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p38 MAPK and Ca^{2+} Contribute to Hydrogen Peroxide-induced Increase of Permeability in Vascular Endothelial Cells but ERK does not

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To examine the involvement of p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal-regulated kinase (ERK) in the oxidative stress-induced increase of permeability in endothelial cells, the effects of a p38 MAPK inhibitor (SB203580) and ERK inhibitor (PD90859) on the H_2O_2-induced increase of permeability in bovine pulmonary artery endothelial cells (BPAEC) were investigated using a two-compartment system partitioned by a semi-permeable filter. H_2O_2 at 1 mM caused an increase of the permeation rate of fluorescein isothiocyanate (FITC)-labeled dextran 40 through BPAEC monolayers. SB203580 inhibited the H_2O_2-induced increase of permeability but PD98059 did not, though activation (phosphorylation) of both p38 MAPK and ERK was observed in H_2O_2-treated cells in Western blot analysis. An H_2O_2-induced increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was also observed and an intracellular Ca^{2+} chelator (BAPTA-AM) significantly inhibited the H_2O_2-induced increase of permeability. However, it showed no inhibitory effects on the H_2O_2-induced phosphorylation of p38 MAPK and ERK. The H_2O_2-induced increase of $[Ca^{2+}]_i$ was not influenced by SB203580 and PD98059. These results indicate that the activation of p38 MAPK and the increase of $[Ca^{2+}]_i$ are essential for the H_2O_2-induced increase of endothelial permeability and that ERK is not.

Keywords: Ca^{2+}; Endothelial cells; ERK; Hydrogen peroxide; Permeability; p38 MAPK

INTRODUCTION

Vascular endothelial cells (EC) constitute a physical barrier between blood and vessel walls.[1] Dysfunction of endothelial permeability induced by oxidative stress has been reported to

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lead to lungs injury[2] and atherosclerosis.[3,4] Pulmonary vascular EC, in particular, are critical targets of oxidants because of their proximity to environmental factors and oxidant-releasing leukocytes. Hydrogen peroxide (H_2O_2) derived from neutrophils[5,6] and/or EC themselves[7] is considered to be causative oxidant in the increase of endothelial permeability, and the rearrangement of cytoskeltal proteins such as actin plays a key role in the regulation of endothelial permeability.[8,9] Therefore, the oxidative stress-induced increase in the endothelial permeability seems to be associated with a dysfunction of the regulatory system for the rearrangement of cytoskeltal proteins.

To study the regulatory mechanisms of endothelial permeability *in vitro*, a two-compartment system partitioned by semi-permeable filter is commonly used. The endothelial monolayer grown on the semi-permeable filter can be regarded as a vessel wall, and the permeation rates of macromolecules such as albumin and dextran through the endothelial monolayer are used as indicators of the endothelial permeability. Using this system, several studies have shown that a rise of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) triggers an increase of the endothelial permeability when EC are exposed to agonists such as thrombin,[10] vascular endothelial growth factor[11] and H_2O_2.[12]

It is widely accepted that the mitogen-activated protein kinases (MAPK), including p38 MAPK, extracellular signal-regulated kinase (ERK) and stress-activated protein kinase/C-Jun N-terminal kinase (SAPK/JNK), play pivotal roles in cell differentiation, cell growth, cell migration and cell death.[13–15] Recently, some MAPK are known to regulate reorganization of actin fibers. For instance, it has been reported that p38 MAPK modulates actin polymerization through the phosphorylation of heat shock protein 27 (HSP27)[16] and that ERK causes cell contraction and membrane ruffling by the phosphorylation of myosin light chain kinase (MLCK) followed by actin redistribution.[17] Our recent experiments with polymorphonuclear leukocytes also showed that SB203580 (a p38 MAPK inhibitor) significantly inhibits opsonized zymosan-induced phagocytosis through the reorganization of actin filaments, suggesting that p38 MAPK regulates the dynamics of the cytoskelton.[18] Moreover, it has been shown that the MAPK is activated by H_2O_2 in several cell types such as kidney epithelial cells,[19] fibroblast cells,[20,21] vascular smooth muscle cells[22,23] and EC.[24] From these facts, it is inferred that the oxidant-induced activation of MAPK plays a cardinal role in the regulation of endothelial permeability.

In the activation mechanisms of MAPK, we have demonstrated that H_2O_2-induced elevation of $[Ca^{2+}]_i$ is essential for the activation of SAPK/JNK but not p38 MAPK and ERK in fibroblast cell line V79.[20,21] In contrast, Zhang *et al.* showed that H_2O_2 induced the Ca^{2+}-dependent activation of ERK in pulmonary arterial smooth muscle cells.[23] Thus, the activation mechanisms of the MAPK family in oxidative stress seem to depend on the cell type. In the present study, we investigated whether p38 MAPK and ERK were involved in the change of endothelial permeability induced by H_2O_2. The relationship between MAPK and Ca^{2+} was also investigated to obtain further insights into the signal transduction mechanisms of H_2O_2-stimulated EC.

MATERIALS AND METHODS

Materials

Iscov's modified Dulbecco's medium (IMDM), fibronectin, fluorescein isothiocyanate (FITC)-dextran 40 (M.W. 40 kDa), SB203580 and PD98059 were purchased from Sigma (MO, USA). Fetal bovine serum (FBS) was from Filtron (Brooklyn, Australia). Penicillin, streptomycin and trypsin were from Gibco (NY, USA). BAPTA-acetoxymethyl ester (AM) and fura 2-AM were from Dojindo (Japan). Millicell-CM was from

Millipore (MA, USA). Six-well culture plates were from Coaster (MA, USA). BCA protein assay reagent was from Pierce (IL, USA). Antibodies to p38 MAPK, phosphorylated p38 MAPK, ERK and phosphorylated ERK were from New England Biolabs (MA, USA). All other reagents were of analytical grade.

Cell Culture

Bovine pulmonary artery endothelial cells (BPAEC) were purchased from Cell Systems (WA, USA). BPAEC were grown in IMDM supplemented with 20% FBS, penicillin (100 IU/ml) and streptomycin (100 μg/ml) in a humidified environment of 5% CO_2, 95% air at 37°C. Culture medium was renewed every 2–3 days. BPAEC at passages 6–10 were used for experiments.

Measurements of BPAEC Monolayer Permeability

Trans-endothelial clearance rates of FITC-dextran 40 were measured as an index of endothelial permeability. For this purpose, a two-compartment system consisting of culture inserts (Millicell-CM) and outer chambers (6-well cell culture plate) was used. The bottom of the culture insert consisted of a semi-permeable filter, the pore size of which was 0.45 μM in diameter. Before cells were seeded, the surfaces of filters were coated with fibronectin. Cells (3×10^5) were grown on the culture inserts, which were placed in 6-well culture plates. The culture inserts were used for experiments when cells had grown to confluence 4–5 days after seeding. The culture inserts were washed triply with phosphate-buffered saline (PBS) and placed in 6-well culture plates. Culture inserts and outer chambers were filled with 2 ml of Krebs-HEPES buffer (KHB [pH 7.4], and 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), respectively, so that there was no difference in the hydrostatic pressure between the culture inserts and the outer chambers.

The systems were placed on a water bath warmed at 37°C and gently agitated at 60 rpm throughout the experiment. After 10 min, FITC-dextran 40 was added to the culture inserts to make a final concentration of 5 μM. H_2O_2 was added to the culture inserts 20 min after the addition of FITC-dextran 40. KHB samples (50 μl) were collected from the outer chambers at the indicated periods of time. Fluorescence of FITC-dextran 40 in the collected samples was measured using a spectrofluorometer (FP-750, Jasco, Japan) at the excitation and emission wavelengths of 495 and 510 nm, respectively.

Drug Treatments

SB203580 or PD98059 was added to culture medium of each dish at a concentration of 20 μM 2–10 min before the addition of H_2O_2 until the cessation of experiments. Cells were pre-incubated with BAPTA-AM at a concentration of 20 μM for 30 min before the of H_2O_2 addition.

Immunoblotting

Cells ($2–4 \times 10^6$) were grown to confluence in culture dishes (10 cm in diameter). After drug treatment was finished, cells were washed with PBS, resuspended in 50 μl of a lysis buffer (20 mM HEPES [pH7.4], 50 mM glycerophosphate, 1% Triton X-100, 10% glycerol, 2 mM EGTA, 1 mM dithiothreitol, 2 mg/ml aprotinin, 2 mg/ml pepstatin A, and 1 mM Na_3VO_4), 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 measured with a commercial kit (BCA protein assay reagent). Proteins in the supernatant were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature with 5% nonfat dry milk powder in TBST (10 mM

Tris–HCl [pH 7.5], 150 mM NaCl, 0.1% Tween 20). The filters were then rinsed triply with TBST and incubated overnight at 4°C with antibodies, which were diluted 1:1000 in TBST plus 5% BSA or nonfat dry milk powder. The filters were washed triply with TBST and then incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit IgG antibody diluted 1:2000 in TBST plus 5% nonfat dry milk powder. The membranes were washed with TBST, and immunoreactive bands were visualized by chemiluminescence detection.

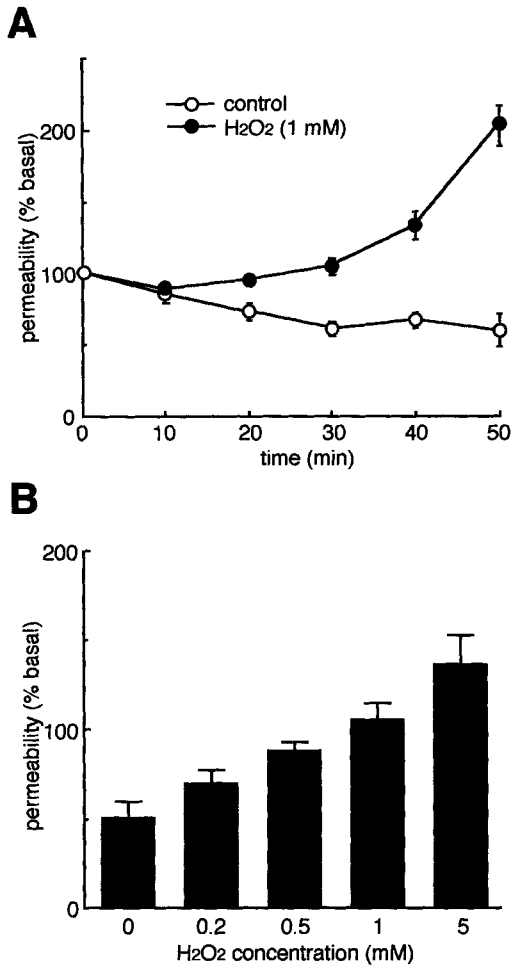

FIGURE 1 (A) Time-dependent changes in the permeability of endothelial monolayers by FITC–dextran 40 when continuously exposed to 1 mM H_2O_2. The vertical axis represents the ratio of intensity of fluorescence from FITC–dextran 40 recovered in the outer chamber at the indicated period of time to that recovered in it at 20 min after the addition of FITC–dextran 40 (zero time), $n = 7$–13. (B) Effects of H_2O_2 at various concentrations on the endothelial permeability. Averaged values of permeability during 40 min after exposure to H_2O_2 are shown, $n = 3$–5.

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

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in a single cell was measured with a fluorescent intracellular Ca^{2+} indicator, fura 2-AM,[25] with the ratio method using dual-wavelength excitation and single emission with a fluorometer (CAM-200, Jasco, Japan). BPAEC were seeded on glass cover slips and grown to confluence. BPAEC attached to coverslips were incubated in KHB containing fura 2-AM (5 μM) for 30 min at 37°C. After incubation, they were stored at 4°C until used. The coverslips were placed on the stage of an inverted microscope (Diaphot 300, Nikon, Japan) and superfused continuously with KHB at a rate of 2 ml/min. Alternate beams of excitation light at 340 and 380 nm were generated by a wheel spinning at 400 Hz from the fluorometer. Fluorescent signals from BPAEC and their ratios were stored in the hard disk of a computer (Macintosh, Apple Japan, Japan). The calibration of fura 2 signals was described in a previous paper.[26]

Statistics

All results were expressed as means ± SEM. Differences in means of groups were determined by Students's *t*-test, and the minimum level of significance was set at $P < 0.05$.

RESULTS

Effects of H_2O_2 on Endothelial Permeability

To investigate the endothelial permeability, BPAEC were grown on fibronectin-coated semipermeable filters. The basal permeation rate of dextran 40 through endothelial monolayers during the initial 20 min was 1.30 ± 0.11 μmol/cm²/min ($n = 23$). Figure 1(A) shows

the time-dependent changes in the endothelial permeability to dextran 40 without and with H_2O_2 (1 mM). Basal permeability without H_2O_2 slightly decreased in a time-dependent manner. When H_2O_2 was added to the medium, a gradual increase up to twice the permeability was observed. Figure 1(B) shows the dose-dependent effects of H_2O_2 on the endothelial permeability. H_2O_2 increased the permeability in a dose-dependent manner.

Roles of MAPK and Ca^{2+} in Endothelial Permeability

We next examined the involvement of MAPK in the regulation of endothelial permeability using specific inhibitors. Figure 2 shows the averaged permeability during 40 min of H_2O_2 stimulation (1 mM). SB203580, an inhibitor of p38 MAPK, and BAPTA-AM, an intracellular Ca^{2+} chelator, significantly attenuated the H_2O_2-induced increase of permeability. On the other hand, PD98059, an inhibitor of ERK, had no effect on the increase of permeability. These results suggested that p38 MAPK and intracellular Ca^{2+}, but not ERK, were involved in the H_2O_2-inudced increase in endothelial permeability.

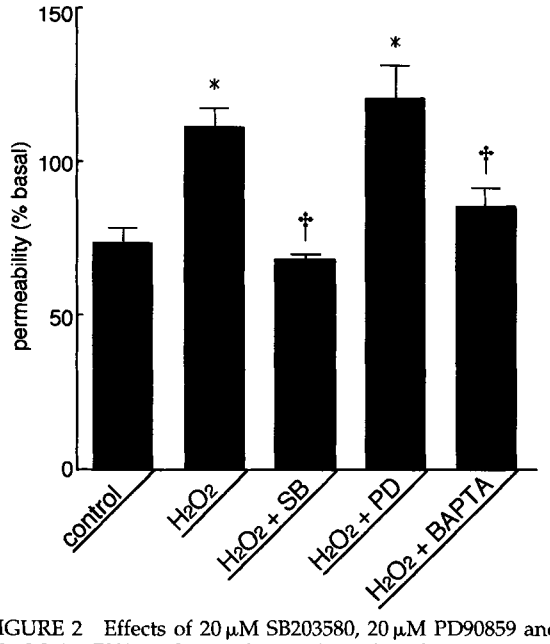

FIGURE 2 Effects of 20 μM SB203580, 20 μM PD90859 and 20 μM BAPTA-AM on the H_2O_2-induced increase of endothelial permeability. Averaged values of permeability during 40 min after exposure to 1 mM H_2O_2 are shown, n = 4–13. *Significantly different from control, †Significantly different from the H_2O_2-treated group.

Relationship between MAPK and $[Ca^{2+}]_i$

We investigated the activation of MAPK in response to H_2O_2. The phosphorylated MAPK, as the activated form, was measured by immunoblotting using specific antibodies to phosphorylated kinases. As shown in Fig. 3, H_2O_2 (1 mM) caused time-dependent phosphorylation of p38 MAPK (Fig. 3(A)) and ERK (Fig. 3(B)) whereas H_2O_2 had no effects on the total amounts of p38 MAPK and ERK as shown in the lower panels of each figure. The maximum phosphorylation was observed in each sample at 20 min after H_2O_2 addition. Several reports showed that the activation of MAPK was regulated by the increase of $[Ca^{2+}]_i$[20,21,27–30] and other reports showed that the activation of MAPK participated in the regulation of $[Ca^{2+}]_i$.[31–33] We therefore, investigated whether there was a relationship between MAPK and $[Ca^{2+}]_i$ in H_2O_2-stimulated BPEAC. Figure 3(C) and (D) show the effects of BAPTA-AM on the phosphorylation of MAPK. H_2O_2-induced phosphorylation of p38 MAPK was unaffected by BAPTA-AM, whereas that of ERK was slightly augmented by it. These results indicated that the activation of MAPK by H_2O_2 was not elicited by the increase of $[Ca^{2+}]_i$.

We also examined whether the H_2O_2-induced $[Ca^{2+}]_i$ increase was mediated by MAPK. Figure 4 shows time-dependent changes in $[Ca^{2+}]_i$ in H_2O_2-stimulated BPAEC. H_2O_2 (1 mM) caused a raped increase in $[Ca^{2+}]_i$, which subsequently remained at the high level (Fig. 4(A)). Figure 4(B)–(D) show the effects of MAPK inhibitors on the H_2O_2-induced increase of $[Ca^{2+}]_i$. The addition of SB203580 caused a gradual increase

in the basal $[Ca^{2+}]_i$ but was found to preserve the H_2O_2-induced $[Ca^{2+}]_i$ increase at the high level (Fig. 4(B)). When the average $[Ca^{2+}]_i$ during 5 min after exposure to H_2O_2 was measured, it was higher in the presence of SB203580 than in

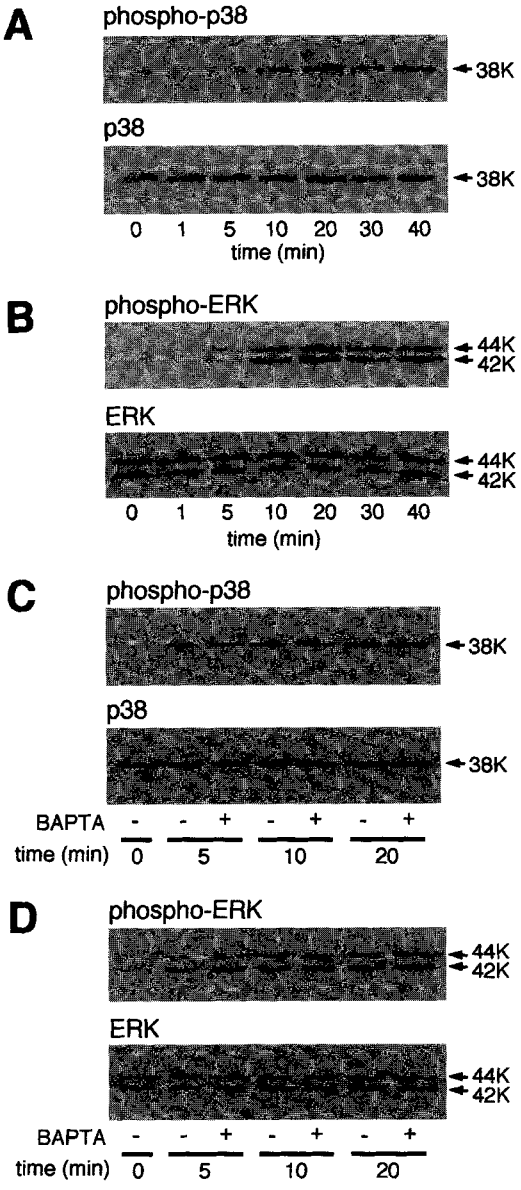

FIGURE 3 Time-dependent changes in the phosphorylation of p38 MAPK (A) and ERK (B) and effects of 20 μM BAPTA-AM on the phosphorylation of p38 MAPK (C) and ERK (D) when cells were continuously exposed to 1 mM H_2O_2. The phosphorylation was detected by immunoblot analysis. Data are representative of 3–6 experiments.

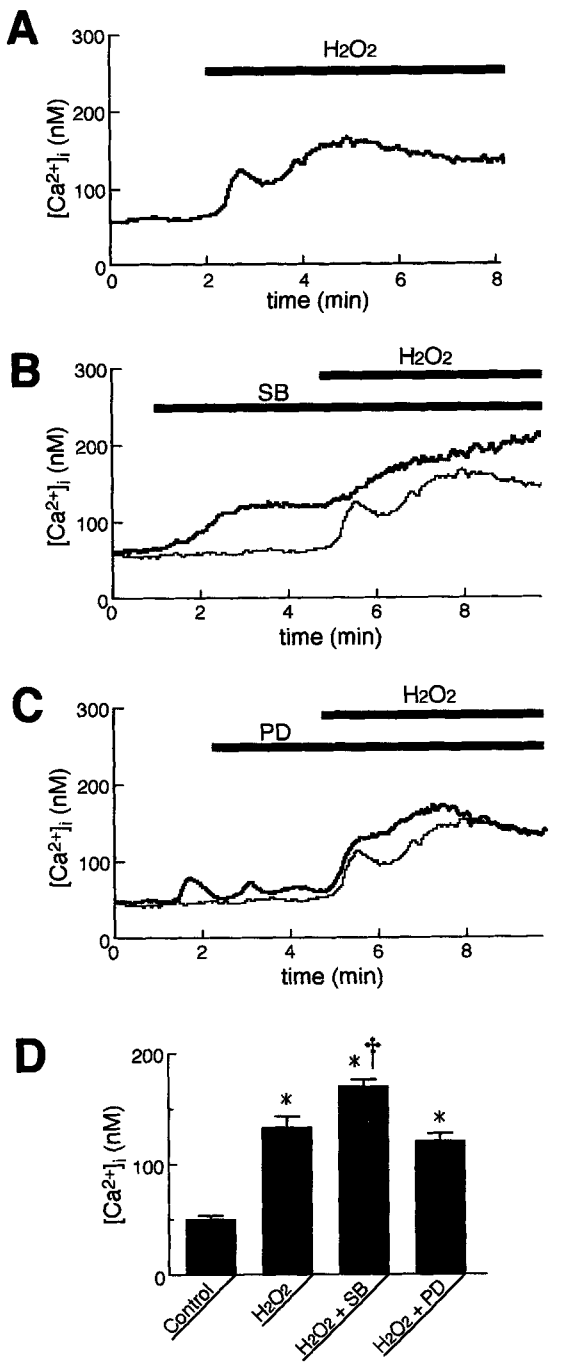

FIGURE 4 Time-dependent changes in $[Ca^{2+}]_i$ (A) and the effects of 20 μM SB203580 (B) and 20 μM PD90859 (C) on the H_2O_2-induced response of $[Ca^{2+}]_i$ when cells were continuously exposed to 1 mM H_2O_2. The fine lines in (B) and (C) represent the control responses to H_2O_2. Data are representative of six experiments. (D) Averaged $[Ca^{2+}]_i$ values during 5 min after H_2O_2 exposure with or without inhibitors, $n = 5–6$. *Significantly different from control, †significantly different from H_2O_2-treated group.

the absence of SB203580 (Fig. 4(D)). This increase of $[Ca^{2+}]_i$ seemed to be due to the additive effect of H_2O_2 and SB203580. PD98059 had no effects on the basal and H_2O_2-stimulated $[Ca^{2+}]_i$ responses (Fig. 4(C) and (D)).

DISCUSSION

In the present study we demonstrated that H_2O_2 caused an increase of dextran flux across the endothelial monolayers. Two pathways for the passage of macromolecules were considered; (1) nonvesicular paracellular pathways that present at cellular junctions or transcellular channels and (2) vesicle-mediated transcellular pathways. When endothelial permeability is increased by inflammatory mediators such as thrombin and histamine, macromolecules are supposed to pass paracellularly through the endothelial monolayer because the contraction or retraction of cells and the interendothelial gap formation are concomitantly observed.[8,9] Furthermore, H_2O_2 has been reported to cause the contraction or retraction and the interendothelial gap formation endothelial cells.[34–36] Therefore, the paracellular pathway for the passage of dextran seems dominant in the present experiments.

We demonstrated that the H_2O_2-induced increase of endothelial permeability was inhibited by SB203580, an inhibitor of p38 MAPK. This result indicates that p38 MAPK regulates an endothelial function such as permeability of macromolecules. Several reports have shown that the activation of p38 MAPK is involved in the rearrangement of actin fibers in various types of cells.[16,24,37] In fact, we recently demonstrated that the activation of p38 MAPK participates in the opsonized zymosan-induced phagcytosis triggered by the rearrangement of actin fibers in bovine polymorphonuclear leukocytes.[18] In human umbilical vein EC (HUVEC), Huot *et al.* showed that H_2O_2 activated p38 MAPK and the inhibition of p38 MAPK by SB203580 led to an inhibition of actin reorganization.[24] Furthermore, it was shown that the rearrangement of actin fibers by p38 MAPK activation was mediated by HSP27.[16,24] These reports suggested that p38 MAPK regulated the H_2O_2-induced increase of endothelial permeability through the phosphorylation of HSP27 followed by actin reorganization.

On the other hand, we observed that the inhibition of ERK by PD98059 did not affect the H_2O_2-induced increase of endothelial permeability. Twenty μm PD98059 inhibited the H_2O_2-induced phosphorylation of ERK to the control level (data not shown). These results lead us to conclude that the H_2O_2-induced activation of ERK did not regulate the endothelial permeability. However, this activation of ERK might participate in the survival responses since the activation of ERK under oxidative stress was recognized to act as protective signal.[13] Recently, Kevil *et al.* demonstrated that the H_2O_2-induced increase in the endothelial permeability of sodium fluorescein (M.W. 376 Da) was inhibited by PD98059 in HUVEC and concluded that the activation of ERK participated in the increased permeability.[36] It was also reported that the activation of protein kinase C was responsible for the H_2O_2-induced increase of endothelial permeability in BPAEC[38] but not in HUVEC.[36] These results suggest that the regulatory mechanisms of the permeability in EC are dependent on the cell type.

We observed that BAPTA-AM significantly inhibited the H_2O_2-induced increase of endothelial permeability as shown in Fig. 2 and that the deprivation of extracellular Ca^{2+} completely inhibited both initial and sustained phases of increase in $[Ca^{2+}]_i$ (data not shown), indicating that the increase in $[Ca^{2+}]_i$ due to an influx of extracellular Ca^2 plays a critical role in the regulation of endothelial permeability. Similar phenomena were observed by Siflinger-Birnboim *et al.*[12] and Shasby *et al.*[39] In their reports, it was shown that the oxidative stress-induced rise of $[Ca^{2+}]_i$ led to an increase of endothelial

permeability by the rearrangement of actin fibers.[12,39] On the other hand, Chang and Wang demonstrated that the increase of $[Ca^{2+}]_i$ existed at the upstream of the phosphorylation of p38 MAPK in rat neutrophils stimulated by formyl-methionyl-leucyl-phenylalanine (fMLP).[30] From these facts, it was inferred that the H_2O_2-induced increase of $[Ca^{2+}]_i$ might trigger the activation of p38 MAPK, leading to a dysfunction of regulation in cytoskeltal proteins of EC. However, our present results ruled out this possibility because BAPTA-AM had no inhibitory effect on the H_2O_2-induced phosphorylation of p38 MAPK, indicating that the increase of $[Ca^{2+}]_i$ did not contribute to the activation of p38 MAPK. These results are consistent with our previous report that the phosphorylation of p38 MAPK induced but H_2O_2 was not inhibited by BAPTA-AM in Chinese hamster fibroblasts.[20,21]

We also addressed the involvement of MAPK in the regulation of $[Ca^{2+}]_i$. In the resting condition without H_2O_2, $[Ca^{2+}]_i$ was increased by SB203580 but not by PD98059, suggesting that p38 MAPK, but not ERK, participates in homeostasis of $[Ca^{2+}]_i$. At this stage, the mechanism of regulation of resting $[Ca^{2+}]_i$ by p38 MAPK is not clear, but might be explained by the fact that SB203580 modifies the Ca^{2+} influx *via* Ca^{2+} channels.[31] In H_2O_2-stimulated BPAEC, an increased response of $[Ca^{2+}]_i$ was observed and SB203580 did not inhibit this response, as shown in Fig. 4(A) and (B). Furthermore, SB203580 and BAPTA-AM significantly attenuated the H_2O_2--induced increase of endothelial permeability (Fig. 2) and BAPTA-AM did not affect the H_2O_2-induced activation of p38 MAPK (Fig. 3(C)). Taken together, these results lead us to conclude that the simultaneous events of two signaling factors, i.e. the phosphorylation of p38 MAPK and the increase of $[Ca^{2+}]_i$, were required for the H_2O_2-induced increase of endothelial permeability. To understand in detail the mechanisms for the H_2O_2-induced increase of endothelial permeability, further experiments will be necessary to clarify the regulatory system of cytoskeltal proteins by p38 MAPK and Ca^{2+}.

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