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BBR Induces Apoptosis in HepG2 Cell Through an Akt-ASK1-ROS-p38MAPKs-Linked Cascade

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ABSTRACT

Berberine (BBR) has indicated significant antimicrobial activity against a variety of organisms including bacteria, viruses, and fungi. The mechanism by which BBR initiates apoptosis remains poorly understood. In the present study, we demonstrated that BBR exhibited significant cytotoxicity in human hepatoma HepG2 cells. Herein, we investigated cytotoxicity mechanism of BBR in HepG2 cells. The results showed that the induction of apoptosis in HepG2 cells by BBR was characterized by DNA fragmentation, an increased percentage of annexin V, and the activation of caspase-3. The expressions of Bcl-2 protein and pro-caspase-3 were reduced by BBR in HepG2 cells. However, Bax protein was increased in the cells. BBR-induced apoptosis was preceded by increased generation of reactive oxygen species (ROS). NAC treatment, a scavenger of ROS, reversed BBR-induced apoptosis effects via inhibition of Bax activation and Bcl-2 inactivation. BBR-induced, dose-dependent induction of apoptosis was accompanied by sustained phosphorylation of MAP Kinases (JNK and p38 MAPK), ASK1, Akt, and p53. Furthermore, SB203580, p38 inhibitor, reduced the apoptotic effect of BBR, and blocks the generation of ROS and NO as well as activation of Bax. We found that the treatment of HepG2 cells with BBR triggers generation of ROS through Akt phosphorylation, resulting in dissociation of the ASK1-mediated activation of JNK and p38 pathways. J. Cell. Biochem. 109: 329–338, 2010.

KEY WORDS: APOPTOSIS; BERBERINE; Akt; ASK1; ROS; p38

BBR, alkaloid isolated from *Berberis* species, is extensively studied for many years. The alkaloid has shown multiple biological and pharmacological activities such as antimicrobial activity [Basha et al., 2002; Hwang et al., 2003], anti-inflammatory agent [Fukuda et al., 1999; Lau et al., 2001], and anti-tumor activity [Chung et al., 2000; Hwang et al., 2006; Lin et al., 2006]. Recently, it has been shown that BBR can cause apoptosis through a mitochondria-caspases-dependent pathway in human HepG2 cells [Hwang et al., 2006]. However, the molecular mechanisms underlying BBR-induced apoptosis are not yet well defined.

Apoptosis is characterized by a number of well-defined features including cellular morphological change, chromatin condensation, DNA fragmentation, and activation of a family of cystein proteases called Caspase [Thornberry and Lazebnik, 1998]. Caspase activation is generally considered to be a key hallmark of apoptosis. Activated caspase-8 then activates downstream events either by directly cleaving and activating procaspase-3 or by cleaving the cytoplasmic protein bid, generating a fragment that activates the mitochondrial pathway [Budihardjo et al., 1999]. Mitochondrial pathway of caspase activation was mediated by Bcl-2 family. The Bcl-2 family of proteins such as anti-apoptotic Bcl-2 and Bcl-XL; pro-apoptotic Bcl-XS and Bax has been suggested to play a role in apoptosis [Kirkin et al., 2004]. In this mitochondrial death pathway, the ratio of expression of the pro-apoptotic Bax protein and the anti-apoptotic Bcl-2 proteins ultimately determines cell death or survival [Liu et al., 1996; Kluck et al., 1997; Lorenzo et al., 2002].

Recent studies have suggested that reactive oxygen species (ROS) may play an important role during apoptosis induction [Jung et al., 2001]. Many stimuli such as anticancer drugs, irradiation, TNF-α, and chemopreventive agents prompt cells to produce ROS [Larrick and Wright, 1990; Simizu et al., 1998]. It has been shown that ROS induces a number of events including mitogen-activated protein kinases (MAPKs) signal transduction pathways in mediating apoptosis [Lee et al., 2000; Aggeli et al., 2006; Benvenisti-Zarom et al., 2006]. JNK and p38 are stress-activated MAPKs that are preferentially activated by cell stress-inducing signal, including oxidative stress, environmental stress, and toxic chemical insults. The MAPKs are induced by stress response as well as cytokines and may mediate a growth arrest and the cell death [Ip and Davis, 1998; Bonni et al., 1999; Chen et al., 2003]. In the MAPK signaling

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cascades, MAPK kinase kinase (MAPKKK) activates MAPK kinase, which subsequently activates MAPK. Each MAPK is activated by distinct upstream kinases [Kyriakis and Avruch, 2001].

ASK1 is multifunctional serine/threonine protein kinase involved in the regulation of diverse physiological processes, including cell differentiation and apoptosis [Takeda et al., 2003]. It was originally discovered as a member of the MAPKKK family that activates two different MAPK cascades, SEK1/MKK7-JNK and MKK3/MKK6-p38 MAPK pathways [Ichijo et al., 1997; Saitoh et al., 1998]. Overexpression of wild-type or the constitutively active form of ASK1 has been reported to induce apoptosis in various cell types [Ichijo et al., 1997; Chang et al., 1998; Saitoh et al., 1998], and the kinase-inactive mutant of ASK1 inhibited apoptosis by tumor necrosis factor-α, Fas ligation, anticancer drugs, or withdrawal of neurotrophic factors [Ichijo et al., 1997; Chang et al., 1998; Chen et al., 1999; Wang et al., 1999; Kanamoto et al., 2000].

Investigations of the signal transduction pathways responsible for such apoptotic mediator induction leading to cell survival or apoptosis have focused on the MAPK and Akt pathway. Intracellular, MAPKs and Akt are the major oxidative stress-sensitive signal transduction pathways [Shi et al., 2003; Cavalho et al., 2004].

Nitric oxide (NO) is a molecular gas that is produced from the amino acid L-arginine by NO syntheses (NOS) [Gustaffson, 1998]. NO displays a variety of biological functions including smooth muscle relaxation, neurotransmission, immune regulation, cellular differentiation, and host defense [Gustaffson, 1998; Jeremy et al., 1999].

However, it is still unknown whether BBR is related with ASK1-p38 MAPK and ROS-mediated apoptosis pathway. In this report, we show that BBR can cause cell cytotoxicity through an Akt-ASK1-p38 MAPK-ROS-caspase-dependent pathway in human hepatoma HepG2 cells.

MATERIALS AND METHODS

CELL CULTURE AND DRUG TREATMENT

Human hepatoma cell line (HepG2) was obtained from the ATCC (Manassas, VA) and cultured in RPMI (Gibco) supplemented with 10% fetal bovine serum (Gibco). The cells were cultured at 37°C in a humidified chamber with 95% air and 5% CO_2. All experiments were performed in plastic tissue culture flasks (Falcon). HepG2 cells were seeded on 24-well plates or 100-mm culture dishes. After plating, cells were allowed to adhere overnight and were then treated with chemical. BBR was purchased from Sigma and stored at −20°C. BBR stock solutions were made in D.W. and diluted in medium prior to use.

DETERMINATION OF CELL VIABILITY (MTT ASSAY)

Cell viability was determined by the MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] assay. The cells were seeded in 24-well plates at a density of 4×10^4 cells/well and treated with BBR at various concentration (0, 10, 30, 50, 100, and 300 μM) for 24 and 48 h. After the exposure period, media were removed. Thereafter, the medium was changed and incubated with MTT (0.1 mg/ml) for 3 h. The viable cell number per dish is directly proportional to the production of formazan, which was solubilized in isopropanol, and measured spectrophotometrically at 570 nm.

DNA FRAGMENTATION

Apoptosis was confirmed by detecting the fragmentation of chromosomal DNA with the classic DNA ladder method. Briefly, both detached and attached cells were harvested by scraping and centrifugation, washed in PBS, resuspended in nuclear lysis buffer with RNase A (200 mg/L), and incubated for 30 min at 37°C. Protein buffer was added and then centrifuge the tubes in cold at 12,000*g* for 30 min. Transfer the samples to new 1.5 ml tubes and then extract the supernatant with 1:1 mixture of phenol: chloroform (gentle agitation for 5 min followed by centrifugation) and precipitate in two equivalence of cold ethanol and one-tenth equivalence of sodium acetate. Following the incubation at −70°C for 30 min, the DNA was pelleted by centrifugation at 12,000 rpm for 20 min, washed once with ice cold 70% ethanol, and centrifuged again. The resulting DNA pellet was allowed to air-dry, and then resuspended in 40 μl of TE (pH 7.5). An equal amount of DNA was electrophoresed in a 2.0% agarose gel and visualized under UV light after staining with ethidium bromide.

HOECHST STAINING

Cells (1×10^5 cells) were plated onto 18-mm^2 coverslips in flasks and cultured with complete medium. After they were treated with BBR, the cells were fixed with 4% formaldehyde for 20 min at room temperature and were then washed with PBS. Cold methanol was added for another 20 min at room temperature followed by washes with PBS by three times. The membrane permeable fluorescent dye Hoechst 33258 (2 μg/ml), which binds to chromatin of cells, was added to the fixed cells, and the cells were examined by an inverted Olympus IX70 microscope (Japan). Apoptotic cells were identified by condensation and fragmentation of nuclei. For each experiment, nuclei from 10 random fields of each coverslip were examined at 200× magnification.

APOPTOSIS ASSAYS

Fluorescence-associated cell sorting (FACS) analysis was performed to discriminate between intact and apoptotic cells. Staining for FITC-labeled annexin V binding to membrane phosphatidylserine and propidium iodide binding for cellular DNA was performed according to the protocol provided by the manufacturer (Boehringer Mannheim). Briefly, cells (1×10^6 cells) were suspended in buffer containing FITC conjugated annexin V and propidium iodide at appropriate concentrations. The samples were analyzed by FACS Vantage using Cell Quest Software (Beckton Dickinson) and 20,000 events from each sample were acquired to ensure adequate data.

NITRITE MEASUREMENT

Cells were cultured in 24-well plates in 1ml of culture medium until confluence. They were treated with BBR, and then the culture media were collected. Nitrite was measured by adding 100 μl of Griess reagent (1% sulphanilamide and 0.1% naphthylethylethylendiamide in 5% (v/v) phosphoric acid) to 100 μl samples of the culture medium. The absorbance at 550 nm was measured using a microplate reader.

WESTERN BLOT ANALYSIS

After the indicated BBR treatment, the medium was removed, and the cells were rinsed with PBS twice. After the addition of 0.6 ml of cold RIPA buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% Na-deoxycholate, 0.1% SDS, 1% Triton X 100) and protease inhibitors, cells were scraped followed at 4°C. Cell lysate was then subjected to a centrifugation of 14,000*g* for 15 min at 4°C. Resultant protein samples were separated by an SDS–PAGE gel and transferred onto a PVDF membrane. The filters first stained by ponceu to confirm uniform transfer of all samples and then incubated in blocking solution (PBS with 0.05% tween 20 and 5% non fat dry-milk) for 1 h at room temperature. The antibodies used in this study, caspase-3, Bcl-2 and anti-Bax were obtained from Santa Cruz Biotechnology Inc and p38, JNK, phospho-p38, and phospho-JNK were purchased from Upstate Cell Signaling. The membrane was reacted firstly with desired primary antibodies for over night at

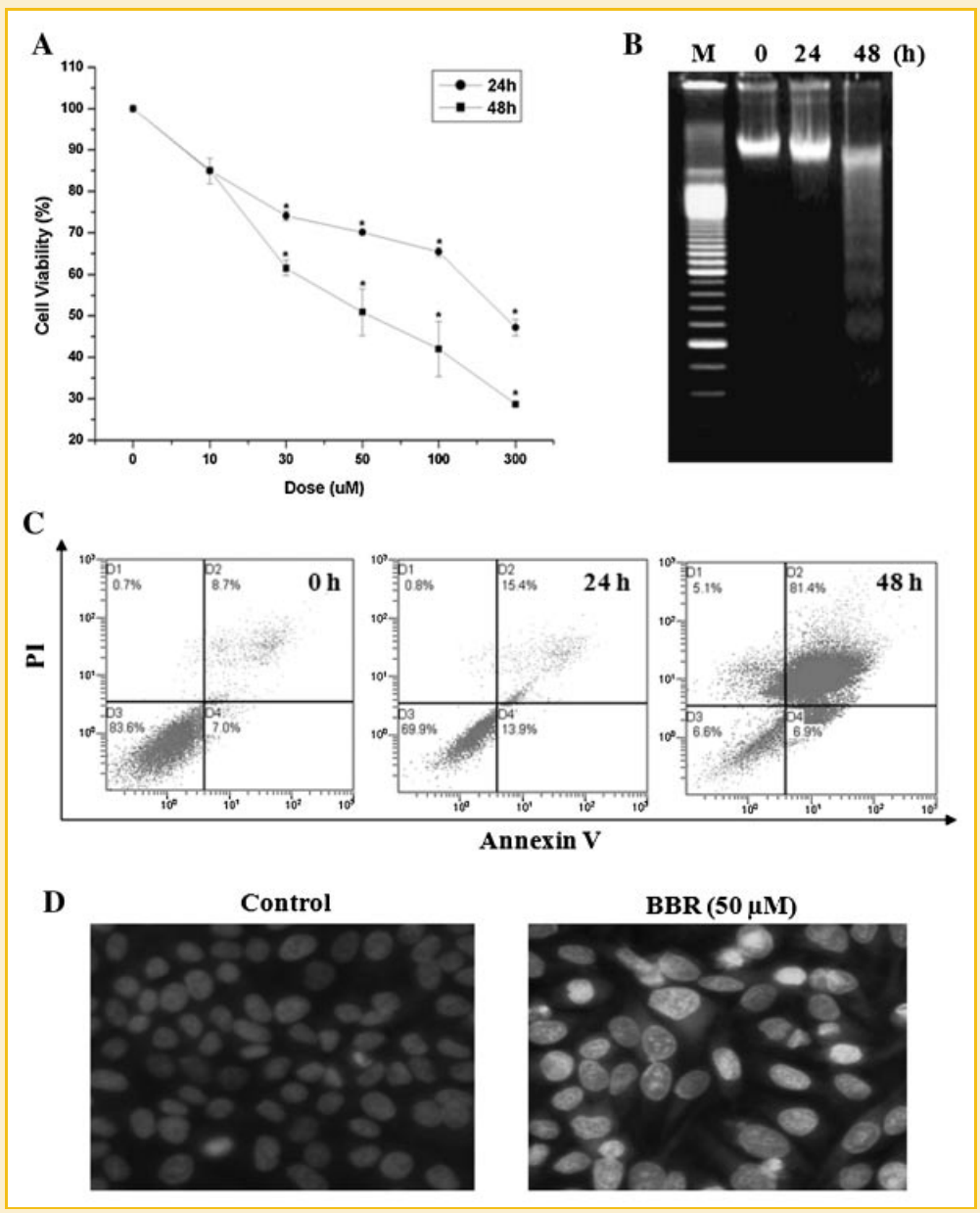

Fig. 1. Induction of apoptosis by BBR. A: HepG2 cells were treated with BBR by dose-dependent manner for 24 and 48 h. The ratios of cell viability were measured by MTT assay. Data are presented as mean ± SD of six replicates from three independent experiments. B: DNA fragmentation was measured by 2.0% agarose gel electrophoresis. C: FACS analyses of Annexin V-FITC/PI staining were performed. Lower right quadrant, early-apoptotic cells, that is, annexin V-FITC-positive/PI-negative cells; upper right quadrant, necrotic or late-apoptotic cells, that is, annexin V-FITC positive/PI-positive cells. D: Apoptosis observed by Hoechst 33258 staining (100×). After cells were treated with 50 μM of BBR for 48 h, marked morphological changes of cell apoptosis such as condensation of chromatin and nuclear fragmentations were found clearly using Hoechst 33258 staining.

−4°C. Membrane was then incubated with appropriate horseradish peroxidase-conjugated secondary antibody (Zymed) for 1 h, washed with PBST, and developed using the ECL kit.

CASPASE-3 ACTIVITY ASSAY

Cells lysates obtained from BBR-treated or untreated cells were tested for caspase-3 activities by addition of a caspase-3-specific peptide substrate conjugated with the fluorescent reporter molecule 7-amino-4-trifluoromethyl coumarin (R&D System Minneapolis, USA). The cleavage of the peptide by the caspase-3 releases the fluorochrome that when excited by light at 400 nm emits fluorescence at 505 nm. The level of caspase enzymatic activity in the cell lysate is directly proportional to the fluorescence signal detected with a fluorescent microplate reader (Fluoroskan Ascent; Labsystems, Finland).

ROS ASSAY

Intracellular generation of ROS was measured with carboxy-H_2DCFDA, which is a cell-permeable and nonfluorescent dye when loaded onto the cells. This compound is oxidized by ROS to fluorescent carboxydichlorofluorescein (DCF) inside the cells. Briefly, the cells seeded in 6-well plates (2×10^5 cells/well) and treated with or without BBR were incubated with 5 μM carboxy-H_2DCFDA for 15 min at 37°C. Then the cells were washed with phosphate buffered saline (PBS) twice, trypsinized, and resuspended in OptiMem I medium. The fluorescence resulting from the rate of oxidation of the dye in the cells was measured using a FACS with an excitation wavelength of 480 nm and an emission wavelength of 530 nm. The generation of ROS in HepG2 cells was also verified by fluorescence microscopy (Nicon, Japan). Cells grown to confluence were treated with or without BBR in the presence of 5 μM carboxy-H_2DCFDA for the indicated time, and resuspended in fresh OptiMem I medium after washing. During fluorescence imaging, the illumination level was reduced to minimal level to prevent photosenitization of the fluorescent probe.

STATISTICAL ANALYSIS

All experiments were performed in triplicates and the results were expressed as mean ± SD. Statistical significances were analyzed by one-way analysis of variance (ANOVA) with Duncan test. P value ≤ 0.05 was considered statistically significant (STATSTICA 2.0, USA).

RESULTS

BBR-INDUCED APOPTOSIS

HepG2 cells were treated with 0–300 μM of BBR, and the cell viability was assayed after 24 and 48 h using the MTT metabolism assay. BBR markedly induced cell death in HepG2 cells in a concentration- and time-dependent manner. LC_{50} was determined to be 50 μM at 48 h of exposure to BBR (Fig. 1A). To confirm the induction of apoptosis by BBR in HepG2 cells, DNA was isolated and analyzed by agarose gel electrophoresis. The gel analyses showed a typical ladder pattern of DNA fragmentation in the treatment of BBR (Fig. 1B). Cell death was also assessed with flow cytometry after double staining with annexin V and PI. The annexin V-FITC-positive/PI-negative population of cells (apoptotic cells; lower right quadrant) was increased in HepG2 cells after 24 h of BBR treatment compared with control cells. The annexin V-FITC-positive/PI-positive population of cells (necrosis and late apoptotic cells; upper right quadrant) also increased in HepG2 cells after 48 h of BBR treatment (Fig. 1C). After cells treated with 50 μM BBR for 48 h, marked morphological changes of cell apoptosis such as condensation of chromatin and nuclear fragmentation were clearly observed by Hoechst 33258 staining (Fig. 1D).

EFFECT OF BBR ON ACTIVATION OF BCL-2 FAMILY AND CASPASE-3

The activation of caspase-3 in response to BBR was examined by using the immunoblotting which is based on the fact that caspase-3 activation is initiated when apoptosis was occurred. Caspase-3 was activated at 24 h after a 50 μM of BBR treatment (Fig. 2A). The activation of caspase-3 in response to BBR was examined by specific fluorogenic peptide substrate for the detection of caspase-3 activity. As Shown in Figure 2B, the treatment of BBR induced a dramatic increase of caspase-3 activity in HepG2 cells. BBR significantly induces the activation of Bax (Fig. 2A). In addition, the expression levels of Bcl-2, an anti-apoptosis protein, was decreased by BBR treatment.

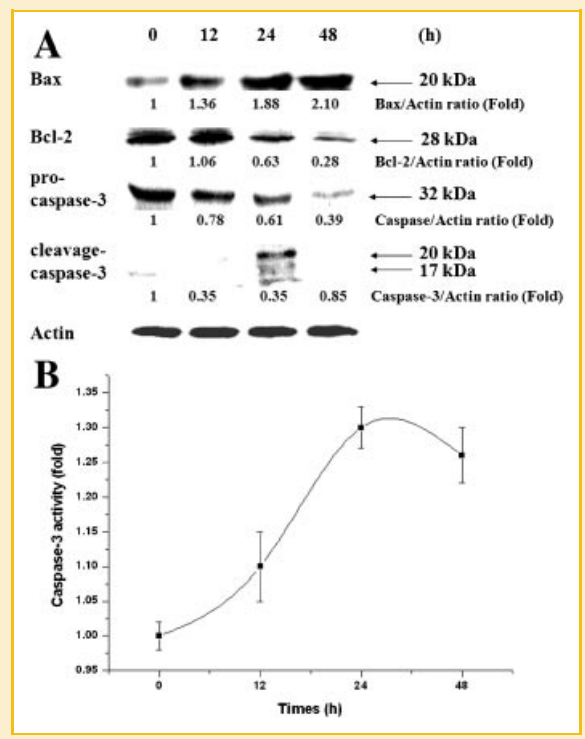

Fig. 2. Western blot analysis of apoptotic related proteins. A: After the treatment with 50 μM of BBR for 12, 24, and 48 h, Western blot was performed to detect the variation of Bax, Bcl-2, and caspase-3 (pro and cleavage). B: Caspase-3 activity by proteolytic fluorogenic substrates. Ac-DEVD-pNA was used as the substrate for caspase-3 and the cleavage of peptide was monitored at 405 nm. The caspase-3 activity of control cells was set to one and the relative changes in the activity were shown.

BBR INDUCED APOPTOSIS BY THE GENERATION OF REACTIVE OXYGEN SPECIES (ROS) IN HepG2 CELLS

As reactive oxygen species (ROS) generation is an important role in apoptosis, we investigated the ability of BBR to generate ROS. Cells were exposed to 50 μM of BBR for 24 and 48 h and analyzed for the presence of ROS by flow cytometry. The generation of ROS by 50 μM BBR was explosively increased at 24 h (Fig. 3A). We addressed this question by examining the effect of NAC, a known antioxidant, on BBR-induced ROS generation, activation of Bcl-2 family, such as Bcl-2 and Bax, and ASK1. The cells were also treated with optimized concentration of NAC (100 and 500 μM) at least 1 h before the treatment of BBR to HepG2 cells. BBR-induced cell death in HepG2 cells was significantly decreased by the pretreatment of NAC. It was further examined whether the pretreatment of NAC blocks BBR-induced activation of Bcl-2 family in HepG2 cells. HepG2 cells were treated with 50 μM of BBR for 24 and 48 h. In other treatment groups, cells were also pretreated with NAC for 1 h before the BBR treatment of HepG2 cells for 24 and 48 h. The pretreatment of NAC (100 μM) markedly inhibited BBR-induced activation of Bax, decline of Bcl-2 (Fig. 3C), and phosphorylation of ASK1 in HepG2 cells (Fig. 3D).

BBR INDUCED TRANSIENT ACTIVATION OF MAPKs PATHWAYS IN HepG2 CELLS

In order to elucidate pathways involved in the cell-death effect of BBR on HepG2 cells, MAPK response to BBR treatment was investigated. MAPKs are a family of protein kinases composed of three sub-families. As illustrated in Figure 4A, p38 MAPK, and JNK activation in HepG2 cells were induced by the treatment with 50 μM of BBR.

Investigations of the signal transduction pathways responsible for such apoptotic mediator induction leading to cell survival or apoptosis have focused on the MAPKs and Akt/protein kinase B pathway. Intracellular MAPKs and Akt are the major oxidative stress-sensitive signal transduction pathways [Shi et al., 2003; Carvalho et al., 2004]. Also, ASK1 regulates the activation of p38 MAPK and JNK pathways. The role of Akt and ASK1-p38 MAPK/JNK cascades was examined in BBR-treated HepG2 cells. The phosphorylation of ASK1 and Akt in the cells was analyzed using

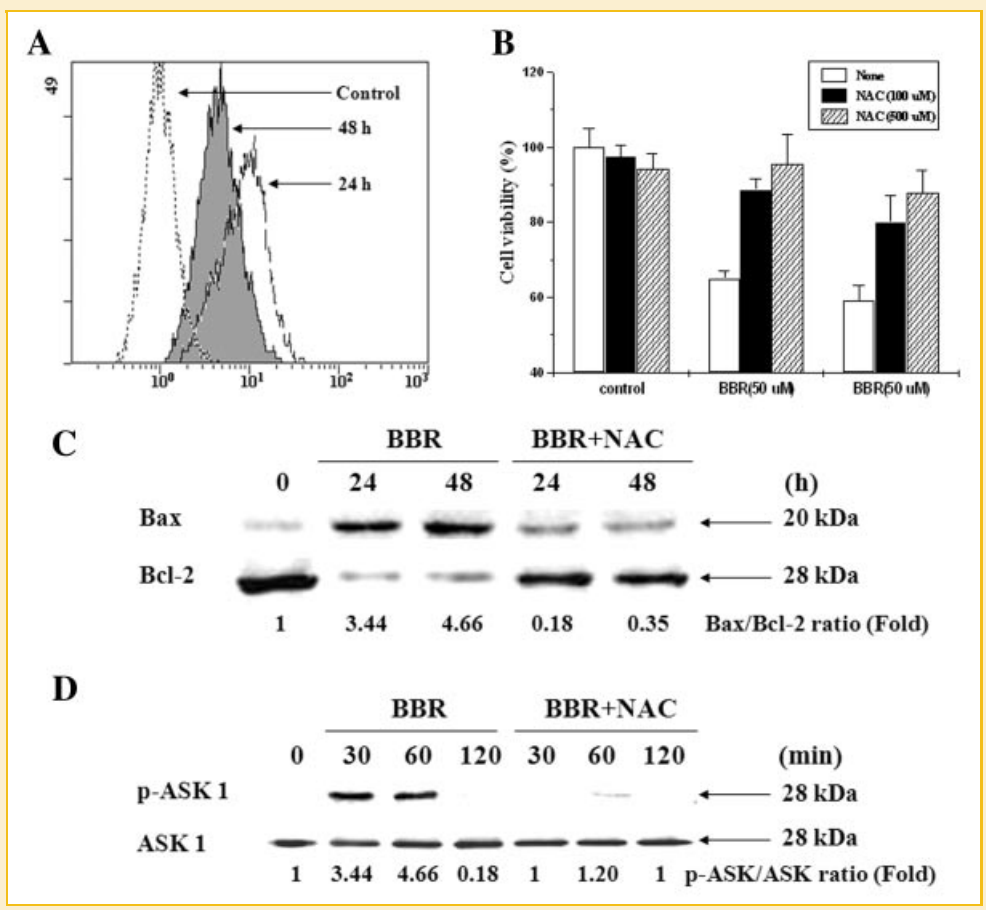

Fig. 3. Effect of BBR on the generation of ROS in HepG2 cells. A: Cells were treated with 50 μM of BBR for 24 and 48 h, and intracellular ROS were measured by FACS analysis. B: Effects of NAC on cell viability. The cells were pretreated with/without NAC (100 and 500 μM) at least 1 h before the treatment of 50 μM of BBR. C: The effects of NAC (100 μM) on the expression levels of Bcl-2 family proteins. HepG2 cells were treated with 50 μM of BBR for 24 and 48 h. Total cell lysates were analyzed by immunoblotting with antibody against Bax and Bcl-2. D: The effects of NAC (100 μM) on the expression levels of ASK1. HepG2 cells were treated with 50 μM of BBR for 30, 60, and 120 min. Total cell lysates were analyzed by immunoblotting with antibody against ASK1 and phspho-ASK1.

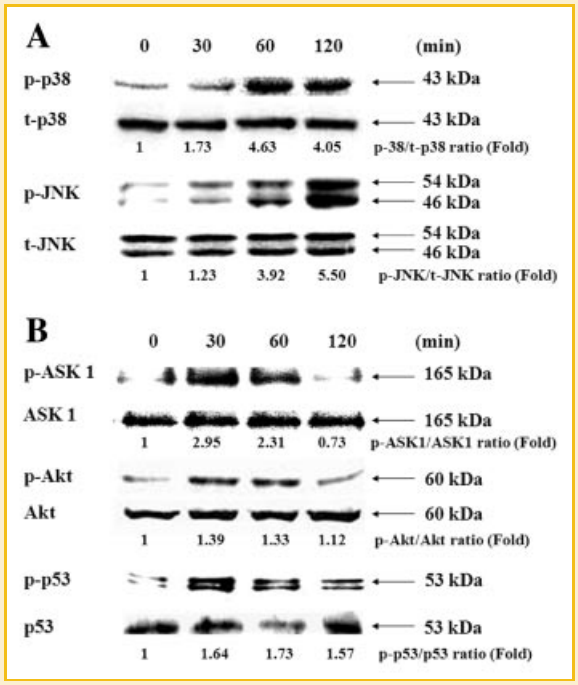

Fig. 4. Effects of BBR on MAPK (A), ASK1, Akt, and p53 (B). HepG2 cells were treated with 50 μM of BBR. Total cell lysates of cells treated with BBR for indicated time were extracted, and the phosphorylated and total proteins of p38, ERK JNK, ASK1, and p53 were immunodetected. Western blot analysis and densitometric analysis of the Western blots.

Western blot at the desired times as indicated. Amounts of ASK1 and Akt phosphorylation were increased at 30 min, and were sustained to 60 min by BBR treated cells (Fig. 4B). The activation of p53 was also studied in BBR-induced apoptosis. The activation of p53 was moderately incited by BBR treatment in time-dependent manner (Fig. 4B).

THE ROLE OF p38 MAPK ON BBR-INDUCED APOPTOSIS IN HepG2 CELLS

To further test the role of p38, the inhibitor of p38, SB203580, was pretreated in BBR treated HepG2 cells. As shown in Figure 5A, the BBR-induced cell death was reduced by SB203580. When the BBR-treated cells were pretreated by SB203580, BBR-induced the activation of Bax was decreased, but the decrease of Bcl-2 was inhibited (Fig. 5B). Furthermore, SB203580 decreased the levels of BBR-induced ROS in HepG2 cells (Fig. 5C).

BBR GENERATES THE PRODUCTION OF NITRIC OXIDE (NO) IN HepG2 CELLS

To evaluate whether NO was related with a cell death by BBR, the NO concentration in a culture media we measured at 48 h after treating cells with BBR at a range from 0 to 200 μM. In the BBR-treated HepG2 cells, NO concentration was increased in a dose-dependent manner as shown in Figure 6A. It was further investigated whether BBR could produce inducible NO synthase (iNOS), an important enzyme in NO production. The enzyme was increased by the treatment of 50 μM BBR in a time-dependent manner (Fig. 6B). Furthermore, BBR-pretreated with SB253580 resulted in inhibition of NO production (Fig. 6C).

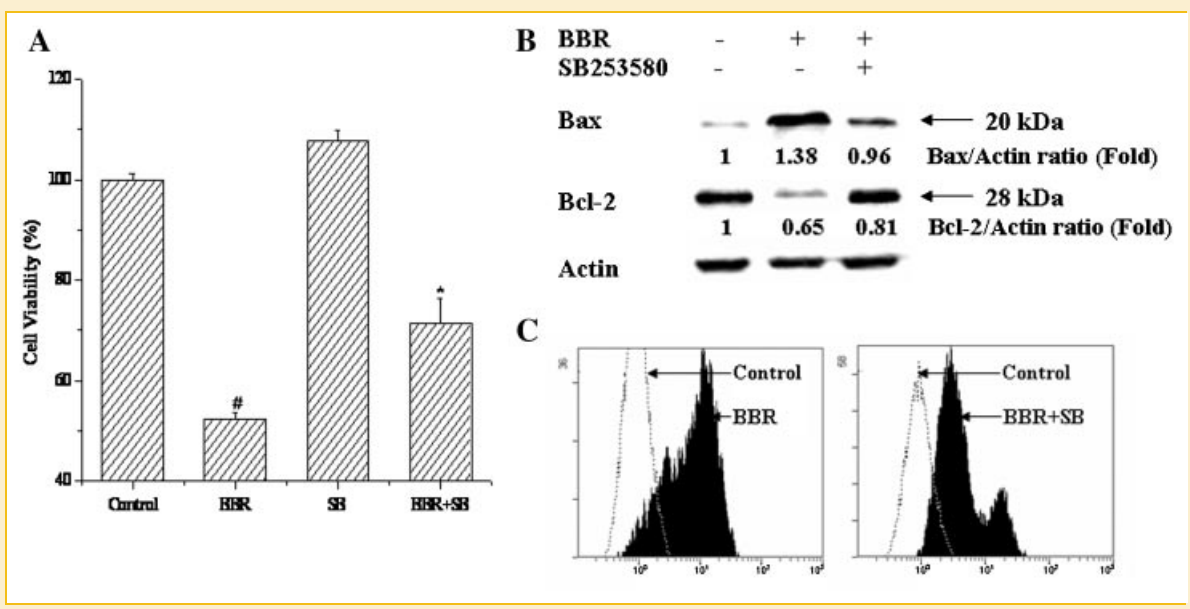

Fig. 5. Effects of p38 inhibitor, SB253580 (SB), on the BBR-induced cell death. A: Effects of SB on the growth of HepG2 cells were determined by MTT assay. SB significantly inhibited BBR-induced cell death in HepG2 cells. B: Effects of SB on BBR-induced pro- and anti-apoptotic proteins. HepG2 cells were pretreated with SB, and then treatment of BBR for 48 h, Western blot analysis was used to detect the Bcl-2 and Bax. C: Effects of SB on BBR-induced generation of ROS. $^{*}P < 0.05$ versus BBR alone. $^{\#}P < 0.05$ versus control.

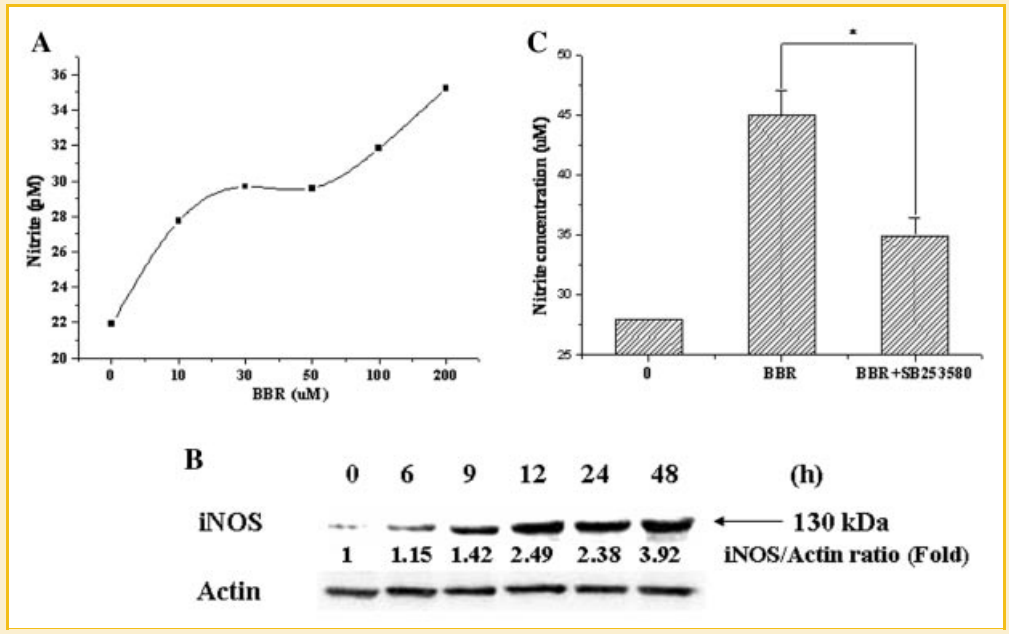

Fig. 6. BBR induces NO production and iNOS expression through p38 MAPKs in HepG2 cells. A: Cells were treated with varying dose of BBR for 48 h. B: The expression of iNOS on BBR treated cells. Cells were treated with 50 μM of BBR for the indicated times, Western blot analysis was used to detect the iNOS. C: Suppression of NO production in BBR-stimulated HepG2 cells by SB203580. $^{*}P < 0.05$ versus BBR alone.

DISCUSSION

BBR is one of the major components of *Coptis chinesis*, which is frequently utilized in proprietary Chinese herbal drugs to have wide range of pharmacological effects. It has been shown that activities of BBR are cell type specific, and that this drug may induce different cellular responses [Zheng et al., 2003; Kuo et al., 2005; He et al., 2006; Lin et al., 2006].

Mitochondria are involved in a variety of key events leading to apoptosis, as releasing of caspase activators, changes in electron transport, the production of ROS, and participation in regulation of both pro- and anti-apoptotic Bcl-2 family proteins [Corbiere et al., 2004]. In addition, BBR had anti-tumor effects in HepG2 cells including a typical ladder pattern of internucleosomal DNA fragmentation, mitochondrial membrane damage, annexin V binding, and activation of caspase indicated by decreased procaspase-8 and increased cleavage of caspase-3 [Hwang et al., 2006]. In the current study, it was confirmed that BBR induced apoptosis by DNA fragmentation and annexin V binding in human hepatoma HepG2 cells. It has been recently reported that BBR-induced the activation of caspase-8 and -3 caused the cleavage of poly ADP-ribose polymerase (PARP) and the release of cytochrome *c*, whereas the expression of bid and anti-apoptosis factor Bcl-XL were decreased [Hwang et al., 2006]. In this study, the activation of caspase-3 and Bax was induced by BBR, whereas the expression of Bcl-2 was decreased. We are not addressed that the potential contribution of Bcl-xL, BH3, and cell cycle proteins. Instead, we focused on the elucidation of related signaling molecules in response to BBR treatment in HepG2 cells. ROS play pivotal roles in DNA damage and apoptosis [Lin et al., 2003]. The ability of BBR to elevate the production of ROS was also monitored in the present study. The results indicate that the treatment of BBR leads to a shift from anti-apoptosis to pro-apoptosis and to the increased generation of ROS by altering the function of the proteins in Bcl-2 family, which results in the activation of capsase-3. These results are in agreement with the previous studies [Hwang et al., 2006; Lin et al., 2006; Jantova et al., 2007], that proposed the involvement of ROS and mitochondrial potential changes in BBR-induced apoptosis.

MAPK family includes the extracellular regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 kinase. The ERK activation has been implicated in cell proliferation and cell cycle progression, while JNK and p38 are more commonly activated in response to stress and cellular damage [Xia et al., 1995]. In this study, the activation of p38 MAPK and JNK was related in BBR-treated HepG2 cells. These results demonstrate that the activation of MAPKs pathway was mediated by BBR-induced apoptosis.

Recently, it was demonstrated that the outcome of elevated Akt in indomethacin-treated 786-O cells can be separated into two consequences: the induction of MAPK phosphorylation leading to cytotoxicity and the inhibition of caspase-8 activation to restrain apoptosis. It was described that mutual amplification between MAPKs and Akt might be the key determinant of apoptosis [Ou et al., 2007]. In the investigations of the signal transduction pathways responsible for such apoptotic mediator induction leading to apoptosis have focused on the MAPKs and AKT pathway. Our data suggest that the signaling molecules including ROS, the MAPK family and AKT are important regulators of cell viability in HepG2 cells.

This finding is consistent with recent studies demonstrating that NH2 [Goping et al., 1998] and COOH termini [Nechushtan et al., 1999] of Bax are rich in glycine and hydroxyl amino acids such as serine and threonine that are potential targets of the p38 MAPK [Ghatan et al., 2000], and critical for regulating the subcellular distribution of Bax.

ASK1 is an upstream kinase of JNK and p38 MAPK [Demoly et al., 1992]. JNK and p38 MAPK are activated through ASK1 in response to various extracellular stimuli, including hydrogen peroxide, tumor necrosis factor-α, and microtubule-disrupting agents [Ichijo et al., 1997; Saitoh et al., 1998; Wang et al., 1999]. We examined the role of ASK1-JNK and p38 MAPK cascade in HepG2 cells by BBR treatment. The results showed that JNK and p38 MAPK is activated through ASK1 in BBR-treated HepG2 cells. Thus, BBR induced the apoptosis by the activation of p38 MAPK pathway and ROS generation. ROS also activate Akt through a PI3-K-dependent mechanism [Konishi et al., 1999; Wang et al., 2000]. Therefore, our results suggest that Akt/ASK1 activity and consequent activation of downstream signaling molecules can be regulated by ROS generation.

In addition, our results show that NAC, ROS inhibitor, suppressed the apoptosis by BBR and the activation of Bax and ASK1. NAC is a potent antioxidant that can inhibit oxidative stress by directly scavenging ROS and replenishing GSH [Zafarullah et al., 2003]. If ROS production mediates BBR-induced senescence via activation of the ASK and Bax pathway, we would expect that NAC should have the ability to inhibit BBR-induced senescence while suppressing the activity of the ASK and Bax pathway. This was confirmed by the study showing that incubation of HepG2 cells with NAC prior to BBR treatment inhibited oxidative stress, attenuated ASK and Bax activation, and finally, reduced senescence induction. Although inhibition of ASK or Bax also abrogates BBR-induced senescence in HepG2 cells, it has no significant effect on BBR-induced increases in ROS production. This suggests that ROS should act upstream of the ASK-Bax pathway to mediate BBR-induced senescence in HepG2 cells (Fig. 7).

One of these pathways involves the activation of JNK/SAPK signaling cascade, which transduces extracellular stimuli into the nucleus. In fact, activated JNK translocates to the nucleus where regulates the phosphorylation of several transcription factors, such as the Jun family proteins, ATF-2, and p53 [Davis, 2000; Vogelstein et al., 2000; Shimokawa et al., 2004]. Nuclear protein p53, the transcription factor and tumor suppressor which integrates numerous signals crucial to the control of life and death of cell, depending on cell type and conditions, is a key sensor of DNA damage [Amundson et al., 1998]. Apoptosis induced by p53 appears to be switched on mitochondrial release of cytochrome *c*, which, once released from the mitochondrion, binds to the WD-40 domain and causes an unfolding of Apaf-1 (apoptosis activating factor), which exposes the nucleotide binding sites to dATP/ATP [Cain et al., 2002]. BBR induced cell cycle arrest and apoptosis of osteosarcoma cells in a dose-, time-, and p53-dependent manner [Liu et al., 2009]. In this study, we found that BBR could induce DNA fragmentation and p53 activation. From these results we can conclude that BBR can cause DNA damage, which has been well documented to be a potent trigger of p53 activation.

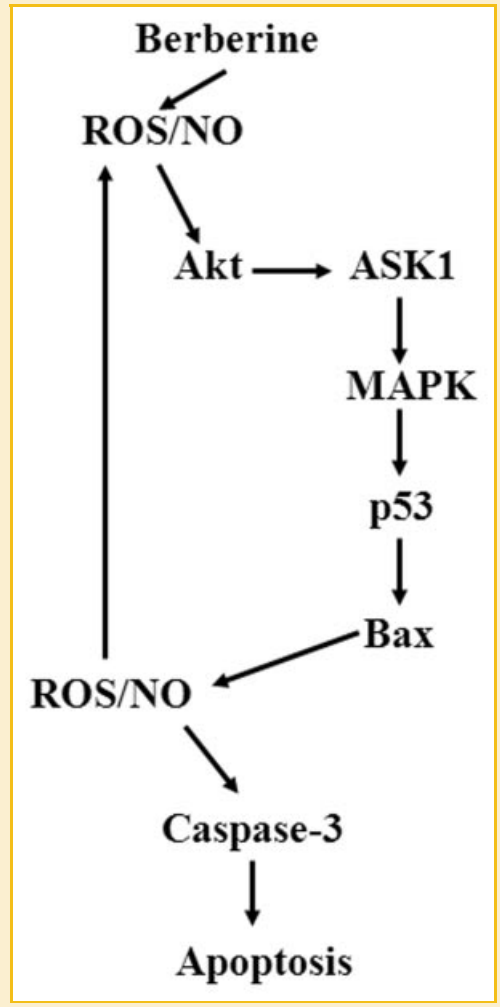

Fig. 7. A model for summarizing BBR-induced apoptosis pathway in human hepatoma HepG2 cells.

NO induces apoptosis via a pathway related to the activation of a caspase cascade [Kim et al., 2000], the release of mitochondrial cytochrome *c* [Brown and Borutaite, 1999], or the regulation of cell survival and the expression of apoptotic gene [Kim et al., 1997; Tamatani et al., 1998]. Our results show that BBR increased the production of NO in HepG2 cells. However, the generation of NO induced caspase-3-dependent apoptotic cell death with strong activation of the p38 kinase, but did not activate JNK/SAPK and in HL60 and dopamine neuronal SH-SY5Y cells [Jun et al., 1999; Ohhashi et al., 1999]. The previous studies demonstrating that NH2 [Goping et al., 1998] and COOH termini [Nechushtan et al., 1999] of Bax are rich in glycine and hydroxyl amino acids such as serine and threonine that are potential targets of the p38 MAPK [Ghatan et al., 2000], and critical for regulating the subcellular distribution of Bax. The inhibition of p38 activity with SB202190 has been known to suppress the activation of caspase-3-like proteases, as well as the cell death [Assefa et al., 2000; Tournier et al., 2000]. Also, the activation of p38 was accompanied by the generation of ROS in

many cell lines treated with cytokine, anticancer drugs, or chemopreventive agents [Chen et al., 2000]. In this study, we observed a rapid phosphorylation of p38 at the time point in BBR-treated HepG2 cells. However, the inhibition of p38 phosphorylation by a specific inhibitor, SB203580, blocked the generation of NO and ROS, as well as the cell death. These results suggested that p38 might be the targets of NO and ROS, and their activations are associated with BBR-induced apoptosis.

In conclusion, based on pharmacological and biochemical studies, we provided evidence of a possible pathway of apoptosis induced by BBR in HepG2 cells. The generation of reactive oxygen species is a crucial event and lies upstream from the signaling molecules including ASK1, AKT, MAPKs, and AKT. Mutual activation occurs between AKT and MAPKs. Cytotoxicity is associated closely with Bax and p53 expression and caspase-3 activation. Both induction and caspase-3 activation are directly or indirectly under the control of MAPKs. These findings establish a mechanistic link between the reactive oxygen species, MAPK pathway, AKT pathway, and BBR-induced cellular alterations and apoptosis.

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