therosclerosis is a major cause of coronary artery disease, which is highly associated with increased circulating cholesterol levels and inflammation in the vascular wall. Recently, phenolic compounds have received considerable attention because of their ability to inhibit lipid peroxidation and lipoxygenase. They function mainly as antioxidants in diseases associated with lipid metabolism, especially atherosclerosis. Ma and Kinneer demonstrated that phenolic antioxidants potently inhibit signal-induced tumor necrosis factor (TNF)-α transcription, suggesting that antioxidants may reduce inflammation through control of cytokine production. Tert-butylhydroquinone (tBHQ), a synthetic phenolic antioxidant, is commonly used as a food preservative because of its potent antilipid peroxidation activity. Several lines of evidence have demonstrated that dietary supplementation with antioxidants has an antiatherogenic function through reducing cholesterol uptake or promoting reverse cholesterol transport. In this study, we investigated whether tBHQ affects expression of ATP-binding cassette transporter A1 (ABCA1) and the potential subsequent effect on cellular cholesterol homeostasis.

Methods and Results: tBHQ increased ABCA1 protein levels and markedly enhanced cholesterol efflux from THP-1 macrophage-derived foam cells. Furthermore, tBHQ reduced calpain-mediated ABCA1 proteolysis via activation of nuclear factor E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1). Inhibition of HO-1 with a pharmacological inhibitor or siRNA and knockdown of Nrf2 suppressed the stimulatory effects of tBHQ on ABCA1 expression and calpain activity.

Conclusions: Nrf2/HO-1 signaling is required for the regulation by tBHQ of ABCA1 expression and cholesterol efflux in macrophage-derived foam cells and an antiatherogenic role of tBHQ is suggested. (Circ J 2013; 77: 2399–2408)

Key Words: Atherosclerosis; ATP-binding cassette transporter A1 (ABCA1); Heme oxygenase-1 (HO-1); Nuclear factor E2-related factor 2 (Nrf2); Tert-butylhydroquinone (tBHQ)
ATP-binding cassette transporter A1 (ABCA1) plays a crucial role in exporting cellular free cholesterol and phospholipids to an extracellular accepter, apolipoprotein A-I (apoA-I), and thus protects against the development of atherosclerosis. Therefore, increasing ABCA1 expression is considered to be an attractive approach to ameliorating excessive cholesterol accumulation and thus atherosclerosis.

ABCA1 expression is upregulated mainly by the liver X receptor (LXR) at the transcription level. Our previous research revealed that Ibrolipim and transforming growth factor-β (TGF-β) upregulate ABCA1 expression in THP-1 macrophage-derived foam cells through the LXRα pathway. In addition, ABCA1 is rapidly degraded by calpains, which appear to be an important system for regulating the activity of ABCA1 in mediating cellular cholesterol efflux. Our group has shown that the apoA-I mimetic peptide D4-F stabilizes ABCA1 protein and enhances ABCA1-dependent cholesterol efflux, but has no effect on ABCA1 mRNA levels.

Adachi et al reported that tBHQ induces translocation of Nrf2 into the nucleus concomitantly with induction of human Nrf2-specific RCT is one of the most important cardioprotective mechanisms. It is widely accepted that removal of excess free cholesterol from arterial cells is very important for maintaining cellular cholesterol homeostasis, decreasing the size of atherosclerotic plaque, and protecting against atherosclerosis. ATP-binding cassette transporter A1 (ABCA1) plays a crucial role in exporting cellular free cholesterol and phospholipids to an extracellular accepter, apolipoprotein A-I (apoA-I), and thus protects against the development of atherosclerosis. Therefore, increasing ABCA1 expression is considered to be an attractive approach to ameliorating excessive cholesterol accumulation and thus atherosclerosis. ABCA1 expression is upregulated mainly by the liver X receptor (LXR) at the transcription level. Our previous research revealed that Ibrolipim and transforming growth factor-β (TGF-β) upregulate ABCA1 expression in THP-1 macrophage-derived foam cells through the LXRα pathway. In addition, ABCA1 is rapidly degraded by calpains, which appear to be an important system for regulating the activity of ABCA1 in mediating cellular cholesterol efflux. Our group has shown that the apoA-I mimetic peptide D4-F stabilizes ABCA1 protein and enhances ABCA1-dependent cholesterol efflux, but has no effect on ABCA1 mRNA levels.

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ATP-binding cassette transporters in HepG2 cells. Tsai et al. showed that HO-1 regulates the expression of scavenger receptor-A (SR-A) and ABCA1 and reduces foam cell formation. However, the effects of tBHQ on ABCA1 and cholesterol efflux have not been well studied. In the present study, we investigated the role of tBHQ in ABCA1 expression and the possible molecular mechanism involved.

Methods

Materials and Preparation of oxLDL

tBHQ (Sigma Aldrich, St Louis, MO, USA), zinc protoporphyrin-IX (ZnPPIX) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were obtained as indicated. 3H-calcium was obtained from Calbiochem (Billerica, MA, USA) and the assay kit for calcium activity was obtained from BioVision (Lyons, France). BCA Protein Assay Kit was purchased from Pierce Chemical (Rockford, IL, USA). TRizol Reagent (Invitrogen, Carlsbad, USA), ReverAid™ First Strand cDNA Synthesis Kit (#k1622) (Fermentas, Burlington, ON, Canada), DyNAmo SYBR® Green qPCR Kits (Finnzymes, Espoo, Finland) and immobilon-P transfer membranes (Millipore, Milford, MA, USA) were obtained as indicated.

Human low-density lipoprotein (LDL; d=1.019–1.063 g/ml) was isolated by density ultracentrifugation from EDTA-treated plasma. The LDL supernatant was dialyzed against isosmotic saline to remove the EDTA. Oxidation of LDL was obtained by incubating it with 5 μmol/L CuSO4 at 37°C for 3 h, and then dialyzing overnight against saline prior to adding to cells. The extent of oxidation of the LDL preparations was determined by measuring the level of thiobarbituric acid reactive substance (TBARS) (PeroxO-quant, Pierce, Rockford, IL, USA). LDL processed in parallel but without CuSO4 was 1.7 TBARS/μg protein and oxidized LDL (oxLDL) was 15.6 TBARS/μg protein.

Cell Culture and Foam Cell Formation Evaluated by Oil Red O Staining

The human monocytic cell line, THP-1, was obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI1640 supplemented with 10% (v/v) fetal bovine serum at 37°C in an atmosphere containing 5% CO2. After 3–4 days, THP-1 cells were treated with phorbol-12-myristate-13-acetate (PMA: 160 mmol/L; Sigma Chemical Co) for 24 h, and then the medium was replaced with a serum-free medium and then the medium was replaced with a serum-free medium containing 0.1% (w/v) BSA, and after 6 h of incubation with medium containing 10 mg/ml apoA-I, efflux medium was collected, centrifuged to remove cell debris as above, and aliquots were taken for extraction and separated by thin-layer chromatography with the use of silica G plates developed in chloroform/methanol/ammonia (25% [w/v]/water (50:65:5:4 [v/v])). Phosphatidylcholine and sphingomyelin spots were visualized by 12 vapors and identified by comigration with standards. Relative radioactivity was measured by Phosphoscreen and quantified by PhosphorImager (Molecular Dynamics Inc). Phospholipid efflux was expressed as percent counts in the supernatant vs. total for each individual lipid.

Western Blot Analyses

Cellular and whole-tissue proteins were extracted. Protein (20 μg lysates) was loaded in each lane of SDS-PAGE using 6% gels, electrophoresed for 2 h at 100 V in buffer, and transferred to polyvinylidene fluoride membranes. After blocking in 5% fat-free dry milk, the membranes were incubated with rabbit antibodies against ABCA1 (Abcam, Cambridge, MA, USA), HO-1 (Abcam), Nrf2 (Santa Cruz Biotechnology) and β-actin (Santa Cruz Biotechnology). Antibody binding was detected by horseradish peroxidase-conjugated goat anti-rabbit antibody and ECL detection chemistry (Amersham Biosciences, Foster City, CA, USA). The relative signal intensities were determined by densitometry using Image-J. The relative densitometry was the ratio of ABCA1 to β-actin densitometry.

RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction (PCR) Analysis

Total RNA was extracted using TRizol reagent in accordance with the manufacturer’s instructions. GAPDH was used as an internal control. Primer sets were as follows: GAPDH sense, 5’-GGTCGCTGAGTATGCTGGAGT-3’ and antisense 5’-CACAGGTCTTCTGGTGGGCACTC-3’; ABCA1 sense, 5’-GTCTCCC-TCCCCGCATCCCTGCG-3’ and antisense 5’-AGTTCCGAA- GTGCTCAGTCGAC-3’; BC01 5’-GTCTTTCCCTGGGCACTCCTGCG-3’. Relative-time quantitative PCR, using SYBR Green detection chemistry, was performed on a Roche Light Cycler Run 5.32 Real-Time PCR System. Melt curve analyses of all real-time PCR products were performed and shown to produce a single DNA duplex. Quantitative measurements were determined using the ΔΔ Ct method, and expression of β-actin was used as the internal control.

Transfection of siRNA

Predesigned small interfering RNAs (siRNAs) against human Nrf2 and HO-1 were obtained from the Santa Cruz Biotechnology and the negative control siRNAs were purchased from Biology Engineering Corporation (Shanghai, China). THP-1 macrophage-derived foam cells (2 × 106 cells/well) were transfected with the siRNA of Nrf2, HO-1 or control using Lipofectamine 2000 (Invitrogen). After 48 h incubation, the siR-
Measurement of Calpain Activity

Calpain activity assay was performed according to the manufacturer’s protocol (BioVision). Briefly, cellular lysates (100 mg) were mixed with reaction buffer and a fluorogenic substrate, Ac-LLY-AFC. The level of released AFC was measured over

NAs of Nrf2 and HO-1 suppressed the expression of Nrf2 and HO-1 proteins by 84% and 86%, respectively, according to Western blot analysis.

Figure 2. Tert-butylhydroquinone (tBHQ) attenuates cholesterol accumulation via promotion of cholesterol efflux in foam cells. (A) Dose-dependent effect of tBHQ on cholesterol efflux. Foam cells were incubated with indicated concentrations of tBHQ for 24 h. Apolipoprotein A-I (ApoA-I)-mediated cholesterol efflux assay was analyzed by liquid scintillation counting assays as described. (B) Time-dependent effect of tBHQ on cholesterol efflux. Foam cells were treated with 100 μmol/L of tBHQ for indicated times, and apoA-I-mediated cholesterol efflux was analyzed as shown. (C, D) Dose- and time-dependent effects of tBHQ on apoA-I-mediated phospholipid efflux. Foam cells were treated with tBHQ as indicated, and apoA-I-mediated phospholipid efflux was calculated by subtracting the efflux to medium and expressed as the percentage of total cellular and medium phospholipids. (E) Effect of tBHQ on cholesterol accumulation in foam cells. THP-1 macrophage-derived foam cells incubated with tBHQ (100 μmol/L) for 24 h were fixed and stained with Oil red O. Cellular nuclei were stained with hematoxylin. The magnification of each panel is ×400. All the results are expressed as mean±SD from 3 independent experiments, each performed in triplicate. *P<0.05 vs. control group, **P<0.01 vs. control group. oxLDL, oxidized low-density lipoprotein.
1 h at 37°C by fluorimetry using 400-nm excitation and 505-nm emission filters.

**High-Performance Liquid Chromatography Assays**

HPLC analysis was conducted as described previously. Cells were washed 3 times with PBS. The appropriate volume (usually 1 ml) of 0.5% NaCl was added to approximately 50–200 μg cellular proteins/ml. Cells were sonicated using an ultrasonic processor for 2 min. The protein concentration in cell solution was measured using BCA kit. A 0.1 ml aliquot cell solution in the same cell lysed buffer was measured for 2 min at 37°C by fluorimetry using 400-nm excitation and 505-nm emission filters.

**Statistical Analysis**

All data were presented as means±SD. All data were evaluated using 1-way ANOVA and Student’s t-test. A value of P<0.05 was considered statistically significant. All the statistical analyses were performed using SPSS 13.0 software (Chicago, IL, USA).

**Results**

**tBHQ Induces ABCA1 Protein But Not mRNA**

To test the effect of tBHQ on ABCA1 expression, THP-1 macrophage-derived foam cells were treated with various concentrations of tBHQ as indicated for 24 h or incubated with 100 μmol/L tBHQ for different time periods. Quantitative real-time PCR and Western blot were performed to measure ABCA1 mRNA abundance and protein levels, respectively. As shown in Figures 1A, B, tBHQ did not significantly alter the levels of ABCA1 mRNA. However, incubating cells with tBHQ for 24 h dose-dependently caused an increase in the protein level of ABCA1 (Figure 1C). Similar results were observed in the later time-course experiment, and tBHQ markedly increased ABCA1 protein levels especially after incubation for 24 h (Figure 1D). These findings suggest that tBHQ upregulates ABCA1 protein expression without a significant effect on ABCA1 mRNA levels.

**tBHQ Enhances Cholesterol and Phospholipid Efflux From THP-1 Macrophage-Derived Foam Cells**

Next, we examined the effects of tBHQ on apoA-I-specific cholesterol efflux from THP-1 macrophage-derived foam cells, and found that, starting at a concentration of 100 μmol/L, tBHQ significantly enhanced cellular cholesterol efflux (Figure 2A).
tBHQ Increases the ABCA1 Protein Level by Inhibiting Calpain Activity

We found that tBHQ increased the ABCA1 protein level, but had no effect on the level of ABCA1 mRNA, implying that tBHQ may have no effect on the transcription of ABCA1 and the stability of its mRNA. Further analysis was then performed to examine the protein stability of ABCA1 with or without tBHQ. We pretreated THP-1 macrophage-derived foam cells with cycloheximide to block de novo protein synthesis, and then incubated the cells with and without tBHQ, followed by monitoring changes in ABCA1 protein levels. Results showed that the degradation rate of ABCA1 protein during 12 h incubation with tBHQ was slower than that of the control group (Figures 3A, B). We further measured the activity of calpain, a protease that promotes ABCA1 degradation. Intriguingly, tBHQ treatment dose-dependently reduced calpain activity (Figure 3C). The addition of μ-calpain dramatically decreased the ABCA1 protein level (Figure 3D); however, tBHQ essentially abolished the effect of μ-calpain on ABCA1 protein (Figure 3D). These results indicate that tBHQ increases ABCA1 protein expression by inhibiting its degrada-

Our results in showed that 100 μmol/L of tBHQ significantly increased cellular cholesterol efflux in a time-dependent manner (Figure 2B). We also examined the effect of tBHQ on phospholipid efflux, because ABCA1 is involved in the regulation of cellular cholesterol and phospholipid homeostasis. Foam cells were incubated with [3H] choline chloride to label choline-containing phospholipids and tBHQ treatment resulted in a significant increase of apoA-I-mediated phospholipid efflux (Figures 2C, D).

Elimination of excessive cholesterol from macrophages in the artery wall is a defense against foam cell formation and the development of atherosclerosis. We thus detected an effect of tBHQ on foam cell formation. Our results for Oil red O staining showed that tBHQ treatment markedly reduced cellular lipid accumulation (Figure 2E). HPLC was then conducted to determine cellular cholesterol content. The concentrations of total cellular cholesterol, free cholesterol and cholesterol ester in tBHQ-treated cells were significantly lower than in the control cells, especially with 100 μmol/L of tBHQ for 24 h (Tables 1, 2). Thus, tBHQ may inhibit foam cell formation by increasing cholesterol and phospholipid efflux.

Figure 3. Effects of tert-butylhydroquinone (tBHQ) on the stability of ATP-binding cassette transporter A1 (ABCA1) protein and calpain activity. (A, B) Effect of tBHQ on ABCA1 stability. Foam cells were preincubated with cycloheximide (CHX, 2 mg/ml) for 1 h, and then treated with tBHQ (100 μmol/L) for the indicated times. Cellular lysates were prepared and the same amount of total proteins was subjected to Western blot assays to determine the protein levels of ABCA1 and β-actin. (C) Effect of tBHQ on calpain activity. Foam cells were treated with the indicated concentrations of tBHQ for 24 h and the calpain activity was determined as described. (D) Effect of tBHQ on calpain-induced ABCA1 degradation. Foam cells were pretreated with 100 μmol/L tBHQ for 12 h and then incubated with and without 0.5 μmol/L μ-calpain following digitonin permeabilization. The protein levels of ABCA1 and β-actin were analyzed by Western blot assays. The data represent the mean ± SD for 3 samples. *P<0.05 vs. control group, **P<0.01 vs. control group.
tBHQ Upregulates ABCA1 Expression

**Figure 4.** Increased ATP-binding cassette transporter A1 (ABCA1) protein expression by tert-butylhydroquinone (tBHQ) is dependent on the Nrf2/HO-1 signaling pathway. (A–D) Foam cells were transfected with control or Nrf2 siRNA, and then incubated with tBHQ (100 μmol/L) for 24 h. (A) Protein samples were immunoblotted with anti-Nrf2 or anti-β-actin antibodies. (B, C) ABCA1 and HO-1 protein expressions were determined using Western blot assays as shown. All the results are expressed as mean±SD from 3 independent experiments. *P<0.05 vs. control group, **P<0.01 vs. control group. (D) Calpain activity was determined as described. (E–G) Foam cells were transfected with control or HO-1 siRNA or pretreated with ZnPPIX for 1 h, and then incubated with tBHQ (100 μmol/L) for 24 h. (E) Protein samples were immunoblotted with anti-HO-1 or anti-β-actin antibodies. (F) ABCA1 protein levels were determined using Western blot assays. (G) Calpain activity was determined as described. The data represent the mean±SD for 3 samples. *P<0.05 vs. control group, **P<0.01 vs. control group. HO-1, heme oxygenase-1; Nrf2, nuclear factor E2-related factor 2; ZnPPIX, zinc protoporphyrin-IX.
PIX (Figure 4G). To confirm these findings, cells were transfected with HO-1 siRNA. As depicted in Figure 4F, silencing HO-1 by more than 85% (Figure 4E) almost completely blocked tBHQ-induced ABCA1 expression. Similarly, the inhibitory effect of tBHQ on calpain activity was abolished in HO-1-silenced cells (Figure 4G). Taken together, these findings demonstrate that tBHQ-induced upregulation of ABCA1 protein expression and cholesterol efflux is dependent on activation of the Nrf2/HO-1 pathway (Figure 5).

Discussion

tBHQ is a synthetic phenolic antioxidant that exhibits anticarcinogenic, anti-inflammatory, antiatherosclerotic, and antidiabetic functions in animals. Because of its potent antilipid peroxidation activity, it has been commonly used as a food antioxidant to prevent oils and fats from oxidative deterioration and rancidity. Many studies have reported the important role of tBHQ as a dietary antioxidant in preventing oxidative damage caused by active oxygen radicals in living systems. Reactive oxygen species (ROS) participate in atherogenesis through different mechanisms, including oxidative stress and inflammation, which can be reduced by antioxidants. Recent research suggests that a tBHQ diet increases the expression of LDL-related protein-1, a multi ligand endocytotic receptor involved in transporting amyloid-beta peptide out of the brain. However, the potential roles of tBHQ in atherosclerosis and lipid metabolism remain unclear.

ABCA1 is a key regulator of macrophage cholesterol efflux to apoA-I, which is a critical step in high-density lipoprotein (HDL) biogenesis and RCT, and has emerged as a potential target for therapies designed to inhibit the development of atherosclerotic vascular diseases. Previous studies from our laboratory and others have shown that several cellular factors
and proteins could interact with ABCA1 and modulate its expression. The expression of ABCA1 is regulated at the transcriptional level by LXR and at the posttranslational level by calpain-mediated proteolysis. The interaction with helical apolipoproteins to generate HDL stabilizes ABCA1 against its degradation, whereas unsaturated fatty acid or excess unesterified cholesterol destabilizes ABCA1 and impairs its dependent cholesterol efflux. Accordingly, pharmacological inhibition of ABCA1 degradation increases HDL biogenesis, leading to a therapeutic benefit by reducing cardiovascular diseases. Present study revealed that tBHQ increases ABCA1 protein levels posttranslationally and enhances cholesterol efflux. However, Lee et al demonstrated that the antioxidant quercetin induced an increase in ABCA1 transcript level, which differs from tBHQ in the regulating mechanism. We consider the difference in chemical structure may be the reason why various antioxidants do not act in a similar way.

tBHQ is a potent inducer of antioxidant protein expression and Nrf2 activation. Nrf2 is a transcription factor that forms a cytoplasmic complex with Keap1. Upon exposure to pheno- lichemicals such as tBHQ, Nrf2 dissociates from Keap1, translocates into the nucleus, and activates the transcription of antioxidant genes through ARE-dependent transcription, thereby protecting cells against the adverse effects of chemical/ radiation exposure. tBHQ exhibits a wide range of pharmacological activities, including antioxidant and anti-inflammatory actions. A recent report suggests that tBHQ induces the Nrf2/ HO-1 axis and protects against oxidative stress-induced cell injury via activation of PI3K/Akt signaling. Cheung et al revealed that tBHQ-induced HO-1 expression is mediated by calcium through the regulation of Nrf2 binding to the enhancer of HO-1. It has been indicated that HO-1 may have an ability to regulate the activity of calpain, which is a cysteine protein- ase, and selectively cleave proteins in response to calcium signals. Calpain induces proteolytic degradation of ABCA1 and disrupts the biogenesis of HDL in hepatocytes. The work reported here showed that tBHQ stimulated nuclear transloca- tion of Nrf2 and subsequently increased the expression of the ARE-dependent gene, HO-1, inhibited calpain activity and then upregulated the ABCA1 protein level. Calpains have more than 10 isoforms; the μ- and m-calpains, the most characterized isoforms, are ubiquitously expressed in mammalian tissues, and tightly regulate functional molecules through limited proteolytic cleavage. Our studies indicate that μ-calpain might be targeted by tBHQ via the Nrf2/HO-1 pathway. However, the exact isoforms involved are unclear. In addition, Wang et al have suggested that Npc1 activity modulates the ABCA1 protein translation rate. Whether tBHQ also affects the ABCA1 protein translation rate is unknown. Thus, additional work is needed to explore whether other mechanisms contribute to the action of tBHQ on ABCA1 protein levels.

In summary, we provide evidence that tBHQ increases ABCA1 protein expression and cholesterol efflux in macrophage-derived foam cells, suggesting that tBHQ may have therapeutic implications in the treatment of cardiovascular diseases by enhancing cellular cholesterol removal. The present study indicates that the mechanism by which tBHQ increases ABCA1 expression and promotes cholesterol efflux is relevant to activation of the Nrf2/HO-1 pathway, and reduction of calpain activity and its mediated protein degradation. This knowledge might turn into new therapeutic concepts for the amelioration of atherosclerosis progression.

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