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Inhibitors of Na^+/Ca^{2+} Exchanger Prevent Oxidant-Induced Intracellular Ca^{2+} Increase and Apoptosis in a Human Hepatoma Cell Line

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Oxidative stress appears to be implicated in the pathogenesis of various diseases including hepatotoxicity. Although intracellular Ca^{2+} signals have been suggested to play a role in the oxidative damage of hepatocytes, the sources and effects of oxidant-induced intracellular Ca^{2+} increases are currently debatable. Thus, in this study we investigated the exact source and mechanism of oxidant-induced liver cell damage using HepG2 human hepatoma cells as a model liver cellular system. Treatment with 200 μM of *tert*-butyl hydroperoxide (tBOOH) induced a sustained increase in the level of intracellular reactive oxygen intermediates (ROI) and apoptosis, assessed by 2′,7′-dichlorofluorescein fluorescence and flow cytometry, respectively. Antioxidants, N-acetyl cysteine (NAC) or N,N′-diphenyl-*p*-phenylenediamine significantly inhibited both the ROI generation and apoptosis. In addition, tBOOH induced a slow and sustained increase in intracellular Ca^{2+} concentration, which was completely prevented by the antioxidants. An intracellular Ca^{2+} chelator, bis-(*o*-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid/cetoxymethyl ester significantly suppressed the tBOOH-induced apoptosis. These results imply that activation of an intracellular Ca^{2+} signal triggered by increased ROI may mediate the tBOOH-induced apoptosis. Both intracellular Ca^{2+} increase and induction of apoptosis were significantly inhibited by an extracellular Ca^{2+} chelator or Na^+/Ca^{2+} exchanger blockers (bepridil and benzamil), whereas neither Ca^{2+} channel antagonists (verapamil and nifedipine) nor a nonselective cation channel blocker (flufenamic acid) had an effect. These results suggest that tBOOH may increase intracellular Ca^{2+} through the activation of reverse mode of Na^+/Ca^{2+} exchanger. However, tBOOH decreased intracellular Na^+ concentration, which was completely prevented by NAC. These results indicate that ROI generated by tBOOH may increase intracellular Ca^{2+} concentration by direct activation of the reverse mode of Na^+/Ca^{2+} exchanger, rather than indirect elevation of intracellular Na^+ levels. Taken together, these results suggest that the oxidant, tBOOH induced apoptosis in human HepG2 cells and that intracellular Ca^{2+} may mediate this action of tBOOH. These results further suggest that Na^+/Ca^{2+} exchanger may be a target for the management of oxidative hepatotoxicity.

Keywords: *tert*-Butyl hydroperoxide, oxidative stress, apoptosis, HepG2 cells, intracellular Ca^{2+}, Na^+/Ca^{2+} exchanger

Abbreviations: BAPTA/AM, bis-(*o*-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid/acetoxymethyl ester;

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BEP, bepridil; BENZ, benzamil; DCF, dichlorofluorescein; DCFH, dichlorofluorescin; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DPPD, N,N′-diphenyl-*p*-phenylenediamine; EGTA, ethylene glycol-bis-(aminoethyl ether)N,N,N′,N′-tetraacetic acid; FA, flufenamic acid; Fura-2/AM, 1-(2,5-carboxyoxazol-2-yl-6-aminobenzfuran-5-oxyl)-2-(2′-amino-methylphenoxy)-ethane-N,N,N′,N′-tetraacetoxylmethyl ester; NAC, N-acetyl cysteine; NIFE, nifedipine; ROI, reactive oxygen intermediates; SBFI/AM, sodium-binding benzofuran isophthalate acetoxylmethyl ester; tBOOH, *tert*-butyl hydroperoxide; VERA, verapamil

INTRODUCTION

Liver produces large amounts of ROI in the course of detoxifying xenobiotic and toxic substances.[1] The ROI produced are normally scavenged by endogenous antioxidants which are abundant in the liver tissue.[2] However, liver injury can occur when large acute doses or chronic exposure to toxic substances overpower the hepatic antioxidant defence system.[3]

ROI readily interact with cellular macromolecules and structures, resulting in changes in membrane permeability, activation of proteases and nucleases, and altered gene expression.[2,4] It is known that these cellular changes induced by ROI lead to apoptotic cell death in a variety of cell types.[5–7] Although intracellular Ca^{2+} has been suggested to play a role in the oxidative damage of hepatocytes,[8] the source and effects of the oxidative stress-induced intracellular Ca^{2+} increase are currently controversial.

Thus, the main purposes of this study are to investigate (1) whether intracellular Ca^{2+} is involved in the oxidant-induced apoptosis of liver cells and (2) what is the mechanism by which an oxidant increases intracellular Ca^{2+} levels, using HepG2 human hepatoma cells as a model liver cellular system. In the experiments we used tBOOH as an oxidant, being frequently employed in similar types of experiments.[9,10]

MATERIALS AND METHODS

Materials

The HepG2 human hepatoma cell line was purchased from American Type Culture Collection (Rockville, MA). The powders Eagle's minimum essential medium, trypsin solution, sodium pyruvate, tBOOH, DPPD, NAC, FA, probenecid and all salt powders were obtained from Sigma Chemical CO. (St. Louis, MO). BENZ, BEP, NIFE and VERA were from RBI (Natick, MA). Fura-2/AM. BAPTA/AM and DCFH-DA were from Molecular Probes, Inc. (Eugene, OR). Fetal bovine serum and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). Fura-2/AM, BAPTA/AM and DCFH-DA were prepared as stock solutions in DMSO, then diluted with aqueous medium to the final desired concentrations. The solution of tBOOH 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 an Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 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.

DNA Isolation and Electrophoresis

DNA isolation was done according to Hockenbery *et al.*[11] HepG2 cells were collected by centrifugation (200× *g*, 10 min), washed twice in phosphate buffered saline (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 mM 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 photographed under UV light.

Intracellular Ca^{2+} Measurement

Aliquots of the HepG2 cells were washed in EBSS. Then, 5 μM Fura-2/AM was added, and the cells were incubated for 30 min at 37°C. Unloaded Fura-2/AM 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/AM-loaded cells were maintained at 25°C for 90 min before fluorescence measurement. For each experiment, 0.5 ml aliquot of Fura-2/AM-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 experiment, fluorescence maximum and minimum values at each excitation wavelength were obtained by 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.

Intracellular Na^+ Measurement

Intracellular Na^+ levels were monitored with the Na^+-sensitive fluorescent dye SBFI.[12] Cells were washed, and resuspended at a density of 4×10^5 cells/ml in Hank's solution. The cells were loaded with 5 μM SBFI/AM in a Hank's solution containing 0.02% pluronic F-127, a nonionic surfactant, for 2 h at 37°C. Unloaded dye was removed by centrifugation at 150× *g* for 3 min. The dual-wavelength excitation method for measurement of SBFI fluorescence was used. Fluorescence was monitored at 500 nm with excitation wavelengths of 340 and 380 nm in a stirred quartz cuvette. In the results relative changes in intracellular Na^+ concentrations were reported as the 340 : 380 fluorescence ratios.

Intracellular ROI Measurement

Relative changes in intracellular ROS in the HepG2 cells were monitored using a fluorescent probe, DCFH-DA.[13] DCFH-DA diffuses through the cell membrane readily and is hydrolyzed by intracellular esterases to non-fluorescent DCFH, which is then rapidly oxidized to highly fluorescent DCF in the presence of ROI. The DCF fluorescence intensity is proportional to the amount of ROI formed intracellularly.[14] Cells were washed twice and resuspended at a concentration of 4×10^5 cells/ml in Hank's solution. For loading DCFH-DA into the cells, cells were incubated with the dye for 2 h at a final concentration of 5 μM at 37°C. Fluorescence (excitation wavelength set at 485 nm and the emission wavelength at 530 nm) was monitored in a well-stirred cuvette. Data are presented as relative fluorescence changes compared to control condition in which the cells were incubated without tBOOH.

Flow Cytometry Assays

For flow cytometry analysis, HepG2 cells were collected and washed twice with PBS buffer (pH 7.4). After fixing in 80% ethanol for 30 min, cells were washed twice, and resuspended in PBS buffer (pH 7.4) containing 0.1% Triton X-100, 5 μg/ml PI and 50 μg/ml ribonuclease A for DNA staining. Cells were then analyzed by a FACScan (BIO-RAD, Hercules, CA). At least 20,000 events were evaluated. All histograms were analyzed using WinBryte software (BIO-RAD, Hercules, CA) to determine percentage of nuclei with hypodiploid content indicative of apoptosis.[15]

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 Apoptotic Cell Death by tBOOH

The effect of tBOOH on apoptotic cell death of HepG2 cells was examined using two independent methods. tBOOH induced DNA fragmentation in a concentration-dependent manner studied by agarose gel electrophoresis as depicted in Figure 1A. This effect of tBOOH was prominent at the concentration of 200 μM. As shown in Figure 1B, tBOOH also induced apoptosis in a time-related manner tested by flow cytometry by determining hypodiploid DNA content stained with PI.[15] These results indicate that tBOOH induced apoptotic cell death in the HepG2 cells.

Inhibitory Effects of Antioxidants on ROI Generation and Apoptosis Induced by tBOOH

In order to determine that apoptosis-inducing activity of tBOOH is due to its ability to generate ROI in HepG2 cells, we investigated the effects of antioxidants, NAC and DPPD, on the tBOOH-induced ROI generation and apoptosis using DCF fluorescence and flow cytometry, respectively.

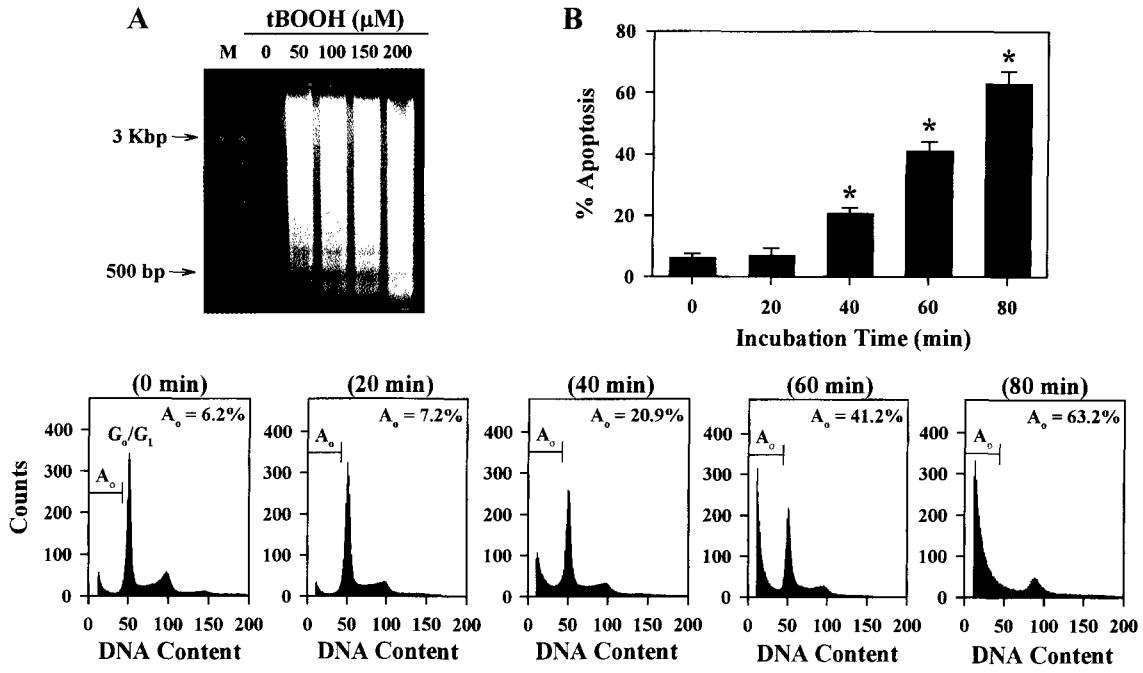

FIGURE 1 tBOOH induces a concentration-dependent (A) and time-dependent (B) apoptotic cell death in HepG2 human hepatoma cells. In the experiments of (A) cells were treated for 1 h with or without each concentration of tBOOH. DNA was isolated from the cells and analyzed by 1.8% agarose gel electrophoresis. Lane M represents DNA marker. In the experiments of (B) the cells were incubated with tBOOH (200 μM) for each designated time. The number of apoptotic cells was measured by flow cytometry as described in text. The region to the left of the G_0/G_1 peak, designated A_0, was defined as cells undergoing apoptosis-associated DNA degradation.[15] In bar graphs the data represent the mean values of four replications with bars indicating SEM. $^*p < 0.05$ compared to control.

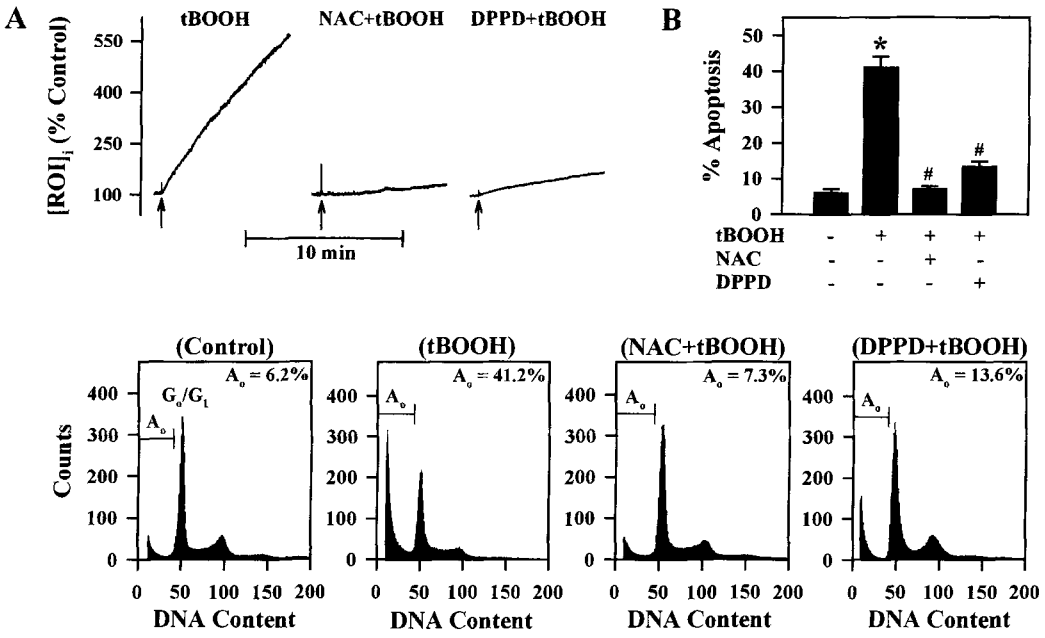

FIGURE 2 Effects of antioxidants on tBOOH-induced generation of intracellular ROI (A) and apoptotic cell death (B) in HepG2 human hepatoma cells. The data (A) show changes in ROI levels induced by tBOOH as a function of time. The arrow shows the time point for addition of tBOOH (200 μM). NAC (50 mM) and DPPD (10 μM) were added 10 min before tBOOH treatment. In the experiments of (B) the cells were incubated with or without tBOOH (200 μM) for 1 h. The number of apoptotic cells was measured by flow cytometry. NAC (50 mM) and DPPD (10 μM) were added 30 min before tBOOH treatment. In bar graphs the data represent the mean values of four replications with bars indicating SEM. $^*p < 0.05$ compared to control. $^\#p < 0.05$ compared to tBOOH alone.

tBOOH induced a slow and sustained increase in intracellular ROI levels in the HepG2 cells (Figure 2A). The apoptosis-inducing activity of tBOOH (200 μM) was significantly suppressed by treatment with either 50 mM NAC or 10 μM DPPD (Figure 2B).

Role of Intracellular Ca^{2+} in the tBOOH-induced Apoptosis

To examine the relationship between the observed apoptosis-inducing action of tBOOH and intracellular Ca^{2+} signaling mechanisms, we measured the effect of tBOOH on intracellular Ca^{2+} concentration using Fura-2 fluorescence technique. As shown in Figure 3A, tBOOH (200 μM) induced a prolonged increase in intracellular Ca^{2+} concentration, which was completely inhibited by 50 mM NAC or 10 μM DPPD. The role of intracellular Ca^{2+} as a signal for the tBOOH-induced apoptosis was further examined by investigating the effect of BAPTA/AM, an intracellular Ca^{2+} chelator. Figure 3B showed that treatment with 1 μM BAPTA/AM significantly suppressed the tBOOH-induced apoptosis. These results suggest that intracellular Ca^{2+} may mediate the tBOOH-induced apoptosis in the HepG2 cells.

Involvement of Na^+/Ca^{2+} Exchanger in Intracellular Ca^{2+} Increase and Apoptosis Induced by tBOOH

To determine the source of the increased intracellular Ca^{2+} concentration induced by tBOOH, we measured intracellular Ca^{2+} concentrations using Ca^{2+}-free medium containing 1 mM EGTA. This experimental protocol can effectively

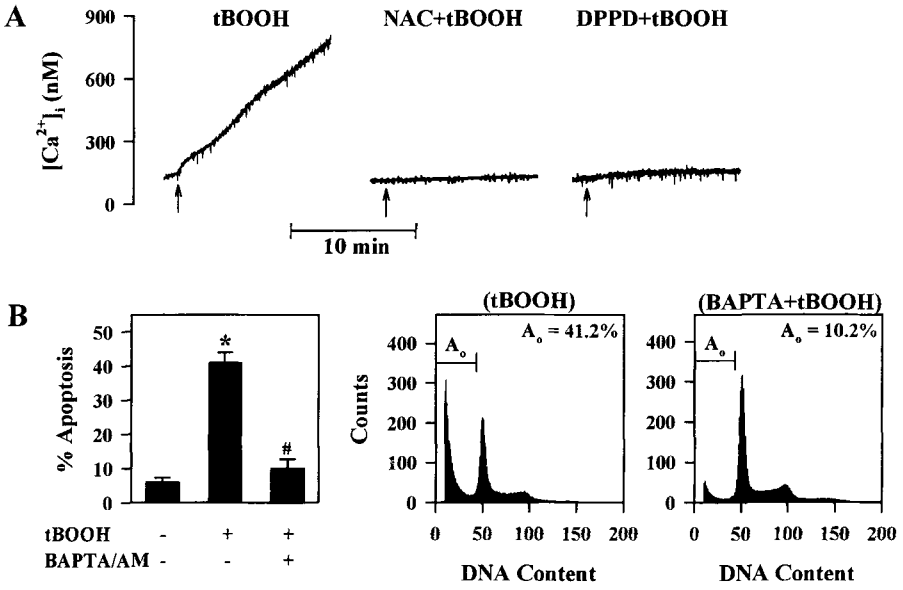

FIGURE 3 tBOOH-induced apoptosis is dependent on intracellular Ca^{2+} increase in HepG2 human hepatoma cells. Intracellular Ca^{2+} concentration was assessed by Fura-2 fluorescence technique (A), and the data represent intracellular Ca^{2+} changes with time. The arrows show the time points for addition of tBOOH (200 μM). NAC (50 mM) and DPPD (10 μM) were added 10 min before tBOOH treatment. Note that these antioxidants completely inhibit the tBOOH-induced increased intracellular Ca^{2+} level. The number of apoptotic cells was measured by flow cytometry (B). BAPTA/AM (1 μM) was added to the cells 30 min before treatment with tBOOH (200 μM). In bar graphs the data represent the mean values of four replications with bars indicating SEM. $^*p < 0.05$ compared to control. $^\#p < 0.05$ compared to tBOOH alone.

reduce extracellular free Ca^{2+} concentration, and thus, blunt available Ca^{2+} influx. Under these conditions intracellular Ca^{2+} increase by tBOOH was completely inhibited as illustrated in Figure 4A. In addition, known inhibitors of intracellular Ca^{2+} release, dantrolene (50 μM) and 3,4,5-trimethoxybenzoic acid-8-(diethylamino)-octyl ester (TMB-8; 20 μM), did not alter the tBOOH-induced intracellular Ca^{2+} increase (data not shown). These results clearly indicate that the tBOOH-induced increased intracellular Ca^{2+} concentration is exclusively due to Ca^{2+} influx from the extracellular site. Furthermore, EGTA completely inhibited the tBOOH-induced apoptosis as shown in Figure 4B, suggesting that Ca^{2+} influx may play a role in the apoptotic action of tBOOH.

However, intracellular Ca^{2+} increase and apoptosis induced by tBOOH were not affected by voltage-sensitive Ca^{2+} channel inhibitors (100 μM VERA and 100 μM NIFE), or by a non-selective cation channel inhibitor (100 μM FA). These results imply that Ca^{2+} influx may be mediated not through these ion channels, but through other mechanisms. Interestingly, inhibitors of Na^+/Ca^{2+} exchanger (50 μM BEP and 50 μM BENZ) completely suppressed both intracellular Ca^{2+} increase and apoptosis induced by tBOOH as illustrated in Figure 5. These results indicate that the reverse mode of the Na^+/Ca^{2+} exchanger may be activated by tBOOH and that Ca^{2+} influx through the exchanger may lead to apoptosis.

Effect of tBOOH on Intracellular Na^+ Concentration

To determine whether the activation of the reverse mode of Na^+/Ca^{2+} exchanger is secondarily achieved by increased intracellular Na^+ concentration, we monitored intracellular Na^+ levels

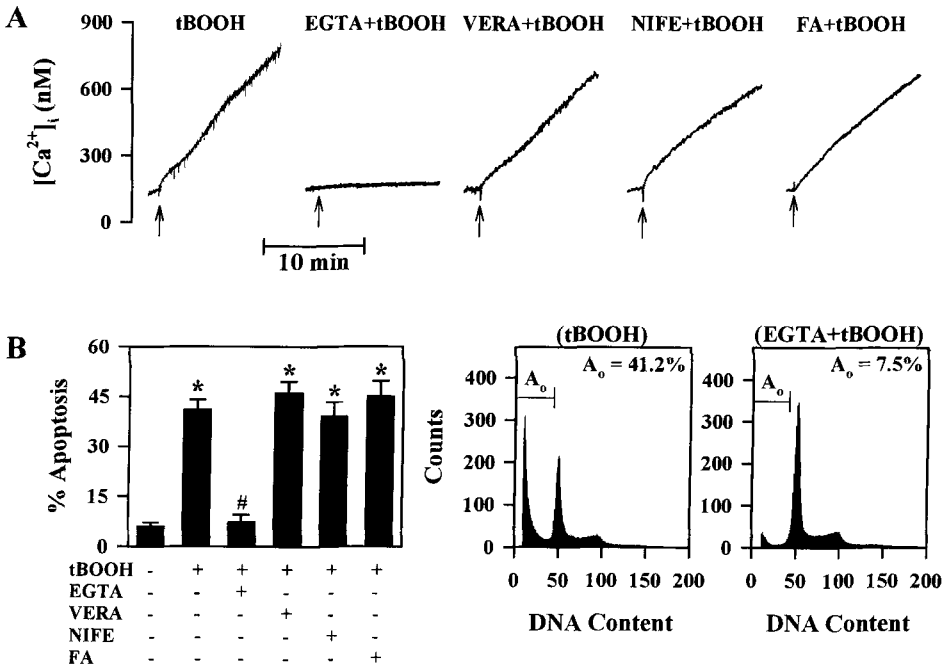

FIGURE 4 Intracellular Ca^{2+} influx is responsible for the tBOOH-induced intracellular Ca^{2+} increase and apoptosis in HepG2 human hepatoma cells. Intracellular Ca^{2+} concentration (A) and apoptosis (B) were assessed by Fura-2 fluorescence technique and flow cytometry, respectively. In the experiments of (A) using EGTA (1 mM), a nominal Ca^{2+}-free medium was used. VERA (100 μM), NIFE (100 μM) and FA (100 μM) were added 10 min before tBOOH application. The data represent intracellular Ca^{2+} changes with time. The arrows show the time points for addition of tBOOH (200 μM). In the experiments of (B), tBOOH (200 μM) was treated for 1 h. EGTA (1 mM), VERA (100 μM), NIFE (100 μM) and FA (100 μM) were added 30 min before tBOOH treatment. In bar graphs the data represent the mean values of four replications with bars indicating SEM. $^*p < 0.05$ compared to control. $^{\#}p < 0.05$ compared to tBOOH alone.

using a Na^+-sensitive fluorescent dye SBFI. As shown in Figure 6, tBOOH indeed decreased intracellular Na^+ concentration, which was completely inhibited by an antioxidant NAC (50 mM). Monensin (50 μM), a Na^+ ionophore which was used as a positive control, showed a rapid increase in intracellular Na^+ concentration. These results suggest that the reverse mode of Na^+/Ca^{2+} exchanger may be activated not by increased intracellular Na^+ concentration, but by other mechanisms, possibly through a direct activation by tBOOH.

DISCUSSION

Numerous studies have demonstrated that oxidative stress is involved in the mechanism of various types of tissue damage.[1,6,16,17] Since hepatocytes generate large amounts of ROI during detoxification of xenobiotics and toxic substances,[1] liver tissues have high probability of the ROI-induced toxicity.[3] In hepatocytes ROI appear to induce cell death through both necrosis[18] and apoptosis[5,7] depending on the applied substances, their doses and duration. The results of the present study clearly showed that tBOOH, an organic peroxide,[9,10] induced apoptotic cell death in the HepG2 human hepatic cell line at the concentration of 200 μM within 1 h (Figure 1A and B). Treatment with antioxidants significantly inhibited the tBOOH-induced increased ROI levels and apoptosis (Figure 2A and B), suggesting that produced ROI may mediate the apoptotic activity of tBOOH. Previous reports have also shown that tBOOH induces apoptosis in

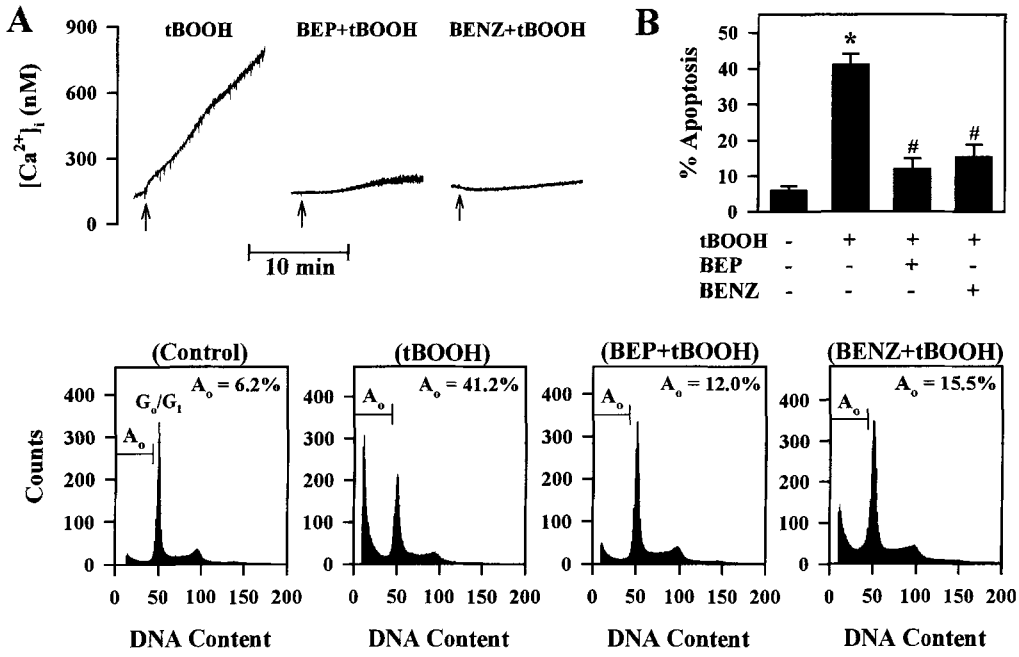

FIGURE 5 tBOOH-induced intracellular Ca^{2+} increase (A) and apoptosis (B) are suppressed by inhibitors of Na^+/Ca^{2+} exchanger in HepG2 human hepatoma cells. Data presentation is the same as Figure 4. In these experiments BEP (50 μM) and BENZ (50 μM) were used as inhibitors of Na^+/Ca^{2+} exchanger. In the experiments of (A) these drugs were added 10 min before tBOOH application. The arrows show the time points for addition of tBOOH (200 μM). In the experiments of (B) the cells were treated with tBOOH (200 μM) for 1 h. BEP (50 μM) and BENZ (50 μM) were added 30 min before tBOOH application. In bar graphs the data represent the mean values of four replications with bars indicating SEM. $^*p < 0.05$ compared to control. $^\#p < 0.05$ compared to tBOOH alone.

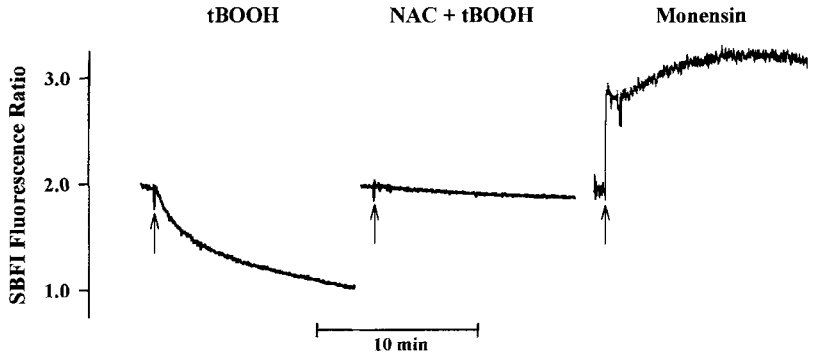

FIGURE 6 The effects of tBOOH on intracellular Na^+ concentration in HepG2 human hepatoma cells. Intracellular Na^+ concentration was measured by using the Na^+-sensitive fluorescent dye SBFI. In the experiments NAC (50 mM) was added 10 min before tBOOH application. The data represent intracellular Na^+ changes with time. The arrows show the time points for addition of either tBOOH (200 μM) or monensin (50 μM). Monensin, a Na^+ ionophore, was used as a positive control.

non-hepatic cell types through the generation of ROI.[19,20]

Intracellular Ca^{2+} has been recognized to act as a common mediator of chemical-induced cell death.[21,22] In many studies using hepatocytes primarily from non-human origin intracellular Ca^{2+} has been shown to mediate cell death induced by oxidative stress.[23,24] Intracellular

Ca^{2+} also acts as an important down-stream signal of the ROI-induced apoptosis in the human HepG2 cells, since antioxidants significantly blocked both intracellular Ca^{2+} increase and apoptosis induced tBOOH (Figures 2B and 3A). Moreover, the tBOOH-induced apoptosis was significantly inhibited by treatment with BAPTA/AM, an intracellular Ca^{2+} chelator (Figure 3B).

Currently, it is not completely understood how ROI increase intracellular Ca^{2+} concentration. ROI may increase intracellular Ca^{2+} through either Ca^{2+} influx or Ca^{2+} release from the internal stores. The Ca^{2+} sources are different depending on the types of cells and ROI used in the experiments. For example, hydrogen peroxide has been shown to induce Ca^{2+} influx in neuronal cells,[25] whereas superoxide anion triggers Ca^{2+} release from cardiac sarcoplasmic reticulum through ryanodine receptor Ca^{2+} channel.[26] Furthermore, tBOOH has been reported to induce Ca^{2+} influx in cardiac myocytes,[27] whereas it induced Ca^{2+} release from internal stores in rat hepatocytes.[28,29] In this study we showed that tBOOH induced Ca^{2+} influx, since the intracellular Ca^{2+} increase by tBOOH was completely abolished using Ca^{2+}-free medium containing 1 mM EGTA (Figure 4A). Significant blockade of the tBOOH-induced apoptosis by pretreatment with EGTA, an extracellular Ca^{2+} chelator (Figure 4B) indicates that Ca^{2+} influx mediated the observed apoptosis by tBOOH.

Extracellular free Ca^{2+} ions may enter into the cells by the following two mechanisms: (i) activation of Ca^{2+} channels, (ii) activation of reverse mode of Na^+/Ca^{2+} exchange mechanism. In this study voltage-dependent Ca^{2+} channel blockers, VERA and NIFE did not significantly alter the tBOOH-induced intracellular Ca^{2+} increase and apoptosis (Figure 4A and B), suggesting no involvement of these ion channels in the activity of tBOOH in the HepG2 cells. Recently, Ca^{2+}-permeable non-selective cation channels have been reported to exist in the HepG2 cells.[30] Since these channels seem to mediate Ca^{2+} influx in many different types of cells,[30–32] we determined this possibility using FA, a known inhibitor of the channels. No significant effects of FA on the tBOOH-induced Ca^{2+} influx and apoptosis (Figure 4A and B) further indicate that the tBOOH-induced Ca^{2+} influx may not be through the activation of the plasma membrane Ca^{2+} channels.

However, BEP and BENZ, known inhibitors of Na^+/Ca^{2+} exchanger significantly suppressed both Ca^{2+} influx and apoptosis induced by tBOOH (Figure 5A and B), implying that the activation of reverse mode of Na^+/Ca^{2+} exchanger may be critical for the actions of tBOOH. Na^+/Ca^{2+} exchanger normally acts to extrude Ca^{2+} ions when intracellular Ca^{2+} rises above certain levels.[33] On the contrary, Ca^{2+} ions enter into the cells under conditions that favor the reverse mode of operation of the Na^+/Ca^{2+} exchanger.[32] 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.[34] ROI also appear to modulate the activity of Na^+/Ca^{2+} exchanger in cardiac myocytes.[35]

The activation of reverse mode of Na^+/Ca^{2+} exchanger may be a second event following to the increased intracellular Na^+ concentration induced by tBOOH. However, this seems not to be the case, because tBOOH did not increase, but decrease intracellular Na^+ level immediately (Figure 6). Treatment with an antioxidant, NAC completely blocked the decrease in intracellular Na^+ level by tBOOH, indicating that tBOOH may directly activate reverse mode of Na^+/Ca^{2+} exchanger through generation of oxidative stress. Currently, we do not know the exact mechanism by which tBOOH activate reverse mode of Na^+/Ca^{2+} exchanger, and it needs more studies in the future.

In this study we did not investigate the mechanisms coupling increased intracellular Ca^{2+} to apoptosis. However, there are several clear possibilities. Increased intracellular Ca^{2+} activates Ca^{2+}/Mg^{2+}-dependent endonuclease,[36]

resulting in DNA fragmentation, the most characteristic biochemical feature of apoptosis.[37] Elevation of intracellular Ca^{2+} level also activates degradative enzymes such as phospholipases and proteinases,[8] and induces a permeability transition in the mitochondrial inner membrane and collapse of the membrane potential.[38]

In conclusion, tBOOH induced apoptotic cell death in a human liver cell line and intracellular Ca^{2+} increase through activation of reverse mode of Na^+/Ca^{2+} exchanger may mediate this action of tBOOH. These results suggest that apoptosis may be a key step in the pathogenesis of liver diseases related with oxidative stress and that Na^+/Ca^{2+} exchanger may be a target for the protection of hepatic damage due to ROI.

Acknowledgements

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