FULL PAPER *Toxicology*

The Effect of *tert*-Butylhydroquinone-Induced Oxidative Stress in MDBK Cells using XTT Assay: Implication of *tert*-Butylhydroquinone-Induced NADPH Generating Enzymes

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ABSTRACT. Tetrazolium salts such as XTT and MTT are widely used to produce formazan for cell proliferation and cytotoxicity assays through bioreductase activity. However, the XTT assay showed significant increase in MDBK cell viability when cells were treated with both 50 and 100 μM of the pro-oxidant, *tert*-butylhydroquinone (*t*-BHQ), although the crystal violet assay showed no cytotoxic effect with these concentrations, and the induction of lipid peroxidation was not observed. We investigated the mechanism of enhancement of XTT substrate reduction after treatment of MDBK cells with *t*-BHQ, leading to apparent increase in cell viability. *t*-BHQ caused an increase in absorbance at 340 nm in culture medium, suggesting that *t*-BHQ increases cellular production and release of NADH and/or NADPH. Although *t*-BHQ did not change the NADH concentration in cell culture medium, the addition of $NADP^+$-dependent glutathione reductase decreased the XTT reduction to the control level, indicating cellular release of NADPH. *t*-BHQ also increased intracellular glucose-6-phosphate dehydrogenase activity, producing NADPH. Taken together, our findings indicate that *t*-BHQ treatment activates NADPH generating enzymes such as glucose-6-phosphate dehydrogenase followed by release of NADPH in the cell culture medium, resulting in direct XTT reduction by NADPH.

KEY WORDS: NADPH, pro-oxidant, reduction, *tert*-butylhydroquinone, XTT assay.

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Oxidative stress is an inevitable problem in almost all living organisms, from microorganisms to mammals, due to the undesirable generation of reactive oxygen species (ROS) during respiratory electron transport energy production [9, 22]. Oxidative stress results from an imbalance between oxidant and antioxidant effects, where excess ROS overcome the detoxication capacity of biological antioxidative functions [9, 22]. ROS lead to the direct oxidation of cellular components, including lipids, proteins and nucleic acids, which results in the alteration of the structure and function of oxidized molecules [9, 14, 22].

Pro-oxidants such as hydrogen peroxide, *tert*-butylhydroquinone (*t*-BHQ), β-naphthoflavone induce oxidative stress [7, 16, 18], whereas these pro-oxidants are characterized as antioxidant to enhance basal transcription of phase II enzymes such as glutathione-*S*-transferase, NAD(P)H:(quinone-acceptor) oxidoreductase, γ-glutamylcysteine synthetase, heme oxygenase, and ferritin [6, 13, 24, 25]. These chemopreventive enzymes which mediate oxidative stress were found to be positively regulated by nuclear factor E2-related factor 2 (Nrf2)-related elcetrophile/antioxidatnt responsive element (EpRE/ARE) activation [6, 13, 20, 24, 25]. Therefore, although pro-oxidant-induced cytotoxicity is dose-dependent, lower doses of pro-oxidants cause cytoprotective responses to a variety of oxidative stressors [11, 16].

Various cytotoxicity tests were developed to evaluate cell viability, from cell staining to detection of cellular enzymes [2, 10, 18]. Tetrazolium salts such as INT(2-[4-indophenyl]-3- [4-nitrophenyl]-5-phenyltetrazolium chloride), XTT (sodium 3'-[1-phenylaminocarbonyl]-3,4-tetrazorium)-bis [4-methoxy-6-nitro] benzene sulfonic acid hydrate), WST-1 ([2-(4-lodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfopheyl)-2H-tetrazolium]), and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were used as chromogens in LDH, XTT, WST-1, and MTT assays, respectively [2, 10]. These tetrazolium salts were reduced by endogenous or exogenous reducing enzymes followed by production of formazan [2, 10]; however, XTT and WST-1 substrates were reduced by NADH and NADPH in the absence of cells and enzyme [2]. In addition, Nrf2 enhanced transcription of NADPH regenerating enzymes including glucose-6-phosphate dehydrogenase (G6P-DH), 6-phosphogluconate dehydrogenase, and NADP-dependent malic enzyme, in addition to phase II enzymes [23]. Thus, cytotoxic assays using tetrazoliums salts may be affected by pro-oxidant-induced cellular responses [2].

In the present study, we demonstrate that *t*-BHQ-induced oxidative stress affected the XTT assay in MDBK cells, and that *t*-BHQ enhanced XTT reduction by production and release of NADPH leading to an apparent enhancement of cell viability.

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MATERIALS AND METHODS

Materials: Crystal violet (CV) and dimethylsulfoxide (DMSO) were purchased from Kanto Chemical Co., (Tokyo, Japan), *t*-BHQ and 1,1,3,3-tetraethoxypropane (TEP) were from Aldrich Chemical Co. (Milwaukee, Wl, U.S.A), 10% neutral formalin buffer solution (pH 7.4) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), L-glutathione oxidized (GSSG) was from Sigma Chemical Co., (St. Louis, MO, U.S.A.), and glutathione reductase (GR) and D-glucose 6-phosphate (disodium salt)(G-6P) were from Oriental Yeast Co., Ltd. (Tokyo, Japan). Mammalian protein extraction reagent (M-PER) and Coomassie Plus-The Better Bradford Assay kit were from Pierce (Rockford, IL, U.S.A.). Bovine serum albumin (BSA) and Cell Proliferation kit (XTT) were from Roche Diagnostics (Lewes, UK). Amplite Colorimetric NAD/NADH Assay Kit Blue color was from ABD Bioquest, Inc. (Sunnyvale, CA, U.S.A.). Other regents used were of the highest grade available.

Cell culture: Madin-Darby Bovine Kidney (MDBK) cells were kindly provided from Prof. Hisaaki Sato (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) at 37°C in an atmosphere of 5% CO_2.

CV and XTT assays: *t*-BHQ was dissolved in DMSO to a concentration of 50 mM and stored at –20°C until required. MDBK cells were seeded in a 96-well plate at a density of $1.5 \times 10^5/cm^2$. After incubation for 24 hr, cell culture medium was aspirated and each well was washed with 100 μl cell culture medium. The culture medium added was aspirated again. After washing the cells three times using identical treatments, 100 μl of cell culture medium containing various concentrations of *t*-BHQ was added to each well and plates were incubated for 24 hr. CV or XTT assay was carried out after incubation as described below. In CV staining, cells were washed three times with PBS (2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 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 using a VERUSAmaxTM Tunable Microplate reader (Molecular Devices Corp., Sunnyvale, CA, U.S.A.). The XTT assay was performed according to manufacturing 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 2 hr incubation, absorbance at 490 nm was measured to determine cell viability with a reference wavelength of 690 nm.

NADH quantification: The cell culture medium from each well was collected for NADH quantification after 24 hr *t*-BHQ treatment, and centrifuged at 1,800 × g for 5 min. Supernatant was measured spectrophotometrically (Shimadzu U-2010 UV-visible, Shimadzu Corp., Tokyo, Japan) at 340 nm, and remaining supernatant was heat-treated at 60°C for 30 min to decompose NAD^+ for NADH detection. After heat treatment, 50-μl aliquots were added to each microtiter plate well followed by addition of 50 μl of NADH sensor buffer, and plates were incubated for 2 hr. After incubation, absorbance at 535 nm was measured to determine NADH quantity.

Effect of GR on XTT assay using t-BHQ treated cells: After 100 μM *t*-BHQ treatment for 24 hr, cells were pretreated for 1 hr with a final concentration of 100 mM Tris (pH 7.5), 1 mM EDTA, 5 mM GSSG and 6.7 U/m*l* GR into culture medium (total: 150 μl) to examine the effect of GR on XTT assay using *t*-BHQ-treated cells. The XTT assay was conducted after pre-treatment, as previously described.

G6P-DH assay: MDBK cells were plated in a 3.5-cm dish at a density of $1.5 \times 10^5/cm^2$ and incubated for 24 hr. Cells were washed 3 times after incubation with 2 m*l* of culture medium and treated with 2 m*l* of cell culture medium containing various concentrations of *t*-BHQ to give a final concentration of 50, 100, and 200 μM for 24 hr. Cells were washed three times 24 hr after *t*-BHQ treatment, lyzed with 200 μl M-PER followed by centrifugation at 14,000 × g for 10 min at 4°C, and G6P-DH activity was determined spectrophometrically according to the method described by Glaser *et al.* [8]. In brief, the G6P-DH assay was performed in a reaction buffer (500 μl) with a final concentration of 50 mM HEPES (pH 7.5), 10 mM $MgSO_4$, 1 mM EDTA, 100 mM KCl, 0.2 mg/m*l* BSA, 1.2 mM G-6P and 0.4 mM $NADP^+$ with the cell lysate (50 μl). Enzyme activity was monitored by following the formation of NADPH at 340 nm (ε=6.22 mM^{-1} cm^{-1}) [5].

Lipid peroxidation assay: Cell lysate was prepared using M-PER, and used to determine thiobarubituric acid reactive substance (TBARS) as described previously [3]. The level of lipid peroxides as nanomoles of malondialdehydes (MDA) per milligram of protein, was calculated from the absorbance at 535 nm using TEP as standard. Protein concentration of cell lysate was determined by Coomassie Plus-The Better Bradford Assay kit with BSA as the standard using the microplate protocol.

Statistical analysis: Unless noted otherwise, all data are expressed as mean ± SD (n=4). Multigroup comparisons were analyzed by two-way ANOVA followed by Student-Newman-Kuel's test.

RESULTS

The effect of t-BHQ-induced oxidative stress on XTT assay in MDBK cells: CV and XTT assays in MDBK cells were used to evaluate *t*-BHQ-induced oxidative stress, with cell viability in the absence of *t*-BHQ defined as 100% in each assay (Fig. 1). Two hundreds μM *t*-BHQ treatment caused cell death in both assays. Although 50 and 100 μM *t*-BHQ treatments showed no cytotoxitic effect in the CV assay, the XTT assay showed a significant increase (180 to 240%) in cell viability. DMSO solvent did not show any

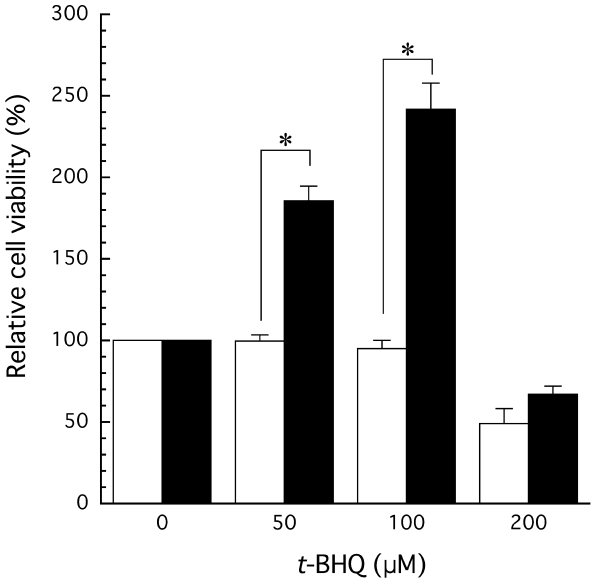

Fig. 1. Effect of *t*-BHQ oxidative stress on XTT and CV assays in MDBK cells. Cell viability was evaluated by specific reduction of XTT and CV staining as described in "Materials and methods". Cells were plated in a 96-well cell culture plate at a density of 4×10^4 cells/well and incubated for 24 hr. After initial incubation and well washing, cells were incubated for 24 hr in 10% FBS-EMEM medium, in the presence of the *t*-BHQ concentration indicated. After the second incubation, cells were subjected to XTT (solid bars) and CV (open bars) assays. The plates were read at 490 nm with a 690 nm reference and 595 nm absorbance in XTT and CV assays, respectively, and the absorbance obtained in the absence of *t*-BHQ in each assay was defined as 100%. Data represent mean ± SD (n=4), and significant difference was determined between two assays with the same dose of *t*-BHQ (* $p<0.01$).

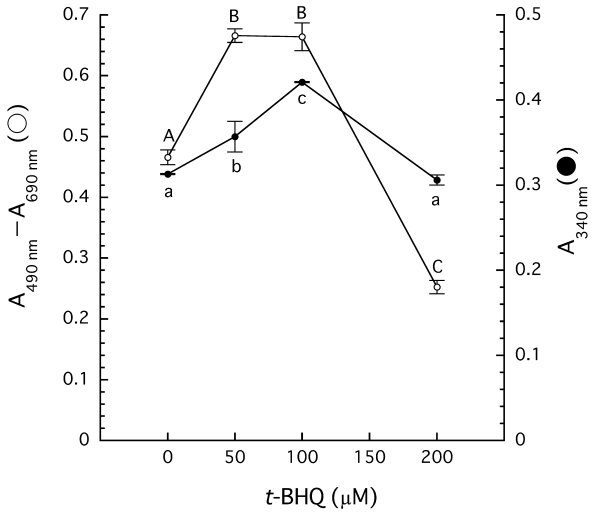

Fig. 2. The relationship between absorbance at 340 nm and XTT reduction activity in *t*-BHQ-treated MDBK cells. Cells were plated in a 96-well plate at a density of 4×10^4 cells/well, and incubated for 24 hr. After initial incubation and well washing, cells were incubated for 24 hr in 10% FBS-EMEM medium, in the presence of the *t*-BHQ concentration indicated. After the second incubation, cells were subjected to the XTT assay and absorbance at 340 nm in culture medium was measured. The plates were read at 490 nm with a 690 nm reference in the XTT assay (open circles), and the supernatant of another well transferred from the incubated well was read at 340 nm (solid circles). Data represent mean ± SD (n=4). Capital and lower case letters indicate significant difference ($p<0.01$) with different letters for each assay.

effect in the assays (data not shown). MDA production was not observed with 100 μM *t*-BHQ treatment compared to control cells (258 ± 63 pmol/mg protein, n=4). Therefore, we proposed that *t*-BHQ caused apparent increase in cell viability or proliferation by XTT reduction enhancement as cell plating was confluent.

The mechanism of XTT reduction enhancement: Although chromogen XTT is converted to colored formazan by endogenous bioreductase activity, the conversion is caused by NADH and NADPH in the absence of cells and enzymes [2], suggesting that *t*-BHQ-enhanced XTT reduction causes the apparent cell proliferation or viability. Therefore, to examine the mechanism of *t*-BHQ-enhanced XTT reduction, the presence of NADH and/or NADPH was detected at 340 nm using culture medium of *t*-BHQ-treated cells [4, 5](Fig. 2). Culture medium spectrophotometric absorbance of 50 and 100 μM *t*-BHQ-treated significantly increased (17 to 34%) concomitant with an increase of XTT reduction (140%); however, the culture medium absorbance of 200 μM *t*-BHQ-treated cells significantly decreased to untreated cell levels. On the other hand, no increase in NADH concentration was observed in culture medium of *t*-BHQ treated cells as compared with untreated cells (0.22 ± 0.03 μM, n=4) using the Amplite Colorimetric NAD/NADH Assay Kit Blue with a detection limit of 0.1 μM. The *t*-BHQ-enhanced XTT reduction was significantly decreased in the co-presence of GR and GSSG, but was not affected in the absence of GR (Fig. 3). The significant decrease of XTT reduction was also observed in the co-presence of GR and GSSG in the *t*-BHQ-absent control. Absorbance increase ($A_{490\ nm}$ to $A_{690\ nm}$) from direct XTT reduction by NADPH positively correlates with the amount of NADPH added (data not shown). From the decrease of absorbance by the addition of GR, the amount of extracellular NADPH produced and released by *t*-BHQ was calculated to be 20.8 ± 4.6 μM (n=4).

The G6P-DH assay was performed after 4, 8, 12, or 24 hr of 50 and 100 μM *t*-BHQ treatment (Table 1). G6P-DH activity was not affected by cell lysate during the course of the assay (data not shown). G6P-DH activity increased after 4 hr of treatment for all *t*-BHQ concentrations indicated; however, the 200 μM *t*-BHQ treatment decreased G6P-DH activity after 8 hr due to cell death. Although 50 and 100 μM *t*-BHQ treatments showed lower activity after 12 and 24 hr, respectively, the G6P-DH activity remained higher than the control level. After 24 hr, G6P-DH activity remained significantly higher in the 100 μM *t*-BHQ treatment.

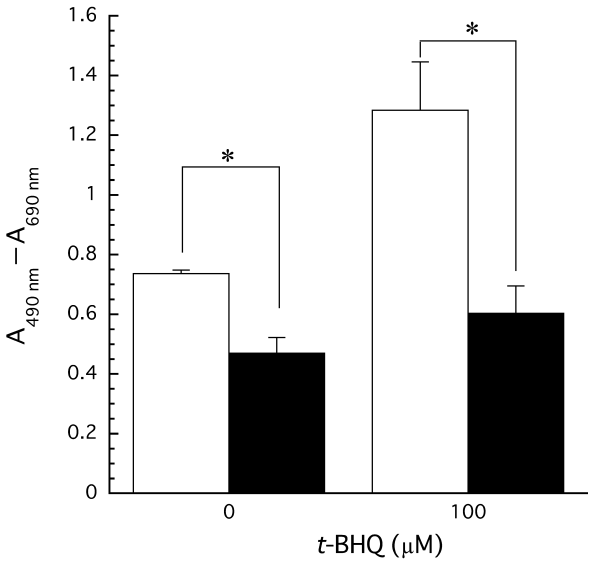

Fig. 3. Effect of GR on XTT assay in *t*-BHQ treated cells. Cells were plated in a 96-well plate at a density of 4×10^4 cells/well, and incubated for 24 hr. After initial incubation and well washing, cells were incubated for 24 hr in 10% FBS-EMEM containing 100 μM *t*-BHQ. After the second incubation, culture medium was brought to a final concentration of 100 mM Tris (pH 8.0), 1 mM EDTA, and 5 mM GSSG in the presence (solid bars) or absence (open bars) of GR (1 U/well), and cells were incubated for an additional 2 hr before being subjected to the XTT assay. Data represent mean ± SD (n=4), and significant difference was determined by two-way ANOVA (* $p<0.01$).

DISCUSSION

Although *t*-BHQ causes metabolism-dependent cytotoxicity in the kidney and bladder as pro-oxidants [15] and generates superoxide radicals in the presence of copper ions [16], it provides cytoprotection from oxidative stress by inducing Nrf2-realted phase II enzyme genes [11–13]. The CV assay is a convenient method to evaluate cell viability [18], and tetrazolium salts are extensively used to evaluate cell growth and cytotoxicity based on reduction of XTT into stable colored formazan by cellular bioreductase activity [21]. Absorbance changes in XTT and CV assays were dependent on cell numbers (data not shown). Although cell plating was confluent, and CV staining revealed no cell death with 50 and 100 μM *t*-BHQ treatments, XTT showed apparent higher cell viability (180 to 240%) in same treatments. In contrast, the 200 μM *t*-BHQ treatment showed strong cytotoxicity in both assays. While H_2O_2 is also typical pro-oxidant, it acts as a signal molecule and stimulates activation of cytokine and growth factor receptors [1]. However, H_2O_2 did not enhance any cell viability as seen in the present study (data not shown).

We proposed that *t*-BHQ treatment enhanced specific XTT reduction [2]. Additionally, the MTT assay showed an increase in apparent MDBK cell viability (180 to 200%) when treated with 50 and 100 μM *t*-BHQ (data not shown) as in XTT assay. MTT are also tetrazolium salts metabolically reduced to highly colored formazans by cellular bioreductase activity [2]; however, the MTT reagent is slowly reduced by microsomal enzymes requiring pyridine nucleotides such as NADH and NADPH [2]. While MTT is not directly reduced by NADH and NADPH, the XTT reagent is reduced by NADH and NADPH in the absence of cells and enzymes [2]. However, the main purpose of the present study is to investigate the mechanism of *t*-BHQ-induced XTT reduction in MDBK cells.

The increase in absorbance of cell culture medium at 340 nm after 24 hr in both 50 and 100 μM *t*-BHQ treatments suggests that *t*-BHQ treatment induced cellular NADH and/or NADPH production and their release. However, an increase of NADH in the cell culture medium was negligible. In this study, we could not measure NADPH content in culture medium. Thus, we examined if $NADP^+$-dependent GR affect *t*-BHQ-induced XTT reduction enhancement due to the consumption of NADPH. Eventually, $NADP^+$-dependent GR decreased XTT reduction to lower control levels (Fig. 3). A higher concentration of NADPH (20.8 ± 4.6 μM) in the cell culture medium was calculated from the decrease of absorbance, and it was concluded that apparent higher cell viability resulted in an increase in formazan from direct XTT reduction by NADPH. Moreover, we measured G6P-DH activity by monitoring NADPH to examine if the enzyme may be involved in increased NADPH production [17]. G6P-DH activity was determined, and the NADPH production was higher than the control level for the duration of the assay with 50 and 100 μM *t*-BHQ treatments, although 50 and 100 μM *t*-BHQ-treatment activity was lower at 8 and 12 hr after treatment.

Table 1. Effect of *t*-BHQ on G6P-DH activity in MDBK cells

t-BHQ (μM)	G6P-DH activity (U/mg) after *t*-BHQ treatment (hr)			
	4	8	12	24
0	0.111 ± 0.01	0.099 ± 0.02	0.090 ± 0.01	0.119 ± 0.01*
50	0.126 ± 0.02	0.106 ± 0.02	0.125 ± 0.03	0.129 ± 0.02
100	0.131 ± 0.01	0.135 ± 0.04	0.122 ± 0.03	0.160 ± 0.02*
200	0.124 ± 0.01	0.071 ± 0.01	ND	ND

Values are presented as mean ± SD (n=4).
Significantly different between 2 groups at the same time ($p<0.01$).
ND: Not detected.

t-BHQ increases intranuclear transcription factor Nrf2 [13], and Nrf2 induces various NADPH regenerating enzymes such as G6P-DH, 6-phosphogluconate dehydrogenase, malic enzymes in addition to phase II enzymes [13, 23]. Further studies need to determine the induction of other NADPH regenerating enzymes such as 6-phosphogluconate dehydrogenase and $NADP^+$-dependant malic dehydrogenase. The TBARS assay showed negligible lipid peroxidation, whereas it remains to be clarified whether *t*-BHQ may release some cellular components such as bioreductive enzymes involved in the reduction of the MTT reagent in addition to the cellular release of NADPH without membrane injury. Another cell line, MDCK cell also showed XTT reduction enhancement in the same concentrations (50 and 100 μM) of *t*-BHQ (data not shown). MDBK cells released NADPH, even in the absence of *t*-BHQ as XTT reduction was observed in the co-presence of GR and GSSG; the release of NADPH in the presence or absence of *t*-BHQ may be peculiar to renal endothelium cells. *t*-Butylhydroperoxide produces NADPH which plays cytoprotective role against oxidative stress in human erythrocytes [17]. On the other hand, in CV test, 200 μM *t*-BHQ treatment caused markedly cell death, suggesting that an increase in G6P-DH activity may activate NADPH-dependent oxidase and enhance oxidative stress-induced cell death [19]. MDBK cells may regulate intracellular NADPH level in cytoprotective role against oxidative stress and enhancement of oxidative stress.

Cytotoxicity tests using tetrazolium salts are affected by pro-oxidant such as *t*-BHQ [2] and may confer false evaluation in apparent cellular proliferation and protection. In the present study, we demonstrate preliminary data on the mechanism of *t*-BHQ-induced XTT reduction enhancement using MDBK cells. However, the XTT reduction enhancement may be due to be involved in NADPH-mediated directed reduction of XTT substrate by NADPH-generating enzymes such as G6-PDH. Further study needs to clarify the mechanism in an increase of G6-PDH activity and other candidates of NADPH-generating enzymes in the *t*-BHQ treatment. Additionally, an alterative approach is to clarify the physiological and pathological events caused by the release of NADPH from *t*-BHQ-treated MDBK cells.

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