Protective effect of butylated hydroxyanisole against hydrogen peroxide–induced apoptosis in primary cultured mouse hepatocytes

Geun Hye Hwang¹, Yu Jin Jeon¹, Ho Jae Han², Soo Hyun Park³, Kyoung Min Baek⁴, Woochul Chang⁵, Joong Sun Kim⁶,⁷, Lark Kyun Kim⁸, You-Mie Lee¹, Sangkyu Lee¹, Jong-Sup Bae¹, Jun-Goo Jee¹, Min Young Lee¹,*¹
¹College of Pharmacy, Research Institute of Pharmaceutical Sciences, Kyungpook National University, Daegu 702-701, Korea
²Department of Veterinary Physiology, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea
³Departments of Veterinary Physiology, and Veterinary Anatomy, College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Korea
⁴Department of Cardiovascular and Neurologic Disease, College of Oriental Medicine, Daegu Haany University, Daegu 706-828, Korea
⁵Department of Biology Education, College of Education, Pusan National University, Busan 609-735, Korea
⁶Research Center, Dongnam Institute of Radiological and Medical Sciences, Busan 619-953, Korea
⁷Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06510, USA

Butylated hydroxyanisole (BHA) is a synthetic phenolic compound consisting of a mixture of two isomeric organic compounds: 2-tert-butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole. We examined the effect of BHA against hydrogen peroxide (H₂O₂)-induced apoptosis in primary cultured mouse hepatocytes. Cell viability was significantly decreased by H₂O₂ in a dose-dependent manner. Additionally, H₂O₂ treatment increased Bax, decreased Bcl-2, and promoted PARP-1 cleavage in a dose-dependent manner. Pretreatment with BHA before exposure to H₂O₂ significantly attenuated the H₂O₂-induced decrease of cell viability. H₂O₂ exposure resulted in an increase of intracellular reactive oxygen species (ROS) generation that was significantly inhibited by pretreatment with BHA or N-acetyl-cysteine (NAC, an ROS scavenger). H₂O₂-induced decrease of cell viability was also attenuated by pretreatment with BHA and NAC. Furthermore, H₂O₂-induced increase of Bax, decrease of Bcl-2, and PARP-1 cleavage was also inhibited by BHA. Taken together, results of this investigation demonstrated that BHA protects primary cultured mouse hepatocytes against H₂O₂-induced apoptosis by inhibiting ROS generation.

Keywords: apoptosis, butylated hydroxyanisole, primary mouse hepatocytes, reactive oxygen species

Introduction

Previous experimental evidences have indicated that the mechanisms underlying various types of hepatic injuries are mediated by robust generation of intracellular reactive oxygen species (ROS) [15,33]. Although ROS play normal physiological roles such as second messengers for normal cellular signaling, excessive ROS generation is known to cause cell damage and tissue injury due to an imbalance between pro- and antioxidants [19,38]. Generation of ROS during metabolism and other activities that exceeds the antioxidant capacity of a biological system gives rise to oxidative stress. Many researches have demonstrated that ROS, such as hydrogen peroxide (H₂O₂), superoxide anions, and singlet oxygen, are involved in many pathological conditions [21,25], and apoptotic cell death could be also promoted depending on the time of exposure and/or concentration of ROS [4,9]. Extracellular H₂O₂ has been used by investigators to inflict oxidative injury in a variety of cell types including neuronal progenitor cells, HepG2 cells, and hepatocytes at concentration ranging from 100 to 1,000 μM [16,22,29]. Previous studies indicated that extracellular H₂O₂ increases intracellular ROS levels via multiple mechanisms including loss of intracellular ROS antioxidants such as glutathione (GSH) [23] or decreased mitochondrial membrane permeability followed by mitochondrial ROS release [8]. Therefore, we used H₂O₂ to induce oxidative injury in primary mouse hepatocytes.

Recent reports suggest that several endogenous and exogenous...
antioxidants neutralize free radicals and protect the body by maintaining redox balance [27,34]. Antioxidants can perform protective roles against free radicals through a variety of different mechanisms including catalytic systems to neutralize or divert ROS, binding or inactivation of metal ions to prevent ROS generation through a Harber-weiss reaction, scavenging and destroying ROS or absorbing energy and electron, and quenching of ROS [28]. Phenolic compounds commonly found in both edible and inedible plants have been reported to have multiple biological or medicinal properties as well as beneficial effects on health [2,17]. Accumulating evidence has indicated that phenolic compounds exert antioxidant effects via a number of mechanisms such as ROS-scavenging activity, regulation of ROS-removing enzymes, antioxidant-synthesizing enzymes, or ROS-forming enzymes [31]. Butylated hydroxyanisole (BHA; 3-tert-butyl 4-hydroxyanisole) is one of the most widely used synthetic phenolic compounds and is regarded as an effective food additive [14]. BHA is known to have various beneficial activities such as antioxidant activity, anti-inflammatory effects, and anticancer potential although some reports suggested that this reagent induces cytotoxicity [26,30,35,39]. However, direct evidence of the effect of BHA on ROS-induced hepatocyte apoptosis has not been obtained. Therefore, we investigated the ability of BHA to protect primary cultured mouse hepatocytes against H$_2$O$_2$-induced apoptosis.

**Materials and Methods**

**Materials**

Eight-week-old male ICR mice were purchased from Daehan Bio Link (Korea). All animal management procedures were conducted in accordance with the standard operation protocols established by Kyungpook National University (permission no. KNU 2012-119). A Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Japan). BHA, H$_2$O$_2$, N-acetyl-L-cysteine (NAC), collagen, recombinant human insulin, dexamethasone, Williams’ E medium and type IV collagenase were obtained from Sigma-Aldrich (USA). 5-(and 6)-dexamethasone, Williams’ E medium and type IV collagenase were supplied by Santa Cruz Biotechnology (USA). Antibodies against Bax and Bcl-2 were obtained from New England BioLabs (USA). Anti-PARP-1, goat anti-rabbit IgG, and goat anti-mouse IgG antibodies were supplied by Santa Cruz Biotechnology (USA).

**Isolation of mouse hepatocytes**

Primary hepatocytes were isolated from mouse liver using the two-step EDTA and collagenase perfusion method. After the mouse was anesthetized, the liver was perfused with Krebs-Henseleit buffer without Ca$^{2+}$ or SO$_4^{2-}$ (115 mM NaCl, 25 mM NaHCO$_3$, 5.9 mM KCl, 1.18 mM MgCl$_2$, 1.23 mM NaH$_2$PO$_4$, 6 mM glucose, and 0.1 mM EDTA) through the hepatic portal vein to remove the blood (flow rate of 7-9 mL/min for 5 min). The liver was then perfused with Krebs-Henseleit buffer without Ca$^{2+}$ or SO$_4^{2-}$ containing 0.02% collagenase and 0.1 mM CaCl$_2$ until the liver appeared soft. Next, the liver was removed and gently minced, and the released cells were dispersed in Dulbecco’s modified Eagle medium (DMEM; Life Technologies) containing 10% FBS and 1% penicillin/streptomycin (Life Technologies). The solution containing the mixed cells and debris was passed through a 100-µm cell strainer (BD Bioscience, USA). Subsequently, the filtrate was centrifuged at 50 × g for 3 min at 4°C. The isolated cells were washed three times with DMEM and then seeded in collagen-coated plates. The cells were maintained in DMEM containing high glucose (4.5 g/L) supplemented with 10% FBS, 1% penicillin/streptomycin, 1 µg/mL insulin, and 10$^{-12}$ M dexamethasone for 24 h at 37°C in a humidified atmosphere (5% CO$_2$). The cells were then incubated with fresh Williams’ E medium without FBS 24 h prior to the experiments.

**Cell viability assay**

Cell viability was measured using a CCK-8 assay. Mouse hepatocytes were cultured in 96-well plates (BD Bioscience) in triplicate for each group. The cells were treated with H$_2$O$_2$ with or without pretreatment with BHA or NAC for 30 min and cultured at 37°C for 24 h. And 10 µL of CCK-8 solution was added to each well followed by further incubation at 37°C for 3 h. Absorbance was measured at 450 nm using a microplate reader (BioTek Instruments, USA). All values are expressed as the mean ± standard error (SE) of triplicate experiments. The values were converted from absolute counts to a percentage of the control.

**Western blot analysis**

Cells were directly lysed in culture dishes with RIPA buffer (Boston BioProducts, USA) supplemented with protease inhibitor cocktail mixture (Thermo Scientific). Cell lysates (30 µg) were separated using 10% or 12% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Germany). The blots were then washed with Tris-buffered solution containing Tween-20 (TBST; 10 mM Tris-HCl [pH 7.6], 150 mM NaCl, and 0.1% Tween-20), blocked with 5% skimmed milk in TBST for 1 h at room temperature, and incubated for 12 h at 4°C with the appropriate primary antibodies against Bax, Bcl-2, and PARP-1 at a 1:1,000 dilution. The membranes were then washed with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti mouse IgG antibodies (1:5,000 dilution) for 12 h at 4°C. The bands were visualized using an enhanced chemiluminescence detection system (Thermo Scientific) according to the manufacturer’s protocols.

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Fig. 1. Effect of hydrogen peroxide (H₂O₂) on the viability of primary cultured mouse hepatocytes. (A) Mouse hepatocytes were incubated with H₂O₂ at the indicated concentrations for 24 h. Cell viability was then measured by a CCK-8 assay. Values are expressed as the mean ± standard error (SE) of three independent experiments with triplicate dishes. *p < 0.05 vs. the control. (B) Cells were incubated with H₂O₂ at the indicated concentrations for 24 h. Cell lysates were subjected to Western blotting to measure the levels of Bax, Bcl-2, and PARP-1 expression. (C) The densities of bands corresponding to Bax and Bcl-2 protein were measured and the Bax/Bcl-2 ratio was calculated. Values are expressed as the mean ± SE of three independent experiments. *p < 0.05 vs. the control.

Detection of intracellular ROS
CM-H₂DCFDA (DCF-DA), which functions as a ROS-sensitive fluorophore, was used to detect intracellular ROS generation. Mouse hepatocytes on 35-mm cell culture dishes (BD Bioscience) were treated with H₂O₂ with or without pretreatment with BHA or NAC for 30 min and cultured at 37°C for 2 h. The cells were moved into the dark and incubated with 5 μM DCF-DA for 30 min at 37°C. Next, the cells were washed three times with PBS and viewed using fluorescence microscopy (DM IL LED Fluo; Leica, Germany) at 100 × magnification. Fluorescence intensity was measured using Tecan Infinite M200 Pro (Life Technologies).

Statistical analysis
All results are expressed as the mean ± SE. Differences between two mean values were analyzed by Student’s t-test. A p value < 0.05 was considered significant.

Results
BHA inhibits H₂O₂-induced apoptosis in primary cultured mouse hepatocytes
To investigate the effect of H₂O₂ on primary mouse hepatocytes, cell viability was measured with a CCK-8 assay. Fig. 1A shows that treatment of mouse hepatocytes with H₂O₂ at concentrations ranging from 100 to 1,000 μM for 24 h led to a significant decrease in cell viability in a dose-dependent manner. We also evaluated the expression of apoptotic marker proteins. H₂O₂ promoted Bax expression, decreased Bcl-2 levels, and increased PARP-1 cleavage. The Bax/Bcl-2 ratio also significantly increased in a dose-dependent manner.

To determine the effect of BHA (panel A in Fig. 2) on decreased cell viability caused by H₂O₂, cells were pretreated with various concentrations (0 ~ 10 μM) of BHA for 30 min and...
BHA ameliorates H2O2-induced oxidative stress in primary cultured mouse hepatocytes

In order to determine whether the protective effects of BHA were associated with antioxidant activities in H2O2-treated mouse hepatocytes, intracellular ROS levels were measured. As shown in panels A-G of Fig. 3, H2O2 significantly increased intracellular ROS levels. This was significantly attenuated by pretreatment with BHA (5 μM) or NAC, a common ROS scavenger (1,000 μM). NAC was used as a positive control.

Protective effect of BHA against H2O2-induced apoptosis is mediated by antioxidant activity in primary cultured mouse hepatocytes

As shown in panel A of Fig. 4, decreased cell viability due to H2O2 was significantly inhibited by pretreatment with BHA or NAC. These results were confirmed by observing cell morphology (panel B in Fig. 4). Increased Bax levels, decreased Bcl-2 expression, and cleavage of PARP-1 promoted by H2O2 were attenuated by pretreatment with BHA (panel C in Fig. 4). Additionally, increases of the Bax/Bcl-2 ratio due to H2O2 treatment were also decreased by BHA (panel D in Fig. 4).

Discussion

Oxidative stress-induced cell damage has been implicated in various disorders. In particular, oxidative stress in the liver is related to apoptotic cell death that is associated with the development of liver disease [5]. H2O2 is a major ROS produced intracellularly during many physiological and pathological processes, and causes oxidative damage. This compound has been extensively used as an inducer of oxidative stress for in vitro models. Therefore, H2O2 was selected to promote oxidative damage in the current investigation.

It has been reported that BHA has an antioxidant effect possibly related to the attenuation of oxidative stress [11]. However, the mechanism underlying the hepatoprotective effect of BHA remains unclear. In the present study, it was demonstrated that BHA protected primary cultured mouse hepatocytes against H2O2-induced cytotoxicity by preventing oxidative stress and apoptosis.

Our results showed that H2O2 treatment significantly decreased cell viability while increasing Bax expression and reducing Bcl-2 levels. Members of the Bcl-2 family, which includes Bcl-2, Bcl-xl, Bad, and Bax, are important regulators of various apoptotic pathways [32]. It has been shown that Bax exerts pro-apoptotic effects whereas Bcl-2 possesses anti-apoptotic activity [37]. In general, Bcl-2 inhibits apoptosis by negatively ...
regulating the apoptotic activity of Bax and forming Bcl-2/Bax heterodimers. The Bcl-2/Bax ratio can help determine whether a cell will live or die after being exposed to an apoptotic stimulus. In this study, a remarkable increase of the Bax/Bcl-2 ratio was observed after H2O2 treatment. Additionally, PARP-1 cleavage increased after H2O2 treatment. The cleavage of PARP is a hallmark of apoptosis [20]. PARP is known to be required for DNA repair. Pro-PARP is cleaved by activated apoptotic enzymes including caspase-3, a major mediator of apoptosis [6,13].

In this study, we demonstrated that H2O2 markedly increased cytotoxicity and intracellular ROS generation in mouse hepatocytes. Pretreatment with BHA significantly inhibited the decrease of cell viability as well as the increased intracellular ROS concentration. Additionally, our results showed that treatment with BHA down-regulates the H2O2-induced increase of the Bax/Bcl-2 ratio and PARP-1 cleavage. Previous studies have clearly shown that ROS induce apoptosis in hepatocytes [10,40]. ROS participate and regulate a diverse number of downstream signaling pathways that govern specific cellular functions [1,12] such as growth, metabolism, cell division, necrosis, apoptosis, and aging [7,18,24]. However, an imbalance in the formation and neutralization of ROS leads to oxidative stress [3]. Oxidative stress caused by ROS is responsible for a
wide variety of cellular damage and is the most widely recognized mechanism of secondary injury [36]. Following oxidative stress, the overproduction of ROS and subsequent antioxidant depletion results in a total breakdown of the endogenous antioxidant defense mechanisms, culminating in the failure to protect cells from oxidative damage [16].

In conclusion, data from this study demonstrated that BHA possesses antioxidant activity. Additionally, pretreatment with BHA prevented apoptotic cell death induced by oxidative stress in mouse hepatocytes. Therefore, BHA may be beneficial for treating liver diseases caused by oxidative stress.

Acknowledgments

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Conflict of Interest

There is no conflict of interest.

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