Effects of Oxidative Stress Caused by tert-Butylhydroquinone on Cytotoxicity in MDCK Cells

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ABSTRACT. Antioxidant and oxidative stress effects of prooxidants are generally dose-dependent, and these effects depend on the prooxidant species and cell type. However, the cellular response to oxidant challenge is a complicated interplay of events involving cellular expression of phase II detoxification enzymes and cellular metal metabolism. This study demonstrates the effect of tert-butylhydroquinone (t-BHQ)-induced oxidative stress on MDCK cells. Cell toxicity tests were carried out using the crystal violet (CV) assay with the following prooxidants: t-BHQ, diethyl maleate (DEM), hydrogen peroxide (H2O2), diquat (DQ) and β-naphthoflavone (β-NF). Except for β-NF, these prooxidants showed dose-dependent cytotoxicity besides the most potent t-BHQ cytotoxicity. Only t-BHQ and DEM caused significant time-dependent expression of ferritin protein as an antioxidant, which segregates Fe2+, causing the Fenton reaction. t-BHQ and DEM increased formation of lipid peroxidation, but DQ showed a tendency to decrease lipid peroxidation levels. In XTT assay, even when substantial cell death was observed in the CV assay, t-BHQ appeared to increase cell viability by enhancing XTT reduction, likely through the production of NADPH. Although curcumin, which induces cytoprotective phase II enzymes and chelates metal ions, decreased cell viability, it inhibited t-BHQ cytotoxicity. These results indicate that t-BHQ exhibits strong cytotoxicity against MDCK cells, an effect mitigated by curcumin, and that t-BHQ-induced oxidative stress activates the pentose phosphate pathway.

KEY WORDS: antioxidant, curcumin, MDCK cell, prooxidant, tert-butylhydroquinone.


Oxidative stress is an inevitable problem in almost all living organisms, from microorganisms to mammals, due to the production of reactive oxygen species (ROS) in the generation of respiratory electron transport energy [17, 25]. Although living organisms developed the detoxification capacity of biological antioxidative functions against ROS, excess ROS results in an imbalance between oxidant and antioxidant effects [17, 25]. Oxidative damage leads to the alteration of the structure and function of oxidized molecules by the direct oxidation of cellular components, including lipids, proteins and nucleic acids [11, 17, 25].

Hydrogen peroxide (H2O2), tert-butylhydroquinone (t-BHQ), diethyl maleate (DEM), diquat (DQ), and β-naphthoflavone (β-NF) are known as prooxidants [3, 5, 7, 13, 18, 21, 23]. These prooxidants, however, can become antioxidants by transcription enhancement of phase II detoxification enzymes, such as glutathione-S-transferase, NAD (P)-H(quinone-acceptor) oxireductase, γ-glutamylicysteine synthetase, heme oxygenase and ferritin [11, 22, 24, 26, 27, 29]. The expressions of these chemopreventive enzymes are associated with nuclear factor E2-related factor 2 (Nrf2)-related electrophile/antioxidant responsive element (EpRE/ARE) activation [12, 14, 26, 28–30]. Therefore, although prooxidant-induced cytotoxicity is dose-dependent, lower doses of prooxidants provide cytoprotection against a variety of oxidative stressors [14, 15, 23, 26].

Although the cellular chemopreventive effect of prooxidants is a complex interplay of events including cellular expression of phase II detoxification enzymes and cellular metal metabolism, antioxidant and oxidative stress seem to be cell- or tissue-specific [7, 22, 27, 28]. The expression of iron storage protein, ferritin, is induced by oxidative stress as an antioxidant, indicating that ferritin has a dual function to store iron in a bioavailable and nontoxic form, as iron (Fe2+) produces the most toxic hydroxyl radical through the Fenton reaction [17, 20]. On the other hand, cytoprotection of ferritin against oxidative stress is controversial due to the release of Fe2+ by superoxide anion and/or radical products [7, 22], suggesting that intracellular ferritin protein and iron levels may affect the detoxification capacity of ROS. Therefore, more detailed studies are required to clarify the effects of prooxidants on cells or tissue, with focus on iron metabolism.

The kidneys are often exposed to a variety of diverse chemicals such as drugs, natural products, industrial products and environmental pollutants [16, 32, 34]. Renal injury is caused by oxidative stress from iron metabolism disorders, such as hemolytic disease [8, 33]. t-BHQ and ferric nitrilotriacetate have also been reported to promote renal carcino-
Materials and Methods

Materials: Crystal violet (CV) and dimethyl sulfoxide (DMSO) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). t-BHQ and 1,1,3,3-tetraethoxypropane (TEP) were purchased from Aldrich Chemical Company Inc. (Milwaukee, WI, U.S.A.). β-NF was purchased from LKT Laboratories, Inc. (St. Paul, MN, U.S.A.). H₂O₂, DEM and 10% neutral formalin buffer solution (pH 7.4) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DQ bromide was purchased from Labor Dr. Ehrenstorfer-Schäfers (Augsburg, Germany). NeutrAvidin conjugated with alkaline phosphatase (ALP), Mammalian Protein Extraction Reagent (M-PER), the Coomassie Plus-The Better Bradford Assay kit and EZ-link N-hydroxysulfoosuccinimide (sulfo-NHS)-Biotin were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.). Bovine serum albumin (BSA) and the Cell Proliferation kit (XTT assay) were purchased from Roche Diagnostics (Lewes, UK). Immuno Plate Maxisorp F96 and assay microplates for the protein assay were purchased from Nunc Techno Glass (Funabashi, Chiba, Japan). Other reagents used were of the highest grade available.

Antibodies and their biotin-labeling: Antibodies to rat liver ferritin were purified from rabbit antiserum by affinity chromatography as described previously [31], and a portion of these antibodies was biotinylated with sulfo-NHS-biotin according to the manufacturer’s instructions.

Cell culture: MDCK cells were kindly provided by Prof. Tsutomu Hodatsu (Kitasato University, Japan), and cultured in Eagle’s minimum essential medium (EMEM, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone, Logan, UT, U.S.A.) at 37°C in an atmosphere of 5% CO₂.

CV and XTT assays: t-BHQ and β-NF were dissolved in DMSO to a concentration of 50 mM and 20 mM, respectively, stored at −20°C until required and used at a final concentration of 1% DMSO in each experiment. MDCK cells were seeded in a 96-well plate at a density of 1.4 × 10⁵/cm². After incubation for 24 hr, cell culture medium was aspirated, and each well was washed with 100 µl of cell culture medium. The added culture medium was aspirated again. After washing the cells three times using identical treatments, 100 µl of cell culture medium containing various concentrations of t-BHQ, DEM, H₂O₂, DQ or β-NF was added to each well, and plates were incubated for 24 hr. The CV or XTT assay was carried out after incubation as described below. For CV staining, cells were washed three times with washing buffer (27 mM KCl, 137 mM NaCl, 15 mM K₂HPO₄, 81 mM Na₂HPO₄, pH 7.2) in place of culture medium and fixed with 10% formalin buffer solution followed by staining with 0.1% CV. After washing dyed cells with tap water, dye was eluted with 100 µl of 50% (w/w) ethanol containing 0.1 M sodium citrate, and absorbance at 595 nm was measured as previously described [19]. The XTT assay was performed based on the metabolic reduction of tetrazolium salt (sodium 3-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) according to the manufacturer’s instructions and methods. Briefly, 50-µl aliquots of XTT labeling and coupling reagents were added directly into each well 24 hr after t-BHQ treatment. After incubation for 3.5 hr, absorbance at 490 nm was measured to determine cell viability with a reference wavelength of 690 nm.

The effect of curcumin on t-BHQ cytotoxicity in MDCK cells: Curcumin was dissolved in DMSO and given a final concentration of 1% DMSO in each experiment. MDCK cells were seeded in a 96-well plate at a density of 1.4 × 10⁵/cm² with culture medium containing 12.5 µM curcumin. After incubation for 24 hr, cells were washed as described above, and 100 µl of cell culture medium containing various concentrations of t-BHQ was added to each well. After incubation for 24 hr, the CV assay was performed as described above.

Measurement of ferritin content and lipid peroxidation: MDCK cells were plated in 100-mm culture dishes at a density of 1.4 × 10⁵/cm² followed by incubation for 24 hr. After washing with 10 ml of culture medium, 10 ml of cell culture medium containing various concentrations of t-BHQ, DEM, H₂O₂, DQ and β-NF, at final concentrations of 0.2, 0.5, 0.2, 0.25 and 0.2 mM, respectively, was added to each culture dish. After the washing steps described above, cells were lysed with 0.5 ml of M-PER, and cell lysates were subjected to sandwich ELISA to measure ferritin content and lipid peroxidation. Briefly, 100 µl of affinity-purified anti-rat liver ferritin antibody (400 ng/ml) in phosphate-buffered saline (PBS, 150 mM NaCl, 20 mM sodium phosphate, pH 7.2) was added to the wells of the immunoplate, and the plate was incubated 4°C overnight. After washing with PBS containing 0.05% Tween 20 (PBST), the plate was masked with gelatin using ELISA buffer (PBS containing 0.1% Tween 20 and 0.1% gelatin, pH 7.2) for 1 hr at room temperature. After washing with PBST, a 100-µl aliquot of canine liver ferritin standards or cell lysate diluted with ELISA buffer (pH 7.2) containing 0.5 M (NH₄)₂SO₄ was added to each well of the plate. The plate was subsequently incubated at 37°C for 2 hr. After washing with PBST, 100 µl of aliquots of 250 ng/ml biotinylated anti-rat liver ferritin antibodies in ELISA buffer were added to the wells of the
plate, and the plate was incubated at 37°C for 1.5 hr. After washing, 100 µl of ALP-labeled avidin (1 µg/ml) in ELISA buffer was added to each well of the plate, and the plate was again incubated at 37°C for 1 hr. After washing, the enzyme reaction was carried out as previously described [31]. The level of lipid peroxides, as picomoles of malondialdehydes (MDA) per milligram of protein, was calculated from the absorbance at 535 nm using TEP as the standard as previously described [19]. The protein concentration of cell lysate was determined using the Coomassie Plus-The Better Bradford Assay kit with BSA as the standard using the microplate protocol.

**Statistical analysis:** All data are expressed as means ± SD. Multiple comparisons were analyzed by one-way or two-way ANOVA followed by Tukey’s test. A P value less than 0.05 was considered statistically significant.

**RESULTS**

The effect of oxidative stress induced by various prooxidants on cytotoxicity in MDCK cells: The CV assay was used to evaluate the effect of oxidative stress induced by various prooxidants by measuring absorbance at 595 nm, as shown in Fig. 1. In this experimental condition, β-NF did not show any cytotoxicity over a concentration range of 0.05 to 0.2 mM (data not shown). The cytotoxicities of the other prooxidants were dose-dependent, but t-BHQ exclusively exhibited the most potent cytotoxicity. Cellular ferritin levels were significantly increased by treatment with t-BHQ or DEM (Fig. 2), but not by treatment with H$_2$O$_2$, DQ and β-NF (data not shown); these expressions of ferritin protein were time-dependent. No significant difference in the levels of MDA was observed in the presence or absence of DMSO (1%), although t-BHQ and β-NF were dissolved with DMSO (Table 1). The treatment with t-BHQ (0.2 mM) and DEM (0.5 mM) tended to increase the levels of MDA (t-BHQ: 274 ± 30 pmol/mg protein; DEM: 188 ± 17 pmol/mg protein). However, H$_2$O$_2$ and DQ did not produce a significant difference as compared with the MDA levels of the control, whereas DQ tended to decrease the level of MDA.

The effect of t-BHQ-induced oxidative stress on the XTT assay in MDCK cells: t-BHQ-induced oxidative stress in-
creased apparent cell viability in MDBK cells in the XTT assay because there was no evidence of any significant increase in cell viability as compared with control values in the absence of t-BHQ in the CV assay, indicating the enhancement of XTT reduction by production of NADPH[19]. MDCK cells also showed significant enhancement of XTT reduction as compared with the CV assay as a result of t-BHQ-induced oxidative stress (Fig. 3). Cell death was observed at the 75 and 100 µM concentrations of t-BHQ in the CV assay, although Fig. 1 shows cell death at 200 µM concentrations, suggesting that these differences may be caused by potent t-BHQ cytotoxicity. Although cell death was observed, an apparent increase in cell viability in the XTT assay was still observed at these concentrations.

**DISCUSSION**

t-BHQ generates superoxide radicals in the presence of copper ions [18]. DEM is known to be a glutathione-depleting agent and to increase ceramide levels by enhancing sphingomyelinase activity [3, 13]. H₂O₂ is an ROS rather than a prooxidant and produces the most toxic hydroxyl radical through the iron-mediated Fenton reaction [17]. DQ is a metabolic oxidant, which results in DQ radical formation by accepting an electron from NADPH-dependent P450 reductase and successive superoxide radical formation by surrendering an electron to the unpaired molecular oxygen from its radical [7, 23]. β-NF is a ligand of the aryl hydrocarbon receptor (AhR), and the AhR ligand generates intracellular ROS [5]. However, although the effects of individual prooxidants on cells are known, few data are available comparing the cellular cytotoxicity of prooxidant species. The prooxidants t-BHQ, DEM, H₂O₂, DQ and β-NF were used in the present study. Under the present

**Table 1. MDA levels in MDCK cells as treated with various prooxidants**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DMSO only</th>
<th>t-BHQ</th>
<th>DEM</th>
<th>H₂O₂</th>
<th>DQ</th>
<th>β-NF</th>
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<tr>
<td>MDA (pmol/mg protein)</td>
<td>192 ± 30</td>
<td>169 ± 30</td>
<td>274 ± 30</td>
<td>188 ± 17</td>
<td>164 ± 18</td>
<td>70 ± 17</td>
<td>102 ± 11</td>
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a) MDCK cells were treated with t-BHQ, DEM, H₂O₂, DQ and β-NF at a final concentrations of 0.2, 0.5, 0.2, 0.25 and 0.2 mM, respectively, as described in “MATERIALS AND METHODS.” t-BHQ and β-NF were dissolved with DMSO. Therefore, two control values are indicated, with or without 1% DMSO. MDA level (pmol/mg protein) in cell lysate was measured with a TEP standard solution. Each value represents the mean±SD of three independent measurements.
experimental conditions, although β-NF did not show any cytotoxicity, the prooxidant effects on MDCK cells by the other prooxidants were dose-dependent in terms of cytotoxicity except for t-BHQ, which exhibited the most potent cytotoxicity to MDCK cells. On the other hand, the most potent t-BHQ cytotoxicity seemed to be affected by experimental conditions, such as serum components and cell conditions, before plating because t-BHQ showed strong cytotoxicity even at a concentration of 100 µM, as shown by data in Figs. 3 and 4, as compared with cytotoxic data seen at a concentration of 200 µM, as shown in Fig. 1.

The presence of cell lysate did not affect ferritin measurements in MDCK cells because ferritin recoveries from cell lysate containing known amounts of canine liver ferritin were almost 100% (data not shown). This suggests that, even if cellular ferritin-binding proteins were present in the cell lysate, they did not affect the ferritin immunoassay, such that ferritin-binding proteins conceal ferritin epitopes directed to anti-ferritin antibodies used in the ferritin immunoassay [22]. Prooxidants provide cytoprotection from oxidative stress by inducing Nrf2-mediated phase II enzyme genes [12, 14, 15]. The ferritin H subunit gene is known to possess ARE, which is located far upstream of ferritin transcription initiation, as in phase II detoxifying enzymes such as glutathione-S-transferase, NAD (P) H:quinone-acceptor oxidoreductase, γ-glutamylcysteine synthetase and heme oxygenase [26–30]. Oxidative stress-induced ferritin is expected to segregate Fe$^{2+}$, which is involved in the Fenton reaction. This iron segregation by ferritin leads to inhibition of the production of the most toxic hydroxyl radical via the oxidation of Fe$^{2+}$ into Fe$^{3+}$ by its ferroxidase. t-BHQ and DEM time-dependently induced ferritin expression, but not H$_2$O$_2$ and DQ. In human cells, ferritin H mRNA expression was significantly induced by t-BHQ and β-NF but to a lesser extent by H$_2$O$_2$ [27], and in mouse cells, ferritin H mRNA was preferentially induced by t-BHQ more than by H$_2$O$_2$ [28]. Multiple pathways may be present in the augmentation of ferritin synthesis in response to various forms of oxidative insult depending on cell or tissue type. Eventually, ferritin protein induction by oxidative stress results in a complicated pathway involving ARE-binding proteins, such as Nrf2, JunD and

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Fig. 3. Effect of t-BHQ-induced oxidative stress on XTT and CV assays in MDCK cells. Cell viability was evaluated by specific reduction of XTT (○) and CV (●) staining as described in "MATERIALS AND METHODS." Cells were plated in a 96-well cell culture plate at a density of 4 × 10$^4$ cells/well and incubated for 24 hr. After initial incubation and well washing, cells were incubated for an additional 24 hr with 10% FBS-EMEM medium, in the presence of the t-BHQ concentration indicated. After the second incubation, cells were subjected to XTT and CV assays. The plates were read at 490 nm with a 690 nm reference and 595 nm absorbance in XTT and CV assays, respectively. Data represent means±SD (n=6), and significant differences were determined as compared with control values in each assay (∗ and **: P<0.05 and P<0.01, respectively). Values sharing identical superscripts (capital and lowercase letters) are not significantly different for different concentrations of t-BHQ in each assay (P>0.05).

Fig. 4. Effect of curcumin on t-BHQ cytotoxicity in MDCK cells. Cells were plated in a 96-well plate at a density of 4 × 10$^4$ cells/well with 10% FBS-EMEM containing 12.5 µM curcumin, and incubated for 24 hr. After initial incubation and well washing, cells were incubated for an additional 24 hr in culture medium containing the various concentrations of t-BHQ indicated. After the second incubation, cell viability was evaluated by CV staining as described in "MATERIALS AND METHODS." The plates were read at 595 nm absorbance. Data represent means±SD (n=4), and significant differences were determined between the two assays in the presence (solid bars) or absence (open bars) of curcumin at each dose of t-BHQ (**) and significant difference (P<0.01) for different concentrations of t-BHQ in each assay.
phosphorylated JunD [27, 28]. In MDCK cells, DEM shows strong ferritin induction as with t-BHQ. However, β-NF does not show any ferritin induction, although β-NF has been reported to be the most potent ferritin inducer [27, 30]. In this study, β-NF did not cause oxidative stress effects including cytotoxicity, ferritin induction and formation of lipid peroxidation products, although β-NF was not used at concentrations higher than 20 mM. β-NF was not prepared at concentrations higher than 20 mM due to difficulty caused by its solubility in 100% DMSO, and so 0.2 mM was used to produce a final concentration of 1% DMSO, which resulted in loss of the cytotoxic effect on the cells (data not shown). Although MDCK cells showed relatively low expression of AhR proteins (16,000/cell) in hepatic and nonhepatic cell culture lines [9], further study is needed to clarify to what degree MDCK cells possess AhR binding to β-NF [5]. To our knowledge, this is the first report that DEM enhances the expression of ferritin protein to the same extent at a higher concentration than t-BHQ. t-BHQ and DEM have been reported to overlap signaling pathways in Nrf2-mediated activation of ARE-driven genes [12]. However, further study is required to characterize and compare transcriptional factors binding to ferritin ARE in t-BHQ and DEM-treated cells.

In MDCK cells, a significant increase in lipid peroxidation was not observed as in MDBK cells [19]. Prooxidants did not seem to cause cell membrane injury due to formation of lipid peroxidation products by prooxidants in kidney cells for an unknown reason. Additionally, DQ showed a decrease in MDA formation, although DQ was shown to cause liver and kidney damage in male Fisher-344 rats [7]. However, quantification of lactate dehydrogenase release is needed in a future experiment.

Recently, we demonstrated preliminary data concerning the prooxidant effects of t-BHQ-induced oxidative stress on MDBK cells [19], showing that t-BHQ enhances XTT reduction. This enhancement of XTT reduction causes an apparent increase in cell viability and may result in NADPH-mediated reduction of the XTT substrate by expression of NADPH-generating enzymes, such as glucose-6-phosphate dehydrogenase [19]. In the present study, MDCK cells still exhibited the enhancement of XTT reduction, probably by production of NADPH, even when cell death was observed. However, XTT reduction by t-BHQ in MDBK cells disappeared with cell death. Cellular NADPH production is likely to be an additive effect, as NADPH is stable in this experimental condition (data not shown). MDCK cells produced more NADPH than MDBK cells under severe oxidative stress conditions for unknown reason, and t-BHQ-induced production of NADPH may be peculiar to kidney cells and species-specific.

Curcumin has shown a remarkably wide range of beneficial pharmacological effects, including anti-inflammatory, antioxidant, antiviral, antiangiogenic and antitumorigenic effects [6, 10]. The pleiotropic activities of curcumin, which has complex chemical properties, are related to multiple signaling pathways [6]. The cytoprotective role of curcumin against oxidative stress is involved in enhancement of the transcriptional activity of Nrf2 leading to expression of phase II detoxifying enzymes [6, 10]. Additionally, curcumin has metal-binding activity to form complexes with the cations, VO$^{2+}$, Mn$^{2+}$, Fe$^{2+/3+}$ and Cu$^{2+}$ [10], and curcumin seems to be a strong iron chelator, although its affinity to other cations is less known [6]. Curcumin caused decrease of cell viability due to its iron chelating action in the range of 12.5 μM to 50 μM [10]. This study also showed that pretreatment with 12.5 μM curcumin did cause a significant inhibition of cell growth compared with data in the presence or absence of t-BHQ (up to 50 μM) because a linear relationship between the absorbance at 595 nm and the numbers of cell plating was observed up to 2.8 × 10$^5$ cells/cm$^2$ (8 × 10$^4$ cells/well) (data not shown). This inhibition of cell growth may be attributed to the shortage of metal ions, especially iron essential for living cells [1]. However, t-BHQ-induced oxidative stress was relieved by pretreatment with curcumin. t-BHQ caused oxidative stress by production of superoxide radicals in the presence of Cu ions [18]. Interestingly, Cu-curcumin complex seems to mimic superoxide dismutase [2]. The antioxidant effects of curcumin in MDCK cells may be due to mutual effects by expressing phase II detoxification enzymes and chelating with Cu ions. Curcumin inhibited ferritin protein expression, although it increased ARE-mediated ferritin mRNA, suggesting that iron chelation activates iron-responsive element-binding protein and translationally suppresses the ferritin level [10]. Although t-BHQ exhibited the most potent cytotoxicity to MDCK cells and induced ferritin expression, iron segregated by the expressed ferritin protein was thought to be released by superoxide radicals formed in the presence of t-BHQ [7, 22]. Therefore, curcumin is also expected to relieve iron-mediated ROS production via its iron-chelating activity.

This study demonstrates that t-BHQ is most potently cytotoxic to MDCK cells. Moreover, t-BHQ-induced oxidative stress affected PPP activity and metal ion metabolism. Renal disease is a complex, multifactorial medical condition. Curcumin may protect against nephrotoxicity caused by exposure to oxidative stress, and help to understand the mechanism of oxidative stress-induced nephrotoxicity. Further clarification of the physiological and pathological effects of t-BHQ-induced oxidative stress on kidney cells is needed.

REFERENCES


