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Tert-butylhydroquinone (tBHQ) protects hepatocytes against lipotoxicity via inducing autophagy independently of Nrf2 activation

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Abstract

Saturated fatty acids (SFAs) induce hepatocyte cell death, wherein oxidative stress is mechanistically involved. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a master transcriptional regulator of cellular antioxidant defense enzymes. Therefore, Nrf2 activation is regarded as an effective strategy against oxidative stress-triggered cellular damage. In this study, tert-butylhydroquinone (tBHQ), a widely used Nrf2 activator, was initially employed to investigate the potential protective role of Nrf2 activation in SFAs-induced hepatotoxicity. As expected, SFAs-induced hepatocyte cell death was prevented by tBHQ in both AML-12 mouse hepatocytes and HepG2 human hepatoma cells. However, the protective effect of tBHQ is Nrf2-independent, because the siRNA-mediated Nrf2 silencing did not abrogate tBHQ-conferred protection. Alternatively, our results revealed that autophagy activation was critically involved in the protective effect of tBHQ on lipotoxicity. tBHQ induced autophagy activation and autophagy inhibitors abolished tBHQ's protection. The induction of autophagy by tBHQ exposure was demonstrated by the increased accumulation of LC3 puncta, LC3-II conversion, and autophagic flux (LC3-II conversion in the presence of proteolysis inhibitors). Subsequent mechanistic investigation discovered that tBHQ exposure activated AMP-activated protein kinase (AMPK) and siRNA-mediated AMPK gene silencing abolished tBHQ-induced autophagy activation, indicating that AMPK is critically involved in tBHQ-triggered autophagy induction. Furthermore, our study provided evidence that tBHQ-induced autophagy activation is required for its Nrf2-activating property. Collectively, our data uncover a novel mechanism for tBHQ in protecting hepatocytes against SFAs-induced lipotoxicity. tBHQ-triggered autophagy induction contributes not only to its hepatoprotective effect, but also to its Nrf2-activating property.

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Keywords

Tert-Butylhydroquinone; autophagy; SFAs; lipotoxicity; AMPK; Nrf2

1. Introduction

Increasing evidence supports that elevated levels of circulating free fatty acids (FFAs) are closely associated with metabolic syndromes, including obesity, insulin resistance, and non-alcohol fatty liver diseases (NAFLD)[1–3]. While adipocytes have a high capacity to store excess FFAs in the form of triglyceride in lipid droplets, non-adipose tissues, such as hepatocytes, skeletal muscle cells, and pancreatic β -cells, are poor in the storage of lipids. Under the circumstance of hyperlipidemia, accumulation of excess lipid in non-adipose tissues leads to cell dysfunction, known as lipotoxicity [4]. In hepatocytes, fatty acid-induced lipotoxicity plays a critical role in the pathogenesis of NAFLD [5]. FFAs are chemically grouped into saturated and unsaturated fatty acids, with different biological functions. Overall, lipotoxicity is most commonly induced by saturated fatty acids (SFAs), such as palmitate acid (16:0), the most abundant saturated fatty acid in the diet and in circulation [6], and stearic acid (18:0), in various experimental settings [7], whereas oleic acid (18:1) in general confers protection against SFAs-induced hepatotoxicity.

The cellular mechanisms underlying SFAs-induced lipotoxicity in hepatocytes are not fully understood; however, it is well accepted that endoplasmic reticulum (ER) stress and oxidative stress are the major intracellular pathways activated by palmitate exposure and contribute to palmitate-induced cellular dysfunction, including cell death [8–13]. Through induction of ER stress, palmitate exposure activates c-Jun N-terminal kinase (JNK) activity, which causes up-regulation of Bim and PUMA, two proapoptotic BH3-only proteins, and subsequent decrease in Bcl-2/Bax ratio, leading to cell death [8, 14, 15]. In addition to ER stress, oxidative stress is also reported to participate in palmitate-induced cell death in a variety of cell types, including hepatocytes [8, 10, 16, 17]. In cardiac myocytes, palmitate increased oxidative stress, leading to JNK activation [18]. Detailed investigation in skeletal muscle cells demonstrated that palmitate-induced mitochondria dysfunction is initiated by the increased production of mitochondrial reactive oxygen species (ROS) under palmitate exposure, which is restored by the addition of ROS scavengers or oleate [19]. In hepatocytes, palmitate, but not oleate, induced ROS generation and reduced cell viability. Similarly, concurrent exposure to low-dose oleate with palmitate alleviated ROS production [17, 20].

Tert-butylhydroquinone (tBHQ) is a synthetic phenolic antioxidant, widely used as a food preservative to extend the shelf life of food. A rich body of evidence has demonstrated that tBHQ is effective in protecting against cellular dysfunction induced by oxidative stress inducers, such as alcohol, dopamine, hydrogen peroxide, and glutamate, in various cell types [21–25]. It has been well-established that tBHQ exerts its antioxidant function through a mechanism whereby it increases nuclear factor (erythroid-derived 2)-like 2 (Nrf2) protein stability via inhibition of the Kelch-like ECH-associated protein 1 (Keap1)-mediated ubiquitination [21, 24, 26]. Based on these observations, tBHQ has become one of widely employed Nrf2 activators in a variety of experimental settings.

Nrf2 activation plays an essential role in the regulation of gene expression of phase II detoxification enzymes and certain antioxidants through the antioxidant response element (ARE) [27]. In unstressed cells, Nrf2 is maintained in latent cytoplasmic complexes via association with the cytoskeleton anchor Keap1. When complex with Nrf2, Keap1 promotes ubiquitin-mediated degradation of Nrf2 [25]. Previous work revealed that oxidative stress

leads to dissociation of this complex via mechanisms which are not fully understood, thereby causing Nrf2 nuclear translocation to activate expression of its target genes [28–30]. Accumulated evidence suggests that Nrf2 activation is an effective strategy for the prevention of oxidative stress-induced cellular damage, and delay of the progress of inflammation [31, 32]. Based on these previous reports, we posited that tBHQ, via activating Nrf2, may confer protective effects on SFAs-induced hepatotoxicity. As expected, our results clearly demonstrated that tBHQ was indeed effective in preventing SFAs-induced cell death in hepatocytes. Unexpectedly, our mechanistic study revealed that the protective effect of tBHQ was independent on its Nrf2 activator property. Detailed investigations in this study uncovered for the first time that tBHQ was a strong inducer of autophagy, whereby tBHQ protects hepatocytes against lipotoxicity in hepatocytes. Furthermore, we found that autophagy induction was required for tBHQ-induced activation of Nrf2.

2. Materials and Methods

2.1. Chemicals

Chemicals, including tBHQ (112941), palmitate acid (P0500), stearic acid (S4751), bovine serum albumin (BSA, A7030), and LY294002 (L9908), were purchased from Sigma-Aldrich. Other chemicals used in this study were purchased from different companies, and were showed as follow: rapamycin (LC Laboratories, R-5000), bafilomycin A1 (Baf, Lclabs, B-1080), chloroquine (CQ, Enco, 0219391910), U0126 (Cell Signaling, 9903), AKT inhibitor IV (Akt 4, CalbioChem, 124011). Amino acid and serum starvation medium (EBSS) was obtained from HyClone Laboratories (SH30029.02). palmitate acid-BSA and stearic acid-BSA conjugates were prepared as described previously [33]. Briefly, palmitate acid or stearic acid was dissolved in ethanol and saponified with sodium hydroxide. The sodium salt was dried, re-suspended in saline and heated at 80 °C until it completely dissolved. While the solution was still warm, isovolumetric 20% (w/v) BSA was added and the mixture was stirred at 50 °C for 4 h to allow palmitate acid or stearic acid to bind to BSA. Palmitate acid-BSA or stearic acid-BSA complex (3 mmol/L fatty acid: 1.5 mmol/L BSA; molar ratio, 2:1) was then sterilized by filtering, and aliquoted for future use. tBHQ was dissolved in ethanol with a stock concentration of 200 mM. In all the experiments, equal amount of solvent (e.g. BSA, ethanol, DMSO) was given to each group.

2.2. Cell culture

Alpha mouse liver (AML)-12 hepatocyte culture was established from a mouse transgenic for human transforming growth factor α , and was obtained from the American Type Culture Collection (ATCC, CRL-2254), and was cultured in Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F-12, 1:1 (DMEM/F-12, Sigma-Aldrich, 051M8322) containing 10% (v/v) fetal bovine serum (PAA Laboratories, A15-701), 5 mg/ml insulin (Sigma-Aldrich, I9278), 5 μ g/ml transferrin (Sigma-Aldrich, T8158), 5 ng/ml selenium (Sigma-Aldrich, 229865), 40 ng/ml dexamethasone (Sigma-Aldrich, D4902), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Life technologies, 15140-122) at 37°C in a humidified O₂/CO₂ (95:5) atmosphere. Primary mouse hepatocytes, which were obtained from Celsis In Vitro Technologies (M91684), and HepG2 cells were cultured in DMEM (Sigma-Aldrich, D5648) containing 10% (v/v) fetal bovine serum, 2 mmol/L glutamine (Sigma-Aldrich, G3126), 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a humidified O₂/CO₂ (95:5) atmosphere. All experiments were repeated at least 3 times.

2.3. Cell death assays

Cell death was determined by the measurement of LDH release and propidium iodide staining. For LDH assay, culture medium was collected and detected using LDH assay kit (Thermo Scientific Inc, NC9674653) according to the manufacturer's instructions. For

propidium iodide staining, cells were trypsinized and stained with propidium iodide staining solution (BD Pharmingen, 556547) according to the manufacturer's instructions. The fluorescence was measured by flow cytometry (accuri C6, BD, CA).

2.4. Analysis of RFP-LC3 puncta

The mRFP-GFP-LC3 plasmid was kindly provided by Dr. Tom Wileman (University of East Anglia) [34]. Cells were transiently transfected with mRFP-GFP-LC3 plasmid using lipofectamine 2000 (Invitrogen, 11668) according to the manufacturer's instructions. Puncta was detected by fluorescent microscope (Nikon eclipse 80i, Nikon Instrument INC. NY). At least 50 cells were counted in each individual experiment.

2.5. Analysis of autophagic flux

The cells were pretreated with CQ (inhibitor of lysosome acidification) or Baf (inhibitor of fusion between autophagosome and lysosome) for 1 hour before tBHQ exposure. The autophagic flux was determined via detecting LC3 by Western-blot and/or using fluorescence microscope for the analysis of RFP-LC3 puncta.

2.6. Western-blot analysis

Western-blot was performed as described previously [35] and the following antibodies were used: Anti-LC3B (3868), anti-AMPK (2603), anti-phospho-AMPK (2531), anti-ERK1/2 (4695), anti-phospho-ERK1/2 (4377), anti-Nrf2 (8882), anti-HO-1 (5061), anti-Akt (9272), anti-keap1 (4617), anti-IRE1 (3294), anti-Bip (3183), and anti-histone H3 (9715) from Cell Signaling; Anti-GADD 153/Chop (SC-793), and anti- β -actin (SC-1615) from Santa Cruz Biotechnology; and anti-phospho-Akt (AF887) from R&D Biosciences.

2.7. RNA interference

Cultured cells were transfected with mouse AMPK siRNA (Santa Cruz Biotechnology, sc-45313), or mouse Nrf2 siRNA (Santa Cruz Biotechnology, sc-37049), using Lipofectamine 2000 according to the manufacturer's instructions. In the control group, cells were transfected with scrambled siRNA (Santa Cruz Biotechnology, sc-37007).

2.8. Statistical analysis

All data were expressed as mean \pm SD of at least three independent experiments. Statistical analysis was performed using a one-way ANOVA and was analyzed by post-hoc test with Fisher's least significant difference (LSD). Differences between treatments were considered to be statistically significant at $p < 0.05$.

3. Result

3.1. tBHQ protects SFAs - induced cell death in hepatocytes

We previously reported that palmitate, a 16-carbon chain saturated fatty acid, caused lipotoxicity in HepG2 cells, a human hepatoma cell line [36]. In the current study, AML-12 cells, a non-transformed mouse hepatocyte cell line, were employed. In consistence with our previous study, palmitate exhibited significant hepatotoxicity in AML-12 cells in both dose- and time- dependent manner, as determined by both morphological observation and LDH release measurement (Fig. 1A & B). To test whether tBHQ, a well-established Nrf2 activator, confers protection against SFAs-induced cell death in hepatocytes, we pretreated AML-12 cells with a progressive doses of tBHQ (0, 10, 50, 100 μ M) for 1 hour before palmitate (0.5 mM) exposure. Hepatotoxicity was assayed by morphological observation, LDH release measurement (Fig. 1C), and propidium iodide staining (Fig. 1D). Our results clearly showed that tBHQ prevented palmitate-induced cell death, with optimal dose at 50

μM tBHQ. To determine the generality of our observations, we also examined the effects of tBHQ on cell death induced by stearic acid, a 18-carbon chain saturated fatty acid. As shown in Fig. 1E & F, stearic acid exhibited marked hepatotoxicity in AML-12 cells in a dose-dependent manner (E), which was similarly alleviated by tBHQ pretreatment (F). To further corroborate our observations, we conducted the similar experiments in HepG2 cells (human hepatoma cells). Our results showed that palmitate-induced cell death in human hepatocytes was also significantly inhibited by tBHQ pretreatment (Supplementary data, Figure S1). At the doses used in this study ($<100 \mu\text{M}$), tBHQ itself did not show hepatotoxicity (Supplementary data, Figure S2).

3.2. The protective effect of tBHQ is independent on Nrf2 activation

tBHQ is one of the widely-used Nrf2 activators [21, 24]. In consistence with many previous reports, exposure of AML-12 cells to tBHQ resulted in a robust Nrf2 activation, evidenced by markedly increased nuclear Nrf2 protein abundance (Fig. 2A). Palmitate slightly attenuated tBHQ-induced Nrf2 activation. To directly determine whether Nrf2 activation contributes to the protective effect of tBHQ, Nrf2 siRNA was transfected into AML-12 cells to induce Nrf2 gene silencing. Nrf2 siRNA transfection efficiency was confirmed by decreased nuclear Nrf2 protein abundance and gene expression of Nrf2 target, HO-1 and NQO1 (Supplementary data, Figure S3). However, the siRNA-mediated Nrf2 gene silencing did not blunt the protective action of tBHQ (Fig. 2B), indicating that tBHQ protects hepatocytes against lipotoxicity via an Nrf2-independent mechanism. Similarly, sulforaphane, another Nrf2 inducer, leading to a strong Nrf2 activation, evidenced by the increased expression of heme oxygenase 1 (HO-1), a target of Nrf2, failed to protect palmitate-induced cell death (Fig. 2C).

3.3. Activation of autophagy contributes to the protective action of tBHQ against palmitate-induced lipotoxicity in hepatocytes

Previous study reported that activation of autophagy protected pancreatic beta-cell against lipotoxicity [37]. In hepatocytes, oleic acid, a 18-carbon unsaturated fatty acid, protects against palmitate-induced cell death via autophagy induction [38]. In the present study, we first examined the effect of autophagy inducers on palmitate-induced cell death in AML-12 cells. To activate autophagy, the cells were either pretreated with rapamycin, a strong autophagy inducer by inhibiting mammalian target of rapamycin (mTOR) pathway, or cultured with amino acid and serum depletion medium (EBSS), reported to induce autophagy by activating adenosine monophosphate-activated protein kinase (AMPK) [39], before palmitate exposure. Concomitant with autophagy activation, rapamycin pretreatment conferred protection against palmitate-induced hepatotoxicity in AML-12 cells (Fig. 3A & B). Similarly, when AML-12 cells were cultured in EBSS medium, lipotoxicity induced by palmitate was significantly alleviated (Fig. 3C). To determine whether the autophagy induction accounts for the anti-lipotoxicity effect conferred by tBHQ, AML-12 cells were pretreated with two autophagy inhibitors, bafilomycin A1 (Baf) and chloroquine (CQ), before tBHQ and palmitate additions. Cell death was analyzed 12 hour later by flow cytometry and LDH release detection. Our results showed that under the circumstance of autophagy inhibition, tBHQ lost its protective capability (Fig. 3D, Supplementary data, Figure S4). Similar result was obtained when palmitate was replaced by stearic acid (Supplementary data, Figure S5). Further investigation demonstrated that tBHQ exposure led to increased accumulation of LC3 (microtubule associated protein 1 (MAP1) light chain 3) puncta and LC3-II conversion in both control and palmitate-treated hepatocytes (Fig. 3E), indicating that autophagy pathway is involved in tBHQ's beneficial effect.

3.4. tBHQ is a strong autophagy inducer

To characterize that tBHQ is an inducer of autophagy, we first evaluated the effect of tBHQ on autophagosome formation via transfection of AML-12 cell with mRFP-GFP-LC3 plasmid before tBHQ exposure. Fluorescent microscopy examination demonstrated that tBHQ exposure induced obvious accumulation of LC3 puncta in both time- and dose-dependent manner (Fig. 4A & B). In parallel with these observations, both immunoblotting and flow cytometry analysis showed that tBHQ markedly increased protein abundance of autophagosome-associated LC3-II and the fluorescence density of LC3 puncta (Fig. 4C). Since the increase in LC3-II may also occur as a consequence of reduced autophagic proteolysis [40]; we subsequently assessed the influence of tBHQ on LC3-II levels in the presence of lysosomal acidification blockers that inhibit proteolysis, Baf and CQ. As shown in Fig. 4D, both Baf and CQ treatment resulted in increased LC3-II conversion. Importantly, tBHQ treatment additionally enhanced LC3-II abundance and the accumulation of LC3 puncta, indicating that tBHQ in fact increased autophagic flux. To further consolidate our observations, we also examined the effect of tBHQ on autophagy induction in mouse primary hepatocytes and human HepG2 cells. As expected, tBHQ similarly induced autophagy activation in these cells (Fig. 4E).

3.5. tBHQ-induced NRF2 activation depends on autophagy induction

tBHQ is a strong Nrf2 activator. To determine whether autophagy and Nrf2 activation, both induced by tBHQ, are interrelated, AML-12 cells were transfected with Nrf2 siRNA before tBHQ exposure. It was observed that Nrf2 gene knockdown did not blunt tBHQ-induced LC-3 puncta accumulation and LC3-II conversion (Fig. 5A). Then, we treated hepatocytes with autophagy inhibitors prior to tBHQ exposure. Our results showed that inhibition of autophagy significantly attenuated tBHQ-induced Nrf2 activation (Fig. 5B). Importantly, we found that the increase in nuclear Nrf2 protein abundance after tBHQ exposure was accompanied by significantly decreased cytosolic Keap1 levels (Fig. 5C), suggesting that autophagy induction contributes, at least partially, to tBHQ-induced Nrf2 activation via increasing Keap1 degradation.

3.6. The protective action of tBHQ is independent on ERK1/2 and Akt pathway

In the present study, we also observed that SFAs, such as palmitate, markedly inhibited the activities of ERK1/2 and Akt (Fig. 6A), two important cellular survival pathways. In contrast, tBHQ robustly activated these two pathways. We therefore postulated that ERK1/2 and/or Akt signal pathways may also involve in the tBHQ-induced protective effect on SFAs-induced lipotoxicity. However, neither U0126, a specific inhibitor for MEK/ERK1/2, nor Akt 4 and LY294002, specific Akt inhibitors, blunted the protection conferred by tBHQ exposure (Fig. 6B), suggesting that these two pathways were not involved in this process. Previous studies reported that ER stress played a critical role in palmitate-induced cell death [8, 14, 15], we therefore also examined the effect of tBHQ on ER stress pathway. Unexpectedly, our result demonstrated that tBHQ increased the expression of ER stress marker proteins, including IRE1, Chop, and Bip (Supplementary data, Figure S6), which ruled out the participation of ER stress in the protective action of tBHQ.

3.7. AMPK-activation contributes to tBHQ-triggered autophagy induction

AMPK activation/phosphorylation activates autophagic pathway [41]. To determine whether AMPK pathway is involved in tBHQ-induced autophagy activation, we first tested the effect of tBHQ on AMPK activation by immunoblot analysis of cellular phosphorylated AMPK levels. Our results revealed that tBHQ enhanced AMPK activities in both time- and dose-dependent manner (Fig. 7A). Palmitate treatment significantly decreased phosphorylated AMPK protein levels, but this reduction was attenuated by tBHQ pretreatment (Fig. 7B).

Moreover, AMPK gene knockdown by siRNA transfection (Fig. 7C) not only aggravated palmitate-induced cell death, but also abrogated the protective function of tBHQ (Fig. 7D). Furthermore, tBHQ-triggered autophagy activation was blocked by AMPK gene knockdown, evidenced by the markedly reduction of the LC3 puncta formation and LC3-II conversion (Fig. 7E). These results altogether indicated that AMPK activation contributes to tBHQ-induced autophagy activation.

4. Discussion

The present study demonstrates for the first time that tBHQ, a widely-used Nrf2 activator confers protection against SFAs-induced cell death in hepatocytes via an Nrf2-independent mechanism. The results in this study reveal that tBHQ exerts its beneficial effect through activating autophagy, which is also required for tBHQ-induced Nrf2 activation. Further investigations demonstrate that the AMPK pathway activation is mechanistically involved in tBHQ-induced autophagy activation.

SFAs-induced lipotoxicity in hepatocytes plays an essential role in the initiation and progression of NAFLD [1–3]. Therefore, strategies aiming to ameliorate lipotoxicity in hepatocytes represent an ideal therapeutic choice for the treatment of NAFLD. Previous work established that oxidative stress is mechanistically involved in the palmitate-induced cell death [8, 10, 16, 20]. Therefore, it is conceivable that the induction of cellular antioxidant defense system using Nrf2 activator may provide protection against lipotoxicity. tBHQ, the major metabolite of butylated hydroxyanisole, has been commonly used as a synthetic food antioxidant to prevent oils and fats from oxidative deterioration and rancidity due to its potent anti-lipid peroxidation activity. At cellular level, Nrf2-activating property renders tBHQ the anti-cytotoxic effect in a variety of pathological processes [21–25]. The neuro-protective effect of tBHQ has been reported in both cell culture and animal studies [23, 42]. In kidney, tBHQ protected against ischemia-reperfusion injury [43]. Moreover, a recent study showed that tBHQ administration suppressed the intestinal inflammation and reduced the mucosal damage following traumatic brain injury [44]. Our study was initially designed to test this hypothesis using tBHQ as an Nrf2 inducer in AML-12 cells, a non-transformed murine hepatocyte cell line. As expected, tBHQ pretreatment efficiently attenuated cell death induced by both palmitate and stearic acid in hepatocytes. However, the data from subsequent mechanistic investigations failed to support our original hypothesis. Although tBHQ strongly induces Nrf2 activation, its protective action is not blunted by Nrf2 siRNA transfection, suggesting that Nrf2 activation is not critically involved in this process. This notion is further supported by our result that sulforaphane, another well-established Nrf2 activator, failed to provide protection against palmitate-induced hepatocyte cell death.

Autophagy is known as a bulk protein degradation system occurred during starvation for the removal and breakdown of cellular components (organelles and proteins), thereby redistributing nutrients for maintaining the cellular energetic balance [45, 46]. Moreover, autophagy also plays a critical role in eliminating proteins and organelles that are damaged due to oxidative stress and aging [47, 48]. Emerging evidence supports that autophagy is critically involved in SFAs-induced cell death [37, 38, 49, 50]. Using INS-1 beta-cells, several groups reported that palmitate induced autophagy, which played a protective role in palmitate-induced beta-cell death [37, 50, 51]. On the contrary, another study using same cells reported that palmitate exposure suppressed autophagic turnover [52]. The similar scenario was also observed in the studies when hepatocytes were used [38, 53]. Although the effects of SFAs, such as palmitate, on autophagy activation are currently controversial, even somehow contradictory, and obviously cell-type specific, the consensus has been reached nevertheless that the induction of autophagy confers protective effect against

palmitate-induced cytotoxicity. In accordance with these previous reports, we showed in the present study that the induction of autophagy, via either rapamycin or culturing cells with amino acid and serum depletion medium, prevented palmitate-induced hepatotoxicity, confirming the important role of autophagy in palmitate-induced hepatotoxicity. The efficient protection of tBHQ against lipotoxicity in hepatocytes prompted us to hypothesize that autophagy induction contributed to tBHQ's beneficial action. This notion was indeed supported by our experimental evidence from subsequent investigations. First of all, our study clearly showed that the protective effect of tBHQ against lipotoxicity was completely abrogated by autophagy inhibitors. Secondly, tBHQ exposure resulted in significant increase in LC3-II conversion and autophagosome formation, indicatives of autophagy induction. LC3 (microtubule associated protein 1 (MAP1) light chain 3) is a homologue of yeast Atg8, widely used as a marker protein for tracing the autophagic process. LC3 is post-translational modified by a ubiquitination-like reaction and cleaved off to become a soluble form, LC3-I, exposing its carboxyl terminal glycine. Upon autophagy induction, LC3-I is modified with phosphatidylethanolamine at the glycine residue, and becomes LC3-II, which is bound to both the outer and inner membrane of the autophagosome. The formation of LC3-II is therefore a good marker to monitor the occurrence of autophagosome formation [54]. However, because of the low pH of the autolysosome, the normally used green fluorescence from the acid-sensitive GFP is lost on fusion of the autophagosome with the lysosome. In this study, we detected LC3 puncta formation based on the red fluorescence from the acid-insensitive mRFP, which is not lost until the proteins are degraded in the autolysosome [34, 45]. Lastly, the autophagic flux assay confirmed that the increased LC3-II conversion and autophagosome formation by tBHQ exposure is not due to its potential suppressive effect on lysosome function, since further increased LC3-II conversion in the presence of lysosome inhibitors was observed.

Although tBHQ has been widely used as a strong Nrf2 inducer, the mechanism by which tBHQ induces Nrf2 activity is not entirely understood. Nrf2 is a ubiquitously expressed transcription factor and highly expressed in the liver [55]. During unstressed conditions, Nrf2 is sequestered in the cytosol via forming a complex with Keap-1, whereby Nrf2 is constitutively degraded through ubiquitin-proteasome system. Keap-1 protein contains many cysteine residues, which can be modified directly through either conjugation or oxidation. Oxidative stress-induced modification causes a Keap1 conformational change, resulting in the release and nuclear translocation of Nrf2 [56–58]. At present, the induction of oxidative stress is generally considered to contribute to tBHQ-triggered Nrf2 stabilization [59]. Concordant with many previous reports, our data showed that in hepatocytes tBHQ exposure increased nuclear Nrf2 translocation. On the other hand, the concomitant inductions of both autophagy and Nrf2 by tBHQ, together with our finding that activation of autophagy, instead of Nrf2, contributes to tBHQ's protective role, implying that autophagy activation may be an upstream regulatory mechanism for tBHQ-induced Nrf2 activation. The inter-modulation between autophagy and Nrf2 activation has been recently reported. A couple of lines of evidence suggest that Nrf2 negatively regulates autophagy [60, 61]. However, in our study, Nrf2 knockdown by siRNA in hepatocytes did not affect tBHQ-induced autophagy induction. This discrepancy implies that the inter-regulation at this direction could be cell-type and/or inducer-dependent. Conversely, we demonstrated that autophagy inhibitors abrogated tBHQ-triggered Nrf2 nuclear translocation. Furthermore, we found that tBHQ exposure decreased cellular Keap1 levels, suggesting that autophagy induction by tBHQ contributes to its Nrf2-activating action via decreasing intracellular Keap1 protein abundance. This notion is indeed supported by a recent study which elegantly showed that Keap1 was in fact a target for autophagy, whose activation led to increased degradation of the Keap1 protein [62].

The mechanisms implicated in autophagy activation are multifactorial. Among these, AMPK has been regarded as a major positive regulator in autophagy. Activation of AMPK leads to inhibition of mTOR, a strong inhibitor of autophagy [41]. The fact that the AMPK activation, by either nutrient starvation or chemical activators, exhibited a protective role against palmitate-induced hepatocyte cell death supported the critical role of AMPK-autophagy axis in this process. At the present study, we demonstrated for the first time that tBHQ exposure led to AMPK activation in hepatocytes in a time- and dose-dependent manner. Knocking down AMPK expression by siRNA not only aggravated palmitate-induced lipotoxicity, but also blocked the protective role of tBHQ. Furthermore, tBHQ-triggered autophagy induction was markedly attenuated by AMPK siRNA transfection. These observations altogether confirmed that AMPK activation was critically involved in tBHQ-induced activation of autophagy and resultant protection against lipotoxicity in hepatocytes.

The underlying mechanism(s) behind the positive effect of tBHQ on AMPK activity as observed in our study are still elusive. In general, AMPK is activated by two distinct signals: a Ca^{2+} -dependent pathway mediated by Calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2)[63] and an AMP-dependent pathway mediated by liver kinase B1 (LKB1) [64]. It has been reported that tBHQ exposure caused transient increase of intracellular calcium concentration in HepG2 cells [65]. In our study, intracellular calcium was not affected by tBHQ exposure; excluding that calcium-regulated mechanisms participate in tBHQ-triggered AMPK activation. The present study did not directly measure the effect of tBHQ on intracellular AMP/ATP ratio and LKB1 activity. Considering the fact that tBHQ exposure affected multiple intracellular signaling pathways, the possibility exists that tBHQ may change intracellular AMP/ATP ratio via altering cellular energetic homeostasis. It is important to determine whether and how tBHQ affects these processes in the future. In addition to direct activation by above-mentioned pathways, phospho-AMPK is also a target of protein phosphatase 2A (PP2A), whose inhibition elicits AMPK activation [66]. In addition to AMPK, both ERK1/2 and Akt is the well-known target of PP2A. In the present study, we observed that tBHQ robustly activates ERK1/2 and Akt, implying that tBHQ may act as a PP2A inhibitor. Further investigation is warranted to elucidate the possible involvement of PP2A in tBHQ-induced AMPK activation.

In summary, our study provides evidence for the first time that via activating AMPK, tBHQ exposure in hepatocytes results in autophagy induction, whereby protecting hepatocytes against lipotoxicity induced by SFAs. Moreover, our results also demonstrate that tBHQ-induced autophagy is essential for its Nrf2-activating property. The present study adds another layer of mechanism to the established beneficial effects of tBHQ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AMPK adenosine monophosphate-activated protein kinase

ARE	antioxidant response element
Baf	bafilomycin A1
BSA	bovine serum albumin
CaMKK2	Calcium/calmodulin-dependent protein kinase kinase 2
CQ	chloroquine
EBSS	amino acid and serum depletion medium
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
FFAs	free fatty acids
JNK	c-Jun N-terminal kinase
HO-1	heme oxygenase 1
Keap1	Kelch-like ECH-associated protein 1
LC3	microtubule associated protein 1 (MAP1) light chain 3
LKB1	liver kinase B1
mTOR	mammalian target of rapamycin
NAFLD	non-alcohol fatty liver diseases
Nrf2	nuclear factor (erythroid-derived 2)-like 2
PP2A	protein phosphatase 2A
ROS	reactive oxygen species
SFAs	SFAs
tBHQ	tert-butylhydroquinone

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Highlights

- tBHQ protects hepatocytes against saturated acids-induced cell death (lipotoxicity).
- The protective action of tBHQ against lipotoxicity is independent of Nrf2 activation.
- tBHQ protects against lipotoxicity in hepatocytes via inducing autophagy.
- Induction of autophagy by tBHQ contributes to its Nrf2-activating property.
- Activation of the AMPK pathway contributes to tBHQ-induced autophagy in hepatocytes.

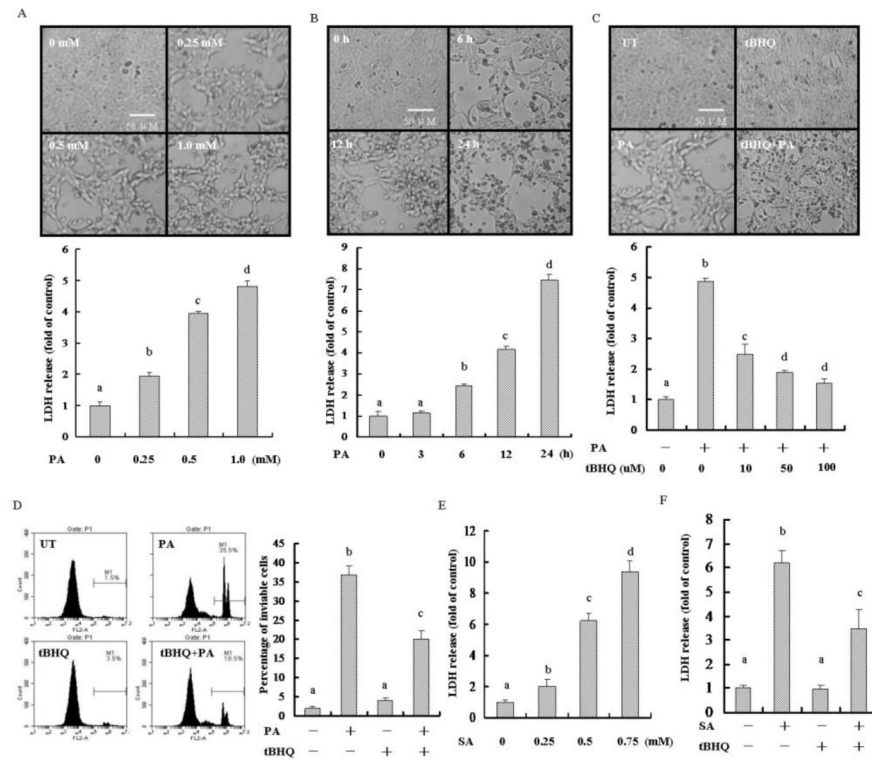


Figure 1.

tBHQ prevents SFAs-induced lipotoxicity in hepatocytes. (A) AML-12 mouse hepatocytes were treated with palmitic acid (PA) at 0, 0.25, 0.5, and 1.0 mM for 12 h. Cellular morphological changes were examined by inverted phase contrast microscope at a scope of 100 ×. LDH release was detected as described in the Methods. All values are denoted as means ± SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$. (B) AML-12 mouse hepatocytes were treated with palmitic acid (PA) at 0.5 mM for 3, 6, 12, and 24 h. Cellular morphological changes were examined by inverted phase contrast microscope at a scope of 100 ×. LDH release was detected as described in the Methods. All values are denoted as means ± SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$. (C) AML-12 mouse hepatocytes were pretreated with tBHQ (50 μM) for 1 h, followed by 0.5 mM palmitic acid (PA) treatment for 12 h. Cellular morphological changes were examined by inverted phase contrast microscope at a scope of 100 ×. LDH release was detected as described in the Methods. All values are denoted as means ± SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$. (D) AML-12 mouse hepatocytes were pretreated with tBHQ (50 μM) for 1 h, followed by 0.5 mM palmitic acid (PA) treating for 12 h. Cell death was detected by propidium iodide staining as described in the Methods. (E) AML-12 mouse hepatocytes were treated with stearic acid (SA) at 0, 0.25, 0.5, and 0.75 mM for 12 h. LDH release was detected as described in the Methods. All values are denoted as means ± SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$. (F) Cells were pre-treated with 50 μM tBHQ for 1 h, followed by 0.5 mM stearic acid (SA) treatment for 12 h. All values are denoted as means ± SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.

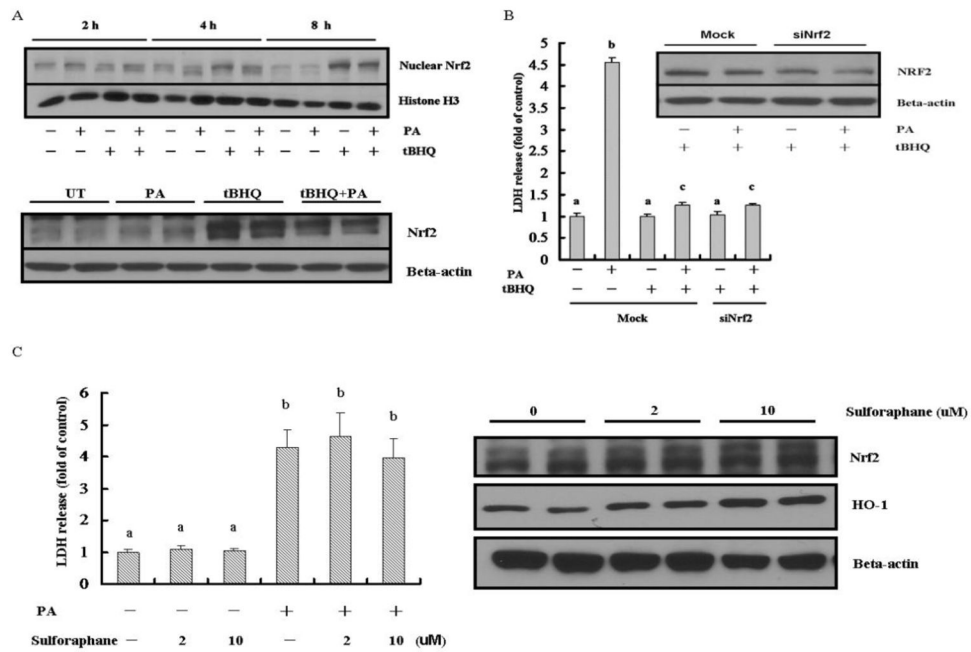


Figure 2. Nrf2 activation is not involved in tBHQ-induced protection against lipotoxicity. (A) AML-12 hepatocytes were treated with 0.5 mM palmitic acid (PA) for indicated durations. For tBHQ pretreatment experiment, tBHQ (50 μ M) was added 1 h before 0.5 mM palmitic acid (PA) exposure. Nuclear protein was extracted using a commercial Nuclear & Cytoplasmic Extraction Kit (G-biosciences, PI-78833). LDH in the cultured medium was detected as described in the Methods. Immunoblotting was performed for Nrf2. (B) AML-12 hepatocytes were transfected with Nrf2 siRNA or scrambled siRNA. LDH in the cultured medium was detected as described in the Methods. All values are denoted as means \pm SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$. (C) Sulforaphane, at indicated doses, was added 1 h before palmitic acid (PA) exposure. LDH in the cultured medium was detected as described in the Methods. Immunoblotting was performed for the detection of Nrf2 and HO-1. All values are denoted as means \pm SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.

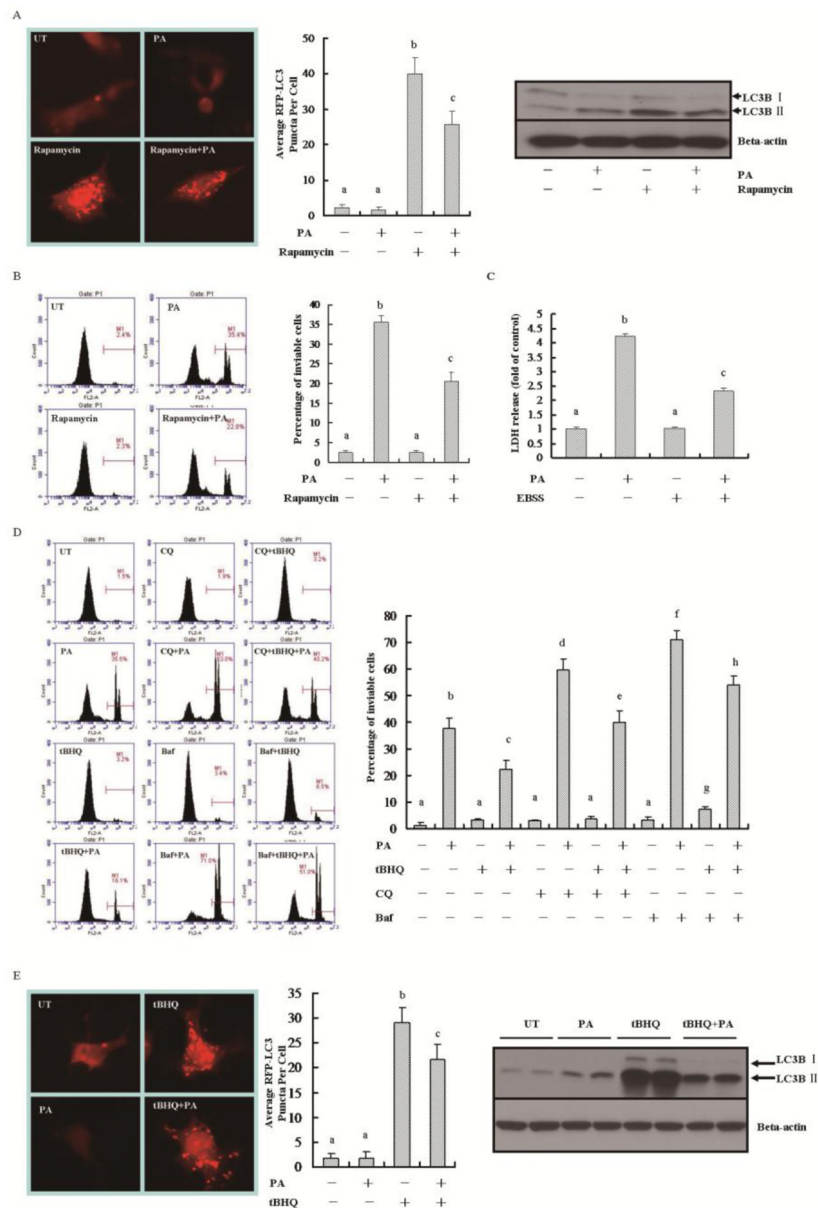


Figure 3. Autophagy induction contributes to the protective effect of tBHQ on palmitate-induced cell death. (A) AML-12 hepatocytes were treated with 0.5 mM palmitic acid (PA) for 12 h with or without rapamycin pre-treatment for 1 h. For LC3 puncta analysis, 2×10^5 /ml cells were seeded into the chamber (BD Falcon, CA) and transfected with mRFP-GFP-LC3 plasmid before palmitic acid (PA) treatment. LC3 puncta formation was examined by fluorescence microscopy. For Western blotting detection, total cell lysates were subjected to immunoblotting assay for LC3B. All values are denoted as means \pm SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$. (B) Cell death was detected by propidium iodide staining. All values are denoted as means \pm SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$. (C) AML-12 hepatocytes were treated with 0.5 mM palmitic acid (PA) for 12 h in either regular or EBSS medium. Cell death was analyzed by LDH release.

All values are denoted as means \pm SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$. (D) AML-12 cells were treated with 0.5 mM palmitic acid (PA) for 12 h with or without 1-h pre-incubation with tBHQ (50 μ M). Autophagy inhibitors, CQ (20 μ M) or Baf (100 nM), were added 1 h before tBHQ addition. Cell death was detected by propidium iodide staining. All values are denoted as means \pm SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$. (E) AML-12 cells were treated with 0.5 mM PA for 12 h. tBHQ (50 μ M) was added 1 h before PA treatment. For puncta analysis, cells were transfected with mRFP-GFP-LC3 plasmid before 0.5 mM palmitic acid (PA) treatment, and examined by fluorescence microscopy. For Western blotting detection, total cell lysates were subjected to immunoblotting assay for LC3B. All values are denoted as means \pm SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.

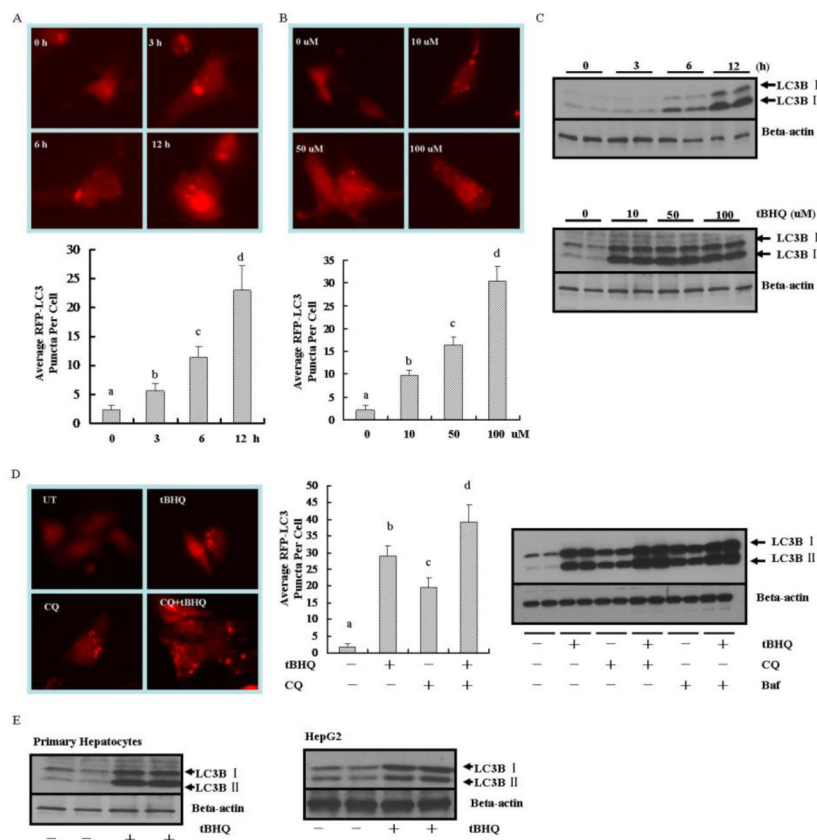


Figure 4.

tBHQ is a strong autophagy inducer in hepatocytes. (A & B) tBHQ induced puncta formation in AML-12 cells in both a time course- and a dose-dependent manner. AML-12 hepatocytes were treated with tBHQ at 50 μ M for indicated durations or at indicated doses for 12 h. For puncta analysis, AML-12 cells were transfected with mRFP-GFP-LC3 plasmid before palmitic acid (PA) treatment, and examined by fluorescence microscopy. All values are denoted as means \pm SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$. (C) tBHQ induced LC3-II conversion in AML-12 cells in both a time course- and a dose-dependent manner. AML-12 hepatocytes were treated with tBHQ at 0.5 μ M for indicated durations or at indicated doses for 12 h. Total lysates were subjected to immunoblotting assay for LC3B. (D) AML-12 cells were pretreated with chloroquine (CQ, 20 μ M) for 1 h, followed by tBHQ treatment for 12 h. Puncta formation and LC3B-II conversion were detected. All values are denoted as means \pm SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$. (E) Primary mouse hepatocytes and HepG2 cells were treated with tBHQ for 12 h. Total cellular lysates were subjected to immunoblotting assay for LC3B. All values are denoted as means \pm SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.

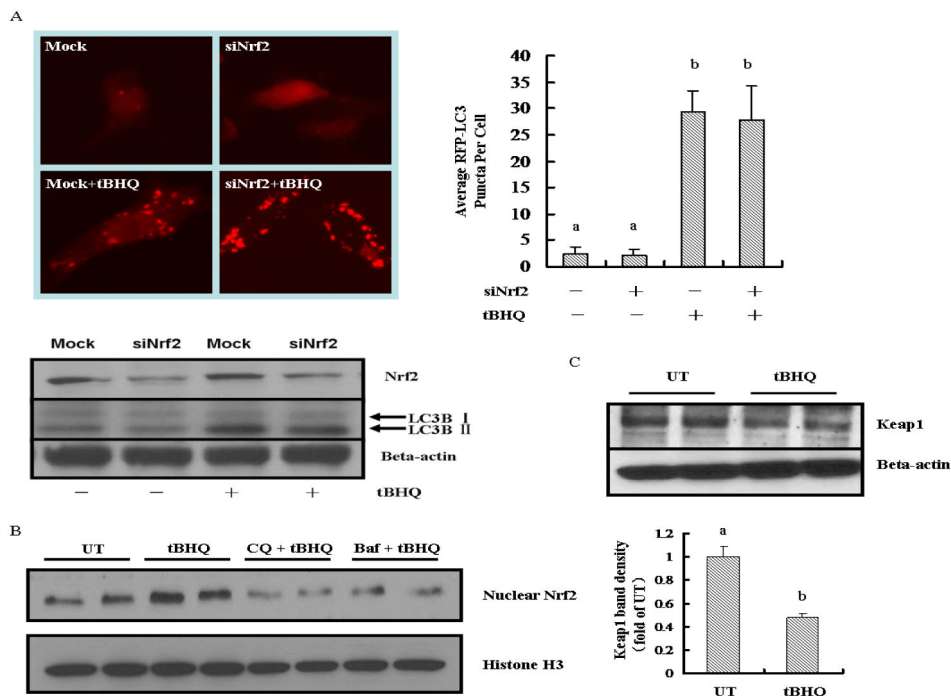


Figure 5.

Induction of autophagy is required for tBHQ-induced Nrf2 activation. (A) AML-12 cells were co-transfected with mRFP-GFP-LC3 plasmid and Nrf2 siRNA or scrambled siRNA. The effect of tBHQ on autophagy induction was examined by fluorescence microscopy for puncta formation and Western blot for LC3B-II conversion. All values are denoted as means \pm SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$. (B & C) AML-12 cells were treated with tBHQ (50 μ M) for 12 h. Autophagy inhibitor, CQ (20 μ M) or Bafilomycin A1 (Baf, 100 nM) was added 1 h prior to tBHQ treatment. Nuclear protein and total cellular lysates were subjected to immunoblotting assay for nuclear Nrf2 and Keap1, respectively. All values are denoted as means \pm SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.

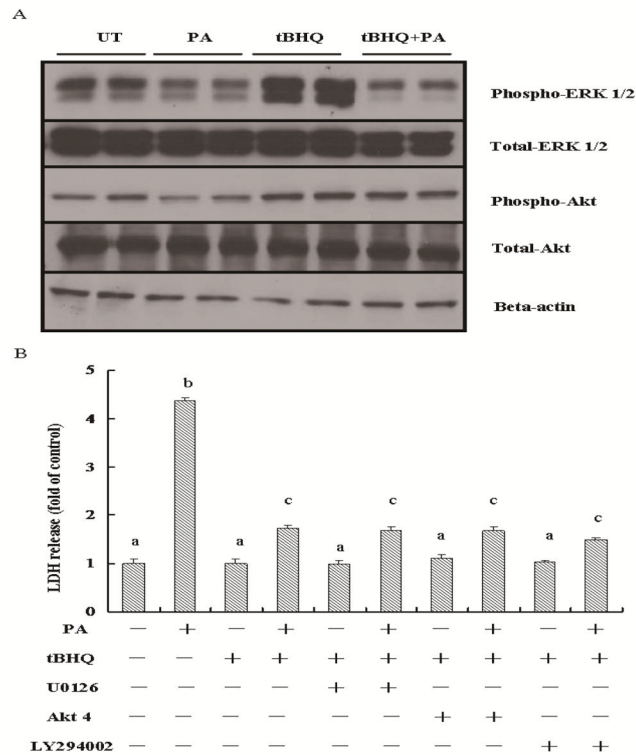


Figure 6. Neither ERK1/2 nor Akt pathway activation contributes to the protective role of tBHQ. (A) tBHQ exposure activated both ERK1/2 and Akt activation. AML-12 cells were treated with 0.5 mM palmitate acid (PA) for 12 h with or without 1 h tBHQ (50 μ M) pretreatment. Total lysates were subjected to immunoblotting assay for ERK1/2 and Akt. (B) U0126 (10 μ M), a specific MEK/ERK 1/2 pathway inhibitor, or AKT inhibitor IV (Akt 4, 50 nM) and LY294002 (10 μ M), two specific Akt inhibitors, were added into the medium 1 h before tBHQ treatment. LDH in the cultured medium was detected as described in the Methods. All values are denoted as means \pm SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.

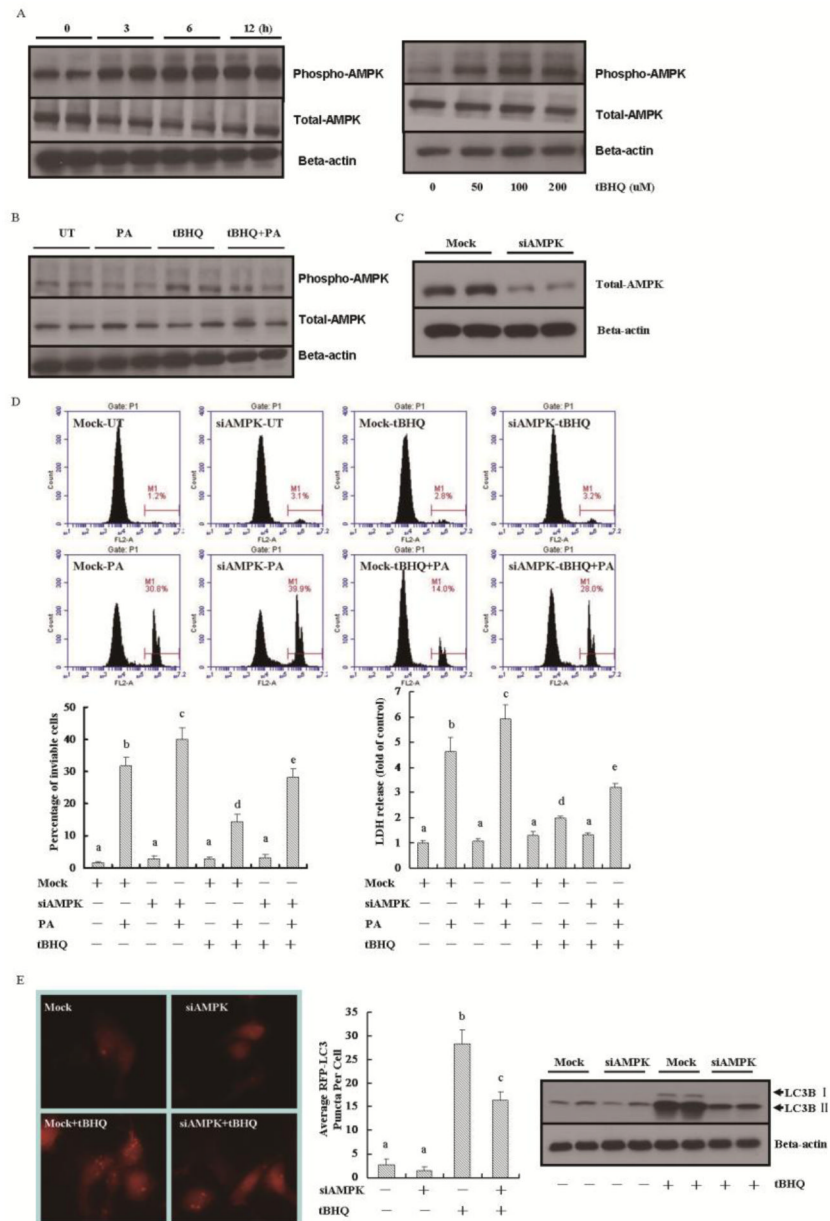


Figure 7. AMPK activation contributes to tBHQ-triggered autophagy induction. (A) AML-12 cells were treated with tBHQ (50 μ M) for indicated time period or for 12 hours at indicated dose. Whole cell lysates were subjected to Western blot for AMPK. (B) AML-12 cells were treated with 0.5 mM palmitate (PA) for 12 h with or without 1 h tBHQ (50 μ M) pretreatment. Whole cell lysates were subjected to Western blot for AMPK. (C) AML-12 cells were transfected with either scrambled or AMPK siRNA. Whole cell lysates were subjected to Western blot for AMPK. (D) AML-12 cells were transfected with either scrambled or AMPK siRNA before tBHQ (50 μ M) and/or palmitic acid (PA) exposure. Cell death was analyzed by detecting LDH release and propidium iodide staining 12 h later. All values are denoted as means \pm SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$. (E) AML-12 cells were co-transfected with mRFP-GFP-LC3 plasmid and AMPK siRNA or scrambled siRNA prior to tBHQ (50

μM) exposure. Puncta formation and LC3B-II conversion were determined by fluorescence microscopy and Western blot, respectively. All values are denoted as means \pm SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.