Induction of metallothionein I by phenolic antioxidants requires metal-activated transcription factor 1 (MTF-1) and zinc

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Phenolic antioxidants, such as tBHQ [2,5-di-(t-butyl)-1,4-hydroquinone], induce *Mt1* (metallothionein 1) gene expression and accumulation of MT protein. Induction of *Mt1* mRNA does not depend on protein synthesis, and correlates with oxidation–reduction functions of the antioxidants. In the present study, we analysed the biochemical pathway of the induction. Induction depends on the presence of MTF-1 (metal-activated transcription factor 1), a transcription factor that is required for metal-induced transcription of *Mt1*, but does not require nuclear factor erythroid 2-related factor 2, a tBHQ-activated CNC bZip (cap 'n' collar basic leucine zipper) protein, that is responsible for regulating genes encoding phase II drug-metabolizing enzymes. Moreover, tBHQ induces the expression of *MRE-βGeo*, a reporter gene driven by five metal response elements that constitute an optimal MTF-1 binding site. Reconstitution of *Mtf1*-null cells with MTF-1 restores induction by both zinc and tBHQ. Unlike activation of phase II genes by tBHQ, induction of *Mt1* expression does not occur in the presence of EDTA, when cells are cultured in zinc-depleted medium, or in cells with reduced intracellular 'free' zinc due to overexpression of ZnT1, a zinc-efflux transporter, indicating that induction requires zinc. In addition, fluorescence imaging reveals that tBHQ increases cytoplasmic free zinc concentration by mobilizing intracellular zinc pools. These findings establish that phenolic antioxidants activate *Mt1* transcription by a zinc-dependent mechanism, which involves MTF-1 binding to metal regulator elements in the *Mt1* gene promoter.

Key words: antioxidants, 2,5-di-(t-butyl)-1,4-hydroquinone (tBHQ), FluoZin-3AM, metal-activated transcription factor 1 (MTF-1), metallothionein, nuclear factor erythroid 2-related factor 2 (Nrf2).

INTRODUCTION

Metallothioneins (MTs) are a group of small, cysteine-rich, metal-binding proteins that are expressed in all eukaryotes and some prokaryotes [1,2]. The cysteine residues of MTs co-ordinate multiple zinc and copper atoms under physiological conditions, but they can also bind toxic metals such as cadmium [3,4]. There are four *Mt* genes in the mouse, two of which, *Mt1* and *Mt2*, are expressed in most cell types [5]. These two genes are transcriptionally regulated by a wide variety of compounds including heavy metals (Zn, Cd, Co, Ni, Ag, Hg and Bi), glucocorticoids [e.g. Dex (dexamethasone)], some alkylating agents (e.g. iodoacetate), oxidants/antioxidants {e.g. H_2O_2, tBHQ [2,5-di-(t-butyl)-1,4-hydroquinone]} and inflammatory signals (lipopolysaccharide) [6–10]. Induction by any of these compounds (except Cu and Cd) results in the accumulation of zinc bound to the newly synthesized apo-MT. The functions of MT are uncertain, but they can detoxify heavy metals, provide a reserve of zinc, and protect against oxidative stress [1,2,9,11,12].

Induction of the *Mt* genes by metals requires multiple MRE (metal response element) sequences located in the promoter region and the zinc-finger transcription factor MTF-1 (metal-activated transcription factor 1) [13–16]. Treatment of cells with metals results in the translocation of MTF-1 to the nucleus and binding to MREs [17]. The mechanism by which MTF-1 is activated by heavy metals is not resolved. Direct binding of zinc to regulatory sites on MTF-1, activation of signal transduction pathways leading to covalent modification of MTF-1 and release of MTF-1 from regulatory molecules have all been proposed [15,16,18–21]. The induction of *Mt1* and *Mt2* by glucocorticoids is mediated by the glucocorticoid receptor binding to a pair of glucocorticoid-response element sequences upstream of the *Mt2* gene [22]. The mechanisms by which other inducers activate *Mt* gene transcription are less well understood. The *Mt1* gene contains an ARE (antioxidant response element) sequence similar to those found in rat phase II genes *Nqo1* [NAD(P)H:quinone oxidoreductase] and *Gsta1* (glutathione *S*-transferase A1). The ARE in the *Mt1* gene promoter overlaps with an upstream stimulatory factor-binding site; multimers of the ARE/upstream stimulatory factor site, upstream of a reporter gene with a minimal promoter, allow weak induction by H_2O_2 in hepa cells; a similar result was obtained with multiple copies of MRE sequences, suggesting that at least two different transcription factors are capable of mediating induction by H_2O_2 [9].

Phenolic antioxidants exhibit multiple protective functions in animals including anti-cancer, anti-diabetic and anti-inflammatory effects [23]. The antioxidants induce a number of genes encoding phase II drug-metabolizing enzymes NQO1 and glutathione S-transferase A1 [24], metal-binding proteins MT-I and MT-II [9] and haem oxygenase-1 (Y. Bi and Q. Ma, unpublished work). In addition, phenolic antioxidants inhibit induction of inflammatory cytokines (i.e. tumour necrosis factor α, interleukin

1β and interleukin 6) in macrophage cells [23,25]. Regulation of gene transcription by phenolic antioxidants is best studied for induction of phase II enzymes. Induction is mediated through an ARE located in the enhancers of the genes and Nrf2 (nuclear factor erythroid 2-related factor 2), a CNC bZip (cap 'n' collar basic leucine zipper) protein [24,26]. Both the MRE and ARE have been suggested to contribute to induction of *Mt1* by tBHQ [27,28]. In the present study, we use genetic and biochemical approaches to show that induction of *Mt1* by tBHQ requires MTF-1 and zinc, but not Nrf2.

MATERIALS AND METHODS

Materials

Restriction endonucleases and other molecular biology reagents were purchased from New England Biolabs (Beverly, MA, U.S.A.), Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.), Invitrogen (Carlsbad, CA, U.S.A.) and Promega (Madison, WI, U.S.A.). DMSO, tBHQ, resorcinol (Res), pBQ (*p*-benzoquinone), BHA (3-t-butyl-4-hydroxyanisole), BNF (β-naphthoflavone), ANF (α-naphthoflavone), cadmium chloride, zinc chloride, Dex and iodoacetic acid were purchased from Sigma (St. Louis, MO, U.S.A.). TCDD (2,3,7,8-tetrachloro-dibenzo-*p*-dioxin) was from AccuStandard (New Haven, CT, U.S.A.). Sulphoraphane was kindly provided by Dr P. Talalay (The Johns Hopkins University, Baltimore, MD, U.S.A.). Cell culture materials were from Invitrogen and Hyclone (Logan, UT, U.S.A.).

Cell culture and treatments

Mouse hepa1c1c7 cells were provided by Dr J. P. Whitlock Jr (Stanford University, Stanford, CA, U.S.A.). The cells were grown as a monolayer in α-minimal essential medium, containing 10 % (v/v) FBS (foetal bovine serum) and 5 % CO_2. Cells were treated with chemicals as described in the Figure legends. In a typical induction experiment, mRNA was measured after 5 or 9 h of treatment as specified in the Figure legends. DMSO was used as a solvent control for TCDD, tBHQ, BNF and ANF. Mouse hepa1A cells were provided by Grechen Darlington (Baylor College of Medicine, Houston, TX, U.S.A.) and were grown in DMEM (Dulbecco's modified Eagle's medium) plus 10 % FBS in 5 % CO_2. Mouse *Nrf2+/+* and *Nrf2−/−* embryonic fibroblast cells were derived from wild-type and *Nrf2*-null mice as described in [29]. The cells were cultured in DMEM with 10 % FBS and 5 % CO_2. Mouse *Mtf1+/+* and *Mtf1−/−* (dko7) cells were provided by Dr W. Schaffner (Universitat Zurich, Zurich, Switzerland) [30]. The cells were cultured as described elsewhere [30]. BHK (baby hamster kidney) cell clones 3038 that were stably transfected with MRE-βGeo, and Zn^r-3038 that over-expresses zinc efflux transporter protein ZnT1 and are resistant to zinc toxicity, were cultured in DMEM with 10 % FBS as described before [16,20]. *Mtf1−/−* cells (dko7) were transfected with plasmids allowing expression of either full-length MTF-1 driven by the CMV (cytomegalovirus) promoter (CMV-MTF-1) or DNA-binding domain of MTF-1 (zinc fingers 1–5) fused to the trans-activator domain of VP$_{16}$ driven by the promoter from the mouse polymerase II gene (Pol II-ZF$_5$VP$_{16}$). In each case, stable clones were isolated after selection for resistance to cadmium toxicity.

Analysis of mRNA

Northern blotting was performed as follows. Total RNA was isolated from cells using a Qiagen total RNA isolation kit (Valencia, CA, U.S.A.). RNA samples (5 μg) were fractionated in a 1 % agarose–formaldehyde gel and transferred to a Nytran membrane. The blot was probed with a DIG (digoxigenin)-labelled ribo-probe prepared with the DIG-labelling kit (Roche Molecular Biochemicals) for mouse *Mt1* or *Nqo1*, according to established procedures [31]. Signals were visualized by chemiluminescence using a DIG RNA detection kit with CDP Star as a substrate (Roche Molecular Biochemicals). Parallel blots of the same samples were probed with DIG-labelled mouse actin probe to ensure equal loading. Results shown were repeated 2–3 times in separate experiments with consistent observations.

Mt1 mRNA abundance was also measured by solution hybridization using an oligonucleotide complementary to the 3′ untranslated region of *Mt1* that was end-labelled with ^{32}P [32]. Cells were lysed with SDS and proteinase K. Aliquots of total nucleic acids were hybridized for approx. 18 h with the labelled probe, followed by digestion with S1 nuclease, precipitation with trichloroacetic acid and quantification by scintillation counting [32]. A standard curve was generated with *Mt1* mRNA standard (calibrated at 10^8 molecules/μl). The number of cells was determined by measuring the amount of DNA in the sample [22]. *Mt2* mRNA was measured in a similar fashion.

MT protein assay

Cells from a nearly confluent 10 cm plate were treated with tBHQ, zinc or CHX (cycloheximide) as indicated for 18 h. Then the cells were harvested, washed once with PBS, resuspended in 1 ml of 10 mM Tris/HCl (pH 7.5) and sonicated briefly. An aliquot was removed to measure DNA and *Mt1* mRNA and the remainder was boiled for 2 min and centrifuged. Aliquots of the supernatant (0.3 ml, equivalent to approx. 7×10^6 cells) were mixed with 4000 pmol of ^{109}Cd (approx. 900 c.p.m./pmol; PerkinElmer, Boston, MA, U.S.A.), incubated for 15 min at 22 °C to allow ^{109}Cd exchange for Zn in MT; then, cadmium not bound to MT was removed by two rounds of haemoglobin extraction [33] and the radioactivity in the supernatant was measured using a scintillation counter. The background with no protein extract (approx. 1 pmol) was subtracted. Cells that do not express MT had < 1 % of the ^{109}Cd binding similar to the hepa1A cells used in these experiments. The number of MT molecules/cell was calculated assuming 7 pmol of Cd/pmol of MT and determining the amount of DNA (number of cells) that gave rise to the aliquot of extract that was assayed.

β-Galactosidase induction and measurement

MRE-βGeo encodes β-galactosidase fused to neomycin phosphotransferase under the control of five MRE-d elements located upstream of the basal mouse *Mt1* promoter (-42 to $+60$) [16]. BHK cell line 3038, which is stably transfected with MRE-βGeo, was treated with tBHQ, Zn, Cd or other agents as described in each Figure. After a brief rinse in PBS, the cells were fixed for approx. 5 min in glutaraldehyde and formaldehyde, rinsed and then incubated with *o*-nitrophenyl β-D-galactopyranoside for approx. 1 h and the absorbance at 405 nm was measured [8].

Zinc depletion, measurement of zinc content and ^{65}Zn uptake

FBS (Hyclone), which contains approx. 55 μM Zn, was mixed with Chelex 100 (Sigma) to generate Zn-depleted FBS according to instructions from Sigma and then sterilized by filtration. Aliquots of DMEM, FBS, Zn-depleted FBS, DMEM with 10 % FBS, and total cell lysate were analysed to determine the Zn and Cd contents using inductively coupled plasma emission spectroscopy by DataChem Laboratories (Morgantown, WV, U.S.A.). Zn uptake by *Mtf1−/−* cells in 6-well dishes was
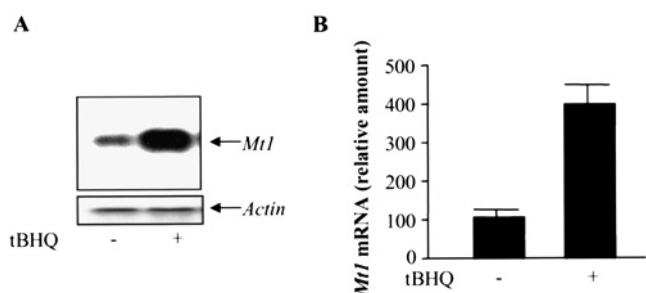
A

← *Mt1*

← *Actin*

tBHQ   −   +

B

Figure 1   Induction of *Mt1* mRNA by tBHQ

Hepa1c1c7 cells were treated with DMSO or tBHQ (100 μM) for 5 h. Total RNA was prepared, fractioned in 1% formaldehyde–agarose gel, transferred to membranes and probed with DIG-labelled riboprobes for mouse *Mt1* and *actin* (**A**). Relative amount of *Mt1* mRNA was measured by quantification of band density using ImageQuant (Molecular Dynamics, San Jose, CA, U.S.A.). The amounts of *actin* in the same samples were used to normalize for loading variations (**B**). Means $\pm$ S.D. were calculated for three separate experiments.

measured by replacing the normal medium with 2 ml of a medium containing Chelex-treated FBS and 4 μM ^{65}Zn (9300 c.p.m.) that was preincubated for several hours to equilibrate temperature and pH. After 1 h, the medium was removed, the cells were washed twice with PBS and then the cell layer was dissolved in 1% SDS. The radioactivity in the medium and the cell layer were determined using a γ counter, and the fraction of total radioactivity in the cell layer was calculated.

Fluorescence imaging of intracellular 'free' zinc

Hepa1c1c7 cells were seeded in a Lab-Tek 4-chamber coverglass systems (Electron Microscopy Sciences, Fort Washington, PA, U.S.A.) in α-minimal essential medium with 10% FBS at a density of 1.5×10^4/ml. Cells were grown at 37°C in 5% CO$_2$ for 24 h to reach 80% confluency. The culture was changed to α-minimal essential medium containing 10% FBS, which was

treated with Chelex 100. FluoZin-3AM and pluronic (Molecular Probes, Eugene, OR, U.S.A) were added to the culture medium at final concentrations of 1 μM or 0.2% respectively. The culture was continued for 30 min, followed by treatment with DMSO, 100 μM tBHQ, 100 μM zinc or 100 μM zinc plus 10 μM pyrithione (Aldrich, St. Louis, MO, U.S.A.) respectively for 50 min in a cell culture incubator. Fluorescence response of the cells was examined using a laser scanning confocal microscope (LSM 510; Carl Zeiss, Thornwood, NY, U.S.A.) fitted with an argon-ion laser ($\lambda_{ex} = 488$ nm; $\lambda_{em} = 520$ nm). Images were scanned at 512×512 pixels. Using only the green channel, the mean grey value of each field was extracted using Optimas 6.51 image analysis software (Media Cybernetics, Silver Spring, MD, U.S.A.). Representative data from three separate experiments are given. Quantitative data represent means $\pm$ S.D. for six separate fields for each treatment. One-way ANOVA analysis and Tukey's multiple comparison test were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, U.S.A.).

RESULTS

Induction of *Mt1* mRNA by tBHQ involves redox cycling

Treatment of hepa1c1c7 cells with tBHQ, a prototype of phenolic antioxidants, at a concentration of 100 μM for 5 h induced the expression of *Mt1* mRNA by approx. 4-fold (Figures 1A and 1B). The EC$_{50}$ for induction of *Mt1* mRNA was approx. 20 μM, compared with approx. 10 μM for *Nqo1* mRNA (Figure 2A). Half-maximal induction was achieved between 2.5 and 5 h for both mRNAs (Figure 2B).

Phenolic antioxidants undergo oxidation–reduction cycling in cells; the redox activity is required for a number of antioxidant functions such as the induction of *Nqo1* [25,34,35]. Therefore we compared *Mt1* mRNA induction by tBHQ analogues with different reduction–oxidation capabilities. Diphenols, quinol and tBHQ, which readily undergo reduction–oxidation, strongly induced *Mt1*; in contrast, resorcinol, which does not undergo
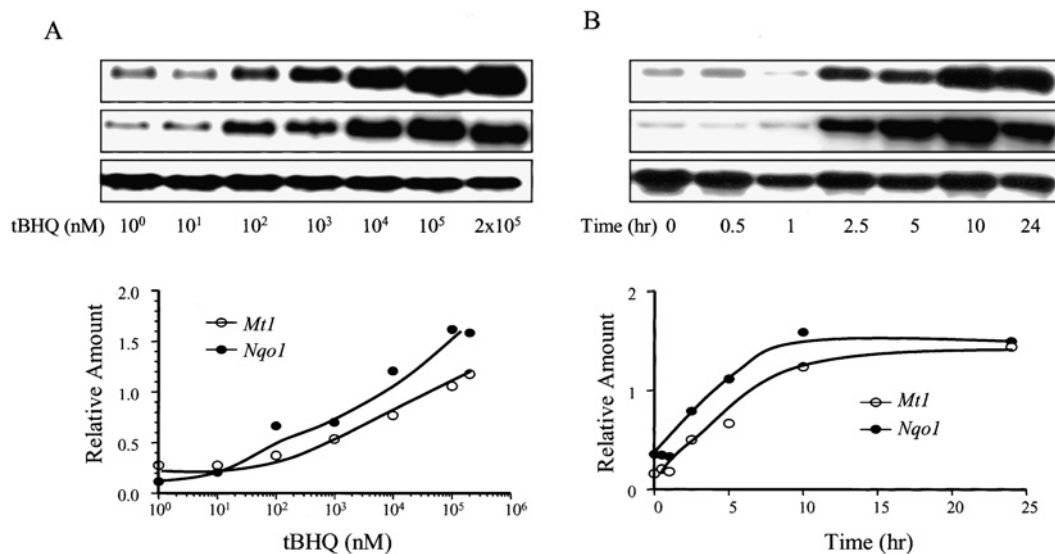
A

tBHQ (nM) 10^0 10^1 10^2 10^3 10^4 10^5 2×10^5

B

Time (hr)   0   0.5   1   2.5   5   10   24

Figure 2   Concentration and time dependence of *Mt1* mRNA induction

(**A**) Hepa1c1c7 cells were treated with increasing amount of tBHQ for 5 h; (**B**) cells were treated with 100 μM tBHQ for various time periods. Total RNA was analysed for the induction of *Mt1* or *Nqo1* by Northern blotting as described in Figure 1. *Actin* was used to control loading variations. From top, the bands represent *Nqo1*, *Mt1* or *actin* respectively. Lower panels show quantification of concentration and time dependence of induction.
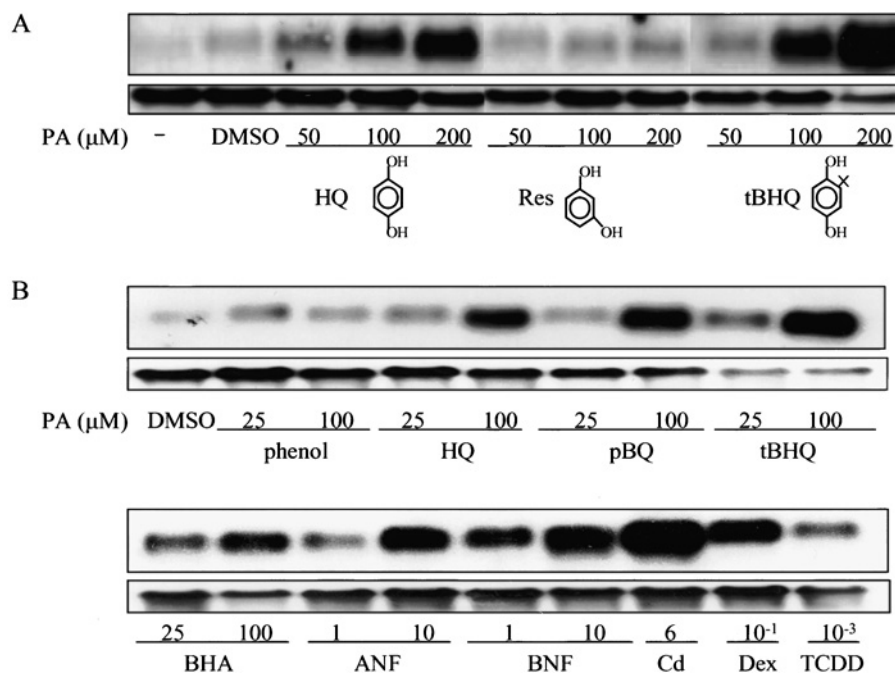
Figure 3   Induction of *Mt1* mRNA by tBHQ analogues

Cells were treated with tBHQ or its analogues for 5 h at the concentrations indicated. Induction of *Mt1* mRNA was examined by Northern blotting as described in Figure 1. Structures of the analogues are shown. *Actin* was used as a loading control. PA, phenolic antioxidants; HQ, quinol (hydroquinone); Res, resorcinol.

redox cycling due to the hydroxy group at the *meso* position on the benzene ring, was inactive (Figure 3A). Similarly, redox-active pBQ and BHA induced *Mt1* mRNA, whereas phenol, which has a single hydroxy group, and benzene, which has none, failed to induce (Figure 3B and results not shown). ANF and BNF, which are metabolizable to phenolic antioxidants, induced *Mt1* mRNA, but TCDD, which is stable in cells and does not possess redox activity, did not. Cadmium and Dex were used as positive controls. Thus induction of *Mt1* mRNA by phenolic antioxidants depends on their redox cycling activities. These observations, along with the similar dose–response and kinetics of *Mt1* and *Nqo1* mRNA induction, suggested that they might be induced by a common mechanism.

Treating mouse hepa1A cells with tBHQ not only induced *Mt1* and *Mt2* mRNAs (results not shown), but MT protein as well (Table 1). The induction of *Mt1* mRNA or MT protein by 100 μM tBHQ was equivalent to that achieved with 30–60 μM zinc. Induction of MT protein by tBHQ was blocked by treatment with CHX, an inhibitor of protein synthesis, but the induction of *Mt1* mRNA was not blocked; in fact, there was a synergistic effect of CHX and tBHQ on *Mt1* mRNA accumulation (Table 1). These results indicate that the mechanism of *Mt1* induction by tBHQ is direct, rather than secondary to induction of some other protein.

Induction of *Mt1* by tBHQ requires MTF-1, but not Nrf2

Induction of *Nqo1* gene expression by tBHQ requires the transcription factor Nrf2 [24,26,29]. Therefore embryonic fibroblasts from *Nrf2*-knockout mice (*Nrf2*−/−) were tested for their ability to support induction of *Mt1*. As shown in Figure 4(A), tBHQ induced *Mt1* mRNA equally well in hepa1c1c7, *Nrf2*+/+ and *Nrf2*−/− cells. As a control, tBHQ induced *Nqo1* in *Nrf2*+/+ but not in *Nrf2*−/− cells as expected (results not shown). Thus Nrf2 is not required for *Mt1* mRNA induction by tBHQ under the experimental conditions in this cell system.

Table 1   Induction of *Mt1* mRNA and MT protein by tBHQ and zinc

Treatment*	*Mt1* mRNA (molecules/cell)†	MT protein (molecules/cell) $\times 10^{-6}$‡
None	84 ± 0.5	3.05 ± 0.16
20 μM tBHQ	118 ± 1	4.44 ± 0.26
100 μM tBHQ	427 ± 7	8.99 ± 0.21
10 μM CHX	380 ± 7	2.71 ± 0.02
100 μM tBHQ + 10 μM CHX	2279 ± 32	3.49 ± 0.15
30 μM zinc	207 ± 3.5	5.77 ± 0.88
60 μM zinc	960 ± 2	16.98 ± 0.74
120 μM zinc	4069 ± 62	52.69 ± 0.09

* Hepa 1A cells were exposed to the indicated chemicals for 18 h; all treatments included 0.1% DMSO.

† *Mt1* mRNA was measured by solution hybridization as indicated in the Materials and methods section. Values represent means $\pm$ range for duplicate cell cultures. *Mt1* mRNA levels were measured in triplicate from each sample. *Mt2* mRNA was induced to a similar extent by tBHQ and zinc (results not shown).

‡ MT protein was measured by ^{109}Cd-binding assay as described in the Materials and methods section. Values represent means $\pm$ range for duplicate cell cultures. MT protein levels were measured in quadruplicate for each sample. MT protein includes both MT-I and MT-II.

Induction of *Mt1* mRNA by metals requires MTF-1 [16,19]. Embryonic fibroblast cells derived from *Mtf1*-knockout (*Mtf1*−/−) cells failed to support tBHQ-mediated induction of *Mt1* mRNA, whereas induction was observed in *Mtf1*+/+ fibroblasts and hepa1c1c7 cells (Figure 4B). As a control, induction of *Nqo1* mRNA by tBHQ was comparable in all three cell lines (results not shown). Furthermore, stable reconstitution of *Mtf1*-null cells by transfection with a plasmid CMV-MTF-1 restored the induction of *Mt1* mRNA by zinc or tBHQ (Figure 4C and Table 1). However, stable reconstitution of the *Mtf1*-null cells with a plasmid, Pol II-ZF$_5$VP$_{16}$, which has only the DNA-binding
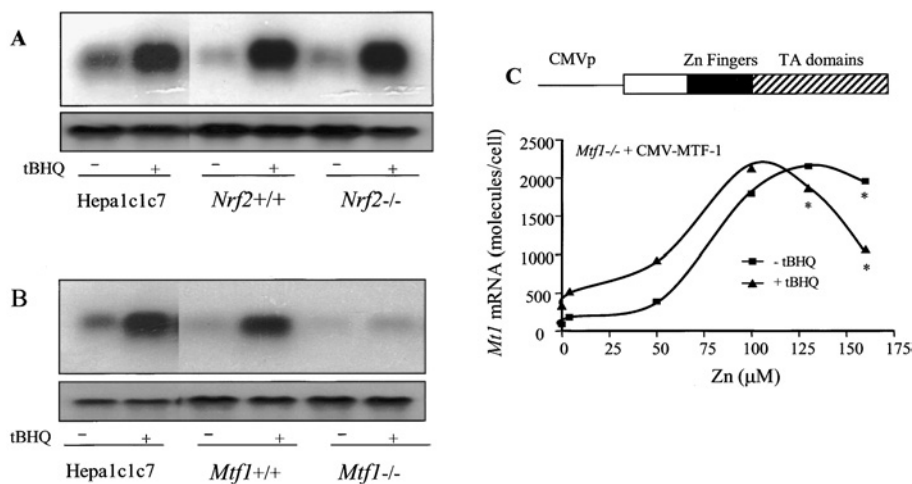
Figure 4   MTF-1 dependence of *Mt1* induction by tBHQ

(**A**) Wild-type (*Nrf2*+/+) and *Nrf2*−/− embryonic fibroblast cells were examined for tBHQ induction of *Mt1* mRNA by Northern blotting. (**B**) *Mtf1*+/+ and *Mtf1*−/− fibroblast cells derived from mouse stem cells were treated with tBHQ and analysed for *Mt1* mRNA induction by Northern blotting. Hepa1c1c7 cells were used as a control for induction. (**C**) *Mtf1*−/− cells were reconstituted with plasmid CMV-MTF-1 allowing expression of full-length mouse MTF-1. The induction of *Mt1* mRNA by zinc with or without 100 μM tBHQ was measured by solution hybridization after 8.5 h. *Visible rounding of cells indicating cell toxicity.

Table 2   Induction of *Mt1* mRNA by tBHQ, iodoacetate, H_2O_2 and zinc depends on MTF-1

Either *Mtf1*−/− cells or a clone of these cells stably transformed with CMV-MTF-1 were treated for 9 h with the indicated compounds. In each case, a range of concentrations of each compound was tested in the cells expressing MTF-1 to determine the optimal concentration for induction and then these concentrations were tested in the *Mtf1*−/− cells. The values shown are the means for duplicate or triplicate determinations of *Mt1* mRNA abundance measured by solution hybridization. DMSO, the solvent for tBHQ, and sulphoraphane had no effect on mRNA abundance.

	Mt1 mRNA (molecules/cell)	
Treatment	*Mtf1*−/− cells	*Mtf1*−/− cells + CMV-MTF-1
None or DMSO	29	95
100 μM tBHQ	37	247
2.5 μM iodoacetate	30	440
500 μM H_2O_2	29	370
2.5 μM sulphoraphane	28	83
100 μM zinc	46	1755

domain of MTF-1 fused to the activation domain of VP$_{16}$, had high basal levels of *Mt1* mRNA but there was little ($< 30\%$) induction by either zinc or tBHQ (results not shown). These findings establish that MTF-1, but not Nrf2, is required for induction of *Mt1* mRNA by tBHQ. Furthermore, some part of MTF-1, other than the DNA-binding domain, is required for tBHQ-mediated induction.

Induction of *Mt1* gene expression by oxidants, such as H_2O_2, or alkylating agents, such as iodoacetate, also depends on MTF-1 as shown by the observation that there was negligible induction by these compounds in *Mtf1*−/− cells, but approx. 3–4-fold induction when these cells were stably reconstituted with CMV-MTF-1 plasmid (Table 2). However, sulphoraphane, which is a potent inducer of *Nqo1* [36] via activation of Nrf2, failed to induce *Mt1* mRNA at any concentration tested (ranging from 0.06 to 20 μM); in fact, it decreased basal levels at higher concentrations. These results suggest that activation of Nrf2 could not induce *Mt1* in the absence of MTF-1.

Induction of *Mt1* mRNA by metals involves binding of MTF-1 to multiple MRE sequences located in the promoter region of the
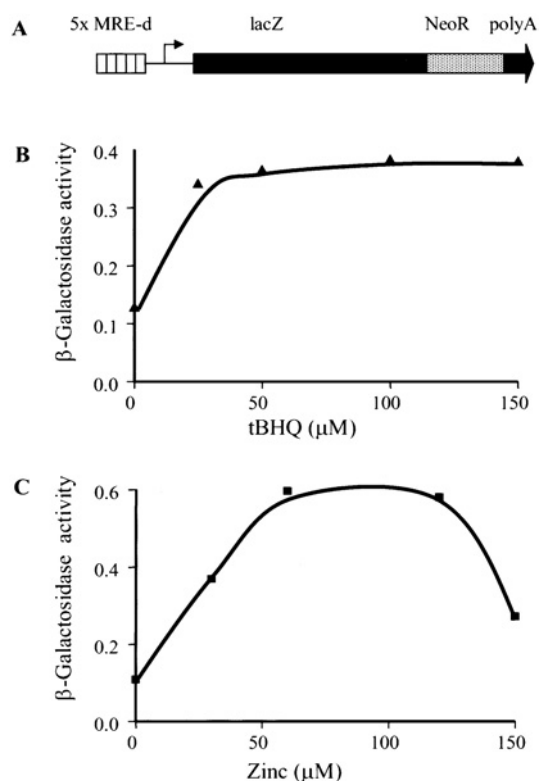

Figure 5   Induction of *MRE-β Geo* reporter gene by tBHQ

(**A**) Schematic representation of *MRE-β Geo* fusion gene. (**B**) BHK 3038 cells were treated with increasing concentrations of tBHQ. β-Galactosidase activity was measured for induction of β-galactosidase. (**C**) Induction of *MRE-β Geo* by zinc was used as a control; induction was for 18 h. Decrease in induction at higher concentrations of zinc (150 μM or higher) reflects cell toxicity.

Mt1 gene [16,19]. Thus we tested the ability of tBHQ to activate MRE-driven reporter gene. BHK cell line 3038 carries a zinc-inducible reporter gene that has β-galactosidase activity driven by five MRE-d sequences upstream of a basal promoter (Figure 5A).
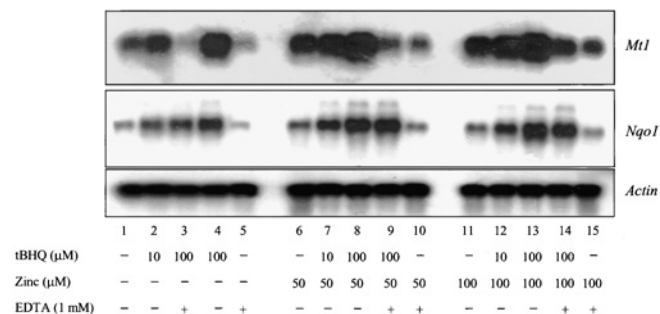
Figure 6   Inhibition of *Mt1* mRNA induction by EDTA

Hepa1c1c7 cells were treated with tBHQ, Zn, EDTA or combinations of the chemicals for 5 h as shown. Total RNA was analysed for *Mt1* mRNA induction by Northern blotting. Induction of *Nqo1* was used as a control.

Addition of tBHQ (25–150 μM) induced β-galactosidase activity approx. 3-fold (Figure 5B), when compared with a maximum of approx. 6-fold for zinc (Figure 5C). These results suggest that tBHQ induces *Mt1* mRNA at the transcriptional level by facilitating MTF-1 binding to MRE sequences.

Induction of *Mt1* mRNA by tBHQ requires zinc

Figure 6 (top panel) shows that 10 and 100 μM tBHQ induced *Mt1* mRNA in the normal medium, which had 4.3 μM zinc. Induction by tBHQ was further increased in the presence of 50 or 100 μM exogenous Zn. EDTA, which chelates extracellular zinc, blocked the inductions by tBHQ with or without added zinc (cf. lane 3 with 4; 9 with 8; 14 with 13). Induction of *Nqo1* was used as a control (Figure 6, middle panel); tBHQ induced *Nqo1* at 10 and 100 μM (lanes 2 and 3); Zn at 50 and 100 μM enhanced the induction. However, EDTA at 1 mM did not block the induction of *Nqo1* by tBHQ (cf. lane 3 and 4; 9 and 8; 14 and 13). Thus inhibition by EDTA is specific for *Mt1* gene transcription. Induction of β-galactosidase by tBHQ in the BHK cells carrying MRE-βGeo was also blocked by EDTA in both the normal medium and medium supplemented with 50 or 100 μM exogenous zinc added (results not shown). As in the previous experiments, there was an additive or synergistic effect of tBHQ and zinc. The combination of 100 μM zinc and 100 μM tBHQ was toxic to BHK cells and this toxicity was prevented by EDTA. These results reveal that induction of both MRE-driven reporter gene and endogenous *Mt1* gene are blocked by EDTA, indicating a role for zinc in the induction by tBHQ.

To ascertain further the requirement of Zn for *Mt1* induction by tBHQ, Zn and other bivalent metals were depleted from the medium by treating the serum with Chelex 100. Induction of *Mt1* in Zn-depleted medium (< 0.7 μM zinc) was compared with a normal medium, which contains approx. 4.3 μM zinc (Figure 7). *Mt1* mRNA could be induced by tBHQ in the normal, but not the Zn-depleted medium. However, cadmium induced *Mt1* mRNA in both a normal and zinc-depleted medium. These results further support the conclusion that induction by tBHQ requires some extracellular zinc and suggests that the mechanisms of induction by tBHQ and cadmium are different.

Zinc-resistant BHK cells (Zn^r-3038) express an excess amount of zinc transporter, ZnT1, which exports zinc from cells and thereby reduces the intracellular 'free' zinc concentration. Figure 8 shows that the basal level of *MRE-βGeo* expression in the Zn^r cells was decreased, and tBHQ failed to induce β-galactosidase to the level observed in the control cells, suggesting that
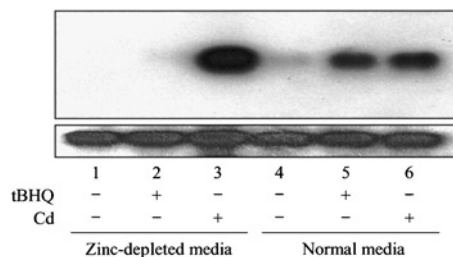

Figure 7   Zn dependence of *Mt1* mRNA induction by tBHQ

Zinc and cadmium were depleted from the culture medium (DMEM with 10 % FBS) by treating the serum with Chelex-100. Hepa1c1c7 cells were cultured in a normal or Zn-depleted medium for 3 days before treatment with tBHQ or Cd for 5 h. Induction of *Mt1* mRNA was analysed by Northern blotting. *Actin* was used as a control for loading variation (lower panel).
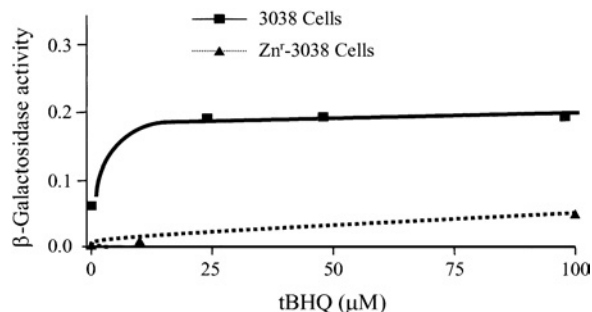

Figure 8   Induction of *MRE-βGeo* in Zn-resistant cells

BHK 3038 cells and their Zn-resistant variant cells Zn^r-3038 were treated with increasing concentrations of tBHQ for 18 h and induction of β-galactosidase was analysed.
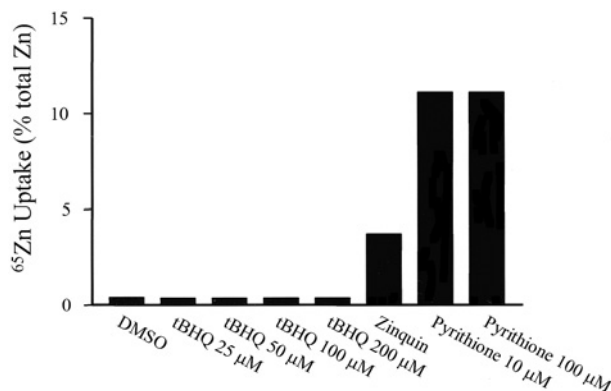

Figure 9   Effect of tBHQ on ^{65}Zn uptake

Mtf1$-/-$ cells (DKO7) were incubated for 1 h in the presence of 4 μM ^{65}Zn plus indicated concentrations of tBHQ, zinquin or pyrithione. The amount of radioactive Zn in the cells and medium was measured and the percentage uptake of radioactivity was calculated.

the basal expression and induction by tBHQ are affected by the activity of ZnT1.

All these observations could be explained if tBHQ increases intracellular 'free' zinc concentration by stimulating zinc uptake from the medium. To test the hypothesis, we examined whether tBHQ affects uptake of ^{65}Zn by the *Mtf1*$-/-$ cells. These results indicate that tBHQ at 25, 50, 100 or 200 μM did not affect zinc uptake, whereas the zinc ionophores, zinquin and pyrithione, at comparable concentrations increased zinc uptake significantly, as expected (Figure 9).
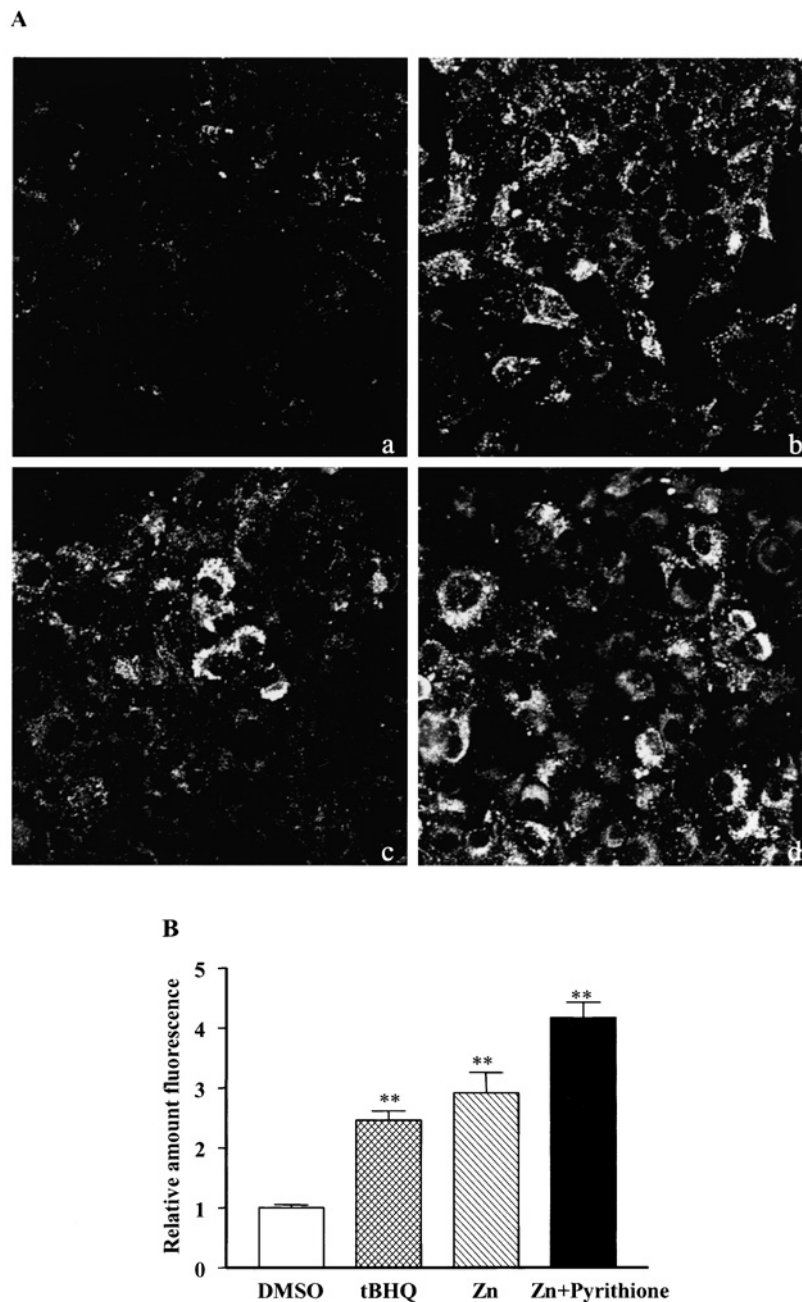
A

B

Figure 10   tBHQ enhances fluorescence of FluoZin-3AM

(**A**) Images show cells treated with (**a**) DMSO, (**b**) 100 μM tBHQ, (**c**) 100 μM zinc or (**d**) 10 μM zinc plus pyrithione for 50 min. Images were taken using a laser scanning confocal microscope as described in the Materials and methods section. (**B**) Quantification of fluorescence. Results are means $\pm$ S.D. for six separate fields for each treatment. **$P < 0.01$ in comparison with DMSO treatment.

Zinc can be mobilized from cytoplasmic proteins, as well as from various organelles in response to stimulation [37]. Therefore we tested if tBHQ mobilizes intracellular zinc to increase cytoplasmic 'free' zinc concentration, which was measured by fluorescence imaging using FluoZin-3AM, a new fluorescence probe with high affinity and selectivity for cytoplasmic Zn^{2+} [38]. To reduce any effect of zinc from the medium, a zinc-free medium was used during the period of treatment. As shown in Figure 10, treatment with 100 μM tBHQ increased cytoplasmic fluorescence by 2.5-fold, and treatment with 100 μM zinc or zinc plus 10 μM pyrithione, which were used as positive controls, increased it by 2.9- and 4.9-fold respectively. Thus tBHQ releases zinc from intracellular pools.

DISCUSSION

MT-I and MT-II are induced in a wide range of cell types by several different classes of inducers, consistent with their roles in zinc and copper homoeostasis, as well as defence against metal overload and oxidative stress [1,2,11]. The regulation of *Mt* gene expression is complex, involving a number of different transcription factors and signalling pathways. Although

the transcription factors and *cis*-acting elements required for induction by metals and glucocorticoids have been identified [13–16,22], the factors and binding sites mediating induction by antioxidants, oxidative signals and inflammatory stimuli are not established. In view of the importance of these inducing signals in the control of cellular homoeostasis and pathogenesis of certain disease states [39–41], elucidating the molecular pathway(s) of *Mt* gene induction by these inducers is important.

In the present study, we exploit the availability of *Nrf2*- and *Mtf1*-null cells obtained by gene targeting to address whether induction of *Mt1* by phenolic antioxidants is mediated by either of the transcription factors. Three lines of evidence support the conclusion that tBHQ induces *Mt1* via MTF-1, but not the Nrf2-signalling pathway. First, loss of *Nrf2* gene function does not affect the induction; however, disruption of the *Mtf1* gene abolishes the induction of *Mt1*. In contrast, reconstitution of *Mtf1*-null cells with full-length MTF-1 restores the induction. Secondly, tBHQ induces the expression of *MRE-βGeo* reporter gene, which is under the control of MRE-d, a DNA-binding site of MTF-1, in agreement with previous results [9]. Finally, the induction of *Mt1* mRNA by tBHQ is strictly dependent on the presence of adequate zinc; this is in agreement with the essential role of zinc in MTF-1 activation. Taken together, these findings suggest that phenolic antioxidants stimulate MTF-1 binding to MRE sequences in the promoter of the *Mt1* gene, and the *Mt2* gene as well, because the two genes are co-ordinately regulated by metals [42]. Lack of induction of *Mt1* by sulphoraphane, a potent activator of Nrf2 [36], suggests that Nrf2 does not participate in *Mt1* induction, e.g. by binding to the ARE element in the promoter [27], even when MTF-1 is present.

The mechanism of MTF-1 activation by phenolic antioxidants remains to be elucidated. Depletion of zinc by either adding EDTA to the medium or by culturing cells in a medium with Chelex-treated serum blocks *Mt1* mRNA induction by tBHQ. Moreover, the expression is reduced in cells that have lower intracellular zinc due to overexpression of the zinc transporter ZnT1 [20]. These findings are consistent with the notion that zinc participates in MTF-1 activation for *Mt1* induction by tBHQ. However, tBHQ does not increase zinc uptake by cells; therefore, it presumably affects the availability of intracellular zinc for MTF-1. This conclusion is consistent with the observation that zinc and tBHQ have additive or synergistic effects (depending on the concentrations of each) and that their combination kills cells under conditions where neither of them is toxic by itself. A simple explanation for these results is that redox cycling initiated by tBHQ releases zinc from intracellular stores, and the signalling pathway that activates MTF-1 detects this transient increase in zinc. Antioxidants activate phase II genes by oxidizing cysteine residues of the regulatory molecule Keap1, which releases Nrf2 so that it can enter the nucleus and bind to ARE sequences in the promoter/enhancer of a number of target genes, including *Nqo1* and genes involved in glutathione synthesis and metabolism [43]. Since cysteine is one of the natural ligands for zinc, this suggests that oxidation of glutathione and possibly other thiol-containing molecules, releases sufficient zinc to activate MTF-1. Oxidation of MT by redox cycling could also contribute to the increase in intracellular zinc, but MT is not an essential source of zinc because the BHK cells used in these experiments do not express their endogenous *Mt* genes [16]. Thus when intracellular zinc levels are decreased (by growing cells in zinc-depleted medium, increasing zinc efflux or changing the equilibrium by chelating extracellular zinc), we postulate that sufficient amount of thiol-bound zinc that can be released by tBHQ redox cycling is not available. The increased fluorescence of the FluoZin3 in response to tBHQ provides direct evidence that this compound can release

zinc from intracellular stores. The observation that alkylating and oxidizing agents also induce *Mt1* in an MTF-1-dependent manner suggests that they may also act by increasing intracellular zinc; thus, these compounds, similar to many metals (e.g. Ag, Hg, Bi, Ni, Co, Cu and Cd), may act by displacing zinc from a variety of binding sites [8]. For this hypothesis to be tenable, one would also have to posit that cadmium is much more effective at increasing intracellular 'free' zinc when compared with tBHQ since it can still induce *Mt1* under zinc-depleted conditions (Figure 7); this could happen if cadmium displaces zinc from molecules (e.g. zinc co-ordinated by histidine residues) that are unaffected by tBHQ.

The MTF-1 molecule contains an N-terminal region that appears to be necessary for optimal *Mt1* gene induction, six (2Cys–2His) zinc fingers that constitute the DNA-binding domain and a modular transcription activation domain in the C-terminal region [30]. We showed that induction of *Mt1* mRNA by tBHQ requires more than the DNA-binding domain (zinc fingers 1–5) of MTF-1. However, the mechanism by which zinc activates MTF-1 is unresolved. Zinc could activate MTF-1 by binding directly to some regulatory domain of MTF-1, by binding to some other molecule thereby releasing MTF-1 [16] or by activating a signal-transduction pathway that results in a covalent modification of MTF-1, e.g. phosphorylation [21,44].

Although the simplest model by which tBHQ activates MTF-1 involves release of zinc from intracellular stores, and the zinc activates MTF-1, it is possible that zinc and tBHQ (or some product generated by redox cycling) act co-operatively by binding to MTF-1 or its regulators. Induction of *Mt1* mRNA by phenolic antioxidants correlates with the redox activities of the antioxidants. Thus biphenols that readily undergo oxidation–reduction cycling strongly induce *Mt1* mRNA, whereas phenolic analogues with low redox capacities are inactive for the induction [23,34,35]. Reduction–oxidation cycling of phenolic antioxidants can generate two types of intermediates, superoxide anion and semiquinone radicals. Either of these intermediates may contribute to antioxidant–target-protein interactions. For the activation of Nrf2, the antioxidants bind covalently to critical cysteine thiols of Keap1 protein, releasing Nrf2 from the cytoplasmic complex and allowing its activation and translocation to the nucleus [45]. The lack of *Mt1* induction by sulphoraphane suggests that MTF-1 is not sequestered by Keap1 [45]. However, by analogy with Nrf2, tBHQ-generated redox products may interact with sensitive residues of some other protein(s), and thereby promote activation of MTF-1 in a zinc-dependent manner.

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