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Hydrogen Peroxide-Induced Activation of SAPK/JNK Regulated by Phosphatidylinositol 3-Kinase in Chinese Hamster V79 Cells

OSAMU INANAMI, KENJI TAKAHASHI, ASUKA YOSHITO, and MIKINORI KUWABARA

ABSTRACT

To clarify activation mechanisms of stress-activated protein kinase/C-Jun N-terminal kinase (SAPK/JNK) during oxidative stress, the roles of phosphatidylinositol 3-kinase (PI 3-kinase), concentration of intracellular calcium ($[Ca^{2+}]_i$), and cyclic AMP-dependent kinase (PKA) in hydrogen peroxide (H_2O_2)-induced SAPK/JNK activation were examined in Chinese hamster V79 cells. SAPK/JNK was dose-dependently activated after H_2O_2 treatment (from 10 μM to 1 mM), and a PI 3-kinase inhibitor (wortmaninn), intracellular calcium chelator (BAPTA-AM), and PKA activator (dibutyl cyclic AMP and forskolin) inhibited this activation. An increase in $[Ca^{2+}]_i$ was observed after treatment with H_2O_2. Immunoprecipitation revealed that a PI 3-kinase regulatory subunit, p85α, was associated with insulin receptor substance 1 (IRS-1) phosphorylated by H_2O_2 treatment. Furthermore, the formation of this complex of p85α and phospho-IRS-1 was abolished by the presence of BAPTA-AM but not forskolin. These results indicated that the PI 3-kinase activated through phosphorylation of IRS-1 upstream of SAPK/JNK after H_2O_2 treatment of V79 cells and that $[Ca^{2+}]_i$ was a regulation factor for phosphorylation of IRS-1. Antiox. Redox Signal. 1, 113–121.

INTRODUCTION

IT IS WELL-DOCUMENTED that cell lines exposed to various stresses such as UV (Derijard *et al.*, 1994; Klotz *et al.*, 1997), X-rays (Kharbanda *et al.*, 1995; Inanami *et al.*, 1999), reactive oxygen species (ROIS) (Qin *et al.*, 1997), and photodynamic action (Tao *et al.*, 1996) induce the activation of stress-activated protein kinase/C-Jun N-terminal kinase (SAPK/JNK) classified into the MAP kinase family. This enzyme is activated by dual phosphorylation at Thr183 and Tyr185 through the cascade of phosphorylation (Derijard *et al.*, 1994). The activation of this kinase was reported to be associated with the induction of stress responses implicated in apoptosis (Kasibhatla *et al.*, 1998; Seimiya *et al.*, 1997; Skov *et al.*, 1997) and the induction of stress-related proteins (Fuchs *et al.*, 1998; Lee and Corry, 1998; Miralles *et al.*, 1998; Reunanen *et al.*, 1998).

Rapid elevation of the concentration of intracellular calcium ($[Ca^{2+}]_i$) was reported in cells exposed to hydrogen peroxide (H_2O_2) (Hiraoka *et al.*, 1997; Qin *et al.*, 1997; Lomonosova *et al.*, 1998; Suzuki *et al.*, 1998), X-rays (Voehringer *et al.*, 1997), and singlet oxygen (Cui *et al.*, 1997). The calcium-dependent SAPK/JNK activation induced by thapsigargin is regulated by PKA in rat liver epithelial cells (Li *et al.*, 1997). Furthermore, the activation of

Laboratory of Radiation Biology, Department of Environmental Veterinary Medicine, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan.

phosphatidylinositol 3-kinase (PI 3-kinase) was also reported to be associated with the activation of SAPK/JNK in γ-irradiated U-937 cells (Kharbanda *et al.*, 1995). Although various regulators of oxidative-stress-induced activation of SAPK/JNK have been reported, the relationship among these regulators for SAPK/JNK remains unclear.

In this experiment, the involvement of PI 3-kinase, cyclic AMP-dependent kinase (PKA), and $[Ca^{2+}]_i$ in the activation of SAPK/JNK induced in H_2O_2-treated Chinese hamster V79 cells was examined.

MATERIALS AND METHODS

Materials and cell culture

Rabbit antibodies recognizing both nonphosphorylated and phosphorylated human SAPK/JNK and exclusively recognizing SAPK/JNK phosphorylated at 183Thr/185Tyr (phospho-SAPK/JNK) were purchased from New England Biolab, Ltd. (Beverly, MA). GST-c-Jun peptide (1–79), monoclonal antibodies for phosphotyrosine (PY-20), and a rabbit antibody for IRS-1 were from Santa Cruz Biotech. Inc. (Santa Cruz, CA). BAPTA-AM and Fura2-AM were obtained from Dojindo Chemical Co. (Kumamoto, Japan). The monoclonal antibodies for bovine p85α, wortmaninn, forskolin, dibutyl cAMP, H_2O_2, and the other reagents were from Wako Pure Chemical Co. (Osaka, Japan).

V79 cells were routinely maintained with α-minimum essential medium (α-MEM) supplemented with 10% bovine fetal serum (FBS). The cultured cells were exposed to phosphate-buffered saline (PBS) containing various concentrations of H_2O_2, and were maintained in a CO_2 incubator at 37°C. After 1 hr, the medium was changed to normal culture medium. An intracellular calcium chelator, 5 μ*M* BAPTA-AM, was added to the normal medium at 1 hr prior to the treatment with H_2O_2.

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

To measure $[Ca^{2+}]_i$, log-phase cells were incubated with 5 μ*M* Fura2-AM for 30 min and collected by trypsinization. The cells (1×10^7) were resuspended in 2 ml of Krebs-HEPES buffer (130 m*M* NaCl, 1 m*M* NaH_2PO_4, 5 m*M* $NaHCO_3$, 5 m*M* KCl, 3 m*M* glucose, 2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 10 m*M* HEPES, pH 7.4) after twice being washed with PBS, and incubated for various times at 37°C after addition of 1 m*M* H_2O_2. $[Ca^{2+}]_i$ was measured by detecting the light emission from internalized Fura2 using a dual-wavelength spectrophotometer (F-2000, Hitachi, Tokyo, Japan). The ratio of the emission intensity of 510 nm at the excitation wavelength of 340 nm vs. emission intensity at the excitation wavelength of 380 nm was used to calculate the molar concentration of $[Ca^{2+}]_i$ as described by Grynkiewicz *et al.*, (1985).

Immunoblot analysis of SAPK/JNK and p85α

Immunoblotting of phospho-SAPK/JNK and p85α was performed as follows: 2×10^6 cells were collected with a cell scraper and twice washed 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 sonicated three times 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 was blocked by TBST (10 m*M* Tris-HCl pH 7.4, 100 m*M* NaCl, 0.1% Tween 20) containing 5% nonfat dry milk and was probed with anti-human phospho-SAPK/JNK and anti-bovine p85α, a chemiluminescence detection kit (Boehringer Mannheim, GmbH, Germany) was then used to detect the proteins.

Kinase assay for SAPK/JNK

For the measurement of the activity of SAPK/JNK, an immune complex kinase assay was employed. Cells (2×10^7) were washed with PBS and lysed in 200 μl of lysis buffer containing 20 m*M* Tris-HCl pH 7.4, 150 m*M* NaCl, 1% Nonidet P-40, 1 m*M* sodium vanadate, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 1 m*M* dithiothreitol (DTT), and 10 μg/ml of leupeptin and aprotinin. After incubation on ice for 30 min, insoluble material was removed by centrifugation at 14,000 rpm for 10 min at 4°C. The lysates were incubated with anti-

SAPK/JNK for 1 hr at 4°C and then for 1 hr after the addition of protein-G–agarose. The protein complexes were washed three times with lysis buffer and once with kinase buffer and resuspended in kinase buffer (20 m*M* HEPES pH 7.0, 10 m*M* $MgCl_2$, 10 m*M* $MnCl_2$, 2 m*M* DTT, and 0.1 m*M* sodium vanadate) containing 0.5 mCi/ml [γ-^{32}P]ATP (800 Ci/mmol; ICN Biochemicals Inc., Costa Mesa, CA) and GST-Jun. The reaction mixture was incubated for 30 min at 30°C, and the reaction was terminated by the addition of 3× Laemmli's sample buffer. The proteins were analyzed by 10% SDS-PAGE and autoradiography.

Immunoprecipitation of p85α, phosphoprotein, and IRS-1

Immunoprecipitation for p85α, phosphoprotein, and insulin receptor substance 1 (IRS-1) was performed by the method described by Kharbanda *et al.*, (1995). In brief, cell lysates (200 mg of protein) precleared by incubation of protein-G–agarose were incubated with anti-p85α, anti-IRS-1, or anti-phosphotyrosine (PY-20) for 1 hr at 4°C and incubated with 40 μl of protein-G–agarose for an additional 1 hr. The resulting immune complexes were washed three times with lysis buffer, separated by 5% SDS-PAGE, and then transferred to nitrocellulose filters. Blocking, treatment with the primary and secondary antibodies and visualization were similar to the procedures used for SAPK/JNK. Exceptionally, in the case of the detection by anti-phosphotyrosine, TBST (10 m*M* Tris-HCl pH7.4, 100 m*M* NaCl 0.1% Tween 20) containing 2% bovine serum albumin (BSA) as a blocking solution was used.

RESULTS

To clarify whether the SAPK/JNK was activated in V79 cells exposed to H_2O_2, Western blot analysis was performed using a specific antibody to SAPK/JNK phosphorylated at 183Thr/185Tyr. The phosphorylated SAPK/JNK is known to be an active form that phosphorylates transcription factors such as ATF2, c-JUN, Elk-1, and p53 (Derijard *et al.*, 1994; Gupta *et al.*, 1996; Bossy-Wetzel *et al.*, 1997; Fuchs *et al.*, 1998). The blotting patterns detected by antibodies to phospho-SAPK/JNK obtained from whole-cell lysates after treatment with various concentrations of H_2O_2 are shown in Fig. 1, top panel. Intense bands of p54 and p46, indicating phosphorylated (activated) SAPK/JNK, were observed at 15 min after the addition of 1 m*M* H_2O_2 (bottom panel in Fig. 1). Faint bands of activated SAPK/JNK were detected at 6 and 3 hr after treatments with low concentrations of 20 and 200 μ*M* H_2O_2, respectively (upper and middle panels in Fig. 1).

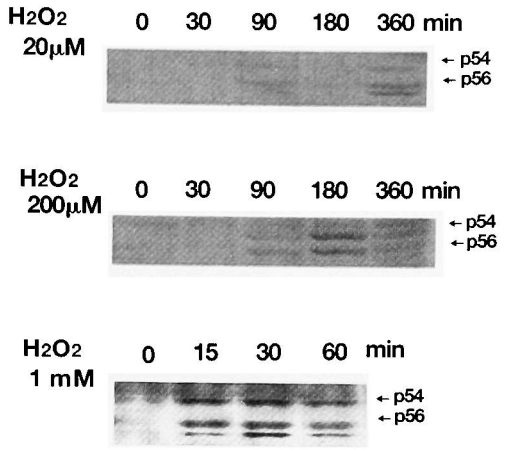

FIG. 1. Immunoblot analysis of phosphorylated SAPK/JNK at various times in Chinese hamster V79 cells exposed to 20 μ*M*, 200 μ*M*, and 1 m*M* H_2O_2.

We examined the relationship between $[Ca^{2+}]_i$ and the activation of SAPK/JNK in H_2O_2-treated V79 cells. Figure 2A shows the effects of pretreatment with 5 μ*M* BAPTA-AM, the intracellular calcium chelator, on the H_2O_2-induced activation of SAPK/JNK. The intensity of bands corresponding to active SAPK/JNK was significantly attenuated by BAPTA-AM, as shown in Fig. 2, suggesting that calcium signals existed in the upstream of SAPK/JNK. Furthermore, immune complex kinase assay for SAPK/JNK revealed that phosphorylation of c-Jun, a substrate for SAPK/JNK, was caused by SAPK/JNK of the lysate of H_2O_2-treated V79 cells (1 m*M* H_2O_2 for 15 min), as shown in lanes 1 and 3 in Fig. 2B. BAPTA-AM-pretreatment of V79 cells induced significant attenuation of the phosphorylation of c-Jun (Fig. 2B, lane 4). These results suggested that $[Ca^{2+}]_i$ increased after the H_2O_2 treatment. In fact, as shown in Fig. 2C (closed circles), at 5

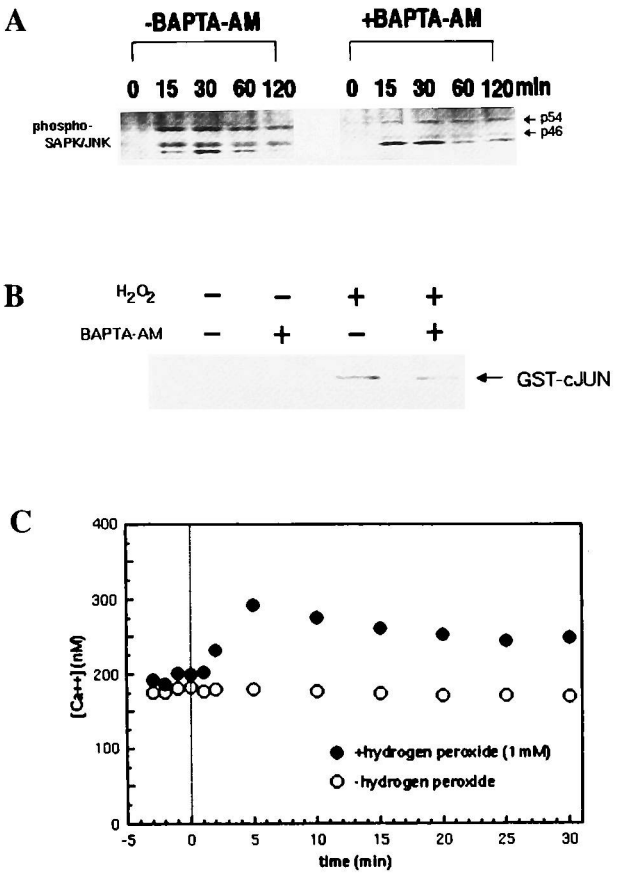

FIG. 2. BAPTA-AM attenuated the H_2O_2-induced accumulation of phosphorylated SAPK/JNK and H_2O_2-induced activation of SAPK/JNK. A. V79 cells were treated with 1 m*M* H_2O_2 for the indicated times without (*left panel*) and with (*right panel*) pretreatment by 5 μM BAPTA-AM. Cell lysates were separated by 10% SDS-PAGE and analyzed by Western blotting using antibody to phospho-SAPK/JNK. **B.** V79 cells were treated with 1 m*M* H_2O_2 for 15 min without and with pretreatment by 5 μM BAPTA-AM. Cell lysates were used for the in vitro kinase assay with GST-c-Jun as the substrate. **C.** Increase in $[Ca^{2+}]_i$ measured by Fura2-AM spectrofluorescence. Open circles and closed circles represent means of three experiments without and with 1 m*M* H_2O_2 stimulation, respectively.

min after the addition of 1 m*M* H_2O_2, $[Ca^{2+}]_i$ increased about twice as much as that obtained under the resting condition (open circles).

Because cAMP elevation and PI 3-kinase were recently reported to be involved in the regulation of the activity of SAPK/JNK (Klippel *et al.*, 1996; Rao and Runge, 1996; Logan *et al.*, 1997; Lopez-Ilasaca *et al.*, 1997; Kita *et al.*, 1998), the effects of reagents for intracellular cAMP elevation, dibutyl cAMP and forskolin, and a PI 3-kinase inhibitor, wortmaninn, on H_2O_2-induced activation of SAPK/JNK in V79 cells were examined. Figure 3 shows complete inhibition of the H_2O_2-induced activation of SAPK/JNK by 10 μM forskolin and 1 m*M* dibutyl cAMP. In the case of PI 3-kinase inhibitor, wortmaninn significantly attenuated the H_2O_2-induced activation of SAPK/JNK. Additionally, the inhibitors did not activate SAPK/JNK by themselves. These results led us to conclude that not only $[Ca^{2+}]_i$ but also PI 3-kinase and intracellular cAMP or PKA are regulators at the upstream of SAPK/JNK during oxidative stress.

PI3-kinase is known to be composed of a 110-kDa catalytic subunit (P110) and an 85-kDa regulatory subunit (p85α) (Shepherd *et al.*, 1998). The formation of the complex of p85α and phosphoproteins such as platelet-derived growth factor (PDGF) receptor, Shc, and IRS-1, which were phosphorylated at tyrosine residues, was a trigger of the activation PI 3-kinase. To examine the phosphoproteins co-immunoprecipitated with anti-p85α in response to oxidative stress, anti-p85α immunoprecipitates from V79 cell lysates, treated with and without 1 m*M* H_2O_2, were subjected to immunoblotting with an anti-phosphotyrosine antibody. As shown in Fig. 4A, the exposure of V79 cells to H_2O_2 induced rapid tyrosine phosphorylation of two proteins corresponding to 90 kDa and 165 kDa within 5 min after H_2O_2 treatment, as indicated by arrows in Fig. 4A. The induction of the H_2O_2-induced phosphoprotein of 90 kDa was still ob-

FIG. 3. H_2O_2-induced accumulation of phospho-SAPK/JNK is dependent on PKA and PI3 kinase. V79 cells were treated with 1 m*M* H_2O_2 for 15 min in the presence of 10 μM forskolin (Fors), 1 m*M* dibutyl cyclic AMP (cAMP), and 50 n*M* wortmaninn (Wort) and cell lysates were used for the in vitro kinase assay with GST-c-Jun as the substrate.

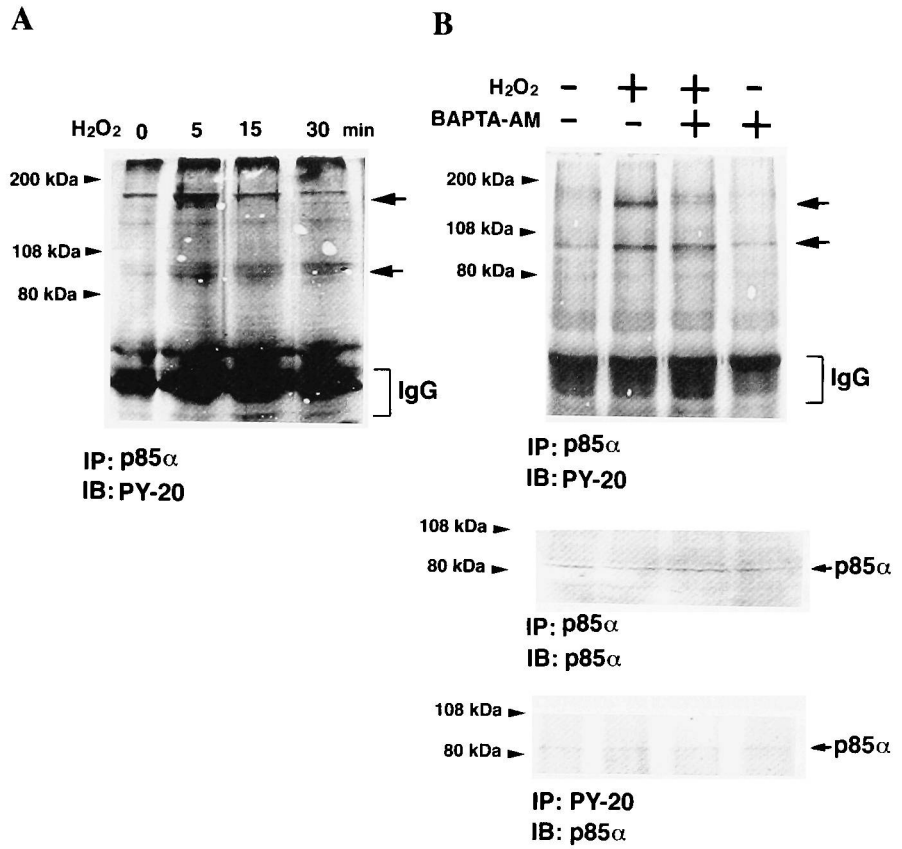

FIG. 4. Requirement of phosphoprotein (p165) association with p85α, a PI 3-kinase regulatory subunit, for H_2O_2-induced SAPK/JNK activation. A. After V79 cells were treated with 1 m*M* H_2O_2 for the indicated times, the cell lysates were immunoprecipitated by anti-p85α. Immunoprecipitates were separated by 5% SDS-PAGE and analyzed by Western blotting using anti-phosphotyrosine (PY-20). **B.** Effects of BAPTA-AM on H_2O_2-induced association of p85α and phosphoprotein. V79 cells without and with pretreatment using 5 μ*M* BAPTA-AM were treated with 1 m*M* H_2O_2 for 5 min. Cell lysates were immunoprecipitated by anti-p85α and immunoprecipitates were separated by 5% SDS-PAGE and analyzed by Western blotting using anti-phosphotyrosine (PY-20) (upper panel) or anti-p85α (middle panel). In the lower panel, the cell lysates were immunoprecipitated by anti-phosphotyrosine (PY-20) and immunoprecipitates were separated by 5% SDS-PAGE and analyzed by Western blotting using anti-p85α.

served in BAPTA-AM-pretreated cells but that of the 165-kDa protein was almost completely eliminated by BAPTA-AM (Fig. 4B, upper panel). Because H_2O_2-induced activation of SAPK/JNK was inhibited by wortmannin and BAPTA-AM in our experimental conditions (Figs. 2 and 3), the tyrosine phosphorylation and association of the 165-kDa protein was important in the activation of PI 3-kinase at the upstream of SAPK/JNK. On the other hand, expression and phosphorylation of the p85α subunit were constant as shown in the middle and bottom panels in Fig. 4B.

To identify the p85α-co-immunoprecipitated phosphoprotein (p165), immunoprecipitation with anti-IRS-1 was performed. IRS-1, like Shc, is reported to be an adaptor protein, and the molecular size of IRS-1 is 165 kDa. This was phosphorylated as a substrate for receptor tyrosine kinase activity and associated with the SH2 domain of p85α. The formation of this complex is considered to be a trigger for the activation of PI 3-kinase (Shepherd *et al.*, 1998). To clarify the induction of tyrosine phosphorylation of IRS-1, the anti-IRS-1 immunoprecipitates from V79 cell lysates, treated with and without 1 m*M* H_2O_2, were analyzed by immunoblotting with an anti-phosphotyrosine antibody. As shown in Fig. 5A, at 5 and 15 min after H_2O_2 treatment, tyrosine-phosphorylated IRS-1 was observed at 165 kDa, whereas the amount of IRS-1 expression was not markedly changed during H_2O_2 stimulation (Fig. 5B). These results strongly indicated that the 165-kDa band that co-precipitated with p85α after H_2O_2 treatment was due to tyrosine-phosphorylated IRS-1.

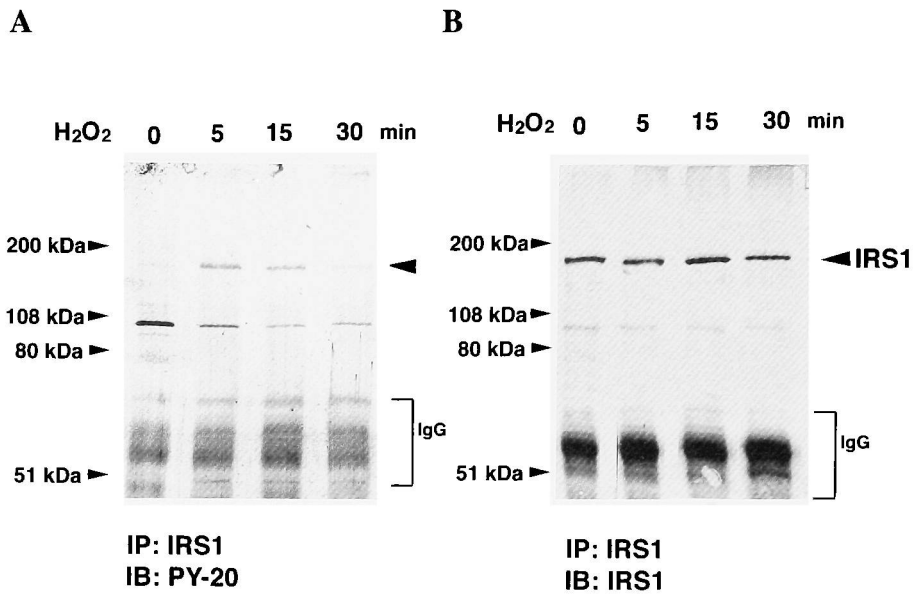

FIG. 5. Phosphorylation of IRS-1 (p165) was induced in H_2O_2-treated V79 cells. V79 cells were treated with 1 m*M* H_2O_2 for the indicated times. Cell lysates were immunoprecipitated by anti-IRS-1 and immunoprecipitates were separated by 5% SDS-PAGE and analyzed by Western blotting using anti-phosphotyrosine (PY-20) (**A**) or anti-IRS-1 (**B**).

Furthermore, to examine the roles of $[Ca^{2+}]_i$ and PKA in H_2O_2-induced formation of p85α/IRS-1 complex, anti-p85α immunoprecipitates from V79 cell lysates, treated with and without 1 m*M* H_2O_2, were subjected to immunoblotting with an anti-IRS-1 antibody. As shown in Fig. 6A, lanes 1 and 2, the formation of the complex of p85α and IRS-1 was induced at 5 min after 1 m*M* H_2O_2 treatment. Furthermore, the formation of this complex was significantly attenuated by BAPTA-AM pretreatment (Fig. 6A, lanes 2 and 3) but not forskolin (Fig. 6B, lanes 2 and 3). These results indicated that $[Ca^{2+}]_i$ acted upstream of the activation of tyrosine kinase for phosphorylation of IRS-1 and that PKA inhibited downstream PI 3-kinase activation in H_2O_2-induced activation of SAPK/JNK of V79 cells.

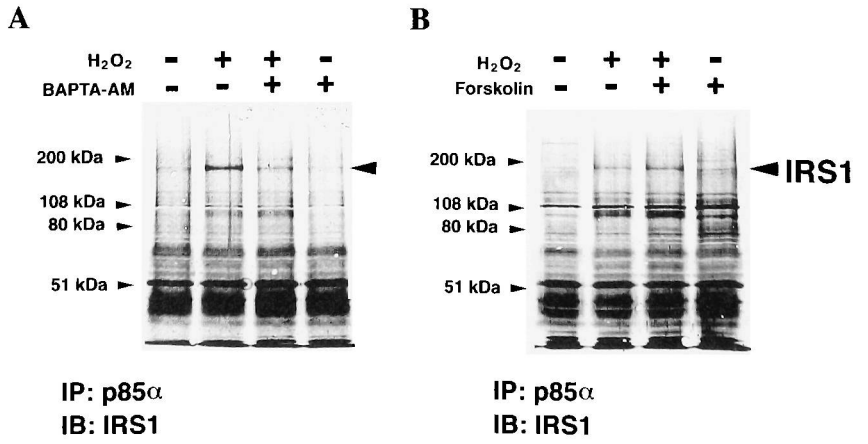

FIG. 6. H_2O_2-induced formation of p85α/IRS-1 complex is inhibited by BAPTA-AM but not forskolin. V79 cells with and without 5 μ*M* BAPTA-AM (**A**) or 10 μ*M* forskolin (**B**) were treated with 1 m*M* H_2O_2 for 5 min. The cell lysates were immunoprecipitated by anti-p85α. Immunoprecipitates were separated by 5% SDS-PAGE and analyzed by Western blotting using anti-IRS-1. Arrowheads indicate IRS-1 (p165).

DISCUSSION

The activation of SAPK/JNK during oxidative stress has been reported to induce apoptosis as well as induction of various genes (Kasibhatla *et al.*, 1998; Seimiya *et al.*, 1997; Skov *et al.*, 1997; Fuchs *et al.*, 1998; Lee and Corry, 1998; Miralles *et al.*, 1998; Reunanen *et al.*, 1998). Our previous work showed that V79 cells exposed to concentrations of H_2O_2 greater than 0.9 m*M* induced apoptotic cell death (Hiraoka *et al.*, 1997). This apoptotic cell death was significantly inhibited by several modifiers, a protein synthesis inhibitor (cycloheximide), a Ca^{2+}-chelator (BAPTA-AM), and an antioxidative compound (PBN), against cell survival and DNA fragmentation. Similar observations about oxidative stress-induced apoptosis and inhibition by a calcium chelator were reported by Voehringer *et al.* (1997) and Lomonosova *et al.* (1998). Although these previous results indicated that protein synthesis and an increase of $[Ca^{2+}]_i$ were required for apoptosis induced by oxidative stress, the downstream site of $[Ca^{2+}]_i$ has not been determined. The data reported here show that H_2O_2-induced SAPK/JNK is mediated via calcium-dependent activation of PI 3-kinase in Chinese hamster V79 cells. This result may indicate that SAPK/JNK underlies the downstream site of $[Ca^{2+}]_i$ to lead to apoptosis, because of the existence of numerous reports concerning the activation of SAPK/JNK linked to induction of apoptosis.

It was reported that oxidative stress-induced increases of $[Ca^{2+}]$ originated from intracellular stores, *i.e.*, endoplasmic reticulum and mitochondria, but not from extracellular fluid (Castilho *et al.*, 1996; Viner *et al.*, 1997). In fact, the addition of BAPTA as an extracellular calcium chelator did not inhibit H_2O_2-induced apoptosis and the activation of SAPK/JNK in V79 cells (data not shown). Recently, Krainev *et al.* (1997) and Morris and Sulakhe (1997) found that there was an oxidant-sensitive cytoplasmic domain of Ca^{2+}-ATPase in sarcoplasmic reticulum. H_2O_2-mediated stimulation of Ca^{2+}-induced Ca^{2+} release was also reported to occur in cardiac myocytes (Suzuki *et al.*, 1998). Although the source of $[Ca^{2+}]_i$ and mechanism of its increase in our experiment were not clarified, it is likely that the H_2O_2-induced increase of $[Ca^{2+}]_i$ originated from the intracellular calcium store as a result of dysfunction of Ca^{2+}-ATPase and/or activation of Ca^{2+}-induced Ca^{2+} release.

The present work showed that inhibition of the H_2O_2-induced increase of $[Ca^{2+}]_i$ by BAPTA-AM abolished the formation of tyrosine phosphorylation of IRS-1. This meant that the increase of $[Ca^{2+}]_i$ activated tyrosine kinases. Yu *et al.*, (1996) and Tokawa *et al.*, (1996) found that an increase of $[Ca^{2+}]_i$ induces the activation of $p21^{(CDC42/(RAC)}$-activated protein kinase (PYK2), which acts as a Ca^{2+}-dependent tyrosine kinase (CADTK), and this CADTK/PYK2 is responsible for the activation of SAPK/JNK. Therefore, it is likely that the H_2O_2-induced increase of $[Ca^{2+}]_i$ activated CADTK/PYK2, resulting in the activation of SAPK/JNK. In this experiment, the increase of $[Ca^{2+}]_i$ seemed to activate these tyrosine kinases and then induce the phosphorylation of IRS-1 because of the inhibition of this phosphorylation by BAPTA-AM-treatment. However, further studies are necessary to prove definitively that these tyrosine kinases can phosphorylate IRS-1.

Because X-rays are known to induce the activation of SAPK/JNK and wortmaninn, a PI 3-kinase inhibitor, inhibited this activation (Derijard *et al.*, 1994), it is possible that PI 3-kinase regulates the activation of SAPK/JNK. The present experiment also confirmed the existence of this regulation in H_2O_2-induced SAPK/JNK activation. PI3-kinase is known to be composed of a 110-kDa catalytic subunit (P110) and an 85-kDa regulatory subunit (p85α). p85α lacks catalytic activity but has one SH3 and two SH2 domains. During the activation of PI 3-kinase by PDGF receptors or insulin receptors, the SH2 domain of p85α associated physically with the PDGF receptor or IRS-1, which was phosphorylated at tyrosine residues. The Src family tyrosine kinases, src, lyn, and fyn, can also associate with the p85α subunit via their SH2 or SH3 domains for activation of PI 3-kinase (Shepherd *et al.*, 1998). Immunoprecipitation and Western blotting in this study demonstrated that tyrosine phosphorylation of IRS-1 initiated activation of PI 3-kinase regulated by an increase of $[Ca^{2+}]_i$, whereas the ac-

tivation of PKA did not inhibit the formation of the complex of IRS-1 and p85α via tyrosine phosphorylation.

These data suggested that the activation of PKA inhibited the SAPK/JNK downstream of PI 3-kinase activation. Because these PKA activators were reported to inhibit oxidative stress-induced apoptosis (Scheid *et al.*, 1999), it is likely that these drugs promoted survival signals through inhibition of the activation of SAPK/JNK to lead to apoptosis. Furthermore, Raynaud *et al.* (1997) reported that H_2O_2 itself attenuated PKA activity as well as 8-azido-[^{32}P]-cAMP binding to the RI and RII regulatory subunits of PKA in human fibroblasts. From these results and our present findings, it also appears likely that the H_2O_2-induced dysfunction of PKA is important in oxidative stress-induced SAPK/JNK activation.

In summary, this experiment clearly demonstrated that H_2O_2 caused Ca^{2+}-dependent activation of SAPK/JNK in V79 cells. The Ca^{2+}-dependent pathway for activation of SAPK/JNK was mediated via this activation and tyrosine kinase and induction of phospho-IRS-1 to activate PI 3-kinase. The activation of PKA appeared to suppress the activation of SAPK/JNK downstream of PI 3-kinase.

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Address reprint requests to:
Dr. M. Kuwabara
Laboratory of Radiation Biology
Department of Environmental
Veterinary Sciences
Graduate School of Veterinary Medicine
Hokkaido Univeristy
Sapporo 060-0818, Japan
E-mail: kuwabara@vetmed.hokudai.ac.jp

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