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Redox Regulation of PI3K/Akt and p53 in Bovine Aortic Endothelial Cells Exposed to Hydrogen Peroxide

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ABSTRACT

To clarify the apoptotic and survival signal transduction pathways in activated vascular endothelial cells exposed to oxidative stress, the effects of inhibitors of signal transduction on hydrogen peroxide (H_2O_2)-induced apoptosis in bovine aortic vascular endothelial cells (BAEC) were examined. Treatment of BAEC with 1 m*M* H_2O_2 caused increases of DNA fragmentation, p53 expression, Bax/Bcl-2 ratio, and the activities of caspases 3 and 9. The increases of DNA fragmentation, Bax/Bcl-2 ratio, and caspase activities were abrogated by BAPTA-AM (an intracellular Ca^{2+} chelator) and *N*-acetyl-L-cysteine (an antioxidant), and augmented by wortmannin [a phosphatidylinositol 3-kinase (PI3K) inhibitor]. The increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) observed in H_2O_2-stimulated cells was unaffected by wortmannin, suggesting that the potentiating effect of wortmannin on the apoptosis was not due to an alteration of $[Ca^{2+}]_i$. H_2O_2 increased the levels of PI3K activity and Akt phosphorylation. Both were attenuated by wortmannin and, to a lesser extent, by genistein (a tyrosine kinase inhibitor) and suramin (a growth factor receptor inhibitor), but not affected by BAPTA-AM. These results suggest that H_2O_2 induces Ca^{2+}-dependent apoptosis and Ca^{2+}-independent survival signals such as redox-regulated activation of PI3K/Akt, which is partly mediated by the activation of growth factor receptors in BAEC. *Antioxid. Redox Signal.* 5, 713–722.

INTRODUCTION

APOPTOSIS plays a crucial role in normal development and in the pathogenesis of several diseases (32). In cardiovascular systems, apoptosis of endothelial cells (EC) is believed to be responsible for the initiation and progress of atherosclerosis because EC function as a biological barrier to protect vessel walls from cholesterol accumulation (8). It has been proposed that reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) derived from activated neutrophils (9, 42) or EC (37) play important roles in endothelial apoptosis. Several studies have shown that exogenous ROS induce endothelial apoptosis characterized by DNA fragmentation (12, 27, 35). Furthermore, it has been demonstrated that the formation of endogenous ROS is involved in EC apoptosis induced by hypoxia (1).

On the other hand, ROS have been shown to induce proliferation in several cell types (3). For instance, Sauer *et al.* demonstrated that treatment of prostate tumor spheroids with H_2O_2 enhanced the cell growth via the expression of c-fos, a growth-related gene (33). In addition, it has been reported that H_2O_2 has not only proapoptotic, but also antiapoptotic effects in several cell types, including EC (14). Phosphatidylinositol 3-kinase (PI3K), a lipid kinase, and Akt, a serine/threonine kinase, have been proposed as candidates for survival signals in mild oxidative stress (23, 36). These enzymes are activated by growth stimuli such as platelet-derived growth factor, epidermal growth factor (EGF), and insulin, and sequential activation of PI3K/Akt is recognized as a survival signal to protect cells from apoptosis (25). Although the activation mechanisms of the PI3K/Akt pathway by oxidative stress are largely unknown, activation of this pathway by

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H_2O_2 is inferred to be partly due to the activation of growth factor receptors because H_2O_2 can phosphorylate these receptors (22). A recent report has demonstrated that H_2O_2 elicits the activation of Akt, leading to phosphorylation of nitric oxide synthase in EC (40). However, it is uncertain whether the activation of PI3K/Akt by H_2O_2 functions as a survival signal in EC exposed to oxidative stress.

It has been shown that ROS induce an increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in several cell types, including EC (26). From the observation that DNA fragmentation induced by H_2O_2 was inhibited when extracellular Ca^{2+} was excluded, a rise in $[Ca^{2+}]_i$ was hypothesized to play a key role in oxidative stress-induced EC apoptosis (12, 35). Our recent study also demonstrated that treatment of bovine aortic EC (BAEC) with 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM), an intracellular Ca^{2+} chelator, inhibited the release of cytochrome *c*, activation of caspases 3 and 9, and apoptosis induced by H_2O_2 (27). The increase of $[Ca^{2+}]_i$ has been also reported to mediate apoptosis induced by other stressors, such as x irradiation (39), photosensitization (19), and serum starvation (20). In addition to apoptosis, many cellular functions are known to be triggered by an increase of $[Ca^{2+}]_i$. In EC, for instance, a $[Ca^{2+}]_i$ increase was reported to be responsible for changes in macromolecular permeability (26) and the release of nitric oxide (2). It is believed that several effects of increased $[Ca^{2+}]_i$ on cellular functions are mediated by phosphorylation of protein kinases such as protein kinase C, Ca^{2+}/calmodulin-dependent kinases, and stress-activated protein kinase (10, 18). It was reported that Akt activation induced by differentiation-inducing factor-1 was sensitive to deprivation of extracellular Ca^{2+} in leukemia cell line K562 (24). In contrast, Craxton *et al.* demonstrated that treatment of B cells with BAPTA-AM did not affect Akt activation induced by stimulation with IgM (7). Thus, the requirement for Ca^{2+} in the activation of the PI3K/Akt pathway is controversial and seems to depend on the cell type.

In the present study, to determine whether oxidative stress promotes cell survival by activating the PI3K/Akt pathway in EC, we tested the effects of several inhibitors of cellular signaling on endothelial apoptosis induced by H_2O_2 and assessed the activities of PI3K and Akt. In addition, the relationship between Ca^{2+} and PI3K/Akt was examined to obtain further insights into the redox regulation of survival signals in EC.

MATERIALS AND METHODS

Materials

Iscove's modified Dulbecco's medium (IMDM), propidium iodide (PI), RNase A, proteinase K, wortmannin, genistein, *N*-acetyl-L-cysteine (NAC), suramin, adenosine, ATP and phosphatidylinositol were purchased from Sigma (St. Louis, MO, U.S.A.). BAPTA-AM and fura 2-AM were from Dojindo (Kumamoto, Japan). Z-VAD-FMK, DEVD-MCA, and LEHD-MCA were from Peptide Institute (Osaka, Japan). Antibodies to p53, Bcl-2, Bax, phosphotyrosine (PY-20), and actin, horseradish peroxidase-conjugated secondary antibodies, and protein A agarose were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibodies to Akt and phosphorylated Akt were from Cell Signaling Technology (Beverly, MA, U.S.A.). [γ-^{32}P]ATP was from ICN Biomedicals (Costa Mesa, CA, U.S.A.). All other reagents and drugs were of analytical grade.

Cell culture and drug treatments

BAEC were purchased from Cell Systems (Kirkland, WA, U.S.A.). BAEC were grown in IMDM supplemented with fetal bovine serum (20%), penicillin (100 IU/ml), and streptomycin (100 µg/ml) in a CO_2 incubator. Culture medium was renewed every 2–3 days. BAEC at passages 6–12 were used for experiments.

Experiments were performed with confluent BAEC 4–6 days after seeding. BAEC were rinsed with phosphate-buffered saline (PBS) and incubated in Krebs-HEPES buffer (KHB; pH 7.4, 130 m*M* NaCl, 5 m*M* $NaHCO_3$, 1 m*M* NaH_2PO_4, 5 m*M* KCl, 1 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, 10 m*M* HEPES, and 5 m*M* glucose) with or without H_2O_2 in a CO_2 incubator for 1 h. Then KHB was changed to IMDM without H_2O_2, and BAEC were further incubated until harvested. BAPTA-AM was added to IMDM for 30 min before H_2O_2 exposure. Other drugs were added to the medium from 30 min before H_2O_2 exposure until the cessation of experiments.

Analysis of DNA fragmentation

DNA fragmentation was assessed by agarose gel electrophoresis. Cells ($2–4 \times 10^6$) were washed with PBS, resuspended in 100 µl of a lysis buffer (pH 7.4, 10 m*M* Tris-HCl, 10 m*M* EGTA, and 0.5% Triton X-100), and kept on ice for 30 min. After centrifugation at 10,000 *g* for 15 min, the supernatant was incubated with RNase A (0.5 mg/ml) for 1 h and further incubated with proteinase K (0.5 mg/ml) for 1 h at 37°C. After the addition of 20 µl of 5 *M* NaCl and 120 µl of 2-propanol, the mixture was centrifuged at 10,000 *g* for 15 min. Then the pellet was resuspended in 20 µl of TE buffer (pH 7.4, 1 m*M* EDTA, 10 m*M* Tris-HCl), mixed with 4 µl of gel-loading buffer (40% sucrose, 0.25% bromophenol blue), and subjected to agarose gel electrophoresis. DNA was visualized by ethidium bromide staining.

Quantitation of apoptosis

Quantification of apoptotic cells was performed by measuring the population distribution of DNA content as described previously (38). Cells (1×10^6) were washed with PBS and fixed in 70% ethanol overnight at 4°C. They were then 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, 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 (Beckman Coulter, Fullerton, CA, U.S.A.).

Immunoblotting

Cells ($2–4 \times 10^6$) were washed with PBS, resuspended in 50 µl of lysis buffer A (pH 7.4, 20 m*M* HEPES, 1% Triton X-100, 10% glycerol, 2 m*M* EDTA, 1 m*M* dithiothreitol,

2 μg/ml aprotinin, 2 μg/ml pepstatin A, 1 m*M* Na_3VO_4, and 1 m*M* phenylmethylsulfonyl fluoride), and kept on ice for 30 min. After centrifugation at 10,000 *g* for 15 min, the protein concentration of the supernatant was determined using the BCA protein assay reagent (Pierce, Rockford, IL, U.S.A.). Proteins in the supernatant were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes. The membranes were incubated in TBST (pH 7.5, 10 m*M* Tris-HCl, 150 m*M* NaCl, and 0.1% Tween 20) plus 5% nonfat milk powder for 1 h at room temperature. They were then incubated overnight at 4°C with primary antibodies in TBST plus 5% nonfat milk powder. Next, they were washed three times and further incubated with horseradish peroxidase-conjugated secondary antibodies in TBST plus 5% nonfat milk powder for 1 hour at room temperature. Finally, the membranes were washed and immunoreactive bands were visualized with chemiluminescence detection.

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

$[Ca^{2+}]_i$ in single cells was measured with a fluorescent Ca^{2+} indicator, fura 2-AM (15). BAEC were seeded on glass coverslips and grown to confluence. BAEC attached to coverslips were incubated in KHB containing fura 2-AM (2 μ*M*) for 30 min at 37°C. When required, wortmannin was concomitantly added to KHB containing fura-2 AM. After incubation, cells were stored at 4°C until used. The coverslips were placed on the stage of an inverted microscope (Diaphot 300; Nikon, Tokyo, Japan) and perfused continuously with KHB at a rate of 2 ml/min. Alternate beams of excitation light at 340 and 380 nm were generated from a fluorometer (CAM-200, Jasco, Tokyo, Japan) by a wheel spinning at 400 Hz. Fluorescent signals through a band-path filter (500 nm) from BAEC were stored in a computer. The calibration of fura-2 signals was described in a previous article (28).

Measurement of caspase activity

Activities of caspases 3 and 9 were determined by measuring hydrolysis rates of fluorescent substrates. Cells were washed, rinsed, and resuspended in lysis buffer A. 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 with a Jasco FP-750 spectrofluorometer (Tokyo, Japan) at the excitation and emission wavelengths of 380 and 460 nm, respectively. The hydrolysis rates of substrates were calculated and expressed as caspase activities.

Measurement of PI3K activity

PI3K activity was determined by TLC as previously described (44). Cells ($2–4 \times 10^6$) were washed with PBS, resuspended in 1 ml of lysis buffer, and kept on ice for 30 min. Cell lysates were centrifuged at 14,000 *g* for 30 min at 4°C, and the supernatant was precleared by incubation with protein A-agarose for 30 min at 4°C. Lysates were incubated with 2 μg of an anti-phosphotyrosine monoclonal antibody (PY-20) on a rotating wheel for 1 h at 4°C. After the addition of 40 μl of a 50% slurry of protein A-agarose, the lysates were further incubated for 1 h at 4°C. The samples were washed three times with a lysis buffer and twice with 10 m*M* Tris-HCl (pH 7.4) containing 1 m*M* Na_3VO_4. PI3K activity was measured by adding 100 μg of sonicated phosphatidylinositol and 10 μCi of [γ-^{32}P]ATP in the presence of 200 μ*M* adenosine (to inhibit phosphatidylinositol 4-kinase activity), 30 m*M* $MgCl_2$, and 35 μ*M* ATP in a total volume of 60 μl. Reactions were performed for 20 min at 25°C and terminated by adding 100 μl of 1 *M* HCl and 200 μl of chloroform/methanol (1:1, vol/vol). After centrifugation and removal of the upper layer, 80 μl of methanol/HCl (1:1) was added to samples. After centrifugation, lipids were separated on TLC plates (Silica gel 60 F_{254}) with a solvent system of chloroform/methanol/H_2O/NH_4OH (45:35:7.5:2.5, by volume). The radioactivities were analyzed with an imaging analyzer Fujix BAS1000 (Fuji Photo Film, Tokyo, Japan).

Statistics

Statistical differences of results were assessed with Student's *t* test.

RESULTS

Induction of BAEC apoptosis by H_2O_2

Figure 1A shows the electrophoretic pattern of DNA after 7 h of H_2O_2 exposure (1 m*M*). Characteristic fragmentation of DNA into oligonucleosomal length was observed. To assess apoptosis quantitatively, flow cytometric analysis with PI staining was performed. The inset in Fig. 1B shows the population distribution of DNA in control cells. When BAEC were exposed to 1 m*M* H_2O_2, the apoptotic population (sub G1) was increased in a time-dependent manner (Fig. 1B). Figure 1C shows the effects of various concentrations of H_2O_2 on apoptosis. Although a slight, but significant, increase in apoptosis was observed when cells were exposed to 0.05 m*M* H_2O_2 and the apoptosis was enhanced in a dose-dependent manner, we mainly used the 1 m*M* concentration of H_2O_2 to show clearly the effects of inhibitors in the following experiments.

Roles of $[Ca^{2+}]_i$ and PI3K in H_2O_2-induced BAEC apoptosis

We next examined the effects of various inhibitors on signaling pathways of EC apoptosis (Fig. 2). H_2O_2-induced increases of DNA fragmentation and the apoptotic fraction were markedly inhibited by the caspase inhibitor Z-VAD-FMK, the intracellular Ca^{2+} chelator BAPTA-AM, and the antioxidant NAC (Fig. 2A and B). These results indicated that H_2O_2 induced caspase-dependent apoptosis and that an increase of $[Ca^{2+}]_i$ and redox regulation were involved in apoptosis. Roles of intracellular Ca^{2+} were further investigated with BAPTA-AM. Figure 2C shows the effects of various concentrations of BAPTA-AM on apoptosis induced by H_2O_2 at 1 m*M* (upper panel) and 0.2 m*M* (lower panel). The effective dose of BAPTA-AM to inhibit the apoptosis induced by 0.2 m*M* H_2O_2 was lower than that by 1 m*M* H_2O_2. These

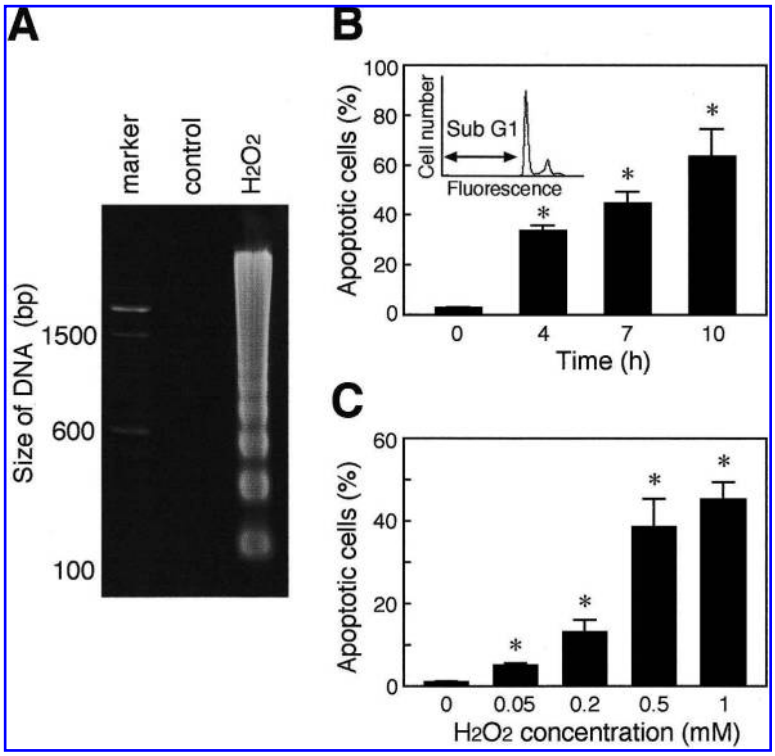

FIG. 1. Effects of H_2O_2 on BAEC apoptosis. (**A**) Agarose gel electrophoresis of DNA. BAEC were exposed to 1 m*M* H_2O_2 in KHB for 1 h and further incubated in normal IMDM for 6 h. Data are representative of three experiments. (**B**) Flow cytometric analysis of DNA ploidity with PI staining. **Inset** shows representative DNA distribution in control cells. BAEC were exposed to 1 m*M* H_2O_2 in KHB for 1 h and further incubated in normal IMDM for indicated periods of time. Data are expressed as means ± SE of four to six experiments. (**C**) Effects of various concentrations of H_2O_2 on apoptosis determined by flow cytometric analysis. BAEC were exposed to H_2O_2 in KHB for 1 h and further incubated in normal IMDM for 6 h. Data are expressed as means + SE of four experiments. *Significantly different from control group ($p < 0.05$).

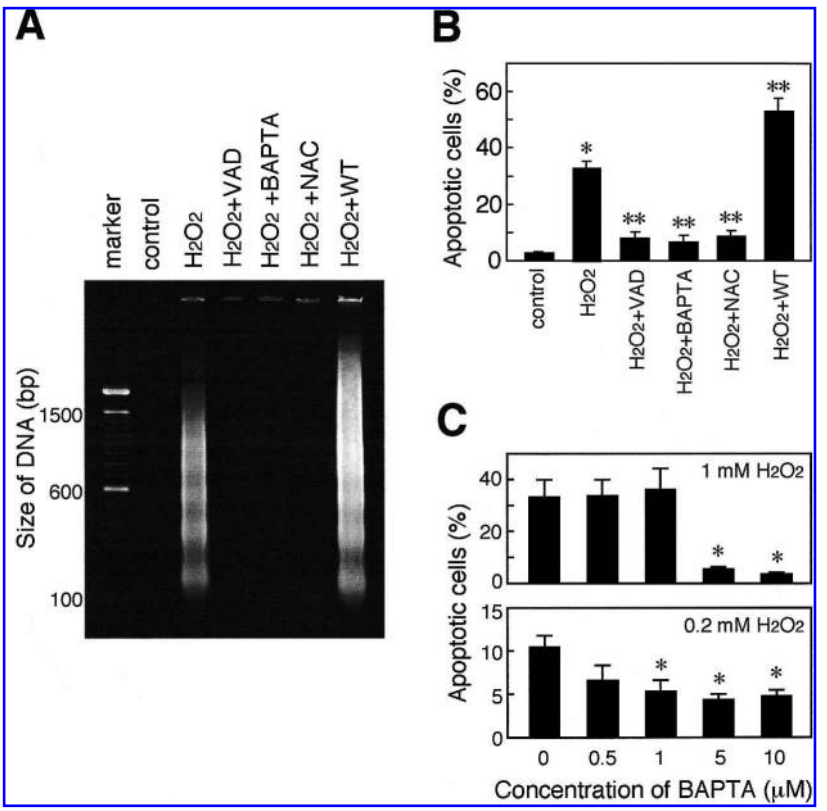

FIG. 2. Effects of inhibitors on H_2O_2-induced BAEC apoptosis. (**A**) Agarose gel electrophoresis of DNA. Data are representative of three experiments. (**B**) Flow cytometric analysis of DNA ploidity with PI staining. Data are expressed as means ± SE of three to six experiments. BAEC were exposed to 1 m*M* H_2O_2 in KHB solution for 1 h and further incubated in normal IMDM for 3 h. BAPTA-AM (10 μ*M*) was added to IMDM for 30 min before H_2O_2 exposure. Z-VAD-FMK (30 μ*M*), NAC (15 m*M*), and wortmannin (0.1 μ*M*) were added to the medium 30 min before H_2O_2 exposure until the cessation of experiments. VAD, Z-VAD-FMK; BAPTA, BAPTA-AM; WT, wortmannin. *Significantly different from control group ($p < 0.05$); **significantly different from H_2O_2-treated group ($p < 0.05$). (**C**) Effects of various concentrations of BAPTA-AM on H_2O_2-induced apoptosis determined by flow cytometric analysis. Data are expressed as means + SE of four experiments. BAEC were exposed to H_2O_2 at 1 m*M* (**upper panel**) or 0.2 m*M* (**lower panel**) in KHB solution for 1 h and further incubated in normal IMDM for 6 h. *Significantly different from BAPTA-free group ($p < 0.05$).

results suggested that the magnitude of H_2O_2-induced apoptosis was dependent on the level of $[Ca^{2+}]_i$ increase.

To test whether the PI3K-mediated survival signal was activated by H_2O_2 treatment, the effects of wortmannin, an inhibitor of PI3K, were examined. As shown in Fig. 2A and B, the increases of the DNA fragmentation and the apoptotic population were augmented by wortmannin, suggesting that H_2O_2 promoted cell survival by activating PI3K.

Because intracellular Ca^{2+} was suggested to play a key role in H_2O_2-induced apoptosis, we measured the dynamics of $[Ca^{2+}]_i$ with fura 2-AM. As shown in Fig. 3A, H_2O_2 caused a transient rise of $[Ca^{2+}]_i$ followed by the sustained increase. To determine the origin of Ca^{2+}, the effects of chelation of extracellular Ca^{2+} were examined. As shown in Fig. 3A and 3B, EGTA completely inhibited both the initial and sustained phases of the $[Ca^{2+}]_i$ increase, suggesting that the increase of $[Ca^{2+}]_i$ was primarily due to Ca^{2+} influx. Because wortmannin augmented H_2O_2-induced apoptosis, we tested the correlation between the potentiation of apoptosis and alteration of $[Ca^{2+}]_i$. Figure 3C shows the effects of wortmannin on the dynamics of $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ response was unaffected by wortmannin, suggesting that the augmentation of apoptosis by wortmannin was not due to alterations of $[Ca^{2+}]_i$.

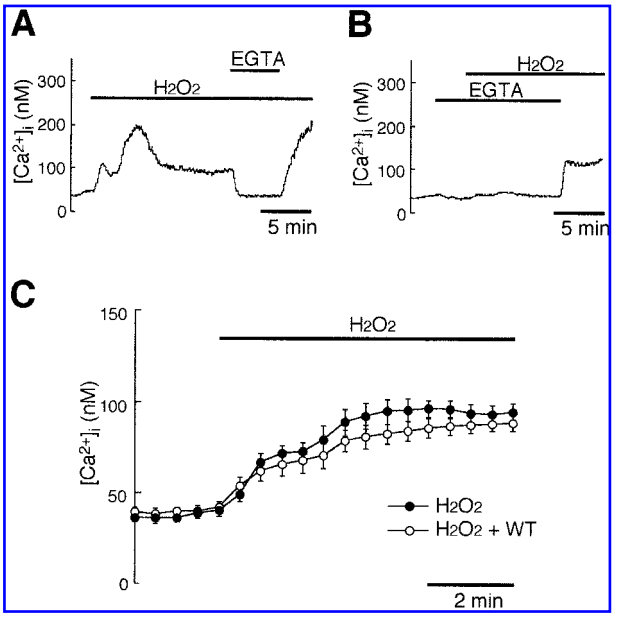

FIG. 3. Dynamics of $[Ca^{2+}]_i$ of BAEC in response to H_2O_2. (**A** and **B**) Effects of EGTA (2 m*M*) on the increase of $[Ca^{2+}]_i$ induced by 1 m*M* H_2O_2. Data are representative of three experiments. (**C**) Effects of wortmannin (0.1 μ*M*) on the increase of $[Ca^{2+}]_i$ induced by 1 m*M* H_2O_2. Wortmannin was added to the perfusing solution (KHB) 30 min before the measurement until the cessation of experiments. Data are expressed as means ± SE of six experiments. WT, wortmannin.

Roles of $[Ca^{2+}]_i$ and PI3K in the expression of p53, Bcl-2, and Bax

To investigate the molecular basis of apoptotic and survival signals in H_2O_2-treated BAEC, protein levels of apoptosis-related factors such as p53, Bcl-2, and Bax were examined. H_2O_2 caused an increase of p53 with a peak 7 h after H_2O_2 exposure (Fig. 4A). Protein levels of Bax were increased, whereas those of Bcl-2 were decreased in a time-dependent manner. Consequently, the ratio of Bax to Bcl-2 was increased (Fig. 4B). Figure 4C and D show the effects of inhibitors on expression of p53, Bcl-2, and Bax. BAPTA-AM did not affect the p53 expression, whereas it inhibited the increase of the Bax/Bcl-2 ratio, suggesting that the increase of $[Ca^{2+}]_i$ played a role downstream of p53 expression. NAC inhibited the

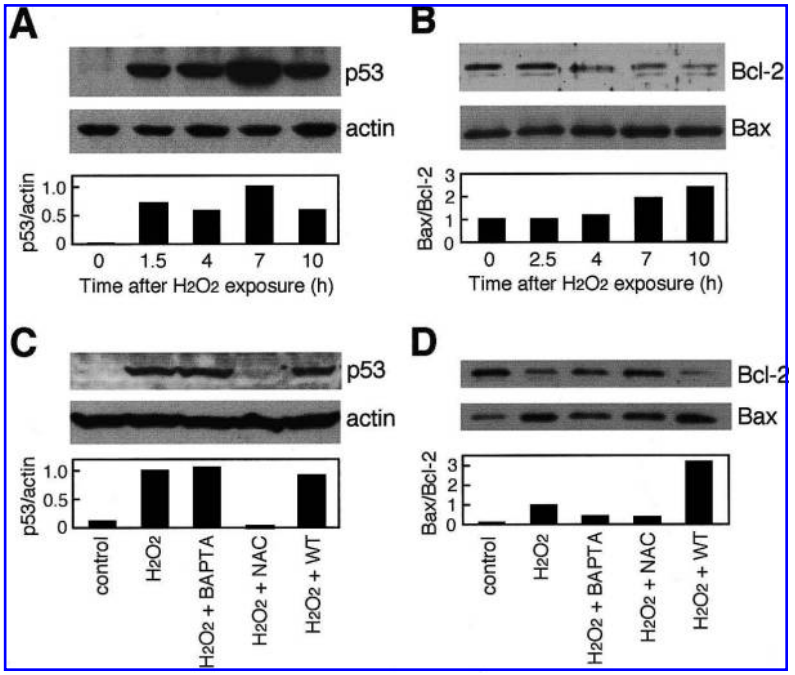

FIG. 4. Immunoblot analysis of the expression of p53, Bcl-2, and Bax. (**A**) Time course of H_2O_2-induced p53 expression. BAEC were exposed to 1 m*M* H_2O_2 in KHB for 1 hour and further incubated in IMDM without H_2O_2. (**B**) Time course of H_2O_2-induced expression of Bcl-2 and Bax. BAEC were exposed to 1 m*M* H_2O_2 in KHB for 1 h and further incubated in IMDM without H_2O_2. (**C**) Effects of inhibitors on the expression of p53. BAEC were exposed to 1 m*M* H_2O_2 in KHB for 60 min. (**D**) Effects of inhibitors on the expression of Bcl-2 and Bax. BAEC were exposed to 1 m*M* H_2O_2 in KHB for 1 h and further incubated in IMDM without H_2O_2 for 6 h. Data are representative of three to four experiments. Drugs were added to the medium as described in the legend to Fig. 2. BAPTA, BAPTA-AM; WT, wortmannin.

increase of both p53 expression and the Bax/Bcl-2 ratio. Wortmannin did not affect the p53 increase, but potently enhanced the increase of the Bax/Bcl-2 ratio.

Because changes in the Bax/Bcl-2 ratio were reported to elicit release of cytochrome *c* from mitochondria to cytosol leading to the sequential activation of caspases 9 and 3 (13), we measured the activities of these caspases (Fig. 5). As expected, caspases 3 and 9 were activated by H_2O_2. The caspase activation was strongly attenuated by BAPTA-AM and NAC, whereas it was augmented by wortmannin. Thus, the effects of these inhibitors on the caspase activation were similar to those on apoptosis (Fig. 2B).

Activation mechanisms of PI3K/Akt pathway

The present results revealed that PI3K played a protective role against apoptosis. Therefore, we next focused on the activation mechanisms of survival signals in H_2O_2-treated BAEC. Figure 6A shows the effects of H_2O_2 on the PI3K activity. The PI3K activity reached the maximum within 10 min after H_2O_2 exposure and decreased gradually. Because PI3K is known to promote cell survival via Akt phosphorylation (25), we examined the serine phosphorylation of Akt. As shown in the upper panels of Fig. 6B, H_2O_2 caused Akt phosphorylation with a peak at 20 min, suggesting that Akt was phosphorylated after the activation of PI3K. We also investigated the various concentrations of H_2O_2 on Akt phosphorylation. H_2O_2 dose-dependently phosphorylated Akt with a threshold of 0.05 m*M* (Fig. 6B, lower panels).

To investigate the involvement of Ca^{2+} and the redox regulation in PI3K activation and Akt phosphorylation, the effects of BAPTA-AM and NAC were examined (Fig. 6C and D). BAPTA-AM had no effect on PI3K activation and Akt phosphorylation, indicating that increased $[Ca^{2+}]_i$ was not required for activation of the PI3K/Akt pathway. NAC almost completely inhibited the activation of PI3K and Akt, suggesting that redox regulation strongly contributed to the survival signals. As expected, the PI3K activation and the Akt phosphorylation induced by H_2O_2 were abrogated by wortmannin.

We finally examined the involvement of tyrosine phosphorylation and growth factor receptors in the activation of PI3K/Akt. As shown in Fig. 6C and D, PI3K activation and Akt phosphorylation were partly inhibited by the tyrosine kinase inhibitor genistein. These results suggested that tyrosine phosphorylation was involved in the activation of PI3K/Akt. Suramin, an inhibitor of growth factor receptor activation, caused partial inhibition of the activation of PI3K/Akt, suggesting that the activation of growth factor receptors was involved in the sequential activation of PI3K and Akt.

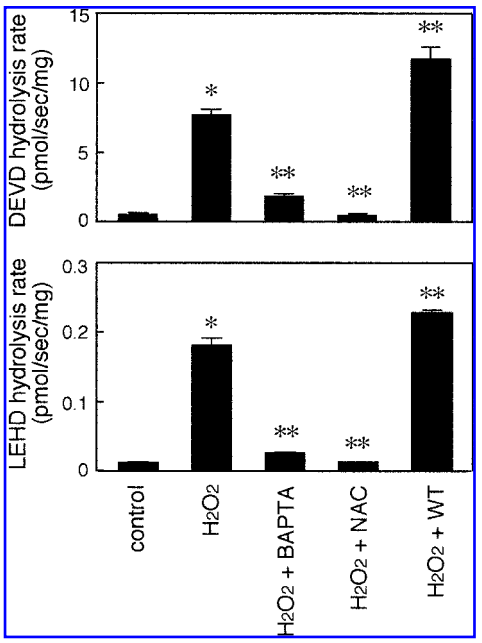

FIG. 5. Effects of inhibitors on caspase activation induced by H_2O_2. Hydrolysis rates of fluorescent substrates that are specific for caspase 3 (Z-DEVD-MCA; **upper panel**) or caspase 9 (Z-LEHD-MCA; **lower panel**) were determined. BAEC were exposed to 1 m*M* H_2O_2 in KHB for 1 h and further incubated in IMDM without H_2O_2 for 3 h. Data are expressed as means ± SE of three experiments. Drugs were added to the medium as described in the legend to Fig. 2. BAPTA, BAPTA-AM; WT, wortmannin. *Significantly different from control group ($p < 0.05$); **significantly different from H_2O_2-treated group ($p < 0.05$).

DISCUSSION

It has been demonstrated that H_2O_2 at low concentrations promotes the proliferation of mammalian cells by mimicking metabolic actions of growth factors such as insulin, EGF, and insulin-like growth factor (3, 21). Although recent evidence suggests that the PI3K/Akt pathway is activated and functions as a survival signal in H_2O_2-stimulated cells (23, 36), the redox regulation of this pathway is largely unknown. In the present study, we demonstrated that H_2O_2 induced the activation of PI3K/Akt and that inhibition of PI3K by wortmannin augmented H_2O_2-induced apoptosis. These results suggested that activation of the PI3K/Akt pathway acted as a survival signal in H_2O_2-treated EC. In apoptosis-related signals examined in the present experiments, the H_2O_2-induced increase of the Bax/Bcl-2 ratio was enhanced by wortmannin, suggesting that the PI3K/Akt pathway acted as a survival signal upstream of Bax and Bcl-2. These results are consistent with the idea that Akt regulates changes in the balance between Bax and Bcl-2 that lead to cell survival (4). We assessed the possibility that the accelerating effect of wortmannin on apoptosis was due to a modification of $[Ca^{2+}]_i$ because this inhibitor was reported to modify the $[Ca^{2+}]_i$ increase induced by thrombin in human platelets (16, 17). This possibility, however, could be excluded due to the finding that the $[Ca^{2+}]_i$ response was unaffected by wortmannin (Fig. 3), suggesting that the augmentation of apoptosis by wortmannin was not due to alteration of $[Ca^{2+}]_i$.

The increase of $[Ca^{2+}]_i$ in oxidative stress-stimulated cells is generally supposed to be due to Ca^{2+} influx from extracellular medium and/or Ca^{2+} release from endoplasmic reticulum (ER) (19). It has been demonstrated that oxidative stress modifies $[Ca^{2+}]_i$ by affecting Ca^{2+}-ATPase (43) or Ca^{2+} channels (11) on ER. Thus, Ca^{2+} release from ER is likely to be involved in the H_2O_2-induced $[Ca^{2+}]_i$ increase observed in the

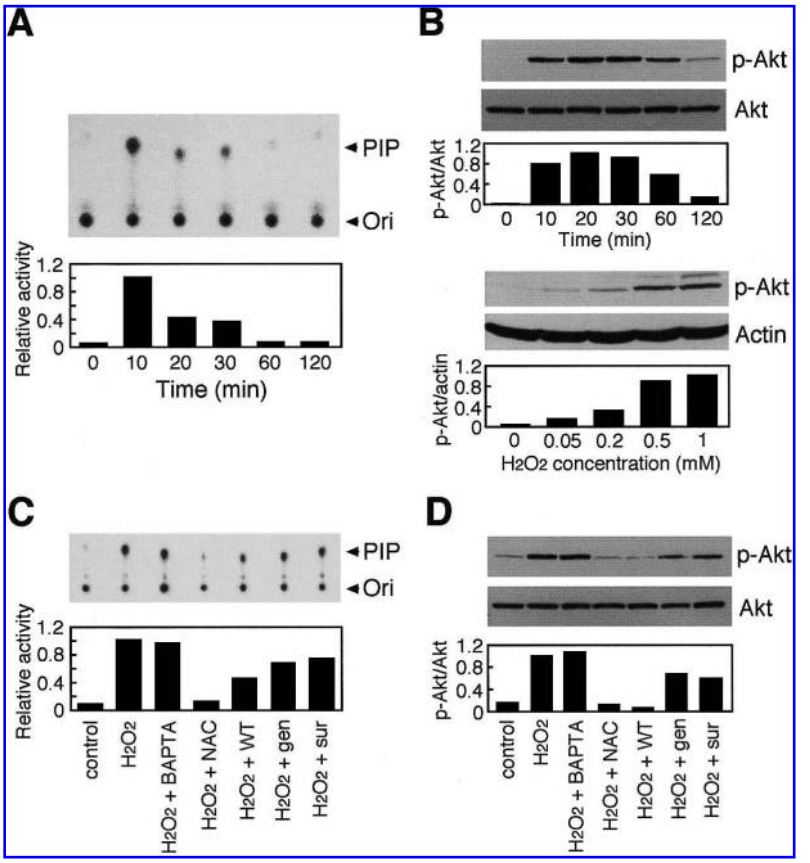

FIG. 6. Activation of PI3K and phosphorylation of Akt induced by H_2O_2. PI3K activity and Akt phosphorylation were analyzed by TLC and immunoblotting, respectively. Relative activity of PI3K and the ratio of phosphorylated Akt to Akt or actin obtained by densitometric analysis are also shown. (**A**) Time courses of PI3K activation induced by 1 m*M* H_2O_2. BAEC were exposed to H_2O_2 in KHB for 60 min and further incubated in IMDM without H_2O_2 for 60 min. (**B**) Time courses (**upper panels**) and dose dependency (**lower panels**) of Akt phosphorylation. In upper panels, BAEC were exposed to 1 m*M* H_2O_2 in KHB for 60 min and further incubated in IMDM without H_2O_2 for 60 min. In lower panels, BAEC were exposed to H_2O_2 in KHB for 60 min. (**C**) Effects of inhibitors on PI3K activation after 20 min of H_2O_2 stimulation. (**D**) Effects of inhibitors on Akt phosphorylation after 30 min of H_2O_2 stimulation. Data are representative of three experiments. Genistein (50 μ*M*) and suramin (0.5 m*M*) were added to the medium 30 min before H_2O_2 exposure until the cessation of experiments. Other drugs were added to the medium as described in the legend to Fig. 2. Phosphorylated phosphatidylinositoland origin are expressed as PIP and Ori, respectively. BAPTA, BAPTA-AM; WT, wortmannin; gen, genistein; sur, suramin.

present study. However, as shown in Fig. 3, deprivation of extracellular Ca^{2+} by addition of EGTA completely abolished the $[Ca^{2+}]_i$ increase. These results suggest that Ca^{2+} release from ER has, if any, minor contribution to the H_2O_2-induced $[Ca^{2+}]_i$ increase in BAEC.

It has been documented that increases in $[Ca^{2+}]_i$ play important roles in the regulation of several protein kinases (10, 18). Therefore, we focused our experiments on the relationship between $[Ca^{2+}]_i$ and the activation of PI3K/Akt. The PI3K activation induced by H_2O_2 was unaffected by treatment with BAPTA-AM. To the best of our knowledge, this is the first report indicating that the increase in $[Ca^{2+}]_i$ is not responsible for the H_2O_2-induced activation of PI3K. We also demonstrated that H_2O_2-induced Akt phosphorylation was independent of the $[Ca^{2+}]_i$ increase. This result is consistent with previous reports that an increase of $[Ca^{2+}]_i$ was not required for Akt activation in EGF-treated 3T3 fibroblasts (6) and anti-IgM antibody-treated murine and chicken B cells (7). In contrast, Ca^{2+}-dependent activation of Akt was reported in a human leukemia cell line (24). In addition, a recent report by Thomas *et al.* showed that the Akt phosphorylation induced by H_2O_2 was inhibited by BAPTA-AM in porcine aortic EC (40). At this stage, we cannot explain the discrepancies in results concerning the Ca^{2+} requirement for Akt phosphorylation. However, it might be explained by the difference in signal transduction mechanisms among cell types.

The H_2O_2-induced activation of the PI3K/Akt pathway was partly inhibited by genistein, suggesting that tyrosine phosphorylation was responsible for the PI3K activation. Although we could not identify the tyrosine kinase contributing to the PI3K activation, growth factor receptor-related tyrosine kinase might be involved because inhibition of the activation of growth factor receptor with suramin (31) inhibited activation of the PI3K/Akt pathway. This speculation may be supported by the report that H_2O_2 induces the tyrosine phosphorylation of EGF receptors, which leads to subsequent signaling events in vascular smooth muscle cells (29). However, this phosphorylation of EGF receptors does not appear to be a direct effect of H_2O_2 on the receptors. Recently, it was proposed that direct phosphorylation of c-Src by H_2O_2 is an upstream event for tyrosine phosphorylation of EGF receptors (41). Further study to identify the primary target of H_2O_2 in survival signals of EC is required.

In a previous study, we reported that H_2O_2 induces apoptosis dependent on expression of p53 and an increase of $[Ca^{2+}]_i$ (27). In that report, it was shown that p53 expression was regulated by protein kinase Cδ, but not by the $[Ca^{2+}]_i$ increase, although the release of cytochrome *c* from mitochondria was dependent on the $[Ca^{2+}]_i$ increase. In the present study, we confirmed that p53 expression did not require a $[Ca^{2+}]_i$ increase and demonstrated that the increase of the Bax/Bcl-2 ratio was dependent on Ca^{2+}. The expression of p53 is known to lead to the up-regulation of Bax and the down-regulation of Bcl-2 (34). Taken together, these results showed that Ca^{2+} acts as a proapoptotic signal downstream from p53 and upstream from Bax/Bcl-2. From these results, it seems possible that Ca^{2+} regulates the transcription levels of several genes, such as Bax and Bcl-2.

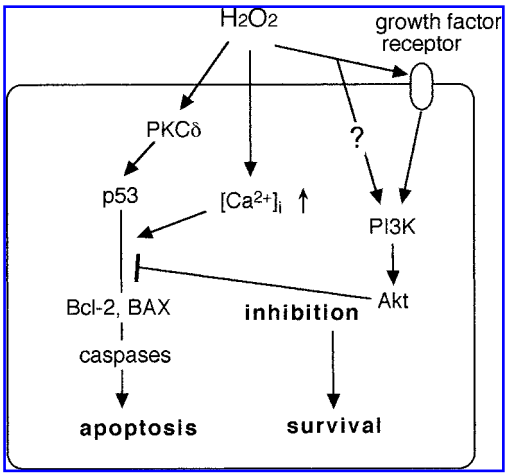

FIG. 7. A schematic model of apoptotic and survival signals in H_2O_2-treated BAEC. PKCδ, protein kinase Cδ.

Recent studies demonstrated that ROS were harmful metabolites, but that they could also act as intercellular messengers to activate different transcription factors. Ruiz-Gines *et al.* showed that H_2O_2 generated from glucose oxidase caused a significant increase in BAEC proliferation and DNA synthesis through the activation of tyrosine kinase (30). In porcine aortic EC stably expressing human vascular endothelial growth factor receptor-2, the receptor activation by vascular endothelial growth factor was followed by a rapid increase in the intracellular generation of H_2O_2 to activate PI3K, Rac, and extracellular signal-regulated kinase, suggesting that H_2O_2 acted as a mediator of angiogenic signals (5). H_2O_2 was also reported to activate Ets-1, a transcription factor that can regulate the angiogenesis-related proteases, including urokinase plasminogen activator, and matrix metalloprotease-1, in BAEC (45). From these reports, it appears that analysis of the oxidative stress and the redox regulation for proliferative signals, as well as apoptotic signals, in EC is very important to understand several cardiovascular diseases, such as hypertension, atherosclerosis, and restenosis. The present results clarified that H_2O_2 not only induced apoptosis, but also promoted the survival of EC, and that the H_2O_2–induced $[Ca^{2+}]_i$ increase was responsible for apoptosis, but not for survival signaling via the PI3K/Akt pathway (Fig. 7). We also demonstrated that the activation of growth factor receptor may be involved in the redox regulation of survival signals. To understand mechanisms of endothelial survival in vascular diseases in detail, further study concerning cellular redox regulation of the PI3K/Akt pathway is required.

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ABBREVIATIONS

BAEC, bovine aortic endothelial cells; BAPTA-AM, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetra(acetoxymethyl) ester; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; EC, endothelial cells; EGF, epidermal growth factor; ER, endoplasmic reticulum; H_2O_2, hydrogen peroxide; IMDM, Iscove's modified Dulbecco's medium; KHB, Krebs–HEPES buffer; NAC, *N*-acetyl-L-cysteine; PBS, phosphate-buffered saline; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; TBST, Tris-buffered saline with Tween 20.

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