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The Mechanism of t-Butylhydroperoxide-Induced Apoptosis in IMR-32 Human Neuroblastoma Cells

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Apoptosis has been implicated in the pathophysiological mechanisms of various neurodegenerative diseases. In a variety of cell types, oxidative stress has been demonstrated to play an important role in the apoptotic cell death. However, the exact mechanism of oxidative stress-induced apoptosis in neuronal cells is not known. In this study, we induced oxidative stress in IMR-32 human neuroblastoma cells with *tert*-butylhydroperoxide (TBHP), which was confirmed by significantly reduced glutathione content and glutathione reductase activity, and increased glutathione peroxidase activity. TBHP induced decrease in cell viability and increase in DNA fragmentation, a hallmark of apoptosis, in a dose-dependent manner. TBHP also induced a sustained increase in intracellular Ca^{2+} concentration, which was completely prevented either by EGTA, an extracellular Ca^{2+} chelator or by flufenamic acid (FA), a non-selective cation channel (NSCC) blocker. These results indicate that the TBHP-induced intracellular Ca^{2+} increase may be due to Ca^{2+} influx through the activation of NSCCs. In addition, treatment with either an intracellular Ca^{2+} chelator (BAPTA/AM) or FA significantly suppressed the TBHP-induced apoptosis. Moreover, TBHP increased the expression of p53 gene but decreased c-myc gene expression. Taken together, these results suggest that the oxidative stress-induced apoptosis in neuronal cells may be mediated through the activation of intracellular Ca^{2+} signals and altered expression of *p53* and *c-myc*.

Key Words: *tert*-butyl hydroperoxide, Oxidative stress, Apoptosis, IMR-32 cells, Intracellular Ca^{2+}, *p53*, *c-myc*, *bcl-2*

INTRODUCTION

Human brain is highly vulnerable to oxidative stress resulting from the generation of reactive oxygen species (ROS) since it consumes a disproportionate amount of oxygen and possesses free radical-generating enzymes such as monoamine oxidase and L-amino oxidase (Coyle & Puttfarcken, 1993; Clemens & Panetta, 1995). ROS have recently been implicated in the pathophysiological mechanisms of neurodegenerative diseases such as stroke and seizure (Choi, 1988).

ROS readily interact with cellular macromolecules and structures, resulting in membrane permeability changes, 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 ROS lead to apoptotic cell death in a variety of cell types (Buttke & Sanstrom, 1994; Jenner & Olanow, 1996; Slater et al, 1995; Stoian et al, 1996). Apoptosis appears to be an active form of cell death which requires the synthesis of mRNA and protein and showes characteristic features of cell shrinkage, chromatin condensation, and DNA fragmentation (Kerr et al, 1972). However, the exact mechanism of apoptosis induction by ROS in the neuronal cells is not completely understood.

In this study, we investigated the mechanism of apoptosis induction by oxidative stress in IMR-32 human neuroblastoma cells as a neuronal model system. In the experiments, we used *tert*-butyl hydroperoxide (TBHP) as an oxidative stress-inducing agent. Since TBHP does not have any neuron specificity in contrast to oxidative stress induced by MPTP in dopaminergic neurons, it is, therefore, useful for studying the more generalized oxidative stress found in many

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neurodegenerative conditions. Moreover, TBHP has been frequently employed in the similar types of experiments (Soszynski & Bartosz, 1997; Gorbunov et al, 1998). In this study, we particularly focused on the possible involvement of intracellular Ca^{2+} signal in the mechanism of the apoptosis-inducing action of TBHP since intracellular Ca^{2+} acts as a common mediator of apoptosis in many cell types (McConkey & Orrenius, 1996). We also examined any change in the expression of apoptosis-related genes, *bcl-2*, *c-myc*, and *p53* (Ryan et al, 1994; Thompson, 1998) in TBHP-treated IMR32 cells.

METHODS

Materials

The powders Eagle's minimum essential medium (MEM) and Earle's basal salt solution (EBSS), 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), flufenamic acid (FA), 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'-tetraacetoxylmethyl 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 Hyclone (Logan, UT) and GIBCO (Grand Island, NY), respectively. Fura-2 and BAPTA/AM were prepared as stock solutions in DMSO and 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

IMR-32 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 $\times$g, 5 min). Cell suspension was aliquot out to measure enzyme activities. To determine glutathione (GSH) peroxidase activity, cells were homogenized with sonicater and centrifuged (15000 $\times$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}/mole · cm and assuming that 2 moles of GSH were formed for each mole of NADPH consumed (Tappel, 1978).

To measure the GSH reductase activity, cell homogenates were centrifuged (15000 $\times$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 & 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 $\times$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 & 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. TBHP was added for 1 hour 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 hr. 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

IMR-32 cells were collected by centrifugation (200 $\times$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 $\times$g for 15 min at 4°C. Fragmented DNA was extracted from the supernatant with phenol-chloroform-isoamylalcohol, precipitated by the 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 hr. The pattern of DNA fragmentation was visualized by electrophoresis in 1.8% agarose gel, containing ethidium bromide and photographed under UV light. (Hockenbery et al, 1990)

Quantitative analysis of fragmented DNA

For quantitative DNA analysis, IMR-32 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 $\times$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 the addition of 1 N perchloric acid. The diphenylamine (DPA) method (Natarajan *et al*, 1994) was used to measure 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) $\times$ 100]

Intracellular Ca^{2+} measurement

Aliquots of the IMR-32 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 $\times$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 experiment, fluorescence maximum and minimum values at each excitation wavelength were obtained by the 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 the F-4500 Intracellular Cation Measurement System, provided by Hitachi.

Northern blot analysis

Cellular total RNAs were extracted using Tri reagent and used for electrophoresis on a 1.3% agarose-6.2% formaldehyde gel. After transfer to a ZetaProbe nylon membrane, genes were cross-linked by UV irradiation. The membrane was prehybridized at 42°C for 1 hr with hybridization buffer (50% deionized formamide, 7% SDS, 0.12 M $NaHPO_4$, and 0.25 M NaCl) and then hybridized at 42°C overnight with hybridization buffer containing denatured [^{32}P]dCTP-labelled cDNA probe. After washed at 42°C in 2xSSC and 0.1% SDS, the membrane was exposed to X-ray film.

Data analysis

All experiments were performed four times. Data were expressed as mean $\pm$ 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 in IMR-32 cells by TBHP

To ensure the induction of oxidative stress by the treatment with TBHP in IMR-32 human neuroblastoma cells, we examined the effects of TBHP on the level of GSH and the activities of GSH peroxidase and reductase. Treatment with TBHP for 1 hour significantly decreased the GSH level and the activity of

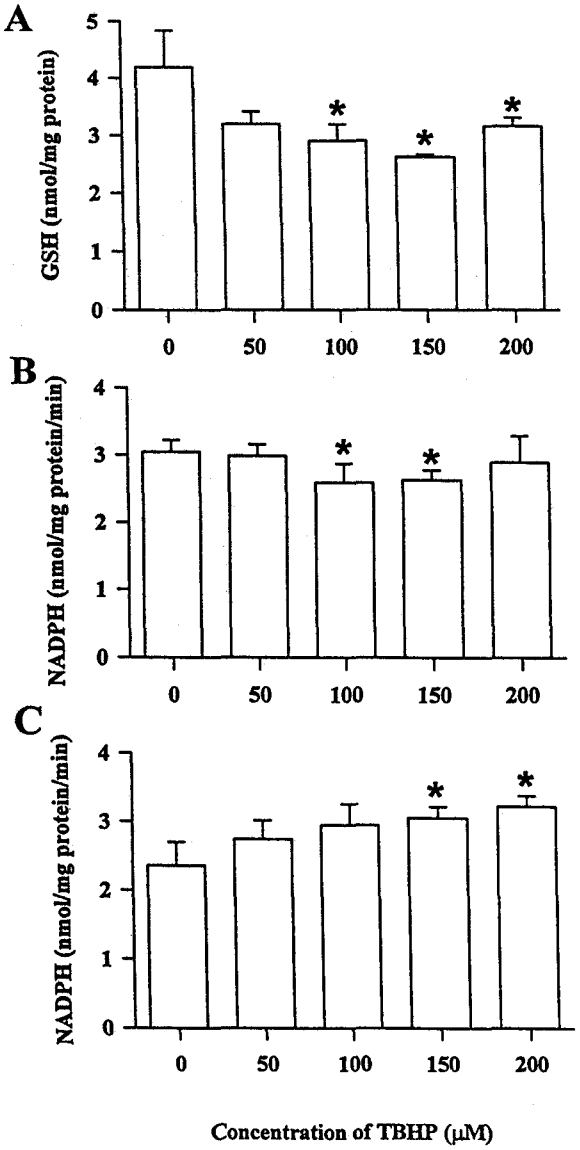

Fig. 1. Induction of oxidative stress by TBHP in IMR-32 human neuroblastoma cells. The data show changes of glutathione (GSH) content (A), GSH reductase activity (B) and GSH peroxidase activity (C) induced by 50-200 μM TBHP for 1 hour. Data points represent the mean values of four replications with bars indicating SEM. *p <0.05 compared to the control condition.

GSH reductase but increased the activity of GSH peroxidase as shown in Fig. 1. These results are comparable to the effects of other oxidative stress inducers (Post et al, 1998) and suggest that TBHP induced oxidative stress in the IMR-32 cells.

Apoptotic cell death by TBHP

The effect of TBHP on cell viability of the IMR-32 cells was examined using the MTT staining method (van de Loosdrecht et al, 1991). TBHP decreased cell viability in a dose-dependent manner as depicted in

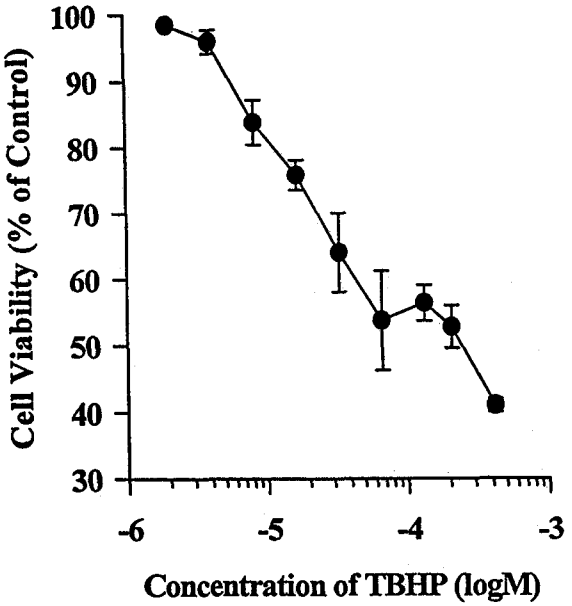

Fig. 2. Effects of TBHP on cell viability in IMR-32 human neuroblastoma cells. Cell viability assay was done by the MTT staining method. Results are expressed as percent change of control condition in which cells were grown in medium without drug. Data points represent the mean values of four replications with bars indicating SEM.

Fig. 2. The concentration ranges of TBHP inducing oxidative stress and cytotoxicity were found to be fairly correlated with each other. To confirm that the TBHP-induced cell death is due to the induction of apoptosis, we observed DNA fragmentation, a hallmark of apoptosis (Wyllie et al, 1984), using agarose gel electrophoresis. As represented in Fig. 3, TBHP induced a dose-dependent DNA fragmentation in the IMR-32 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

To examine the relationship between the observed apoptosis-inducing action of TBHP and intracellular Ca^{2+} signaling mechanisms, we measured intracellular Ca^{2+} concentration changes using Fura-2 fluorescence technique. As shown in Fig. 4A-a, TBHP (200 μM) induced a prolonged increase in intracellular Ca^{2+} concentration. To determine the source of the increased intracellular Ca^{2+} concentration induced by TBHP, we measured intracellular Ca^{2+} concentrations using a nominal Ca^{2+}-free medium containing 1.0 mM EGTA. This experimental protocol can effectively reduce extracellular free Ca^{2+} concentration, and

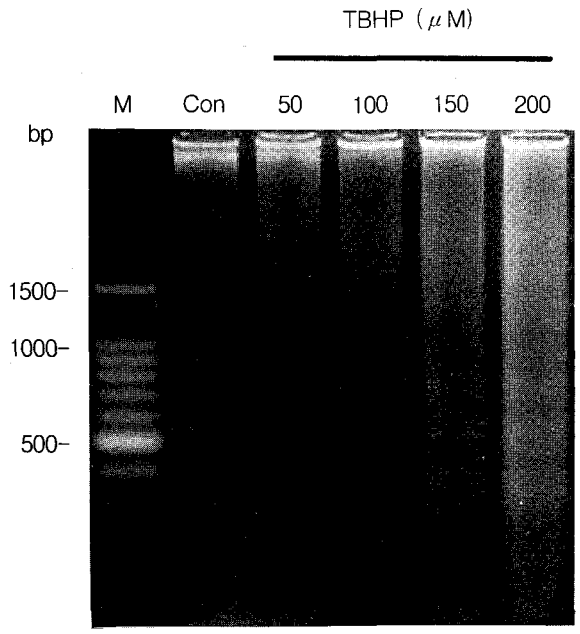

Fig. 3. TBHP-induced apoptosis in IMR-32 human neuroblastoma cells. Cells were treated without or with each concentration of TBHP for 1 hr. Fragmented DNAs were isolated from the cells and analyzed by 1.8% agarose gel electrophoresis. Lane M represents DNA marker.

thus, blunt available Ca^{2+} influx. Under these conditions, cellular response to TBHP was completely inhibited as illustrated in Fig. 4A-b. These results indicate that the TBHP-induced increase in intracellular Ca^{2+} concentration is exclusively due to Ca^{2+} influx from the extracellular site. In addition, the increased intracellular Ca^{2+} was significantly blocked by flufenamic acid (FA), a non-selective cation channel (NSCC) blocker. These results further suggest that TBHP may induce Ca^{2+} influx through the activation of NSCCs.

Role of intracellular Ca^{2+} in the TBHP-induced apoptosis

To determine the role of intracellular Ca^{2+} as a signal for the apoptotic cell death, we investigated the effects of BAPTA/AM, an intracellular Ca^{2+} chelator (Jiang et al, 1994) and FA on the TBHP-induced apoptosis in the IMR-32 cells by quantifying DNA fragmentation using DPA method. Figure 5 showed that treatment with either 2 μM BAPTA/AM or 50 μM FA significantly suppressed the TBHP-induced DNA fragmentation in the IMR-32 cells. These results suggest that intracellular Ca^{2+} signals may mediate the TBHP-induced apoptosis in the IMR-32 cells.

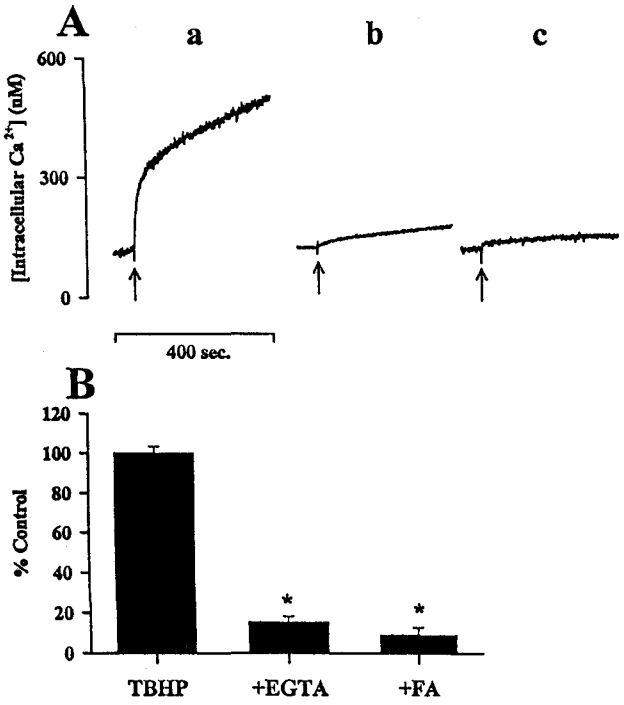

Fig. 4. TBHP induces Ca^{2+} influx in IMR-32 human neuroblastoma cells. Intracellular Ca^{2+} concentration was assessed by Fura-2 fluorescence technique. The data (A) represent intracellular Ca^{2+} changes with time measured in normal Ca^{2+}-containing medium (a), in nominal Ca^{2+}-free medium containing 1.0 mM EGTA (b), or in the presence of 50 μM flufenamic acid (c). The arrows show the time points for addition of 200 μM TBHP. The data (B) show quantitative changes of the increased intracellular Ca^{2+} concentration induced by the drug compared to TBHP alone. Each column represents the mean value of four replications with bars indicating SEM (*$p < 0.05$ compared to TBHP alone).

Effects of TBHP on the gene expression of p53, c-myc, bcl-2

To examine whether TBHP has an effect on the expression of apoptosis-regulatory genes, we measured the expression of *p53, c-myc, bcl-2* mRNA level. As shown in Fig. 6, TBHP induced increase in the level of *p53*, decrease in *c-myc*, and no change in *bcl-2* mRNA levels.

DISCUSSION

Oxidative stress appears to be involved in the mechanism of various types of cell injury (Shlafer et al, 1982; Stohs, 1995; Davis, 1996; Jenner & Olanow, 1996). Particularly, neuronal cells are highly likely to suffer ROS-induced cytotoxicity (Coyle & Puttfarc-

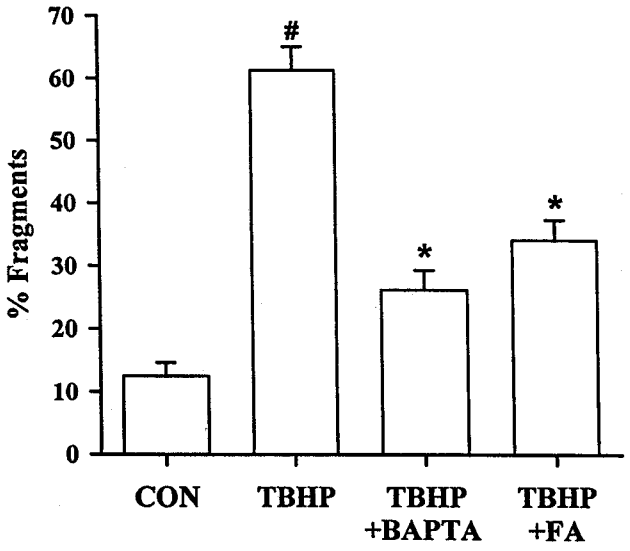

Fig. 5. Role of intracellular Ca^{2+} in the TBHP-induced apoptosis in IMR-32 human neuroblastoma cells. BAPTA/AM (2 μM), an intracellular Ca^{2+} chelator, and FA (50 μM), a nonselective cation channel blocker, were added to the cells 4 hr and 1 hr before treatment with 200 μM TBHP for 1 hour, respectively. 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.

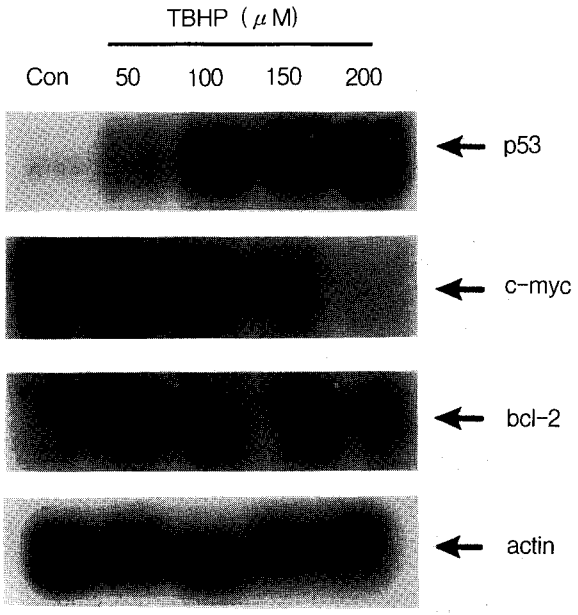

Fig. 6. Northern blot analyses on *p53, c-myc, and bcl-2* in TBHP-treated IMR-32 cells. Total RNA from the cells treated with TBHP for 1 hr was electrophoresed and transferred to ZetaProbe membrane. Northern blots were performed by hybridizing the membrane with ^{32}P-labeled cDNAs of *p53, c-myc, bcl-2*. Actin gene was used for verifying even loading of RNAs.

ken, 1993) because neurons consume large amounts of oxygen during normal metabolism and possess free radical-generating enzymes such as monoamine oxidase, tyrosine hydroxylase, and L-amino oxidase. Recently, ROS 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 mechanism of ROS-induced apoptosis in neuronal cells has not been fully discovered yet.

In the present study, we showed that TBHP, used as an oxidative stress inducer (Soszynski & Bartosz, 1997; Gorbunov et al, 1998), produced oxidative stress (Fig. 1) and induced apoptotic cell death (Fig. 3) in IMR-32 cells, which are consistent with other previous reports using other cell types (Langley et al, 1993; Kondo et al, 1997; Kim et al, 1998).

Intracellular Ca^{2+} has been shown to act as a common mediator of chemically induced cell death (Harman & Maxwell, 1995). Intracellular Ca^{2+} also appears to play a role as a signal transducer in the mechanism of apoptosis (Distelhorst & Dubyak, 1998). Thus, in this study, we investigated the possible involvement of intracellular Ca^{2+} signals in the TBHP-induced apoptosis in the IMR-32 cells. The results showed that TBHP increased intracellular Ca^{2+} concentration through Ca^{2+} influx (Fig. 4). Significant blockade of the TBHP-induced DNA fragmentation by pretreatment with BAPTA/AM, an intracellular Ca^{2+} chelator (Fig. 5), indicates that induced Ca^{2+} influx may mediate the observed apoptotic action of TBHP. Previously, we have also reported that in human hepatoma cells, TBHP induced intracellular Ca^{2+} increase via the influx mechanism (Kim et al, 1998).

Extracellular Ca^{2+} ions appear to enter the cells by the following two mechanisms: i) activation of Ca^{2+} channels and ii) activation of reverse mode of Na^{+}/Ca^{2+} exchange mechanism. It has been reported that voltage-dependent nonselective cation channels (NSCCs) exist in various cell types (Chen et al, 1997). Although the presence of the NSCCs in IMR-32 cells has not been studied yet, TBHP may induce Ca^{2+} influx through the activation of these NSCCs since the NSCCs seem to be invlolved in the regulation of Ca^{2+} influx (Chen et al, 1997). Interestingly, in cardiac myocytes, the activity of NSCCs has been shown to be increased by treatment with ROS (Jabr & Cole, 1995). Na^{+}/Ca^{2+} exchanger normally acts to extrude Ca^{2+} ions when intracellular Ca^{2+} rises above certain

levels (DiPolo & Beauge, 1987). However, Ca^{2+} ions enter the cells under conditions that favor the reverse mode of operation of the Na^{+}/Ca^{2+} exchanger (DiPolo & 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). ROS 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 has any effect on reverse operation of Na^{+}/Ca^{2+} exchanger.

The induction of oxidative stress by TBHP (Fig. 1) seems to be mediated by ROS released from TBHP. Thus, the TBHP-induced Ca^{2+} influx (Fig. 4) may be due to the actions of ROS. In other studies, exogenous administration of ROS such as hydrogen peroxide, increased intracellular Ca^{2+} concentration in neuronal cells (Tretter & Adam-Vizi, 1996) and in human endothelial cells (Dreher & Junod, 1995). However, the Ca^{2+} source appears to be different, depending on the types of cells and ROS used in these experiments. For example, hydrogen peroxide has been shown to induce Ca^{2+} influx in neuronal cells (Tretter & Adam-Vizi, 1996), whereas superoxide anion triggers Ca^{2+} release from cardiac sarcoplasmic reticulum through ryanodine receptor Ca^{2+} channels (Kawakami & Okabe, 1998).

Accumulating evidence indicates that intracellular Ca^{2+} signal is involved in the mechanism of apoptosis (McConkey & Orrenius, 1996). One of the targets for the elevation of intracellular Ca^{2+} concentration is the activation of Ca^{2+}-dependent protein kinases and phosphatases. Direct activation of the Ca^{2+}-dependent neutral proteinase, calpain, may represent another target for intracellular Ca^{2+} action in apoptosis. Increased intracellular Ca^{2+} also activates Ca^{2+}/Mg^{2+}-dependent endonuclease (Cohen & Duke, 1984), resulting in DNA fragmentation, the most characteristic biochemical feature of apoptosis (Wyllie et al, 1984).

Decreased expression of *c-myc* in our study (Fig. 6) is relevant to the previous reports that reduced expression of *c-myc* mRNA and its inappropriate expression are associated with cellular apoptosis (reviewed by Thompson, 1998). Induction of p53 can lead to either cell cycle arrest or cell death depending on the cell types and insult level. Our results of increased expression of *p53* by TBHP treatment in the IMR-32 cells (Fig. 6) are consistent with other reports in which *p53* was induced during the apoptosis by ROS (Lotem et al, 1996; Kinscherf, 1998).

Bcl-2 and its family members have been recently identified as dual acting genes in apoptosis. Whereas *bcl-2, bcl-XL, mcl-1* and *BAG-1* act as inhibitors of apoptosis (Boise et al, 1993; Reynolds et al, 1994; Takayama et al, 1995), *bad, bax, bak and bik* act as promoters of apoptosis (Oltvai et al, 1993; Boyd et al, 1995; Yang et al, 1995). Bcl-2 is localized in the outer mitochondrial membranes, nuclear envelope, and endoplasmic reticulum (Krajewski et al, 1993), where ROS are generated. There has been a controversy that Bcl-2 acts as an antioxidant (Hockenbery et al, 1993) or as a pro-oxidant (Steinman, 1995). Although the expression of bcl-2 was not changed in the present study (Fig. 6), we can not exclude the possibility that other bcl-2 family members such as *bax* may be involved in the TBHP-induced apoptosis. Moreover, transcription factor *p53* whose expression was increased by TBHP (Fig. 6), has been shown to be a direct transcriptional activator of *bax* genes (Miyashita et al, 1994).

In this study, we did not investigate the interrelationship between the TBHP-induced activation of intracellular Ca^{2+} signals and altered expression of these genes. No information is available for that. We are currently pursuing more studies to elucidate the molecular mechanism underlying altered expression of *p53* and *c-myc* during TBHP-induced apoptosis in relation to intracellular Ca^{2+} increase.

In conclusion, TBHP induced the apoptotic cell death in a human neuroblastoma cell line, and intracellular Ca^{2+} signals and altered expression of *p53* and *c-myc* may mediate this action of TBHP. These results suggest that apoptosis may be a key step in the pathogenesis of various neurodegenerative diseases related with oxidative stress.

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REFERENCES

Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turk LA, Mao X Nunez G, Thompson CB. bcl-x, a bcl-2 related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 74: 597, 1993

Bonnefoy-Berard N, Genestier L, Flacher M, Revillard JP. The phosphoprotein phosphatase calcineurin con-

trols calcium-dependent apoptosis in B cell lines. *Eur J Immunol* 24: 325−329, 1994

Boyd JM, Gallo GJ, Elangovan B, Houghton AB, Malstrom S, Avery BJ, Ebb RG, Subramanian T, Chittenden T, Lutz RJ. Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 femily proteins and interacts with viral and cellular survival-promoting proteins. *Oncogene* 11: 1921−1928, 1995

Chen WH, Yeh TH, Tsai MC, Chen DS, Wang TH. Characterization of Ca^{2+}- and voltage-dependent non-selective cation channels in human HepG2 cells. *J Formos Med Assoc* 96: 503−510, 1997

Choi DW. Glutamate neurotoxicity and diseases of the nervous system. *Neuron* 1: 623−34, 1988

Clemens JA, Panetta JA. Free radicals in central nervous system diseases. *eds. Blake D. and Winyard P.G., Immunopharmacology of free radical species,* Academic Press, London, UK, p73−83, 1995

Cohen JJ, Duke RC. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J Immunol* 132: 38−42, 1984

Coyle JT, Puttfarcken P. Oxidative stress, glutamate, and neurodegenerative disorders. *Science* 262: 689−694, 1993

Davis JB. Oxidative mechanisms in β-amyloid cytotoxicity. *Neurodegeneration* 5: 441−444, 1996

Demaurex N, Monod A, Lew DP, Krause KH. Characterization of receptor-mediated and store-regulated Ca^{2+} influx in human neutrophils. *Biochem J* 297: 595−601, 1994

DiPolo R, Beauge L. The squid axon as a model for studying plasma membrane mechanisms for calcium regulation. *Hypertension* 10: I15−I19, 1987

Distelhorst CW, Dubyak G. Role of calcium in glucocorticosteroid-induced apoptosis of thymocytes and lymphoma cells: resurrection of old theories by new findings. *Blood* 91: 731−734, 1998

Dreher D, Junod AF. Differential effects of superoxide, hydrogen peroxide, and hydroxyl radical on intracellular calcium in human endothelial cells. *J Cell Physiol* 162: 147−153, 1995

Goldhaber JI. Free radicals enhance Na^+/Ca^{2+} exchange in ventricular myocytes. *Am J Physiol* 271: H823−H833, 1996

Gorbunov NV, Tyurina YY, Salama G, Day BW, Claycamp HG, Argyros G, Elsayed NM, Kagan, V. E. Nitric oxide protects cardiomyocytes against *tert*-butyl hydroperoxide-induced formation of alkoxyl and peroxyl radicals and peroxidation of phosphatidylserine. *Biochem Biophys Res Commun* 244: 647−651, 1998

Grynkiewicz G, Poene M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260: 3440−3450, 1985

Harman AW, Maxwell MJ. An evaluation of the role of calcium in cell injury. *Annu Rev Pharmacol Toxicol* 35: 129−144, 1995

Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348: 334−336, 1990

Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsmeyer SJ. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* 75: 241−251, 1993

Ishii H, Kurose I, Kato S. Pathogenesis of alcoholic liver disease with particular emphasis on oxidative stress. *J Gastroenterol Hepatol* 12: S272−S282, 1997

Jabr RI, Cole WC. Oxygen-derived free radical stress activates nonselective cation current in guinea pig ventricular myocytes. Role of sulfhydryl groups. *Circ Res* 76: 812−824, 1995

Jenner P, Olanow CW. Oxidative stress and the pathogenesis of Parkinson's disease. *Neurology* 47: S161−S170, 1996

Jiang S, Chow SC, Nicotera P, Orrenius S. Intracellular Ca^{2+} signals activate apoptosis in thymocytes: Studies using the Ca^{2+}-ATPase inhibitor thapsigargin. *Exp Cell Res* 212: 84−92, 1994

Kawakami M, Okabe E. Superoxide anion radical-triggered Ca^{2+} release from cardiac sarcoplasmic reticulum through ryanodine receptor Ca^{2+} channel. *Mol Pharmacol* 53: 497−503, 1998

Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26: 239−257, 1972

Kigoshi T, Nakano S, Okamoto H, Imaizumi N, Kitazawa M, Ishii T, Morimoto S, Uchida K. Enhancement of potassium-, and angiotensin II-stimulated aldosterone production by the calcium chelator EGTA in bovine adrenal glomerulosa cells in vitro. *J Steroid Biochem Mol Biol* 60: 215−220, 1997

Kim J-A, Kang YS, Kim YO, Lee SH, Lee YS. Role of Ca^{2+} influx in the tert-butyl hydroperoxide-induced apoptosis of IMR32 human hepatoblastoma cells. *Exp Mol Med* 30: 137−144, 1998

Kinscherf R, Claus R, Wagner M, Gehrke C, Kamencic H, Hou D, Nauen O, Schmiedt W, Kovacs G, Pill J, Metz J, Deigner HP. Apoptosis caused by oxidized LDL is manganese superoxide dismutase and p53 dependent. *FASEB J* 12: 461−7, 1998

Kondo Y, Rusnak JM, Hoyt DG, Settineri CE, Pitt BR, Lazo JS. Enhanced apoptosis in metallothionein null cells. *Mol Pharmacol* 52: 195−201, 1997

Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, Reed JC. Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res* 53: 4701−4714, 1993

Langley RE, Palayoor ST, Coleman CN, Bump EA. Modifiers of radiation-induced apoptosis. *Radiat Res* 136: 320−326, 1993

Lotem J, Peled-Kamar M, Groner Y, Sachs L. Cellular oxidative stress and the control of apoptosis by wild-type p53, cytotoxic compounds, and cytokines. *Proc Natl Acad Sci USA* 93: 9166−9171, 1996

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265−275, 1951

McConkey DJ, Orrenius S. The role of calcium in the regulation of apoptosis. *J Leukoc Biol* 59: 775−783, 1996

Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B, Reed JC. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and invivo. *Oncogene* 9: 1799−1805, 1994

Natarajan N, Shambaugh GE 3rd, Elseth KM, Haines GK, Radosevich JA. Adaptation of the diphenylamine (DPA) assay to a 96-well plate tissue culture format and comparison with the MTT assay. *Biotechniques* 17: 166−171, 1994

Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 74: 609−619, 1993

Post A, Holsboer F, Behl C. Induction of NF-kappaB activity during haloperidol-induced oxidative toxicity in clonal hippocampal cells: suppression of NF-kappaB and neuroprotection by antioxidants. *J Neurosci* 18: 8236−46, 1998

Reynolds JE, Yang T, Qian L, Jenkinson JD, Zhou P, Eastman A, Craig RW. Mcl-1, a member of the bcl-2 family, delays apoptosis induced by c-Myc overexpression in Chiness hamster ovary cells. *Cancer Res* 54: 6348−6352, 1994

Rossi L, Silva JM, McGirr LG, O'Brien PJ. Nitrofurantoin-mediated oxidative stress cytotoxicity in isolated rat hepatocytes. *Biochem Pharmacol* 37: 3109−3117, 1988

Ryan JJ, Prochownik E, Dottlieb CA, Apel IJ, Merino R, Nunez G, Clarke MF. *c-myc* and *bcl-2* modulates p53 function by altering p53 subcellular trafficking during the cell cycle. *Proc Natl Acad Sci USA* 91: 5878−5882, 1994

Salkie ML, Simpson E. The erythrocyte glutathione reductase activity (E.C. 1.6.4.2) of healthy blood donors. *J Clin Pathol* 23: 708−710, 1970

Schiaffonati L, Tiberio L. Gene expression in liver after toxic injury: analysis of heat shock response and oxidative stress-inducible genes. *Liver* 17: 183−191, 1997

Shlafer M, Kane PF, Wiggins VY, Kirsh MM. Possible role for cytotoxic oxygen metabolites in the pathogenesis of cardiac ischemic injury. *Circulation* 66: I85−I92, 1982

Slater AF, Nobel CS, Orrenius S. The role of intracellular oxidants in apoptosis. *Biochim Biophys Acta* 1271: 59−62, 1995

Soszynski M, Bartosz G. Decrease in accessible thiols as an index of oxidative damage to membrane proteins. *Free Radic Biol Med* 23: 463−469, 1997

Srivastava SK, Beutler E. Accurate measurement of oxidized glutathione content of human, rabbit, and rat red blood cells and tissues. *Anal Biochem* 25: 70−76, 1968

Steinman HM. The bcl-2 oncoprotein functions as a prooxidant. *J Biol Chem* 270: 3487−3490, 1995

Stohs SJ. The role of free radicals in toxicity and disease. *J Basic Clin Physiol Pharmacol* 6: 205−228, 1995

Stoian I, Oros A, Moldoveanu E. Apoptosis and free radicals. *Biochem Mol Med* 59: 93−97, 1996

Stys PK, Waxman SG, Ransom BR. Na^+-Ca^{2+} exchanger mediates Ca^{2+} influx during anoxia in mammalian central nervous system white matter. *Ann Neurol* 30: 375−380, 199

Takayama S, Sato T, Krajewski S, Kochel K, Irie S, Millan JA, Reed JC. Cloning and functional analysis of BAG-1: a novel bcl-2-binding protein with anti-cell death activity. *Cell* 80: 279−284, 1995

Tappel AL. Glutathione peroxidase and hydroperoxides. *Methods Enzymol* 52: 506−513, 1978

Thompson EB. The many roles of c-Myc in apoptosis. *Annu Rev Physiol* 60: 575−600, 1998

Tretter L, Adam-Vizi V. Early events in free radical-mediated damage of isolated nerve terminals: effects of peroxides on membrane potential and intracellular Na^+ and Ca^{2+} concentrations. *J Neurochem* 66: 2057−2066, 1996

van de Loosdrecht AA, Nennie E, Ossenkoppele GJ, Beelen RH, Langenhuijsen MM. Cell mediated cytotoxicity against U 937 cells by human monocytes and macrophages in a modified colorimetric MTT assay. A methodological study. *J Immunol Methods* 141: 15−22, 1991

Wyllie AH, Morris RG, Smith AL, Dunlop D. Chromatin cleavage in apoptosis: Association with condensed chromatin morphology and dependence on macromolecular synthesis. *J Pathol* 142: 67−67, 1984

Yang E, Jha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* 80: 285−291, 1995

Yu BP. Cellular defenses against damage from reactive oxygen species. *Physiol Rev* 74: 139−162, 1994