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Roles of Protein Kinase C δ in the Accumulation of P53 and the Induction of Apoptosis in H_2O_2-treated Bovine Endothelial Cells

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To clarify the signaling pathways of oxidative stress-induced apoptosis in bovine aortic endothelial cells (BAEC), we treated cells with 1 mM H_2O_2 and investigated the roles of protein kinase C δ (PKCδ) and Ca^{2+} in the accumulation of p53 associated with apoptosis. The treatment of cells with H_2O_2 caused the accumulation of p53, which was inhibited by rottlerin (a PKCδ inhibitor) but not by BAPTA-AM (an intracellular Ca^{2+} chelator). PKCδ itself was activated through the phosphorylation at tyrosine residues. H_2O_2 induced the release of cytochrome *c* and the activation of caspases 3 and 9, and these apoptotic signals were inhibited by rottlerin and BAPTA-AM. These results suggest that PKCδ contributes to the accumulation of p53 and that Ca^{2+} plays a role in downstream signals of p53 leading to apoptosis in H_2O_2-treated BAEC.

Keywords: PKCδ; Ca^{2+}; p53; H_2O_2; Apoptosis; Endothelial cells

INTRODUCTION

Apoptosis plays a critical role in the normal development and pathogenesis of several diseases.[1] In the cardiovascular system, apoptosis of endothelial cells (EC) is hypothesized to contribute to the initial endothelial injury that leads to atherosclerosis.[2,3] When EC are exposed to reactive oxygen species (ROS) derived from leukocytes[4,5] or generated after ischemia/reperfusion[6] in pathological conditions, ROS are considered to play a causative role in apoptosis of EC. In fact, several studies have shown that exogenous ROS triggers apoptosis of EC.[7,8] Therefore, it is important to clarify the molecular mechanisms of oxidative stress-induced apoptosis of EC.

It has been shown that ROS induce apoptosis through the accumulation of tumor suppressor protein p53, which regulates the expression levels of anti-apoptotic proteins such as Bcl-2 and pro-apoptotic proteins such as BAX.[9–12] A failure in the balance between Bcl-2 and BAX causes the translocation of BAX to mitochondria and triggers the release of cytochrome *c* followed by the activation of caspases 9 and 3, leading to apoptosis. Since the upregulation and activation of p53 are regulated by its phosphorylation, several protein kinases, including mitogen-activated protein kinase (MAPK) and protein kinase C (PKC), have been reported to phosphorylate it.[13–16] Recently, Aoki *et al.*[12] showed that oxidative stress-induced apoptosis in human aortic EC through the upregulation of p53. However, the mechanisms of p53 phosphorylation in oxidative stress-stimulated EC still remain unclear.

ROS have been shown to induce an increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).[17,18] A rise of $[Ca^{2+}]_i$ is prerequisite for many biological responses, including apoptosis.[19–21] From the observation that H_2O_2-induced apoptosis was inhibited by the removal of extracellular Ca^{2+}, a rise of $[Ca^{2+}]_i$ was inferred to play an important role in

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the oxidative stress-induced apoptosis of EC.[7] On the other hand, several investigators showed that the activation of protein kinase C δ (PKCδ) was involved in H_2O_2-induced apoptosis using various cell lines[22–24] and that it was independent of Ca^{2+}.[25,26] These results illustrated the complicated nature of the relationship between $[Ca^{2+}]_i$ and PKCδ in oxidative stress-induced apoptosis.

In the present study, to clarify the roles of Ca^{2+} and PKCδ in oxidative stress-induced endothelial apoptosis, we examined the effects of chelation of intracellular Ca^{2+} and inhibition of protein kinases on both p53 accumulation and apoptosis in H_2O_2-treated bovine aortic endothelial cells (BAEC). Furthermore, roles of Ca^{2+} and PKCδ in downstream signals of p53 such as cytochrome *c* release and caspase activation were also investigated.

MATERIALS AND METHODS

Materials

Bovine aortic endothelial cells (BAEC) were purchased from Cell Systems (Washington, USA). Iscov's modified Dulbecco's medium (IMDM), propidium iodide (PI), RNase A, *N*-acetyl-L-cysteine (NAC), wortmannin, SB203580 and PD98059 were purchased from Sigma (Missouri, USA). KN93 and GF109203X were from Calbiochem (California, USA). BAPTA-acetoxymethyl ester (AM) was from Dojindo (Kumamoto, Japan). z-VAD-FMK, Ac-DEVD-MCA, AcLEHD-MCA were from Peptide Institute (Osaka, Japan). Protein A-agarose, p53 recombinant protein and antibodies to p53, cytochrome *c*, PKCδ, phosphotyrosine (4G10) and actin were from Santa Cruz Biotechnology (California, USA). Antibodies to phosphorylated p38 MAPK and the phospho-serine motifs at Ser-15, Ser-20 and Ser-385 in bovine p53 were from Cell Signaling Technology (Massachusetts, USA). [γ-^{32}P]ATP (370 MBq) was from ICN Biomedicals (California, USA). All reagents were of analytical grade.

Cell Culture and Drug Treatments

BAEC were grown in IMDM supplemented with 20% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml) in a humidified environment of 5% CO_2, 95% air at 37°C. Confluent BAEC at passages 6–12 were used for experiments. BAEC were rinsed with phosphate-buffered saline (PBS) and incubated in Krebs-HEPES buffer (KHB; pH 7.4, 130 mM NaCl, 5 mM $NaHCO_3$, 1 mM NaH_2PO_4, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-2-ethanesulfonic acid [HEPES], 5 mM glucose) with or without 1 mM H_2O_2 in a CO_2 incubator at 37°C. After 1 h-incubation, KHB was substituted for IMDM without H_2O_2 and BAEC were further incubated until cells were harvested at the indicated periods of time. BAPTA-AM was added to IMDM and kept for 30 min before H_2O_2 exposure. Other drugs were added to medium 30 min before H_2O_2 exposure throughout the experiments.

Immunobloting

Cells were washed with PBS, suspended in 50 μl of a lysis buffer (20 mM Tris–HCl, pH 7.4, 1 mM EGTA and EDTA, 2.5 mM sodium pyrophosphate, 1 mM glycerolphosphate, 1 μg/ml leupeptin, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonylfluoride [PMSF] and 1% Triton X-100) and kept on ice for 30 min. After centrifugation at 10,000*g* for 15 min, the supernatant was collected and the protein concentration was determined using a commercial kit (BCA protein assay reagent; Pierce, Illinois, USA). Proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with 3% nonfat milk powder in TBST (10 mM Tris–HCl, pH 7.6, 150 mM NaCl and 0.1% Tween-20), rinsed triply with TBST, incubated overnight at 4°C with primary antibodies diluted 1:1000 in TBST plus 3% nonfat milk powder, washed triply with TBST and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies diluted 1:1000 in TBST plus 3% nonfat milk powder. The membranes were again washed with TBST, and immunoreactive bands were visualized by chemiluminescence detection.

Immunoprecipitation and Kinase Assay for PKCδ

Immunoprecipitation was performed as described[27] with a slight modification. In brief, cells were washed twice with PBS, suspended in a lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM dithiothreitol (DTT), 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1% Triton X-100) and sonicated. After incubation on ice for 30 min and centrifugation at 10,000*g* for 15 min, supernatants were incubated with the antibody to PKCδ for 1 h at 4°C and then further incubated for 1 h at 4°C with protein-A-agarose. The resulting immune complexes were washed triply with a lysis buffer and used for immunobloting or kinase assay.

PKCδ activity was measured with the immune complex kinase assay. The immune complexes of PKCδ prepared as described above were washed once with kinase buffer and suspended in 30 μl of the buffer (20 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 2 mM DTT, 0.1 mM Na_3VO_4 and 20 μM ATP) containing 370 kBq of [γ-^{32}P]ATP and 3 μg of p53 recombinant protein. When required,

phosphatidylserine (PS, 0.8 μg/ml) and diacylglycerol (DG, 8.0 μg/ml) were added to the reaction mixture. The reaction mixture was incubated for 30 min at 30°C and analyzed by SDS-PAGE and autoradiography.

Translocation of PKCδ

Translocation of PKCδ was assessed as previously described.[28] In brief, cells were washed, suspended in homogenization buffer A (20 mM HEPES–HCl, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 250 mM sucrose, 1 mM PMSF, 25 μg/ml aprotinin, and 1 μg/ml leupeptin) for 30 min at 4°C and sonicated. Nuclei, granules and unbroken cells were removed by centrifugation at 1000*g* for 10 min at 4°C. The supernatant was centrifuged at 40,000*g* for 30 min in a HITACHI CS 100 ultracentrifuge (Tokyo, Japan) at 4°C to separate cytosol (supernatant) and membrane (pellet) fractions. The pellet was dissolved in a lysis buffer. PKCδ in cytosol and membrane fractions were detected by immunoblotting.

Release of Cytochrome *c*

Release of cytochrome *c* into cytosol was assessed as previously described.[29] In brief, cells were washed twice with PBS and suspended in 200 μl of homogenization buffer A. The suspension was disrupted by nitrogen cavitation (400 pounds/square inch for 5 min at 4°C, Parr Instrument, Illinois, USA) and centrifuged at 40,000*g* for 30 min at 4°C. The supernatant was collected as the cytosol fraction. Immunoblotting was performed as described in section of immunoblotting except that PBST (0.1% Tween-20 in PBS) was used instead of TBST.

Measurement of Caspase Activity

Activity of caspases 3 and 9 was determined as described previously.[21] In brief, cells were washed, rinsed and resuspended in a lysis buffer. The lysates were mixed with PBS containing a fluorescent substrate for caspase 3 or 9 (Ac-DEVD-MCA or Ac-LEHD-MCA, respectively) at room temperature. Fluorescence was continuously measured using a JASCO FP-750 spectrofluorometer (Tokyo, Japan) at the excitation and emission wavelengths of 380 and 460 nm, respectively. The hydrolysis rates of the fluorescent substrates were calculated and expressed as the caspase activities.

Analysis of Apoptotic Cells

Quantification of apoptosis was performed by measuring the population distribution of cells in the sub G_1 phase as previously described.[27] Cells were washed with PBS and fixed in 70% ethanol overnight at 4°C. Cells were washed, resuspended in 100 μl of PBS, and incubated with RNase A (0.5 mg/ml) for 1 h at 37°C. After being washed and resuspended in 1 ml of PBS, the cells were stained with PI (50 μg/ml) for 30 min at 4°C. The population distribution of DNA content was analyzed using an EPICS XL flow cytometer (Florida, USA).

RESULTS AND DISCUSSION

Effects of Various Inhibitors on H_2O_2-induced p53 Accumulation

It has been shown that oxidative stress induces apoptosis through accumulation of p53 in several cell types, including EC,[9–12] although posttranscriptional modification and accumulation of p53 in EC under oxidative stress have not yet been demonstrated.

Figure 1A shows that H_2O_2 caused time-dependent accumulation of p53 in BAEC. To determine

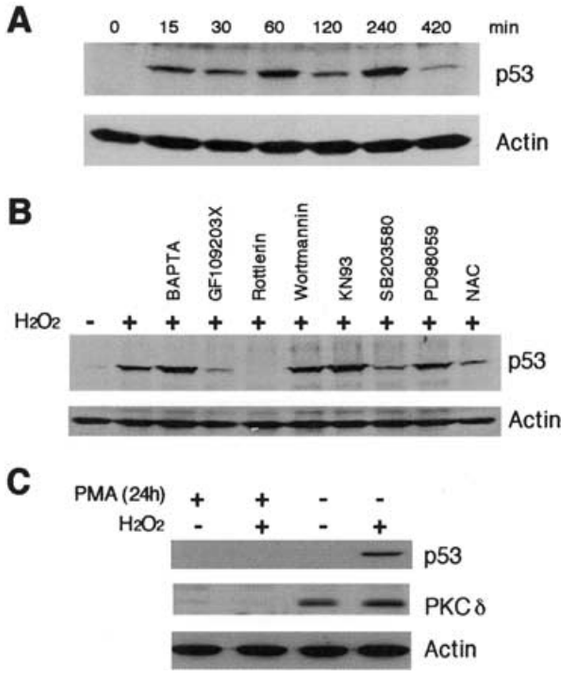

FIGURE 1 Immunoblot analysis of the expression of p53. (A) Time course of H_2O_2-induced p53 accumulation. BAEC were initially exposed to 1 mM H_2O_2 in KHB for 60 min and further incubated in IMDM without H_2O_2. (B) Effects of inhibitors on H_2O_2-induced accumulation of p53. BAEC were exposed to 1 mM H_2O_2 in KHB for 60 min. The procedure used for drug treatment is described in "Materials and methods" section. (C) Effects of PKC downregulation by long-term treatment with PMA on the accumulation of p53 and PKCδ. BAEC were pre-incubated with 0.1 μM PMA for 24 h and exposed to 1 mM H_2O_2 in KHB for 60 min.

the signaling pathways of the H_2O_2-induced increase of p53, we examined the effects of a Ca^{2+} chelator (BAPTA-AM), kinase inhibitors (GF109203X for PKC, rottlerin for PKCδ, wortmannin for phosphatidylinositol 3-kinase [PI3-K], KN93 for CaM kinase II, SB203580 for p38 MAPK and PD98059 for extracellular signal-regulated kinase [ERK]) and an H_2O_2 scavenger (*N*-acetyl-L-cysteine [NAC])[30] on them. As shown in Fig. 1B, the suppression of p53 accumulation was closely associated with the inhibition of PKC by GF109203X but not with the chelation of Ca^{2+} by BAPTA-AM, suggesting that the Ca^{2+}-independent PKC family is involved in p53 accumulation. Since rottlerin, an inhibitor of PKCδ, inhibited p53 accumulation, it was inferred that PKCδ was related to the accumulation. To further prove the contribution of PKCδ to p53 accumulation, long-term treatment of BAEC with phorbol 12-myristate 13-acetate (PMA) was performed since this treatment was reported to induce the downregulation of the PKC family.[31] Incubation of BAEC with 0.1 μM PMA for 24 h resulted in the complete disappearance of not only PKCδ but also p53 accumulation (Fig. 1C).

The increase in accumulation of p53 by H_2O_2 was also suppressed by scavenging H_2O_2 by NAC and, to a lesser extent, by inhibition of p38 MAPK by SB203580 (Fig. 1B), suggesting that H_2O_2 is a trigger of p53 accumulation and that p38 MAPK plays a role in it. On the other hand, PI3-K, CaM kinase II and ERK were not associated with H_2O_2-induced accumulation of p53 because wortmannin, KN93 and PD98059 showed no inhibitory effects.

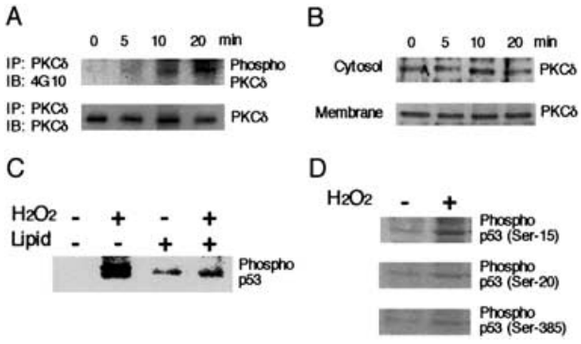

FIGURE 2 H_2O_2 phosphorylates p53 through the activation of PKCδ. (A) Phosphorylation of PKCδ by H_2O_2. BAEC were exposed to 1 mM H_2O_2 in KHB for the indicated periods of time. Anti-PKCδ immunoprecipitates from total cell lysates were analyzed by immunoblotting with antibodies to phosphotyrosine (4G10) or PKCδ. (B) Localization of PKCδ at membrane and cytosol fractions. BAEC were exposed to 1 mM H_2O_2 in KHB for the indicated periods of time. Cytosol and membrane fractions were analyzed by immunoblotting with anti-PKCδ. (C) Kinase assay for PKCδ. BAEC were exposed to 1 mM H_2O_2 in KHB for 60 min. PKCδ immunoprecipitates from total cell lysates were incubated for 30 min at 30°C with p53 recombinant protein and [γ-^{32}P]ATP in the presence or absence of lipid activators. Reaction products were analyzed by SDS-PAGE and autoradiography. (D) Immunoblot analysis of phosphorylated p53 with specific antibodies. BAEC were exposed to 1 mM H_2O_2 in KHB for 60 min. Cell lysates were analyzed by immunoblotting with specific antibodies to phosphorylated p53 at Ser-15, -20 and -385.

Involvement of PKCδ in H_2O_2-induced Phosphorylation of p53

We next examined the activation of PKCδ in H_2O_2-treated BAEC. Exposure of BAEC to H_2O_2 caused tyrosine-phosphorylation of PKCδ in a time-dependent manner (Fig. 2A). Levels of PKCδ in cytosol and membrane fractions remained unchanged (Fig. 2B), suggesting that no translocation of PKCδ from the cytosol to the membrane occurred. To confirm that the phosphorylation of PKCδ at the tyrosine residue induced its activity, we performed an *in vitro* kinase assay of PKCδ on p53 recombinant protein as a substrate. H_2O_2 induced the ability of PKCδ to directly phosphorylate the recombinant p53 even in the absence of lipid activators like phosphatidylserine (PS) and diacylglycerol (DG) (second lane of Fig. 2C). No enhancement of the H_2O_2-induced activation of PKCδ by them was observed (fourth lane of Fig. 2C), though the lipids slightly activated PKCδ without H_2O_2 (third lane of Fig. 2C). These results suggested that PKCδ was changed to a constitutively active state through phosphorylation of tyrosine residues after H_2O_2 treatment and that this activation was a lipid-independent phenomenon. Konishi *et al.*[32] reported a similar observation that PKCδ overexpressed in COS cell line was phosphorylated at tyrosine residues and constitutively active after H_2O_2 treatment. Immunoblot analysis using specific antibodies to phosphorylated p53 was performed to identify the phosphorylation sites of p53. H_2O_2 strongly phosphorylated p53 at Ser-15 and, to a lesser extent, at Ser-20 and Ser-385 (Fig. 2D).

PKCδ is known as a member of the novel PKC (nPKC) family and is activated via a Ca^{2+}-independent mechanism.[25,26] In contrast to anti-apoptotic PKC isoforms such as PKCα, PKCβII and PKCε, PKCδ is recognized as a mediator of pro-apoptotic pathways.[25,26] A recent report showed that PKCδ mediated a mitochondrion-dependent apoptotic pathway in H_2O_2-treated human breast cancer cells.[24] However, the role of PKCδ in p53-dependent apoptosis of EC has not been established. The present study showed that the H_2O_2-induced accumulation of p53 was inhibited not only by the broad-spectrum PKC inhibitor GF109203X, but also by the specific inhibitor of PKCδ rottlerin. Furthermore, the recombinant p53 was directly phosphorylated by immunoprecipitated PKCδ after H_2O_2 treatment. These results provided the first evidence that PKCδ contributes to the p53 accumulation in H_2O_2-stimulated BAEC.

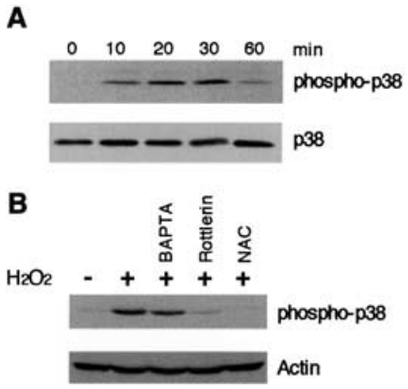

FIGURE 3 PKCδ mediates the H_2O_2-induced activation of p38 MAPK. (A) Time course of phosphorylation of p38 MARK induced by H_2O_2. BAEC were exposed to 1 mM H_2O_2 in KHB for the indicated periods of time. Cell lysates were analyzed by immunoblotting with antibodies to phosphorylated or nonphosphorylated p38 MAPK. (B) Effects of inhibitors on H_2O_2-induced phosphorylation of p38 MAPK. BAEC were exposed to 1 mM H_2O_2 in KHB for 30 min in the presence of inhibitors. Cell lysates were analyzed by immunoblotting with anti-phosphorylated p38 MAPK and anti-actin.

Involvement of PKCδ in Phosphorylation of p38 MAPK

Since p38 MAPK was partly involved in the accumulation of p53 as described above (Fig. 1B), the next step was to investigate the relationship between p38 MAPK and PKCδ. H_2O_2 caused time-dependent phosphorylation of p38 MAPK (Fig. 3A) and it was inhibited by rottlerin (Fig. 3B), indicating that PKCδ was responsible for it. BAPTA-AM did not affect phosphorylation of p38 MAPK (Fig. 3B), but NAC abolished it (Fig. 3B). These results indicated that H_2O_2 induced the activation of p38 MAPK through Ca^{2+}-independent activation of PKCδ.

Cytochrome *c* Release, Caspase Activation and Apoptosis Regulated by PKCδ and $[Ca^{2+}]_i$

It has been reported that the sequential activation of caspases 9 and 3 following the release of cytochrome *c* occurs in oxidative stress-induced apoptosis.[33] Therefore, we measured the levels of cytosolic cytochrome *c*, caspase activities and apoptosis (Figs. 4 and 5). H_2O_2 induced the release of cytochrome *c* from mitochondria and the activation of caspases 3 and 9 (Fig. 4A and B). H_2O_2 also caused a time-dependent increase of apoptotic cells (Fig. 5A). The increase of apoptosis was inhibited by z-VAD-FMK, a broad-spectrum caspase inhibitor (Fig. 5B). These results indicated that H_2O_2 caused caspase-dependent apoptosis through a mitochondrial pathway in BAEC. Furthermore, rottlerin inhibited the accumulation of p53,

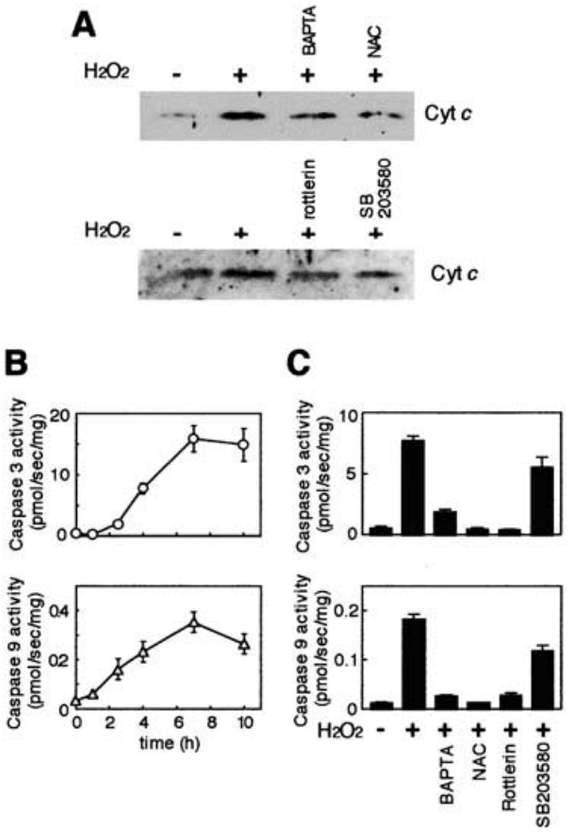

FIGURE 4 Release of cytochrome *c* and the activation of caspases by H_2O_2. (A) Effects of inhibitors on cytochrome *c* release. BAEC were exposed to 1 mM H_2O_2 in KHB for 1 h and further incubated in IMDM without H_2O_2 for 3 h. The procedure used for drug treatment is described in "Materials and methods" section. Cytochrome *c* in the cytosol fraction was detected by immunoblot analysis. (B) Time course of caspase activation induced by H_2O_2. Hydrolysis rates of fluorescent substrates that were specific for caspase 3 (Z-DEVD-MCA) or caspase 9 (Z-LEHD-MCA) in cell extracts were determined. BAEC were initially exposed to 1 mM H_2O_2 in KHB for 60 min and further incubated in IMDM without H_2O_2. Results are expressed as Mean ± SEM of three experiments. (C) Effects of inhibitors on caspase activation induced by H_2O_2. BAEC were exposed to 1 mM H_2O_2 in KHB for 1 h and further incubated in IMDM without H_2O_2 for 3 h. The procedure used for drug treatment is described in "Materials and methods" section. Results are expressed as Mean ± SEM of three experiments.

the release of cytochrome *c* (Fig. 4A), the activation of caspases (Fig. 4C) and apoptosis (Fig. 5B). These results led us to conclude that the accumulation of p53 caused by PKCδ contributed to apoptosis of BAEC.

In contrast, BAPTA-AM inhibited the release of cytochrome *c*, the activation of caspases and apoptosis without the inhibition of p53 accumulation. These results suggested that Ca^{2+} contributed to the downstream signals of p53. Ca^{2+} is recognized as an important signal transduction molecule in apoptosis. Our previous studies showed that an increase in $[Ca^{2+}]_i$ induced the release of cytochrome *c* and the activation of caspase 3 and resulted in

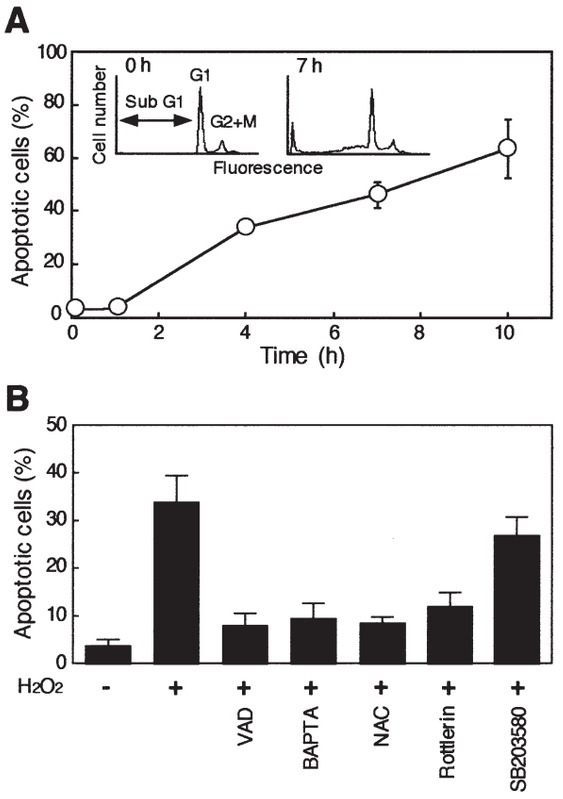

FIGURE 5 Flow cytometric analysis of apoptotic cells in H_2O_2-treated BAEC. (A) Time course of changes in the sub-G_1, population (apoptotic cells) induced by H_2O_2. BAEC were exposed to 1 mM H_2O_2 in KHB for 60 min and further incubated in IMDM without H_2O_2. Results are expressed as Mean ± SEM of four to twelve experiments. The insets indicate representative results of flow cytometry. (B) Effects of inhibitors on the percentages of sub-G_1 populations observed in flow cytometric analysis. The procedure used for drug treatment is described in "Materials and methods" section. Results are expressed as Mean ± SEM of four to twelve experiments.

apoptosis in X-irradiated MOLT-4 cells[20] and photosensitized Chinese hamster V79 cells.[21] A sustained increase of $[Ca^{2+}]_i$ caused by 1 mM H_2O_2 was observed in the present study (data not shown). However, the exact mechanism for the release of cytochrome *c* from mitochondria by Ca^{2+} in H_2O_2-treated BAEC was not elucidated. It is plausible that Ca^{2+} promotes the activation of pro-apoptotic molecules such as BAX to induce the opening of permeability transition (PT) pores.[34]

As shown in Figs. 4 and 5, the release of cytochrome *c*, the activation of caspases and apoptosis were partly inhibited by SB203580, suggesting that p38 MAPK played some part in the induction of apoptosis in H_2O_2-treated BAEC. As for the involvement of p38 MAPK in the phosphorylation of p53, Huang *et al.*[35] reported that a p38 MAPK inhibitor or expression of a dominant negative mutant of p38 MAPK impaired the phosphorylation of p53 in a UVC-irradiated mouse epidermal cell line. Recently, Rahman *et al.*[36] showed that rottlerin prevented the thrombin-induced phosphorylation of p38 MAPK, suggesting that p38 MAPK was activated at the downstream of PKCδ. The relationship between PKCδ and p38 MAPK in oxidative stress-induced apoptosis was clarified in the present study. The inhibition of PKCδ prevented the H_2O_2-induced phosphorylation of p38 MAPK and the inhibition of p38 MAPK partly abrogated the accumulation of p53, showing that p38 MAPK partly played a role downstream of PKCδ in the accumulation of p53 and apoptosis. The weak inhibitory effects of SB203580 on the p53 accumulation, cytochrome *c* release and apoptosis might be explained by transient activation of p38 MAPK within 30 min after H_2O_2-treatment (Fig. 3A).

In conclusion, PKCδ mediates the phosphorylation and accumulation of p53 leading to apoptosis in H_2O_2-treated BAEC and p38 MAPK, as a downstream kinase of PKCδ, also partly contributes to the accumulation of p53. The H_2O_2-induced increase in $[Ca^{2+}]_i$ contributes to downstream signals of p53. However, further study is required to clarify the regulatory mechanisms of accumulation and phosphorylation of p53 by PKCδ and Ca^{2+} in oxidative stress-induced endothelial apoptosis.

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