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Role of Ca^{2+} influx in the tert-butyl hydroperoxide-induced apoptosis of HepG2 human hepatoblastoma cells

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Abbreviations: Fura-2, 1-(2,5-carboxyoxazol-2-yl-6-aminobenzfuran-5-oxyl)-2-(2'-amino-methylphenoxy)-ethane-N,N,N'N'-tetraacetoxylmethyl ester; GSH, glutathione; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NSCCs, nonselective cation channels; OFRs, oxygen free radicals; TBHP, *tert*-butyl hydroperoxide; BAPTA/AM, bis-(*o*-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid/acetoxymethyl ester

Abstract

Oxidative stress appears to be implicated in the pathogenesis of various diseases including alcoholic liver injury. In this study we investigated the mechanism of apoptosis induced by *tert*-butyl hydroperoxide (TBHP) in HepG2 human hepatoblastoma cells. Treatment with TBHP significantly reduced glutathione content and glutathione reductase activity, and increased glutathione peroxidase activity, indicating that TBHP induced oxidative stress in the HepG2 cells. TBHP also induced reduction of cell viability and DNA fragmentation, a hallmark of apoptosis, in a dose-dependent manner. In addition, TBHP induced a sustained increase in intracellular Ca^{2+} concentration, which was completely prevented by the extracellular Ca^{2+} chelation with EGTA. TBHP also induced Mn^{2+} influx. These results indicate that the intracellular Ca^{2+} increase by TBHP is exclusively due to Ca^{2+} influx from the extracellular site. Treatment with either an extracellular (EGTA) or an intracellular Ca^{2+} chelator (BAPTA/AM) significantly suppressed the TBHP-induced apoptosis. Taken together, these results suggest that TBHP induced the apoptotic cell death in the HepG2 cells and that Ca^{2+} influx may play an important role in the apoptosis induced by TBHP.

Keywords: *tert*-butyl hydroperoxide, oxidative stress, apoptosis, HepG2 cells, intracellular Ca^{2+}, Mn^{2+} influx

Introduction

Liver produces large amounts of oxygen free radicals in the course of detoxifying xenobiotic and toxic substances (Stohs, 1995). Normally, the produced OFRs may be scavenged by endogenous antioxidants which are abun-dant in the liver tissue (Yu, 1994). However, liver injury can occur when large acute doses or chronic exposure to toxic substances overpower the hepatic antioxidant defense system (Ishii *et al.*, 1997).

readily interact with cellular macromolecules and structures, resulting in changes in membrane permeability, activation of proteases and nucleases, and altered gene expression (Yu, 1994; Schiaffonati and Tiberio, 1997). It is well known that these cellular changes induced by OFRs lead to apoptotic cell death in a variety of cell types (Slater *et al.*, 1995; Jenner and Olanow, 1996; Stoian *et al.*, 1996). However, the mechanism of induction of apoptosis by OFRs in the hepatocytes is incompletely understood.

Thus, in this study we investigated the apoptosis induced by oxidative stress in HepG2 human hepatoblastoma cell line as a model human liver cell. In the experiments we used *tert*-butyl hydroperoxide (TBHP) as an oxidative stress inducer, since TBHP has been frequently employed in the similar types of experiments (Soszynski and Bartosz, 1997; Gorbunov *et al.*, 1998). In addition, we tested the possible involvement of intracellular Ca^{2+} signal in the action mechanism of TBHP, since intracellular Ca^{2+} acts as a mediator of apoptosis in many cell types (McConkey and Orrenius, 1996).

Materials and Methods

Materials

HepG2 human hepatoblastoma cell line was purchased from American Type Culture Collection (Rockville, MA). The powders Eagle's minimum essential medium and Earle's basal salt solution , trypsin solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), ethylene glycol-bis-(aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), sodium pyruvate, *tert*-butyl hydroperoxide (TBHP), probenecid and all salt powders were obtained from Sigma Chemical CO. (St. Louis, MO). 1-(2,5-Carboxyoxazol-2-yl-6-aminobenzfuran-5-oxyl)-2-(2'-amino-methylphenoxy)-ethane-N,N,N'N'-tetra-acetoxylmethyl ester (Fura-2) and bis-(*o*-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid/acetoxymethyl ester (BAPTA/AM) were from Molecular Probes, Inc. (Eugene, OR). Fetal bovine

serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). Fura-2 and BAPTA/AM were prepared as stock solutions in DMSO, then diluted with aqueous medium to the final desired concentrations. The solution of TBHP was diluted immediately prior to the start of the experiments and stored in ice during the experiments. The stock solution of drugs was sterilized by filtration through 0.2 μm disc filters (Gelman Sciences: Ann Arbor, MI).

Cell culture

HepG2 cells were grown at 37˚C in a humidified incubator under 5% CO_2/95% air in a MEM supplemented with 10% FBS, 200 IU/ml penicillin, 200 μg/ml of streptomycin and 1 mM sodium pyruvate. Culture medium was replaced every other day. After attaining confluence the cells were subcultured following trypsinization.

Enzyme assay

Cells were quickly collected in ice-cold 0.1 M Tris-HCl (pH 7.6) by scraping and centrifuge (1000 *g*, 5 min). Cell suspension was aliquot out to measure enzyme activities, For determination of the glutathione (GSH) peroxidase activity, cells were homogenized with sonicater and centrifuged (15000 *g*, 40 min) at 4˚C. The supernatant containing the enzyme was incubated with 0.015% H_2O_2, 1 mM GSH and 0.2 mM NADPH for 5 min at 25˚C. The enzyme activity was calculated from change in optical density (OD)/min at 340 nm using a molar extinction coefficient for NADPH of 6.22×10^3 $cm^2/\mu mol$ and assuming 2 moles of GSH formed for each mole of NADPH consumed (Tappel, 1978).

To measure the GSH reductase activity, cell homogenates were centrifuged (15000 *g*, 40 min) at 4˚C. The supernatant was incubated with 6 mM NADPH, 0.9 mM EDTA and 0.54 mM oxidized GSH for 5 min at 25˚C. The enzyme activity was calculated based upon the level of NADPH consumed (Salkie and Simpson, 1970). Total protein content was measured according to the method of Lowry *et al.* (1951).

Determination of GSH content

Harvested cells were quickly homogenized in ice-cold 8% sulfosalicylic acid and centrifuged (15,000 *g*, 30 min) at 4˚C. Then, the supernatant was incubated with 0.1 M sodium phosphate buffer (pH 7.5) containing 6 mM 5,5'-dithio-bis-2-nitrobenzoic acid, 0.3 mM NADPH and 50 units of the GSH reductase for 6 min at 30˚C. The ODs of *p*-nitrothiophenol produced were measured at 412 nm, and converted to the concentrations of GSH (Srivastava and Beutler, 1968).

Cell viability assay (MTT staining)

Cell viability was assessed by the MTT staining method (van de Loosdrecht *et al.*, 1991). Cells from 4-5-day old cultures were incubated in 1 ml of media in 24-well plates at an initial density of 5×10^4 cells/ml. Drugs to be tested were added to cultures 2 days after seeding in order to ensure uniform attachment of cells at the onset of the experiments. Culture medium was replaced every day. In control experiments cells were grown in the same media containing drug-free vehicle. After a period of incubation, 100 μl of MTT (5 mg MTT/ml in H_2O) were added and cells incubated for a further 4 h. Two hundred microliters of DMSO were added to each culture and mixed by pipetting to dissolve the reduced MTT crystals. Relative cell viability was obtained by scanning with an ELISA reader (Molecular Devices, Menlo Park, CA) with a 540 nm filter.

DNA isolation and electrophoresis

HepG2 cells were collected by centrifugation (200 *g*, 10 min), washed twice in PBS (pH 7.4) and resuspended at a density of 4×10^6 cells/400 μl in hypotonic lysing buffer (5 mM Tris, 20 mM EDTA, pH 7.4) containing 0.5 % Triton-X 100 for 30 min at 4˚C. The lysates were centrifuged at 13,000 *g* for 15 min at 4˚C. Fragmented DNA was extracted from the supernatant with phenol-chloroform-isoamylalcohol, precipitated by addition of 2 volume of absolute ethanol and 0.1 volume of 3 M sodium acetate, and treated with RNAse A (500 U/ml) at 37˚C for 3 h. The pattern of DNA fragmentation was visualized by electrophoresis in 1.8 % agarose gel containing ethidium bromide and photo-graphed under UV light. (Hockenbery *et al.*, 1990)

Quantitative analysis of fragmented DNA

For quantitative DNA analysis, HepG2 cells were collected and washed twice with PBS. Cells were resuspended in lysis buffer containing 1 mM EDTA, 10 mM Tris-HCl (pH 8.0) and 0.2% Triton X-100 and incubated on ice for 30 min. Low and high molecular weight DNAs were separated by centrifugation at 15,000 *g* at 4˚C. The supernatant was collected and the pellet resuspended in 0.5 ml of lysis buffer. DNAs from both the supernatant and the pellet were precipitated by addition of 1 N perchloric acid. The DPA method (Natarajan *et al.*, 1994) was used for measurement of DNA content. The percent change of DNA fragments was calculated with the following equation: % Fragment = [A_{570} of small DNA/(A_{570} of small and large DNA) × 100]

Intracellular Ca^{2+} measurement

Aliquots of the HepG2 cells were washed in EBSS. Then, 5 μM Fura-2 was added, and the cells were incubated for 30 min at 37˚C. Unloaded Fura-2 was removed by centrifugation at 150 *g* for 3 min. Cells were resuspended at a density of 2×10^6/ml in Krebs-Ringer buffer (KRB)

containing 125 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 25 mM HEPES, 6 mM glucose and 2.5 mM probenecid (pH 7.4). Fura-2-loaded cells were maintained at 25˚C for 90 min before fluorescence measurement. For each experiment, 0.5 ml aliquot of Fura-2-loaded cells was equilibrated to 37˚C in a stirred quartz cuvette. Fluorescence emission (510 nm) was monitored with the excitation wavelength cycling between 340 and 380 nm using a Hitachi F4500 fluorescence spectrophotometer. At the end of an experi-ment, fluorescence maximum and minimum values at each excitation wavelength were obtained by first lysis of cells with 20 μg/ml digitonin (maximum) and then adding 10 mM EGTA (minimum). With the maximum and minimum values, the 340:380 nm fluorescence ratios were converted into free Ca^{2+} concentrations using a software, F-4500 Intracellular Cation Measurement System, provided by Hitachi.

Measurement of Mn^{2+} influx

Mn^{2+} influx measurement was performed under the same conditions as described for intracellular Ca^{2+} measurement except that the excitation wavelength was 360 nm. Fluorescence of Fura-2 is quenched by Mn^{2+} at this wavelength, whereas it is not altered by changes of intracellular Ca^{2+} concentration (Grynkiewicz *et al.*, 1985). Mn^{2+} influx was started by adding 300 μM $MnCl_2$.

Data analysis

All experiments were performed four times. Data were expressed as mean ± standard error of the mean (SEM) and were analyzed using one way analysis of variance (ANOVA) and Student-Newman-Keul's test for individual comparisons. P values less than 0.05 are considered statistically significant.

Results

Induction of oxidative stress and cytotoxicity by TBHP

To confirm that TBHP induces oxidative stress in HepG2 human hepatoblastoma cells, we examined the effects of TBHP on the levels of GSH and the activities of GSH peroxidase and reductase. TBHP (200 μM) significantly decreased the GSH level and the activity of GSH reductase, but increased the activity of GSH peroxidase as shown in Figure 1. These results are comparable to the effects of other oxidative stress inducers in hepatocytes (Rossi *et al.*, 1988). These results suggest that TBHP induced oxidative stress in the HepG2 cells.

The effect of TBHP on cell viability of the HepG2 cells was also examined usig the MTT staining method (van de Loosdrecht *et al.*, 1991). TBHP decreased cell viability in a dose-dependent manner as depicted in Figure 2. The concentration of TBHP exerting a significant cytotoxicity was about 100-200 μM. The concentration ranges of TBHP inducing oxidative stress and cytotoxicity were found to be fairly correlated with each other.

Apoptosis-inducing activity of TBHP

The effect of TBHP on apoptosis of the HepG2 cells was also studied using agarose gel elctrophoresis of fragmented DNA, and the results are depicted in Figure 3. TBHP induced a dose-dependent apoptosis in the HepG2 cells. The apoptosis-inducing activity of TBHP was observed at the concentration of 200 μM at which TBHP induced oxidative stress and a significant cytotoxicity.

Effects of TBHP on intracellular Ca^{2+} concentration and Mn^{2+} influx

To examine the relationship between the observed apoptosis-indicing action of TBHP and intracellular Ca^{2+} signaling mechanisms, we measured the change of intracellular Ca^{2+} concentration using Fura-2 fluorescence technique. As shown in Figure 4A, TBHP (200 μM) induced a prolonged increase in intracellular Ca^{2+}

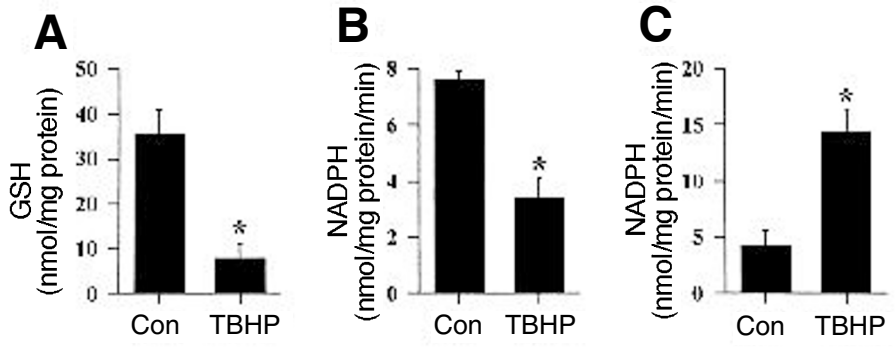

Figure 1. Effects of TBHP on oxidative stress in HepG2 human hepatoblastoma cells. The data show changes of glutathione (GSH) content (A), GSH reductase activity (B) and GSH peroxidase activity (C) induced by 200 μM TBHP. Data points represent the mean values of four replications with bars indicating SEM. *$p<0.05$ compared to the control condition.

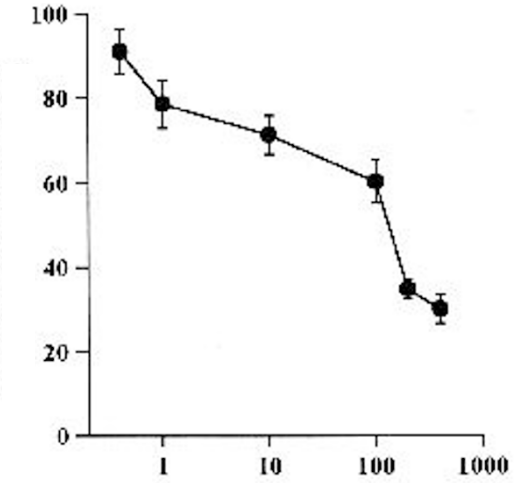

Figure 2. Effects of TBHP on cell viability in HepG2 human hepatoblastoma cells. Cell viability assay was done by the MTT staining method. Results are expressed as percent change of control condition in which cells were grown in medium without drug. Data points represent the mean values of four replications with bars indicating SEM.

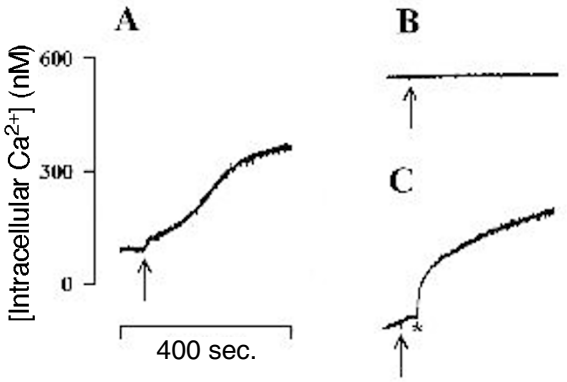

Figure 4. TBHP induces Ca^{2+} influx in HepG2 human hepatoblastoma cells. Intracellular Ca^{2+} concentration was assessed by Fura-2 fluorescence technique in normal Ca^{2+}-containing medium (A), or Ca^{2+}-free medium containing 2 mM EGTA (B and C). The data represent intracellular Ca^{2+} changes with time. The arrows and asterisk show the time points for addition of 200 μM TBHP and 1 mM $CaCl_2$, respectively.

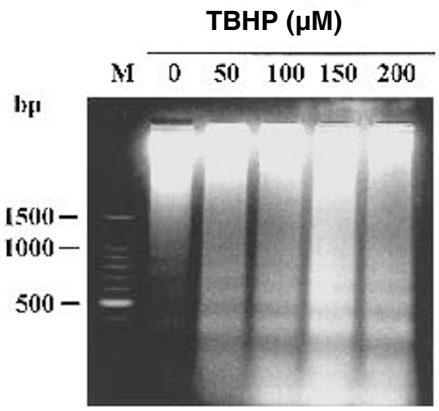

Figure 3. TBHP induces apoptotic cell death in HepG2 human hepatoblastoma cells. Cell were treated for 1 h without or with each concentration of TBHP. DNA was isolated from the cells and analyzed by 1.5% agarose gel electrophoresis. Lane M represents DNA marker.

concentration. To determine the source of the increased intracellular Ca^{2+} concentration induced by TBHP, we measured intracellular Ca^{2+} concentrations using Ca^{2+}-free medium containing 2 mM EGTA. The experimental protocol can effectively reduce extracellular free Ca^{2+} concentration, and thus, blunt available Ca^{2+} influx. Under these conditions cellular response to TBHP was completely inhibited as illustrated in Figure 4B. Furthermore, in these conditions addition of 1 mM $CaCl_2$ following treatment with TBHP induced a rapid increase in intracellular Ca^{2+} concentration as shown in Figure 4C. These results indicate that the TBHP-induced increased intracellular Ca^{2+} concentration is exclusively due to Ca^{2+} influx from the extracellular site. To confirm the Ca^{2+} influx by TBHP, we measured Mn^{2+} influx after treatment with TBHP. The Ca^{2+} influx pathway in many different cell types is also permeable for Mn^{2+} (Grynkiewicz *et al.*, 1985). As the binding of Fura-2 with Mn^{2+} results in quenching of the fluorescence of the dye, Mn^{2+} entry into the cells is represented by and proportional to the detected decrease of Fura-2 fluorescence (Demaurex *et al.*, 1994). As shown in Figure 5, TBHP (200 μM) rapidly decreased Fura-2 fluorescence, indicating that TBHP induced a rapid Mn^{2+} influx. These results further suggest that TBHP increased intracellular Ca^{2+} concentration through Ca^{2+} influx mechanism.

Effects of Ca2+ chelators on the TBHP-induced apoptosis

The role of intracellular Ca^{2+} as a signal for the TBHP-induced apoptosis of the HepG2 cells was further examined by investigating the effects of BAPTA, an intracellular Ca^{2+} chelator (Jiang *et al.*, 1994) and EGTA, an extracellular Ca^{2+} chelator (Kigoshi *et al.*, 1997), on the TBHP-induced apoptosis. Figure 6 showed that treatment with either 2 μM BAPTA or 2 mM EGTA significantly suppressed the TBHP-induced DNA fragmentation in the HepG2 cells. These results suggest that Ca^{2+} influx may mediate the TBHP-induced apoptosis of the HepG2 cells.

Discussion

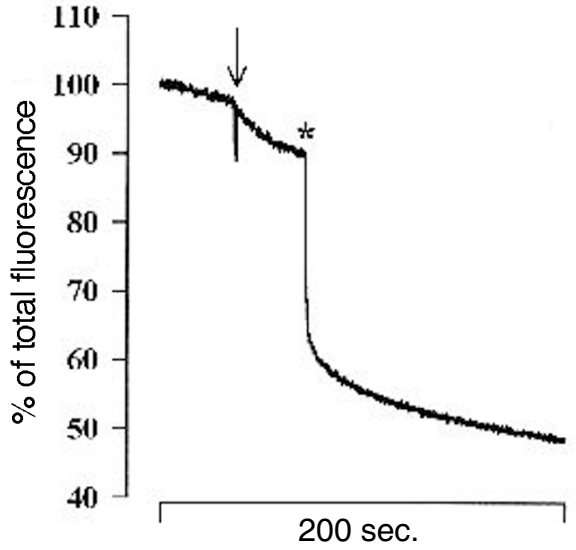

Figure 5. Effect of TBHP on the Mn^{2+} influx in HepG2 human hepatoblastoma cells. After Fura-2 loaded cells were suspended in KRB buffer solution, fluorescence emission (510 nm) was measured with the excitation wavelength of 360 nm. Results are expressed as percent change of total Fura-2 fluorescence. The cells were stimulated by 200 μM TBHP as indicated by the arrow, and then 300 μM $MnCl_2$ was added as indicated by the asterisk.

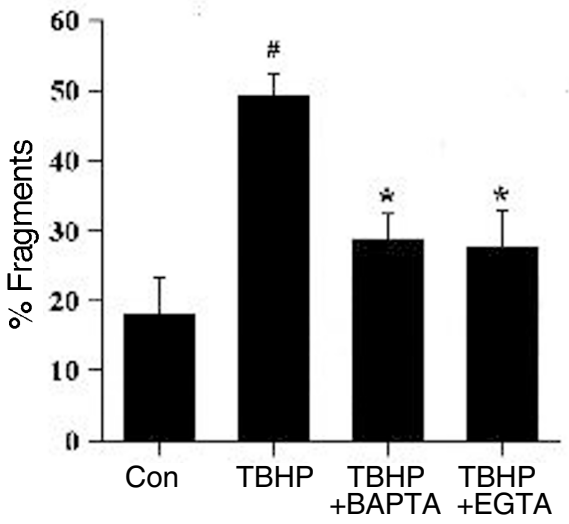

Figure 6. Inhibitory effects of Ca^{2+} chelators on the TBHP-induced DNA fragmentation in HepG2 human hepatoblastoma cells. BAPTA/AM (2 μM) and EGTA (2 mM), an intracellular and extracellular Ca^{2+} chelators, respectively, were added to the cells 4 hr before treatment with 200 μM TBHP. The amount of fragmented DNA was measured by DPA method. Results are expressed as the percent change of DNA fragments compared to the control condition in which the cells were grown in medium containing drug-free vehicle. Data points represent the mean values of four replications with bars indicating SEM. $^{\#}p<0.05$ compared to control. $^{*}p<0.05$ compared to TBHP alone.

Oxidative stress appears to be involved in the mechanism of various types of cell injury (Shlafer *et al.*, 1982; Stohs, 1995; Davis, 1996; Jenner and Olanow, 1996). Particularly, liver cells have high probability of the OFR-induced toxicity (Ishii *et al.*, 1997), because hepatocytes produce large amounts of OFRs during detoxification of xenobiotics and toxic substances (Stohs, 1995). Recently, OFRs have been shown to induce apoptosis, a naturally occurring form of cell death, in many different types of cells (Slater *et al.*, 1995; Stoian *et al.*, 1996). However, the apoptosis-inducing activity of OFRs in human hepatocytes has not been much studied yet. Thus, the main purpose of this study was to determine whether OFRs induce apoptotic cell death in a human hepatocyte.

In the present study we used TBHP as an oxidative stress inducer which has been frequently employed as an oxidative stress inducer (Soszynski and Bartosz, 1997; Gorbunov *et al.*, 1998). The results showed that TBHP produced oxidative stress (Figure 1) and induced apoptotic cell death in the HepG2 cells (Figure 3). These results are consistent with previous other reports that in non-hepatocytes TBHP induced apoptosis through the induction of oxidative stress (Langley *et al.*, 1993; Kondo *et al.*, 1997).

Intracellular Ca^{2+} has been shown to act as a common mediator of chemical-induced cell death (Harman and Maxwell, 1995). Intracellular Ca^{2+} also appears to play a role as a signal transducer in the mechanism of apoptosis (Distelhorst and Dubyak, 1998). Thus, in this study we investigated the possible involvement of intracellular Ca^{2+} signals in the TBHP-induced apoptosis of the HepG2 cells. The results showed that TBHP increased intracellular Ca^{2+} concentration through Ca^{2+} influx, since the increased intracellular Ca^{2+} induced by TBHP was completely abolished using Ca^{2+}-free medium containing 1.0 mM EGTA (Figure 4). The results of Mn^{2+} influx induced by TBHP (Figure 5) further confirmed the activation of Ca^{2+} influx pathway by TBHP. Moreover, Mn^{2+} influx study has been widely employed to monitor Ca^{2+} influx in many different cell types (Chen and Rembold, 1992; Khodorov *et al.*, 1996; Geiszt *et al.*, 1997). Significant blockade of the TBHP-induced DNA fragmentation by pretreatment with either BAPTA/AM, an intracellular Ca^{2+} chelator, or EGTA, an extracellular Ca^{2+} chelator (Figure 6), indicates that increase of Ca^{2+} influx may mediate the observed apoptosis by TBHP. However, Bird *et al.* (1993) have reported that in isolated rat hepatocytes TBHP induced only intracellular Ca^{2+} release through enhancing the sensitivity of inositol 1,4,5-trisphosphate (IP_3) to its recep-tors. We do not exactly know this discrepancy between their and our

results, and it remains to be determined.

Although speculated, extracellular Ca^{2+} ions appear to enter into the cells by the following two mechanisms: i) activation of Ca^{2+} channel ii) activation of reverse mode of Na^{+}/Ca^{2+} exchange mechanism. Recently, it has been reported that voltage-dependent nonselective cation channels (NSCCs) are prensent in the HepG2 cells (Chen *et al.*, 1997). Since the NSCCs seem to be invlolved in the regulation of Ca^{2+} influx (Chen *et al.*, 1997), TBHP may induce Ca^{2+} influx through the activation of these NSCCs. Interestingly, in cardiac myocytes the activity of NSCCs has been shown to be increased by treatment with OFRs (Jabr and Cole, 1995). Na^{+}/Ca^{2+} exchanger normally acts to extrude Ca^{2+} ions when intracellular Ca^{2+} rises above certain levels (DiPolo and Beauge, 1987). However, Ca^{2+} ions enter into the cells under conditions that favor the reverse mode of operation of the Na^{+}/Ca^{2+} exchanger (DiPolo and Beauge, 1987). Reverse operation of the Na^{+}/Ca^{2+} exchanger during anoxia has been reported to be a critical mechanism of Ca^{2+} influx and subsequent neuronal cell injury (Stys *et al.*, 1991). OFRs also appear to modulate the activity of Na^{+}/Ca^{2+} exchanger (Goldhaber, 1996). Thus, the possibility also exists that TBHP induced Ca^{2+} influx through the activation of the reverse mode of Na^{+}/Ca^{2+} exchanger. Further studies are required to elucidate whether TBHP induces Ca^{2+} influx through either NSCC activation, reverse operation of Na^{+}/Ca^{2+} exchanger, or both.

The induction of oxidative stress by TBHP (Figure 1) may be mediated by OFRs released from TBHP. Thus, it can be speculated that the Ca^{2+} influx induced by TBHP (Figure 4) may be due to the actions of OFRs. In other studies exogenous administration of OFRs, such as hydro-gen peroxide, increased intracellular Ca^{2+} concentration in neuronal cells (Tretter and Adam-Vizi, 1996) and in human endothelial cells (Dreher and Junod, 1995). However, the Ca^{2+} source appears to be different, depending on cell types and reactive oxygen species used in these experi-ments. For examples, hydrogen peroxide has been shown to induce Ca^{2+} influx in neuronal cells (Tretter and Adam-Vizi, 1996), whereas superoxide anion triggers Ca^{2+} release from cardiac sarcoplasmic reticulum through ryanodine receptor Ca^{2+} channel (Kawakami and Okabe, 1998).

Accumulating evidence indicates that intracellular Ca^{2+} signal is involved in the mechanism of apoptosis (McConkey and Orrenius, 1996). One of targets for the elevation of intracellular Ca^{2+} concentration is the activation of Ca^{2+}-dependent protein kinases and phosphatases. Activation of calcineurin, a Ca^{2+}/calmodulin-dependent protein serine/threonine phosphatase, induced by intracellular Ca^{2+} elevation (Bonnefoy-Berard *et al.*, 1994) has been shown to induce apoptosis through regulating the activity of the transcription factor, NF-AT (nuclear factor of activated T cells) (Shibasaki *et al.*, 1997). Direct activation of the Ca^{2+}-dependent neutral proteinase, calpain, may represent another target for intracellular Ca^{2+} action in apoptosis. Calpain is rapidly activated in apoptotic cells (Squier *et al.*, 1994), and specific inhibitors of calpain block apoptosis in many different types of cells (Jordan *et al.*, 1997; Squier *et al.*, 1997). Increased intracellular Ca^{2+} activates Ca^{2+}/Mg^{2+}-dependent endonuclease (Cohen and Duke, 1984), resulting in DNA fragmentation, the most characteristic biochemical feature of apoptosis (Wyllie *et al.*, 1984). Ca^{2+}-dependent transglutaminase which catalyzes the post-translational coupling of amines to proteins and the crosslinking of proteins, also appears to be a target for Ca^{2+} action. The enzyme is highly acti-vated in apoptotic cells (Fesus *et al.*, 1987), and over-expression of the enzyme triggers apoptotic cell death (Melino *et al.*, 1994).

In conclusion, TBHP induced the apoptotic cell death in a human liver cell line and intracellular Ca^{2+} signals may mediate this action of TBHP. These results suggest that apoptosis may be a key step in the pathogenesis of various liver diseases related with oxidative stress.

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