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Original Research Communication

Elevation of Intracellular Calcium Ions Is Essential for the H_2O_2-Induced Activation of SAPK/JNK but Not for That of p38 and ERK in Chinese Hamster V79 Cells

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ABSTRACT

The mitogen-activated protein kinases (MAPK), including stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), p38, and extracellular signal-related kinase (ERK), are believed to be important biomolecules in cell proliferation, survival, and apoptosis induced by extracellular stimuli. In Chinese hamster V79 cells exposed to hydrogen peroxide (H_2O_2), we recently demonstrated that SAPK/JNK was activated by tyrosine kinase and intracellular Ca^{2+} ($[Ca^{2+}]_i$). In this study, we report that $[Ca^{2+}]_i$ release from intracellular stores is important in the activation of SAPK/JNK but not p38 and ERK. H_2O_2-induced elevation of $[Ca^{2+}]_i$ was observed in Ca^{2+}-free medium. Pretreatment with thapsigargin, a Ca^{2+}-ATPase inhibition of endoplasmic reticulum (ER), did not influence H_2O_2-induced elevation of $[Ca^{2+}]_i$ in the absence of external Ca^{2+}. An intracellular Ca^{2+} chelator (BAPTA-AM) inhibited H_2O_2-induced phosphorylation of SAPK/JNK, but an extracellular Ca^{2+} chelator (EDTA) or a Ca^{2+} entry blocker ($NiCl_2$) did not. Activation of p38 and ERK in V79 cells exposed to H_2O_2 was observed in the presence of these inhibitors. These results suggest that $[Ca^{2+}]_i$ release from intracellular stores such as mitochondria or nuclei but not ER, occurred after H_2O_2 treatment and Ca^{2+}-dependent tyrosine kinase-induced activation of SAPK/JNK, although $[Ca^{2+}]_i$ was unnecessary for the H_2O_2-induced activation of p38 and ERK. Antiox. Redox Signal. 1, 501–508.

INTRODUCTION

In oxidative stress caused by reactive oxygen intermediate species (ROIs) (Hiraoka *et al.*, 1997; Morris and Sukakhe, 1997; Krainev *et al.*, 1997; Viner *et al.*, 1997; Inanami *et al.*, 1999b; Suzuki *et al.*, 1998), ionizing radiation (Voehringer *et al.*, 1997; Takahashi *et al.*, 1999), peroxide (Kim *et al.*, 1998), UV (Wang *et al.*, 1999), and photodynamic action (Inanami *et al.*, 1999c), the elevation of $[Ca^{2+}]_i$ occurs and it is believed to be an important factor in both apoptosis and necrosis (Lemasters *et al.*, 1998). In the mechanism of Ca^{2+}-induced cell death, the dysfunction of mitochondria, for example, their swelling and a decline of membrane potential (Petit *et al.*, 1998; Robb *et al.*, 1999), as well as the release of various apoptotic factors, *i.e.*, cytochrome *c* (Inanami *et al.*, 1999c), caspase-9 (Krajewski *et al.*, 1999), and apoptosis-inducing factor (AIF) (Petit *et al.*, 1998) by mitochondria, were recently reported to be followed by elevation of $[Ca^{2+}]_i$. As another important biomolecule involved in cell death and survival, mitogen-activated protein kinases (MAPK), including SAPK/JNK, p38, and extracellular signal-related kinase (ERK), are known to be activated by oxidative stress (Cuvillier *et al.*, 1996; Inanami *et al.*, 1999a,b; Wang *et al.*, 1998). Cuvillier *et al.* (1996) demonstrated that the balance between ERK and SAPK/JNK determined the fate of cells (cell growth or cell death).

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There is much evidence that the activation of SAPK/JNK is deeply associated with apoptosis and that of ERK with proliferative and survival signals (Cuvillier *et al.*, 1996; Inanami *et al.*, 1999a; Wang *et al.*, 1998). However, the interaction between $[Ca^{2+}]_i$ and MAP kinases in oxidative stress is still uncertain, although both molecules are recognized as key factors in oxidative stress-induced cell death. In a previous study, we found that $[Ca^{2+}]_i$ elevation, Ca^{2+}-dependent tyrosine kinase and phosphatidylinositol 3-kinase (PI3 kinase) played essential roles in the upstream signals of the activation of SAPK/JNK after H_2O_2 treatment of Chinese hamster V79 cells (Inanami *et al.*, 1999b). In this communication, we report that $[Ca^{2+}]_i$ elevation originated from internal Ca^{2+} stores is essential for the activation of SAPK/JNK but not p38 and ERK, in hydrogen peroxide (H_2O_2)-treated V79 cells and discuss the relationships among cell death, $[Ca^{2+}]_i$, and these kinases.

MATERIALS AND METHODS

Materials

Rabbit polyclonal antibodies recognizing phosphorylated human SAPK/JNK (Thr183/Tyr185), p38 (Thr180/Tyr182), and ERK (Thr202/Tyr204) were purchased from New England Biolab, Ltd. (Beverly, MA). BAPTA-AM and fura2-AM were obtained from Dojindo Chemical Co. (Kumamoto, Japan). Horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine antibody (PY-20) was purchased from Signal Transduction Laboratories (Lexington, KY). H_2O_2 and the other reagents were from Wako Pure Chemical Co. (Osaka, Japan).

Cell culture

V79 cells were maintained routinely with α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS). The cultured cells were exposed to phosphate-buffered saline (PBS; 138 m*M* NaCl, 2.7 m*M* KCl, 8.1 m*M* Na_2HPO_4, 1.15 m*M* KH_2PO_4, 1 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$) containing 10 m*M* H_2O_2, and were maintained in a CO_2 incubator at 37°C. For evaluation of Ca^{2+} entry from extracellular fluid, 5 m*M* EDTA or 2 m*M* $NiCl_2$ was added to the H_2O_2-containing solution. An intracellular Ca^{2+} chelator, 5 μM BAPTA-AM, was added to the culture medium 1 hr prior to the treatment with H_2O_2.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ in one colony (4–5 cells) was measured with a fluorescent Ca^{2+} indicator, fura2 (Grynkiewicz *et al.*, 1985) by a ratio method using dual-wavelength excitation and single emission with a fluorometer (CAM-200, Jasco, Japan). V79 cells attached to a coverslip were incubated with medium containing 5 μM fura2-AM for 0.5–1 hr at room temperature. After incubation, the coverslip with cells was set in a small chamber (0.2 ml in volume) on the stage of an inverted microscope (TMD, Nikon, Japan) equipped with a fluorometer (CAM-200, Jasco, Japan). Fura2-loaded cells were illuminated by alternate beams of excitation light at 340 and 380 nm. The fluorescent signal was detected with a CF UV lens (Nikon Fluor x40 oil-immersion objective). The emission light passing through a pinhole diaphragm slightly larger than the colony was collected by a photomultiplier through a 500-nm filter. Fluorescent signals and their ratios were stored on the hard disk of a computer (Macintosh, Apple, Japan) through an A/D converter (MacLab 4e, AD Instruments, Australia). The calibration of fura2 signals is described elsewhere (Ohta *et al.*, 1996). Cells were continuously prepared with PBS by a peristaltic pump at a flow rate of 2 ml/min at 37°C.

Immunoblot analysis of SAPK/JNK, p38, and ERK

Immunoblotting of phospho-SAPK/JNK was performed as follows: 2×10^6 cells with or without treatment using H_2O_2 were collected with a cell scraper and washed twice with ice-cold PBS. Then 200 μl of Laemmli's sample buffer (125 m*M* Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 1% β-mercaptoethanol, 0.006% bromophenol blue) was directly added to the cell pellet. The solution was triply sonicated for 20 sec each time on ice, boiled for 3 min, and subjected to 10% SDS-PAGE. The proteins in the gel were electrotransferred to a nitrocellulose membrane. The membrane blocked by TBST

(10 m*M* Tris-HCl, pH 7.4, 100 m*M* NaCl, 0.1% Tween 20) containing 5% skim milk was probed with anti-human phospho-SAPK/JNK (1/1,000), anti-human p38 (1/1,000), and anti-human phospho-ERK (1/1,000), and was detected with a chemiluminescence detection kit (Boehringer Mannheim, GmbH, Germany). To detect phosphotyrosine using PY-20 (1/2,000), TBST containing 5% bovine serum albumin (BSA) was used for blocking and staining the solutions.

RESULTS

Ca^{2+}-dependent activation of SAPK/JNK and no activation of p38, ERK, and tyrosine kinase by H_2O_2

The activities of three MAP kinases, SAPK/JNK, p38, and ERK, and phosphotyrosine of proteins were determined by western blotting in V79 cells exposed for 15 min to 10 m*M* H_2O_2. Because the MAP kinases, such as MEK, SEK, MKK3, and MKK6, activate MAP kinase by phosphorylating at threonine and tyrosine residues, the existence of the phosphorylated MAP kinases indicates the activation of the kinases. The results presented in lane 2 of Fig. 1 show the H_2O_2-induced activation of three MAP kinases and the accumulation of phosphotyrosine due to the activation of tyrosine kinase. The activation of SAPK/JNK by H_2O_2 was inhibited by pretreatment with BAPTA-AM, an intracellular Ca^{2+} chelator. In contrast, it did not affect the activation of p38, ERK, and tyrosine kinases, as shown in lane 3 of Fig. 1. These results indicated that $[Ca^{2+}]_i$ was an important factor in the oxidative stress-induced activation of SAPK/JNK but not in the activation of p38, ERK, and global tyrosine kinases. To examine the origin of this intracellular Ca^{2+} responsible for the activation of SAPK/JNK by H_2O_2, the effects of the extracellular Ca^{2+}-chelator EDTA and nonselective Ca^{2+} channel blocker $NiCl_2$ on the activation of SAPK/JNK by H_2O_2 were examined. Treatments with EDTA (lane 4) and $NiCl_2$ induced no changes in the activation of SAPK/JNK (lane 5). These results suggest that the elevation of $[Ca^{2+}]_i$ from the intracellular stores is important in the activation of SAPK/JNK by H_2O_2.

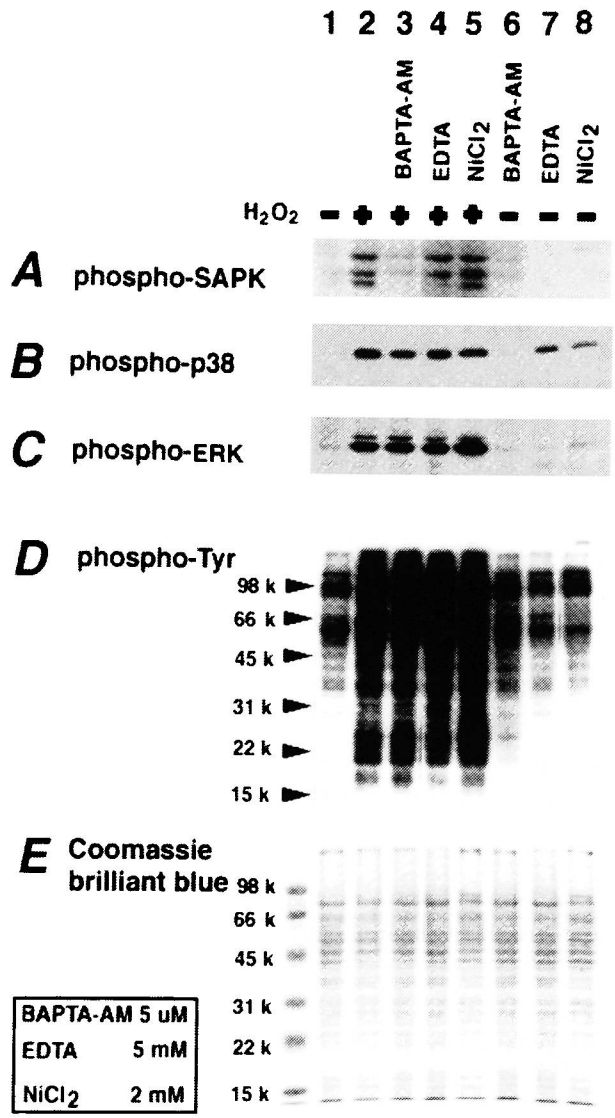

FIG. 1. Immunoblot analysis of phosphorylated SAPK/JNK (A), phosphorylated p38 (B), phosphorylated ERK (C), and phosphotyrosine (D) at 15 min in Chinese hamster V79 cells exposed to 10 m*M* H_2O_2 and effects of various Ca^{2+}-related inhibitors on their phosphorylation. (E) Coomassie brilliant blue stain. Lane 1, Untreated cells; lane 2, cells treated with 10 m*M* H_2O_2; lane 3, cells treated with 10 m*M* H_2O_2 + 5 μ*M* BAPTA-AM; lane 4, cells treated with 10 m*M* H_2O_2 + 5 m*M* EDTA; lane 5, cells treated with 10 m*M* H_2O_2 + 2 m*M* $NiCl_2$; lane 6, cells treated with 5 μ*M* BAPTA-AM; lane 7, cells treated with 5 m*M* EDTA; lane 8, cells treated with 2 m*M* $NiCl_2$.

H_2O_2-induced elevation of $[Ca^{2+}]_i$ by release from the intracellular stores and entry from extracellular fluid

To confirm the elevation of $[Ca^{2+}]_i$ due to release from the intracellular stores, V79 cells loaded with fura2-AM, and the effect of H_2O_2

on the fura2 ratio ($[Ca^{2+}]_i$) was monitored. In the presence of 2.5 m*M* $CaCl_2$, H_2O_2 produced a biphasic elevation of $[Ca^{2+}]_i$ (Fig. 2A). Namely, rapid elevation occurred within 1 min, followed by the gradual elevation of $[Ca^{2+}]_i$. In Ca^{2+}-free solution containing 0.5 m*M* EGTA, the H_2O_2-induced initial elevation of $[Ca^{2+}]_i$, but not the gradual phase, was abolished (Fig. 2B). The elevation of $[Ca^{2+}]_i$ evoked by H_2O_2 in the absence of extracellular Ca^{2+} was about one-third that in the presence of extracellular Ca^{2+} (2.5 m*M*) (Fig. 2D). These results indicated that the H_2O_2-induced elevation of $[Ca^{2+}]_i$ resulted from both extracellular and intracellular sources. Because the H_2O_2-induced activation of SAPK/JNK still occurred even under conditions in which extracellular Ca^{2+} was removed by addition of EDTA, the elevation of $[Ca^{2+}]_i$ from the intracellular stores seemed to play an important role in the activation of SAPK/JNK by H_2O_2. Furthermore, this increased-response of $[Ca^{2+}]_i$ in extracellular Ca^{2+}-free condition was observed in 1 m*M* H_2O_2 treatment (data not shown).

To clarify the intracellular source of $[Ca^{2+}]_i$, cells were pretreated with the Ca^{2+}-ATPase inhibitor thapsigargin in Ca^{2+}-free medium. This treatment is known to achieve depletion of Ca^{2+} stores of Ca^{2+}-ATPase-rich organelles such as endoplasmic reticulum (ER) (Thastrup *et al.*, 1990). Following treatment with thapsigargin, a transient elevation of $[Ca^{2+}]_i$ due to impairment of Ca^{2+}-ATPase occurred and then it returned to the control level as shown in Fig. 2C. The subsequent addition of 10 m*M* H_2O_2 resulted in a response indistinguishable from

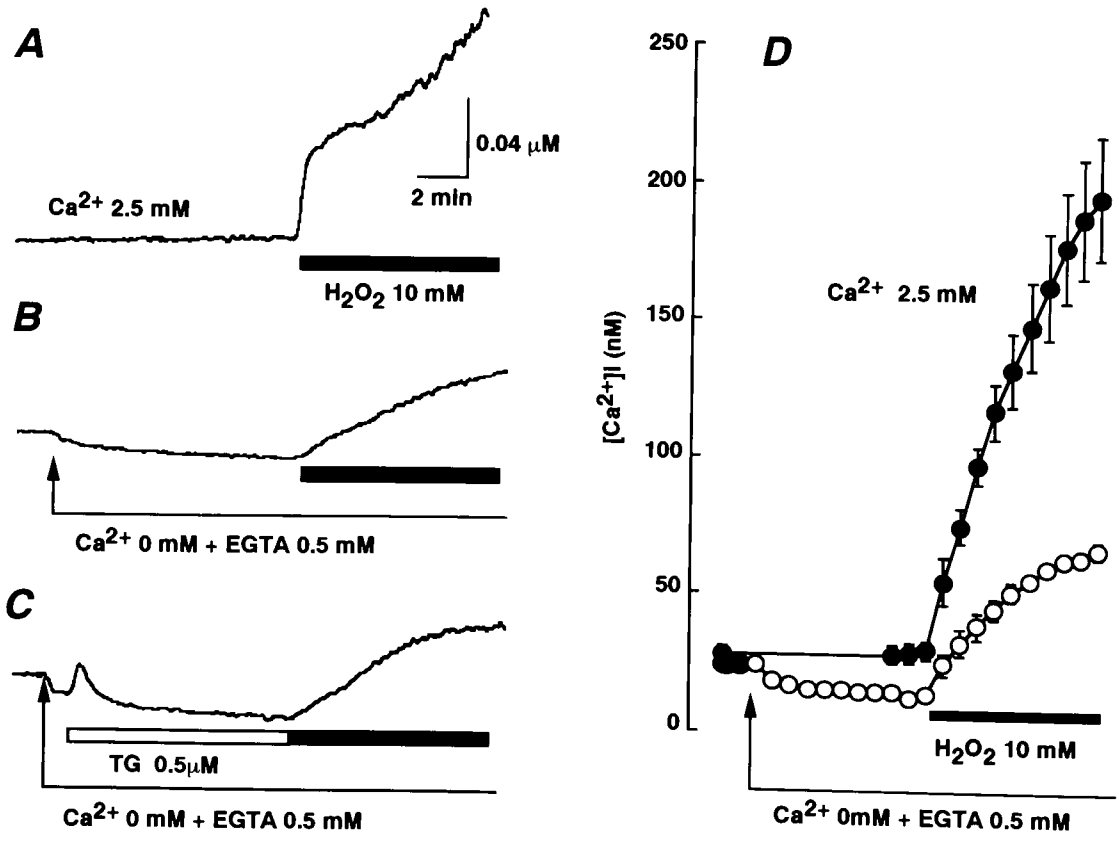

FIG. 2. Change in the $[Ca^{2+}]_i$ in H_2O_2-treated Chinese hamster V79 in the presence (A) and absence (B) of extracellular Ca^{2+}. Fura2-loaded cells were challenged with 10 m*M* H_2O_2 as shown by solid bars. (C) To inhibit the activity of Ca^{2+}-ATPase, 0.5 μM TG was added prior to exposure of cells to 10 m*M* H_2O_2 without extracellular Ca^{2+}, as indicated by the open bar. (D) Summary of change in $[Ca^{2+}]_i$ evoked by H_2O_2 obtained in the presence (●) and absence (○) of extracellular Ca^{2+} were plotted at time (every 1 min). Each symbol and error bar represents mean ± SEM of $n = 8$ (●) and $n = 6$ (○).

that of cells without and with thapsigargin. Thus, the ER is not likely to function as an intracellular store responsible for H_2O_2-induced elevation of $[Ca^{2+}]_i$.

DISCUSSION

A previous study demonstrated that 20 μM–1 mM H_2O_2 activated SAPK/JNK through a tyrosine kinase/PI 3 kinase pathway regulated by $[Ca^{2+}]_i$ (Inanami *et al.*, 1999b). Here we described the effects of 10 mM H_2O_2 on the activation of three MAP kinases (SAPK/JNK, p38, and ERK) and the relationship between these kinases and $[Ca^{2+}]_i$. As shown in Fig. 1, H_2O_2-treatment of V79 cells induced the activation of three MAP kinases. However, from the results with the Ca^{2+} chelator BAPTA-AM, the requirement of H_2O_2-induced elevation of $[Ca^{2+}]_i$ was clearly demonstrated for the activation of SAPK/JNK but not that of p38, ERK, and tyrosine kinases. Similar results were observed in lower concentrations (1 mM) of H_2O_2 (data not shown). Furthermore, this H_2O_2-induced activation of SAPK/JNK is not required for Ca^{2+} influx from extracellular medium, because the extracellular Ca^{2+} chelator EDTA and nonselective Ca^{2+} channel blocker $NiCl_2$ did not affect the SAPK/JNK activation. In addition, p38 was activated by EDTA and $NiCl_2$ alone but not BAPTA-AM, as shown in lanes 6–8 of Fig. 1B. It seems that the elimination of extracellular Ca^{2+} rather than that of intracellular Ca^{2+} induces a stress response and specifically activates p38.

In fura2-loaded cells, H_2O_2 evoked biphasic $[Ca^{2+}]_i$ increase in the presence of external Ca^{2+}. The rapid phase of $[Ca^{2+}]_i$ increased by H_2O_2 was abolished under the external Ca^{2+}-free condition. On the other hand, the gradual phase was still observed in the absence of external Ca^{2+}. These results clearly show that the effusion of Ca^{2+} from the intracellular Ca^{2+} stores as well as Ca^{2+} influx from extracellular fluid, significantly contributed to the H_2O_2-induced elevation of $[Ca^{2+}]_i$. Although many reports concerning the elevation of $[Ca^{2+}]_i$ induced by oxidative stress exist, there is no clear consensus about the mechanism responsible for the elevation of $[Ca^{2+}]_i$ by H_2O_2. Influx through voltage-dependent Ca^{2+} channels (Roveri *et al.*, 1992), nonspecific disruption in membrane Ca^{2+} permeability (Jabr and Cole, 1993; Clague and Langer, 1994), depression of Na^{2+}-Ca^{2+} pump activity (Kaneko *et al.*, 1989), and Ca^{2+} release from intracellular stores (Suzuki and Ford 1991, 1992; Nicotera and Rossi, 1994; Favero *et al.*, 1995) have been proposed. Concerning the elevation of $[Ca^{2+}]_i$ due to Ca^{2+} release from the intracellular stores, Suzuki and Ford (1991) reported that the Ca^{2+}-ATPase of vascular smooth muscle sarcoplasm reticulum (SR) was inhibited by ROIs, resulting in the release of Ca^{2+} to elicit Ca^{2+}-mediated signal transduction. Furthermore, Suzuki and Ford (1992) reported that the IP_3-induced Ca^{2+} release in an SR preparation was potentiated by ROS. However, from the present experiment, the possibility that H_2O_2-induced inhibition of Ca^{2+}-ATPase triggered the elevation of $[Ca^{2+}]_i$ was ruled out, because pretreatment with thapsigargin to abrogate the function of Ca^{2+}-ATPase and to deplete Ca^{2+} in the ER store did not influence the H_2O_2-induced elevation of $[Ca^{2+}]_i$ (Fig. 2C).

Recently, Herson *et al.* (1999) found that 10 mM H_2O_2 induced the elevation of $[Ca^{2+}]_i$ due to the release of Ca^{2+} from a thapsigargin-insensitive Ca^{2+} stores and the influx of extracellular Ca^{2+} through a nonselective Ca^{2+} channel in the insulin-secreting cell line CRI-G1. The intracellular organelles in the nucleus (Nicotera and Rossi, 1994), mitochondria (Boquist, 1984), and calcium-binding cytoskeletal protein such as annexin VI (Hoyal *et al.*, 1996), as thapsigargin-insensitive intracellular stores, may be responsible for H_2O_2-induced $[Ca^{2+}]_i$ elevation in V79 cells. Further experiments will be required to clarify the mechanism of Ca^{2+} effusion from the intracellular stores.

In a previous study (Hiraoka *et al.*, 1997), 10 mM of H_2O_2 used in this experiment was reported to induce apoptotic cell death in V79 cells. This apoptotic cell death was partially inhibited by BAPTA-AM treatment or the protein synthesis inhibitor cycloheximide. Recently, in apoptosis induced in H_2O_2-treated HeLa cells, the activation of SAPK/JNK and ERK was found to act in opposition to influence cell survival, and the activation of p38 and NF-κ B did

not influence the fate of the cells (Wang *et al.*, 1998). Maundrell *et al.* (1997) found that SAPK/JNK could modulate the phosphorylation of BCL-2, which is an important molecule for regulation of apoptosis.

In this study, when cells were pretreated with BAPTA-AM to chelate $[Ca^{2+}]_i$, the activation of ERK as a survival signal was found to be preserved in H_2O_2-treated V79 cells and that of SAPK/JNK as an apoptotic signal was completely inhibited by Ca^{2+} chelation. This may be the reason that BAPTA-AM treatment can attenuate apoptosis in various cell lines exposed to oxidative stress, as observed by many investigators (Baek *et al.*, 1997; Hiraoka *et al.*, 1997; Toborek *et al.*, 1997; Voehringer *et al.*, 1997; Kim *et al.*, 1998; Reader *et al.*, 1999; Takahasi *et al.*, 1999). Furthermore, Ca^{2+} itself has been also reported to be able to induce dysfunction of mitochondria (Petit *et al.*, 1998; Robb *et al.*, 1999) and release of apoptotic factors such as cytochrome *c*, caspase-9, and AIF (Inanami *et al.*, 1999c; Krajewski *et al.*, 1999; Petit *et al.*, 1998). These facts led to the speculation that the elevation of $[Ca^{2+}]_i$ in cells exposed to oxidative stress triggers the simultaneous activation of several apoptotic signals, which is an early key event leading to cell death.

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ABBREVIATIONS

AIF, apoptosis-inducing factor; BAPTA-AM, *O,O'-bis* (2-aminophenoxyl)ethaneglycol-*N,N,N',N'*-tetraacetic acid tetraacetoxymethyl ester; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; BSA, bovine serum albumin; EDTA, ethylenediamine-*N,N,N',N'*-tetraacetic acid; ER, endoplasmic reticulum; ERK, extracellular signal-related kinase; FBS, fetal bovine serum; Fura2-AM, 1-[6-amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)-ethane-*N,N,N',N'*-tetraacetic acid tetraacetoxymethyl ester; HRP, horseradish peroxidase; H_2O_2, hydrogen peroxide; MAPK, mitogen-activated protein kinases; α-MEM, α-minimum essential medium; PBS, phosphate-buffered saline; PI3 kinase, phosphatidylinositol 3 kinase; ROIs, reactive oxygen intermediate species; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase.

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