Attempts were made to reduce the NQO1 activity by using siRNA or the inhibitor dicumarol. Unfortunately, both of these strategies resulted in either cell death, or no significant inhibition of the enzyme activity at concentrations that were well-tolerated by the cells through mechanisms that are not understood.

We tested whether other transition metals could increase induction by tBHQ. Exposure of AREc32 cells to 50 μmol/l of $CoCl_2$, $NiSO_4$, or $FeCl_2$ for 24 hr enhanced induction of luciferase activity by tBHQ (10 μmol/l) from 20-fold to 35-, 37-, and 38-fold, respectively. This implied that, in addition to Cu^{2+}, other redox-active metals can also mediate the oxidation of tBHQ to tBQ. Of note, serum reduced the Cu^{2+}-mediated potentiation of the tBHQ-induced luciferase activity (see Figure S1A available online), possibly as a result of chelating or sequestering the Cu^{2+} by serum proteins, suggesting that such processes will affect the intracellular copper levels.

Cu^{2+} Is a Very Efficient Catalyst of the Oxidation of tBHQ

In Tris-HCl buffer (pH 8.0), tBHQ has a UV absorption maximum at 290 nm (a_m ~3000 M^{-1} cm^{-1}) (Figure 4A, black line). Addition of $CuCl_2$ caused a 38-nm blue shift, to 252 nm, and a hyperchromism (Figure 4A, red line), suggesting that Cu^{2+} catalyzed the oxidation of tBHQ to tBQ according to the reaction shown in the inset. Indeed, examination of the UV spectrum of authentic tBQ showed that it absorbs maximally at 252 nm with a molar absorptivity of ~20,000 M^{-1} cm^{-1} such that the spectra of tBHQ upon addition of $CuCl_2$ and the spectrum of tBQ were virtually identical (Figure 4A, red line and Figure 4B). We reasoned that, under aerobic conditions, the oxidation of tBHQ to tBQ should also occur when Cu^{+} is used in place of Cu^{2+}, since in the presence of O_2, Cu^{+} is easily oxidized to Cu^{2+}. Indeed, although the reaction initially proceeded at a slightly slower rate, Cu^{+} was also a very efficient catalyst (Figure S2). Bubbling N_2 reduced the rate of Cu^{+}-dependent formation of tBQ by 40% (Figure S3A), confirming that O_2 is required to accept electrons from Cu^{+} and generate Cu^{2+}, which in turn oxidizes tBHQ. As expected, bubbling N_2 had no effect on the initial rate of the Cu^{2+}-dependent formation of tBQ (Figure S3B).

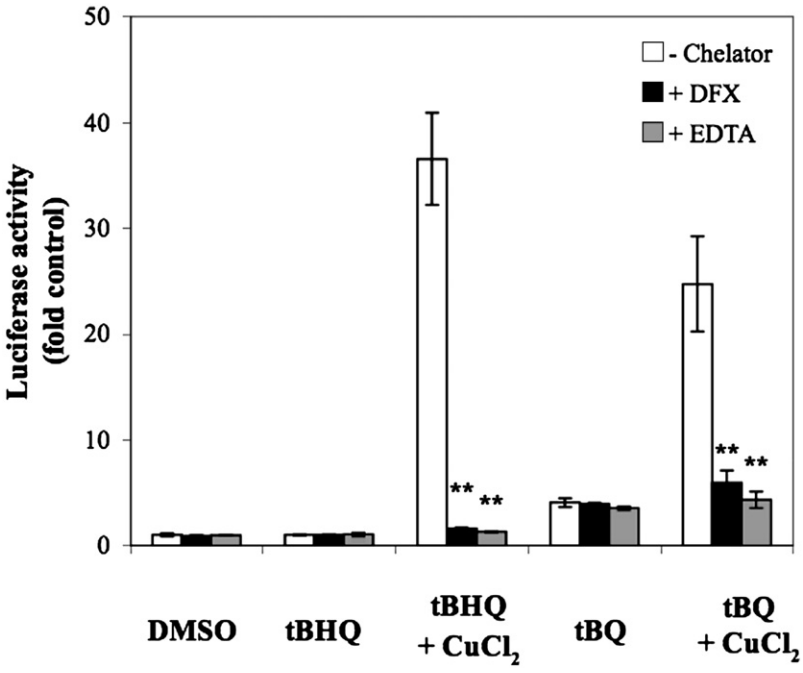
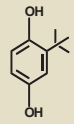
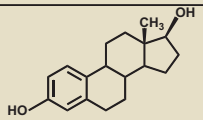
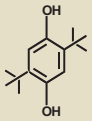
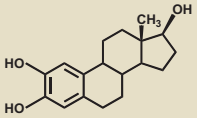
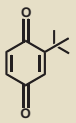
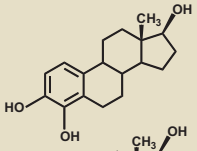
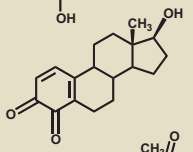
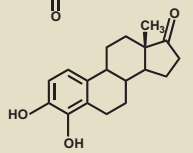
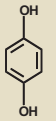
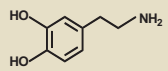
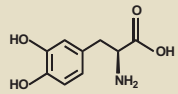


Figure 3. The Metal Chelators DFX and EDTA Have Distinct Effects on the Induction of Luciferase Activities by tBHQ and tBQ

AREc32 cells were exposed to 5 μM tBHQ or 2.5 μM tBQ in PBS with or without 50 μM $CuCl_2$ in the presence or absence of 100 μM DFX or 100 μM EDTA. After 30 min, the PBS was replaced with DMEM plus serum. The cells were further incubated for 24 hr before measuring luciferase activity. The luciferase activity of cells treated with 0.1% DMSO (control) was set to 1. Values are mean ± SD. Results are representative of three separate experiments. $^{**}p < 0.001$.

Table 1. The Effect of $CuCl_2$ on the Induction of Luciferase Activity by Diphenols

Phenolic compound	Con. (μmol/l)	Structure	ARE-Luciferase activity (fd to control)		Phenolic compound	Con. (μmol/l)	Structure	ARE-Luciferase activity (fd to control)	
			- $CuCl_2$	+ $CuCl_2$				- $CuCl_2$	+ $CuCl_2$
tBHQ	5		1.1 ± 0.1	32.6 ± 2.5 **	E2	10		1.3 ± 0.1	1.5 ± 0.1
dtBHQ	5		1.0 ± 0.1	1.3 ± 0.1	2-OH-E2	5		1.5 ± 0.1	6.3 ± 1.3 **
tBQ	2.5		4.1 ± 0.3 *	24.7 ± 4.5 *	4-OH-E2	5		1.2 ± 0.1	6.9 ± 1.5 *
Resorcinol	20		1.3 ± 0.1	1.5 ± 0.1	E_2-3,4-Q	25		5.8 ± 0.4**	12.5 ± 2.7 *
1,4-BQ	20		16 ± 3.8 **	33 ± 0.7 **	4-OH-E1	5		1.5 ± 0.1	6.3 ± 0.2 *
1,4-HQ	20		1.4 ± 0	39 ± 13 *	Dopamine	12.5		1.0 ± 0.02	5.5 ±1.9 *
Catechol	20		1.5 ± 0.2	11 ± 2.6 **	L-DOPA	50		1.0 ± 0.07	2.3 ± 0.6*

AREc32 cells were incubated for 30 min in PBS containing each test compound in the presence or absence of 50 μM $CuCl_2$. Thereafter, the PBS was removed and replaced with DMEM plus serum. The cells were further incubated for 24 hr before luciferase activity was measured. The concentration of tBHQ, dtBHQ, tBQ, 2-OHE2, 4-OHE2, and 4-OH-E1 in PBS was 5 μmol/l; the concentration of E2 was 10 μmol/l; the concentration of E_2-3,4-Q was 2.5 μmol/l; and the concentration of all of the other compounds was 20 μmol/l. The value of luciferase activity of cells treated with vehicle (control) was set at 1. Values are mean ± SD. *$p > 0.05$; **$p > 0.005$.

The possibility that other (than O_2) endogenous electron acceptors from Cu^+ could be important for this reaction was next considered. Perhaps the most relevant one in vivo is H_2O_2, as it can efficiently generate Cu^{2+} from Cu^+ according to the reaction: $Cu^+ + H_2O_2 \rightarrow Cu^{2+} + OH^{\bullet} + OH^-$ (Halliwell and Gutteridge, 2007). Adding H_2O_2 to the mixture of tBHQ and Cu^+ under N_2 accelerated the reaction even further than when the same reaction was carried out at ambient conditions (Figure S4), confirming that H_2O_2, in addition to O_2, constitutes another physiologically relevant electron acceptor contributing to the copper-catalyzed oxidation of hydroquinones.

We also asked whether other divalent cations could catalyze the oxidation of tBHQ to tBQ and found that Mn^{2+} was also effective (Figure S5). In contrast, Fe^{2+} was only weakly active, and Zn^{2+} was essentially inactive. Thus, it appears that both in vitro and in cell culture, copper is the most efficient catalyst of this reaction, but other transition metals are also capable of mediating the oxidation of tBHQ.

tBQ but Not tBHQ Reacts with Cys Residues in Glutathione and Keap1

We next examined the ability of tBHQ and tBQ to react with sulfhydryl groups. When GSH was added to the solution containing tBHQ and $CuCl_2$, a time-dependent 50-nm red shift, to 303 nm, and a hypochromism were noted, presumably reflecting the formation of a thioether bond between the quinone and the cysteine residue of glutathione (Figure 4A, purple and brown lines); this observation was confirmed in a reaction of tBQ with GSH (Figure 4B, blue line). Titration of small aliquots of substoichiometric quantities of GSH into a solution containing a constant amount of tBQ resulted in incremental increases in the absorbance at 303 nm, proportional to each aliquot that was added (Figure 4C). Once the concentration of GSH was equal to the concentration of tBQ, there was no further change, indicating a reaction stoichiometry of 1:1. In contrast to the reaction of tBQ with GSH, no spectral changes were observed when tBHQ was used in place of tBQ, showing that tBHQ itself

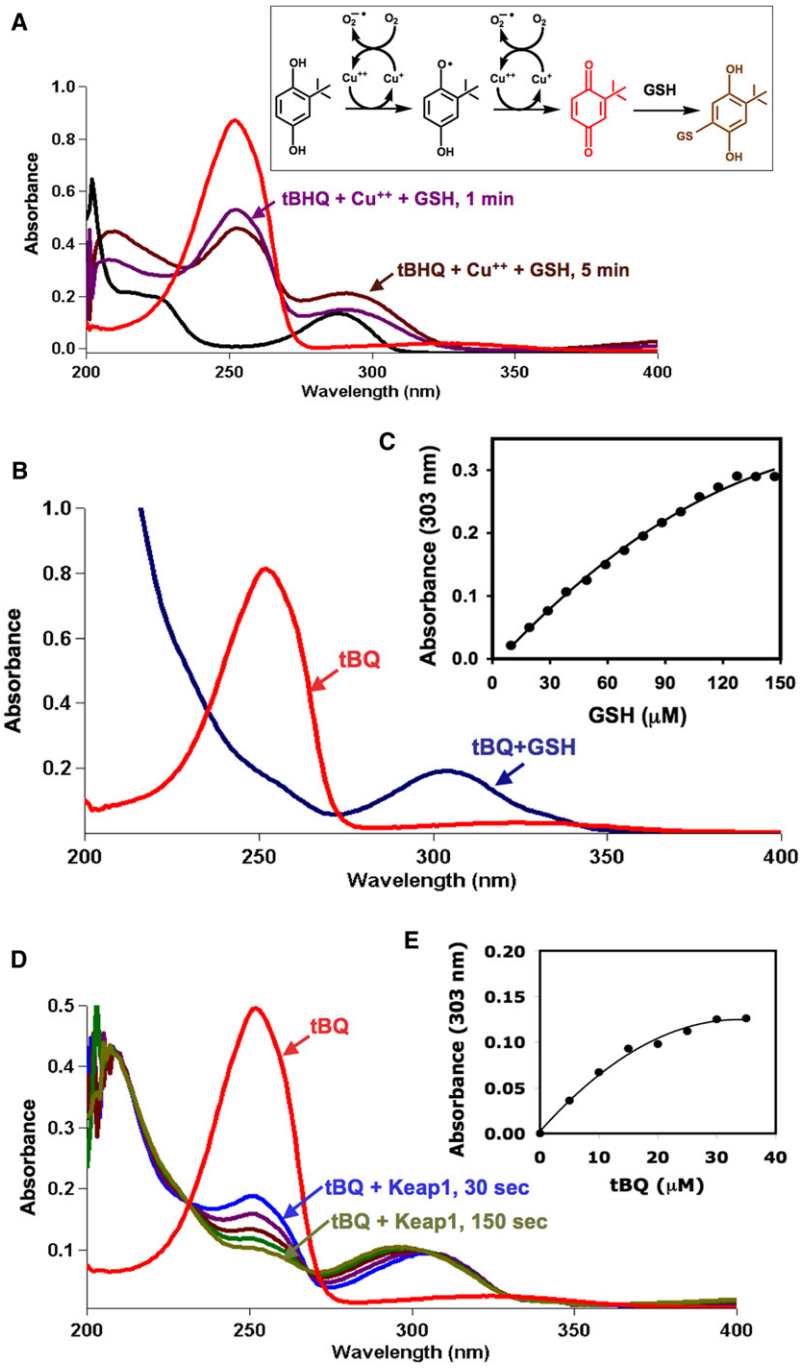


Figure 4. tBQ but Not tBHQ Reacts with Glutathione and Cysteine Residues in Keap1

(A) Absorption spectra in 20 mM Tris-HCl/0.005% Tween 20 (pH 8.0) at 25°C of 100 μM tBHQ before (black line) and after (red line) addition of 100 μM $CuCl_2$, and following incubation with 100 μM GSH for 1 min (purple line) and 5 min (brown line).

(B) Absorption spectra of 100 μM tBQ before (red line) and after 5 min incubation with 200 μM GSH (blue line) in the same buffer.

(C) Titration of 2.0-μl aliquots each delivering 5 nmol of GSH into a solution of 100 μM tBQ in a final volume of 500 μl under the same reaction conditions.

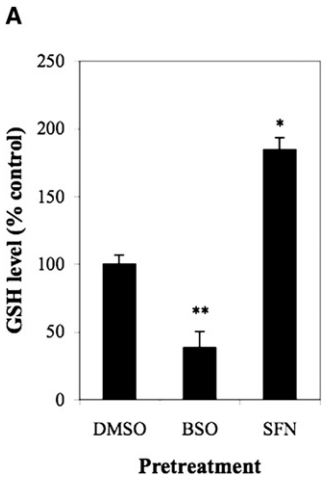
(D) Absorption spectrum kinetics of the reaction of 50 μM tBQ with 5 μM Keap1. Spectra were recorded every 30 s.

(E) Titration of 0.5-μl aliquots each delivering 2.5 nmol of tBQ into a solution of 5 μM Keap1 in a final volume of 500 μl.

does not react with GSH (Figure S6). Overnight incubation of tBQ with GSH resulted in the formation of two mono- and one bis-substituted glutathione conjugates, in agreement with a previous report (van Ommen et al., 1992). The conjugates were separated by HPLC and analyzed by mass spectrometry, revealing two identical molecular ions at m/z 470 for the mono-thioethers, and a molecular ion at m/z 775 for the bis-thioether (Figure S7).

The reaction of Keap1 with inducers of ARE-driven gene expression [e.g., SFN, the Michael acceptors bis(2-hydroxybenzylidene) acetone, and the oleanane dicyanotriterpenoid 2-cyano-3, 12-dioxooleana-1,9(11)-dien-28-onitrile] leads to changes in the spectra of these molecules that are very similar to the changes observed when they react with sulfhydryl groups in GSH and DTT (Dinkova-Kostova et al., 2002, 2005). In the present study, we also observed similar time-dependent spectral changes when tBQ was allowed to react with recombinant murine Keap1 (Figure 4D). Titration experiments revealed that the stoichiometry of the reaction of Keap1 with tBQ is 1:4, indicating that four cysteine residues were modified by the quinone under these reaction conditions (Figure 4E). Taken together, our experiments demonstrate that Cu^{2+} catalyzes the oxidation of tBHQ to tBQ, and it is the resultant quinone that subsequently reacts with cysteine residues in Keap1.

ROS are generated during Cu^{2+}-mediated redox-cycling of tBHQ (Li et al., 2002) and the closely related 2,5-di-*tert*-butylhydroquinone (dtBHQ) (Nakamura et al., 2003). dtBHQ can undergo a similar one-electron oxidation as tBHQ, including conversion to its benzoquinone form, and Cu^{2+}-dependent superoxide generation, but unlike tBQ, it has been reported not to react with GSH (Nakamura et al., 2003). As described above, the addition of GSH to a solution containing $CuCl_2$ and tBHQ leads to a rapid decrease in absorbance at 252 nm, the λ_{max} for tBQ, and an increase in absorbance at 303 nm, reflecting the formation of quinone-glutathione conjugate (Figure 4A and Figure S8A). In sharp contrast, the corresponding decrease in absorbance was much less pronounced when dtBHQ was used in place of tBHQ (Figure S8B), indicating that, under these reaction conditions, formation of a glutathione conjugate with dtBHQ is inefficient, most likely due to steric hindrance of the second *tert*-butyl group. These findings are consistent with the reported chemoprotective activity of tBHQ against benzo[*a*]pyrene-induced neoplasia of the forestomach of ICR/Ha mice,



B

Pretreatment	30 min treatment in the presence of $CuCl_2$ (50 μM)		
	tBHQ (5 μM)	4OHE2 (5 μM)	Dopamine (50 μM)
DMSO	25.9 ± 0.5	5.5 ± 0.8	10.5 ± 2.5
BSO (20 μM)	70 ± 7.6**	15.4 ± 3.3*	18.0 ± 0.4*
SFN (2 μM)	9.0 ± 0.4**	2.8 ± 0.2*	5.4 ± 2.1*

Figure 5. Cellular GSH Levels Influence the Cu^{2+}-Potentiated Activation of Nrf2 by Oxidizable Diphenols

(A) GSH levels after 24 hr pretreatment with 20 μM BSO or 2 μM SFN. Cells were incubated with 20 μM BSO or 2 μM SFN in DMEM plus serum for 24 hr. After this pretreatment period, cells were exposed to PBS containing 5 μM tBHQ and 50 μM $CuCl_2$ for 30 min. The PBS was then removed and the cells were further incubated in DMEM plus serum for 24 hr before luciferase activity was measured. The GSH level of the cells pretreated with 0.1% DMSO (control) was set to 100%. (B) Cellular glutathione levels affect the potentiation by $CuCl_2$ of tBHQ-, 4OHE2-, and dopamine induced luciferase activity. AREc32 cells were incubated with 20 μM BSO or 2 μM SFN in DMEM plus serum for 24 hr. After this pretreatment period, cells were exposed to PBS containing 5 μM tBHQ, 5 μM 4OHE2, or 50 μM dopamine in the presence of 50 μM $CuCl_2$ for 30 min. The PBS was then removed and the cells were further incubated in DMEM plus serum for 24 hr before luciferase activity was measured. The value from the cells pretreated and treated with 0.1% DMSO (control) was set to 1.0. Values are mean ± SD. Results are representative of three separate experiments. *$p < 0.05$; **$p < 0.005$.

and the lack of protection by dtBHQ in the same model (Wattenberg et al., 1980).

We then evaluated the relative contributions of ROS and the electrophilic quinone reacting with intracellular thiols in the induction of ARE-dependent gene expression by tBHQ in the presence of $CuCl_2$. Under our culture conditions, where the cells were treated with 5 μmol/l or 10 μmol/l of either tBHQ or dtBHQ in the presence of $CuCl_2$, ROS were not detectable using the fluorescent probe 2′,7-dichlorodihydrofluorescein diacetate, though they were detected when the cells were treated with 10-fold higher concentrations of tBHQ or dtBHQ (data not shown). Furthermore, only tBHQ was able to induce luciferase activity in the presence of $CuCl_2$, whereas dtBHQ was essentially inactive as an inducing agent in the absence of copper and only very weakly active in the presence of copper (Table 1). Taken together, our results strongly suggest that the major species that stimulates Nrf2-mediated gene induction is the electrophilic quinone, and that ROS that are formed during the redox cycling play a much smaller role in the induction process.

Cellular GSH Levels Influence the Cu^{2+}-Potentiated Activation of Nrf2

As demonstrated above, the quinone forms of oxidizable diphenols react stoichiometrically with GSH in vitro. To examine the significance of this reaction in cells, we evaluated the effect that manipulating the intracellular GSH level had on the ability of tBHQ to induce ARE-driven genes. Thus, we first pretreated the cells with BSO to deplete GSH. As shown in Figure 5A, exposure to 20 μM BSO for 24 hr reduced the intracellular GSH level by 50%. This decrease in GSH markedly increased the induction of luciferase activity by treatment with 5 μM tBHQ for 30 min in the presence of 50 μM $CuCl_2$ from 26-fold to 70-fold. On the other hand, a 200% increase in the level of intracellular GSH (following 24 h-exposure to 2 μM SFN) decreased the induction of luciferase activity by 5 μM tBHQ in the presence of 50 μM $CuCl_2$, from 26-fold to 9-fold (Figure 5B). Although these data could also be explained by competition of sulforaphane and tBQ for binding to Keap1, we think that this interpretation is unlikely, on the basis of the known instability of Keap1-sulforaphane complexes and the rapid metabolism and excretion of sulforaphane (Ye et al., 2002; Zhang and Callaway, 2002), the administration of which preceded exposure to tBHQ and $CuCl_2$ by 24 hr. These data suggest that the intracellular thiol concentration influences the ability of tBHQ to activate the Keap1/Nrf2/ARE pathway, providing further support for the hypothesis that the thiol-reactive quinone metabolite represents the actual proximal inducing agent.

Induction of ARE-Reporter Luciferase Activity by Catechol Estrogens and Catecholamines Requires Oxidation to Their Quinone Derivatives

Cu^{2+} mediates the oxidation of catechol estrogens (Li et al., 1994; Thibodeau and Paquette, 1999). Lee et al. (2003) reported that 2-OHE2 and 4-OHE2 induced ARE-driven gene expression. On the basis of our findings, we predicted that Cu^{2+} will potentiate the activation of Nrf2 by catechol estrogens. To test this hypothesis, AREc32 cells were treated with 2-OHE2, 4-OH-E1, and 4-OHE2 in PBS for 30 min. As expected, 2-OHE2, 4-OHE2, and 4-OH-E1 were unable to induce ARE-luciferase activity in the absence of $CuCl_2$ (Table 1). However, in the presence of 50 μM $CuCl_2$, each of these catechol estrogens induced the luciferase activity markedly, and at a concentration of 5 μmol/l, they induced luciferase activity >6-fold. To determine whether the quinone formed from catechol estrogens was the

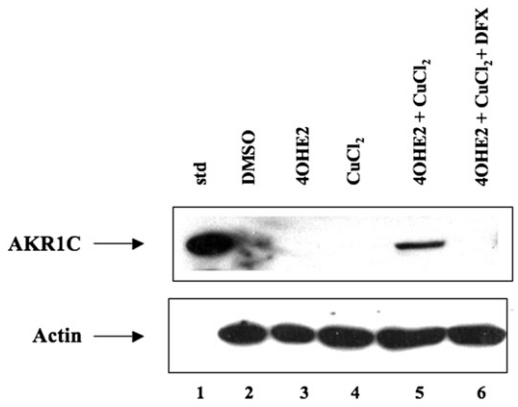


Figure 6. $CuCl_2$ Is Required for the Induction of AKR1C by 4OHE2

Cells were exposed to PBS containing DMSO, 5 μM 4OHE2, 50 μM $CuCl_2$, or 5 μM 4OHE2 and 50 μM $CuCl_2$ in the presence or absence of 100 μM DFX for 30 min. The PBS was then removed and the cells were further incubated in DMEM plus serum for 24 hr. Whole cell extracts (30 μg of protein) were separated by 15% SDS-PAGE and the levels of AKR1C analyzed by western blotting. Immunoblotting for actin was used as the loading control. Results are representative of two separate experiments.

active species, we obtained E_2-3,4-Q by oxidation of 4-OHE2. E_2-3,4-Q induced ARE-luciferase in the absence of $CuCl_2$ by ~6-fold (Table 1). Immunoblotting revealed that 4-OHE2 induced endogenous AKR1C expression only in the presence of $CuCl_2$. However, induction of AKR1C by this steroid was completely abolished by DFX (Figure 6). Our results demonstrate that, like the synthetic diphenols, endogenous catechol estrogens are not inducers, but oxidation to their quinone derivatives is the requisite step in the activation of Nrf2.

High concentrations of dopamine activate Nrf2 in astrocytes and meningeal cells (Shih et al., 2007) and in PC12 dopaminergic neuronal cells (Jia et al., 2008). Because dopamine and its precursor L-DOPA, possess two hydroxyl groups on the benzene ring and can undergo oxidation to become catechol quinones, we postulated that Cu^{2+} might increase the activation of Nrf2 by dopamine and L-DOPA. We exposed AREc32 cells to dopamine or L-DOPA for 30 min and found that neither of the compounds had any effect on the luciferase activity in the absence of Cu^{2+}. However, in the presence of 50 μmol/l $CuCl_2$, 30 min treatment with 12.5 μmol/l dopamine resulted in 6-fold increase in the luciferase activity (Table 1), and 30 min treatment with 50 μmol/l L-DOPA produced a 2-fold increase in the luciferase activity (Table 1). In addition, pretreatment of AREc32 cells with BSO that decreased the intracellular glutathione by 50% nearly doubled the induction of luciferase activity by dopamine and $CuCl_2$ (Figure 5B); in contrast, an ~2-fold increase in the cellular glutathione content, achieved by pretreatment with SFN, diminished the induction by nearly 50% (Figure 5B). Our data suggest that oxidation of dopamine and L-DOPA is the key step in determining their ability to activate Nrf2-regulated neuroprotective pathways.

DISCUSSION

This study establishes the chemical and molecular mechanisms whereby hydroquinone-type compounds activate the Keap1/Nrf2/ARE signaling pathway. Under aerobic conditions, Cu^{2+} catalyzes the oxidation of *ortho*- and *para*-hydroquinones to their corresponding quinones, which, in turn, react with cysteine residues in Keap1, resulting in derepression of Nrf2. This study also highlights the importance of transition metals and oxygen in the activation of the Keap1/Nrf2/ARE pathway by oxidizable phenolic compounds that are ubiquitous in our environment.

We used tBHQ as a model compound to demonstrate that its oxidation to tBQ is the rate-limiting step that enables the hydroquinone to activate the Keap1/Nrf2/ARE pathway. Furthermore, we have shown that induction of ARE-driven gene expression by a series of compounds that contain *para*- or *ortho*-hydroquinone moieties is similarly potentiated by Cu^{2+}. Oxidizable diphenols are a ubiquitous class of compounds, to which we are exposed continuously. Many are metabolic products of dietary plant constituents or of environmental pollutants such as polycyclic aromatic hydrocarbons (Jin and Penning, 2007; Palackal et al., 2002; Park et al., 2008). Some are produced during metabolism of endogenous precursors such as catechol estrogens and dopamine. The ability of Cu^{2+} and other transition metals to potentiate the activation of the Keap1/Nrf2/ARE pathway implies that any alteration in the homeostasis of copper or other transition metals will have a significant impact on the biological effects of hydroquinones and quinones in vivo.

We have demonstrated that the capacity of hydroquinones to activate the Keap1/Nrf2/ARE pathway is closely correlated with the efficiency of their conversion to eletrophilic metabolites and their potencies as inducers are dependent on the concentration of Cu^{2+} and oxygen. As the copper content in some tissues exceeds 100 μmol/l (Linder and Hazegh-Azam, 1996), and the doses used in the present study are within physiological levels, it seems likely that a significant fraction of the hydroquinones will be converted to their respective quinones, especially in tissues, which are normally exposed to high levels of oxygen. Recent studies have shown that *para*-hydroquinones, such as tBHQ and the *ortho*-hydroquinone carnosic acid, protect neuronal cells against oxidative stress in a manner associated with activation of ARE-driven gene expression (Satoh et al., 2006, 2008, 2009). Our findings indicate that the oxidation of hydroquinones is a prerequisite for their protective effects.

It should be noted, however, that the overproduction of quinones has detrimental effects, including acute cytotoxicity, immunotoxicity, and carcinogenicity, and our findings imply that high levels of oxygen and transition metals are important determinants. Thus, aldo-keto reductase catalyzed formation of the electrophilic and redox-active *ortho*-quinones 7,12-dimethylbenz[*a*]anthracene-3,4-dione and benzo[*a*]pyrene-7,8-dione has been shown to occur in human lung adenocarcinoma cells exposed to the polycyclic aromatic hydrocarbons 7,12-dimethylbenz[*a*]anthracene and benzo[*a*]pyrene, causing reactive oxygen species generation, oxidative DNA damage, and DNA strand breaks (Jin and Penning, 2007; Palackal et al., 2002; Park et al., 2008). Similarly, the *ortho*-quinone products of dopamine have been implicated in the cytotoxicity of this neurotransmitter in dopaminergic neuronal cells (Asanuma et al., 2004; Miyazaki and Asanuma, 2009). Patients with Wilson's disease (carrying a genetic mutation in the copper transporter that results in copper accumulation) have high frequency of Parkinsonism (Taly et al., 2007). In addition, the fact that iron and copper levels

are relatively high in the alveolar epithelial cells in the breast (Vorherr, 1974) may partly explain why 4OHE2 plays a role in the etiology of breast cancer (Cavalieri et al., 2000). Copper metabolism is altered in tumor-bearing mice and rats and also in humans (Apelgot et al., 1986; Semczuk and Pomykalski, 1973; Tani and Kokkola, 1972). Additionally, serum and tumor copper levels are significantly elevated in patients with cancer, compared with healthy subjects (Kuo et al., 2002), and positively correlate with tumor incidence, burden, malignant progression, and recurrence in many human and animal cancers. On the basis of our findings, we hypothesize that elevated endogenous copper levels will result in enhanced activation of the Keap1/Nrf2/ARE pathway by oxidizable diphenols from both exogenous and endogenous origin in an effort to protect against potential deleterious effects of quinones. A prominent example is the up-regulation of NQO1, a prototypical Nrf2-regulated enzyme that catalyzes the obligatory 2-electron reduction of *ortho*- and *para*-quinones, thereby diverting them from participating in redox cycling and sulfhydryl depletion reactions. Notably, although under our experimental conditions copper is most efficient in the hydroquinone to quinone conversion, we do not think that our findings are restricted to diseases associated with increased copper concentration, but will also be valid for other pathologic abnormalities linked to excessive exposures to metals from contaminated water or food.

In summary, the ability of hydroquinones to undergo redox cycling and to form quinones provides the basis for their biological activity—either to activate the Nrf2 cytoprotective pathway or, at high concentrations, to create oxidative stress and damage macromolecules, ultimately causing toxicity and/or carcinogenicity. In this study, we demonstrate an alternative, nonenzymatic, oxygen- and copper-dependent pathway for the activation of the Keap1/Nrf2/ARE pathway by hydroquinones. This knowledge is essential for designing novel chemopreventive strategies and for developing new approaches against drug resistance.

SIGNIFICANCE

***Para*- and *ortho*-hydroquinones represent a ubiquitous class of oxidizable diphenols. Among them are metabolites of exogenous dietary constituents and environmental pollutants, as well as endogenous catechol estrogens and dopamine. Paradoxically, both cytoprotective and cytotoxic properties have been attributed to such molecules. At low concentrations, they activate transcription factor Nrf2 that controls the expression of a battery of >100 cytoprotective proteins, whereas at high concentrations, they lead to acute cytotoxicity and carcinogenicity. In this study, we demonstrate that a large series of oxidizable diphenols are able to activate Nrf2-dependent gene expression only in the presence, but not in the absence, of transition metals and oxygen. Under aerobic conditions, Cu^{2+} catalyzes the oxidation of *ortho*- and *para*-hydroquinones to their corresponding quinones, which subsequently react with cysteine residues of the protein sensor for inducers Keap1, resulting in activation of Nrf2. Furthermore, oxidation to quinones is both necessary and sufficient for activation of the Keap1/Nrf2 pathway by *ortho*- and *para*-hydroquinones. To our knowledge, this is the first experimental evidence that oxidizable hydroquinones are not Nrf2 activators themselves, but their corresponding quinones are the ultimate inducers. Our data also imply that hydroquinones will not stimulate Nrf2-mediated gene induction in the absence of transition metals and oxygen. These findings are of particular importance for certain human conditions in which the levels of transition metals are increased, such as Wilson's disease (genetic mutation in the copper transporter resulting in copper accumulation) or patients with cancer (who have increased plasma and tumor copper levels), and especially in tissues that are exposed to relatively high concentrations of oxygen, such as the lung, the brain, and the alveolar epithelium of the breast.**

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

All chemicals were of analytical grade and were from Sigma-Aldrich (Dorset, UK.). E_2-3,4-Q was prepared as described elsewhere (Zahid et al., 2006). The media supplements for cell culture were from Life Technologies. The antisera against AKR1C and AKR1B10 have been described elsewhere (MacLeod et al., 2009; O'Connor et al., 1999). The antibody against HO-1 was from BIOMOL International.

Cell Culture and Evaluation of ARE-Driven Luciferase Activity

The stable human mammary ARE-reporter cell line, AREc32 (Wang et al., 2006), was maintained in growth medium (Dulbecco's MEM with glutamax [DMEM] supplemented with 10% fetal bovine serum [FBS] and penicillin-streptomycin) containing 0.8 mg/ml G418, at 37°C, in 95% air and 5% CO_2.

4-OHE1, 4-OHE2, and tBQ were dissolved in acetonitrile. L-buthionine-*S*,*R*-sulfoximine (BSO), $CuCl_2$, and CuCl were prepared in H_2O. Dopamine and 3,4-dihydroxy-L-phenylalanine (L-DOPA) were freshly prepared in ethanol and 0.5 N HCl, respectively. All other chemicals were dissolved in DMSO. For xenobiotic treatments, AREc32 cells were seeded in 96-well plates at a density of 1.2×10^4 cells/well and were allowed 24 hr to recover. Typically, cells were treated with the xenobiotic of interest for further 24 hr in DMEM. However, on occasions, treatments were for 30 min in PBS. For treatments in medium, cells were left for 24 hr in the presence of the xenobiotic before being harvested, and the firefly luciferase activity in cell lysates was measured using a luminometer (Turner Designs Model TD-20/20, Promega) following addition of Luciferase Assay Reagent (Promega). For short-term treatment in PBS, the culture medium was replaced with PBS containing xenobiotics (in 0.1% vehicle by vol.). After 30 min, PBS was replaced with growth medium, and the cells were further incubated for 24 hr. In experiments involving metal chelators, each chelator was mixed with $CuCl_2$ prior to being mixed with tBHQ or tBQ.

To culture cells under low O_2 conditions, a hypoxia workstation (Biotrace Fred Baker) gassed with 1% (v/v) O_2, and 5% (v/v) CO_2 balanced with ~94% N_2 was used. Cells were seeded at 1.2×10^4 cells per well in growth medium in 96-well plates and first incubated for 24 hr at 95% air and 5% CO_2. Cells and culture medium were equilibrated in the hypoxic workstation for 2 hr prior to induction for further 24 hr.

Purification of Keap1 and Binding of Quinones

Recombinant Keap1 was expressed and purified as described elsewhere (Dinkova-Kostova et al., 2002). Binding studies were performed at 25°C by adding a 5-μl aliquot of a solution containing the relevant compound (dissolved in acetonitrile) to a 500 μl solution of 5 μM Keap1 in 20 mM Tris·HCl/0.005% (v/v) Tween 20 (pH 8.0) in 0.5-cm pathlength quartz cuvettes. Immediately after mixing, the UV-VIS spectra were recorded by using a double-beam spectrophotometer. To determine the reaction stoichiometry, tBQ was titrated in 0.5-μl aliquots delivering 2.5 nmol of tBQ per aliqout into 5 μM Keap1 in the same buffer in a final 500-μl reaction volume. The increase in absorbance at 303 nm was monitored after addition of each aliquot. Similar experiments were performed with reduced glutathione in place of Keap1.

Determination of GSH

Reduced glutathione was measured as described elsewhere (Kamencic et al., 2000). Cells were seeded at 0.5 × 10^5 cells per well in 24-well plates. After overnight incubation, cells were treated with 20 μM BSO or 2 μM SFN for 24 hr and then were lysed. Cell lysates (100 μl) were incubated with 100 μl PBS containing 80 μM monochlorobimane (mCB) and 1 U/ml glutathione *S*-transferase for 1 hr at 25°C. Formation of the GS-mCB adduct was quantified by its fluorescence with excitation at 390 nm and emission at 490 nm.

Western Blot Analysis

Whole-cell extracts were prepared as described elsewhere (Wang et al., 2006). Briefly, cells were lysed in 0.1 M HEPES (pH 7.4), containing 0.5 M KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, and 20% glycerol that was supplemented with protease inhibitors (Roche Diagnostics). Proteins (30 μg) were separated by SDS-PAGE. Immunoblotting was performed as described elsewhere (O'Connor et al., 1999).

Statistical Analysis

Statistical comparisons were performed by the unpaired Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures and can be found with this article online at doi:10.1016/j.chembiol.2009.12.013.

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