Oxidative stress activates various signal transduction pathways, including Jun N-terminal kinase (JNK) and its substrates, that induce apoptosis. We reported here the role of angiopoietin-1 (Ang1), which is a prosurvival factor in endothelial cells, during endothelial cell damage induced by oxidative stress. Hydrogen peroxide (H$_2$O$_2$) increased apoptosis of endothelial cells through JNK activation, whereas Ang1 inhibited H$_2$O$_2$-induced apoptosis and concomitant JNK phosphorylation. The inhibition of H$_2$O$_2$-induced JNK phosphorylation was reversed by inhibitors of phosphatidylinositol (PI) 3-kinase and dominant-negative Akt, and constitutively active-Akt attenuated JNK phosphorylation without Ang1. These data suggested that Ang1-dependent Akt phosphorylation through PI 3-kinase leads to the inhibition of JNK phosphorylation. H$_2$O$_2$-induced phosphorylation of SAPK/Erk kinase (SEK1) at Thr$^{386}$, which is an upstream regulator of JNK, was also attenuated by Ang1-dependent activation of the PI 3-kinase/Akt pathway. In addition, Ang1 induced SEK1 phosphorylation at Ser$^{360}$, suggesting the existence of an additional signal transduction pathway through which Ang1 attenuates JNK phosphorylation. These results demonstrated that Ang1 attenuates H$_2$O$_2$-induced SEK1/JNK phosphorylation through the PI 3-kinase/Akt pathway and inhibits the apoptosis of endothelial cells to oxidative stress.

It has become increasingly apparent that oxidative stress is an important component of aging and is involved in the pathogenesis of many diseases. For example, high glucose levels stimulate reactive oxygen species production through both mitochondria and NADPH oxidase, which might lead to the progression of diabetic complications (1). The effects of oxidative stress on vascular cells have now been elucidated and show that it promotes both angiogenic and apoptotic activity in vascular endothelial cells. Oxidative stress, increased by modulating insulin signaling in both in vitro (3) and in vivo (4), leads to the up-regulation of vascular endothelial growth factor KDR/Flik-1 signaling (2) and the concomitant proliferation of endothelial cells. Moreover, oxidative stress has also been shown to increase cell death and apoptosis in vascular endothelial cells in vitro (3) and in vivo (4).

In the view of intracellular molecules, oxidative stress could cause activation of numerous major signal transduction pathways, including specific kinases such as extracellular signal-regulated kinases (5), Jun N-terminal kinase (JNK, also called stress-activated protein kinase) (6), and also up-regulation of transcription factors such as nuclear factor-kB (NF$\kappa$B) (7) and c-Jun (8). In particular, hydrogen peroxide (H$_2$O$_2$) is one of the major reactive oxygen intermediates that can activate many of these pathways and induces the phosphorylation and activation of JNK in vascular endothelial cells (8) and in other cell types.

JNK, initially identified by Kyriakis and Avruch (9), is activated by various stimulatory signals, resulting in apoptotic and inflammatory responses via various its substrate effectors. Tumor necrosis factor (TNF) induces JNK activation, resulting in the suppression of E2F1 (10), and lipopolysaccharide and H$_2$O$_2$ also induce the phosphorylation and activation of JNK and up-regulate the inflammatory cytokine, interleukin-6 (IL-6), and vascular permeability (11). Furthermore, JNK has been shown to play an important role in the pathogenesis of diabetes by modulating insulin signaling in both in vitro (12) and in vivo experiments (13).

Angiopoietin-1 (Ang1) was isolated as a ligand of Tie2 (14), which had already been identified as an orphan receptor (15). Among the various angiogenic cytokines, Ang1 is one of the most important factors, as data from gene-targeting experiments have shown that the Ang-Tie2 system is necessary for vascular remodeling, maturation and stabilization, and normal lymphangiogenesis (16, 17). Additional facts show that this system may also be associated with pathogenesis of angiogenic disorders, such as diabetic retinopathy (18, 19), pulmonary hypertension, and tumor angiogenesis. A recent study (20) has also shown that Ang1 up-regulation by Src-suppressed protein oxidase, leads to the up-regulation of vascular endothelial growth factor KDR/Flik-1 signaling (2) and the concomitant proliferation of endothelial cells. Moreover, oxidative stress has also been shown to increase cell death and apoptosis in vascular endothelial cells in vitro (3) and in vivo (4).
kinase C substrate in astrocytes contributes to tight junction formation in the blood-brain barrier. In proteome experiments, we showed that Ang1 induces autophosphorylation of Tie2, which recruits and activates intracellular signaling molecules, including p85 (a subunit of phosphatidylinositol (PI) 3-kinase), Grb3, Snp-2, and Dok-R (21). Ang1 has also been reported to induce specific effects, such as chemotactic responses through ShcA (22) and sprouting through either Dok-R or Akt activation (23). In addition, Ang1 has been demonstrated to be a potent survival factor against apoptosis induced by serum starvation (24), irradiation, and mannitol (25) through the activation of Akt in a PI 3-kinase-dependent manner. Tie2 also interacts with ABIN-2 in a ligand-dependent manner and inhibits NFκB activity and concomitant endothelial cell death (26).

Ang1 is now thought to be a promising factor for the development of new therapeutic strategies against vascular diseases, as vascular endothelial growth factor-induced vascular hyperpermeability is reversed by Ang1 (27), and in diabetes animal models, hyperpermeability and apoptosis of vascular cells are reduced by Ang1 (28). Moreover, ocular angiogenesis is suppressed in transgenic mice in which Ang1 is overexpressed in the retina (29). However, the effects of Ang1 upon vascular injury, induced by hydrogen peroxide, have not been investigated.

In this study, we investigated Ang1 suppression of the biological effects induced by oxidative stress by analyzing the intracellular signaling pathways involved. We demonstrate that Ang1 attenuates H2O2-induced SEK1/JNK phosphorylation through the PI 3-kinase/Akt pathway and decreases apoptosis of vascular endothelial cells, induced by oxidative stress.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sodium pyrophosphate, sodium fluoride, sodium orthovanadate, aprotinin, leupeptin, and phenylmethylsulfonyl fluoride were obtained from Sigma. Reagents for SDS-PAGE were obtained from Bio-Rad. Protein A-Sepharose was purchased from Amersham Biosciences. All other materials were ordered from Sigma.

**Cell Culture**—Primary cultures of porcine retinal endothelial cells (PRECs) were isolated by homogenization and a series of filtration steps as described previously (30). Primary PRECs were grown on collagen-coated dishes (Iwaki Glass Inc.) containing Dulbecco’s modified Eagle’s medium supplemented with 5.5 mM glucose, 10% porcine serum, 50 μg/ml heparin, and 50 mmol/liter endothelial cell growth factor. Cells were cultured in 5% CO2 at 37 °C with media replenishment every 3 days. Endothelial cell homogeneity was confirmed by immunoreactivity with anti-factor VIII antibodies and analysis by confocal microscopy. Cells growing between passages 7 and 11 were used in these experiments and were treated with hydrogen peroxide (Wako, Osaka, Japan), angiopoietin-1, TNFα (R & D Systems, Minneapolis, MN), wortmannin, and LY294002 (Calbiochem), as indicated in the text. Human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bio Science (Baltimore, MD) and cultured on collagen-coated dishes with EGM-2 BulletKit® (Cambrex Bioscience) until the third passage, and this was followed by study on apoptosis and signal transduction.

**Recombinant Adenoviruses**—cDNA of constitutively active Akt (CA-Akt, Gag protein fused to the N terminus of wild type Akt) and cDNA of dominant-negative Akt (DN-Akt, containing Thr308→Ala and Ser473→Ala substitutions) have been described previously (31). cDNAs of wild-type and DN JNK (K55R) have also been described previously (32). Recombinant adenoviruses were constructed by homologous recombination between the parental virus genome and either expression cosmid cassettes or shuttle vectors as described previously. Adenovirus constructs were cloned at a concentration of 1 × 10⁶ plaque-forming units/ml, and vectors with the same parental genome carrying the lacZ gene or the green fluorescent protein (GFP) gene were used as controls. Expression of each recombinant protein was confirmed by Western blot analysis, and a 10-fold increase was observed, compared with cells infected with the control adenovirus.

**siRNA-mediated Gene Silencing**—Validated stealth RNAi against Akt1, Akt2, JNK, and SEKI were purchased from Invitrogen. HUVECs were transfected with siRNA against them or Stealth RNAi-negative control (Invitrogen) using Targetfect-siRNA transfection kit (Targeting Systems, Santee, CA) according to the manufacturer’s instructions. To be brief, 1 ml of Opti-MEM (Invitrogen) containing 100 pmol of Stealth RNAi duplex and transfection solution was incubated in a 35-mm dish at 37 °C for 2 h, followed by addition of growth media. 12 h later, media were refreshed with growth media, and after a further 36-h incubation, apoptotic study and signal transduction analysis were performed. The specific gene silencing was confirmed by Western blotting by using antibodies against Akt (New England Biolabs, Beverly, MA), JNK1, SEKI (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma).

**Induction and Quantitative Determination of Apoptosis**—PRECs were plated onto 24-well plates and incubated for 18 h with varying concentrations of H2O2 in the presence or absence of Ang1 pretreatment or of adenoviruses encoding DN-JNK or GFP, as indicated in the text. HUVECs transfected with siRNA against JNK or SEKI or control siRNA were treated with 0.75 mM H2O2. Floating cells were collected by two PBS washes, and adherent cells were harvested by trypsinization. Trypan blue number and size distribution of floating and adherent cells were determined using a fluorescence microscope (Nikon Eclipse TE300, Tokyo, Japan). More than 95% of floating cells were deemed to be apoptotic, as confirmed by TdT-mediated dUTP nick-end labeling (TUNEL) assays using an in situ cell death detection kit (Roche Applied Science) according to the manufacturer’s instructions. To detect apoptosis in adherent cells, parallel wells were washed with PBS, fixed for 15 min with 4% paraformaldehyde, and subjected to the TUNEL assay. Apoptotic cells were subjected to three independent blind counts by investigators in five different locations. The percentage of apoptotic cells was based on the sum of the floating cells plus the apoptotic adherent cells in a given cell population.

**Caspase-3 Activity**—PRECs were treated for 6 h with H2O2 in the presence or absence of either Ang1 or of adenoviruses encoding DN-JNK or GFP, as indicated in the text. To assay for caspase-3 activity, floating and adherent cells were harvested by trypsinization, followed by neutralization in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and further washing with PBS. The cells were then lysed, and the lysates were used in colorimetric assays for caspase-3 activity (ApoAlert caspase-3 colorimetric assay kit; Clontech), according to the manufacturer’s instructions.

**Immunoblot Analysis**—PRECs were washed with cold PBS and lysed in 1× Laemmli buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol) containing protease inhibitors (10 mM sodium pyrophosphate, 100 mM NaF, 1 mM Na3VO4, 1 μM aprotinin, 1 μg/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride). Protein concentrations were determined using a Bio-Rad protein assay. Total cell lysate (30 μg) was subjected to SDS-PAGE under reducing conditions, and proteins were transferred to nitrocellulose membranes (Bio-Rad). The blots were incubated with primary antibodies followed by incubation with horse-radish peroxidase-conjugated secondary antibody (Amersham Biosciences). Visualization was performed using the enhanced chemiluminescence detection system (ECL, Amersham Biosciences) according to the manufacturer’s instructions.

**Immunoprecipitation**—Cells were washed with cold PBS and lysed in 1× Laemmli buffer containing protease inhibitors as described above. Cell lysates were heated to 95 °C for 2 min, and equal volumes of lysates were subjected to SDS-PAGE under reducing conditions. The blots were incubated with anti-phospho-specific JNK, anti-phospho-specific SEKI (Cell Signaling Technology, Beverly, MA), or anti-phospho-specific Akt antibodies (New England Biolabs). Lane loading differences were normalized by reblotting with nonphosphorylation-specific (total) anti-JNK1, anti-SEKI1 (total) antibody, or anti-Akt (total) antibody.

**Immunoprecipitation**—Cells were washed three times with cold PBS and solubilized in 200 μl of lysis buffer (1% Triton X-100, 50 mM HEPES, 10 mM EDTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM sodium orthovanadate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride). After centrifugation at 12,000 rpm for 10 min, 1.0 mg of protein was subjected to immunoprecipitation. To clear protein extracts, protein A-Sepharose (20 μl of a 50% suspension) was added to the cell lysates, after which they were incubated for 1 h, followed by centrifugation and collection of the supernatant. A rabbit anti-Tie2-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was then added followed by incubation at 4 °C for 2 h with rocking. 20 μl of protein A-Sepharose was then added, and the sample was incubated for another 2 h at 4 °C with rocking. To denature proteins, protein A-Sepharose antigen-antibody conjugates were separated by centrifugation, washed five times, and boiled for 3 min in Laemmli sample buffer. Immunoblotting was performed with anti-mouse monoclonal anti-phosphotyrosine antibody (4G10) (Upstate Biotechnology, Inc., Lake Placid, NY).
RESULTS

H$_2$O$_2$ Promotes Apoptosis in Vascular Endothelial Cells through JNK, and Ang1 Inhibits the Apoptosis—To determine whether H$_2$O$_2$ promotes apoptosis in PRECs, the cells were treated for 18 h with various concentrations of H$_2$O$_2$ (Fig. 1A), and apoptotic cells were then analyzed by a TUNEL assay. The results indicate that H$_2$O$_2$ increases the number of apoptotic PRECs in a dose-dependent manner, from 14.8 ± 1.1% of cells under control conditions to 36.4 ± 4.5% of cells treated with 1.0 mM H$_2$O$_2$ (Fig. 1A). We then investigated whether Ang1 would attenuate H$_2$O$_2$-induced apoptosis, and PRECs were therefore treated with 0.75 mM H$_2$O$_2$ in the presence or absence of the indicated doses of Ang1 (Fig. 1, B and C). Ang1 treatment was found to reduce the number of apoptotic cells in a dose-dependent manner, with a maximum decrease of 45% detected at a dose of 150 ng/ml (p < 0.001) (Fig. 1, B and C).

Because several publications reported that H$_2$O$_2$ increases JNK activation and concomitant apoptosis in endothelial cells (3, 8), we performed the experiments using adenoviral vectors encoding either dominant-negative (DN)-JNK or green fluorescent protein (GFP). PRECs were treated with 0.75 mM H$_2$O$_2$ for 15 h, which resulted in an increase in the number of apoptotic cells to 30.2 ± 3.2% (p < 0.001) (Fig. 1D). This apoptotic response was inhibited by DN-JNK to a lower level of 18.7 ± 3.3% (p < 0.001) (Fig. 1D), indicating that H$_2$O$_2$ induces apoptosis in PRECs through JNK activation. For further confirmation, we studied the apoptotic pathway by using gene silencing by siRNA. HUVECs were transfected with siRNA against JNK and SEK1, or scramble siRNA (ctl), were incubated with 0.75 mM H$_2$O$_2$ for 12 h, and TUNEL assay was performed. F, 48 h after transfection, HUVECs were harvested, and the obtained total cell lysates were subjected to Western blotting analysis.
results shown above indicated that H$_2$O$_2$ increases endothelial cell apoptosis and caspase-3 activity through JNK and that these were attenuated by Ang1. These findings prompted us to extend the mechanisms underlying the anti-apoptotic effects of Ang1 and to examine the signal transduction pathways involved. To determine whether the anti-apoptotic effect of Ang1 is indeed mediated through JNK, we performed experiments with preadipocytes (PRECs) treated with H$_2$O$_2$, and the activity levels of caspase-3 were determined.

Ang1 Attenuates H$_2$O$_2$-induced JNK Phosphorylation—The results shown above indicated that H$_2$O$_2$ increases endothelial cell apoptosis and caspase-3 activity through JNK and that these were attenuated by Ang1. These findings prompted us to study further the mechanisms underlying the anti-apoptotic effects of Ang1 and to examine the signal transduction pathways involved. To determine whether the anti-apoptotic effect of Ang1 is indeed mediated through JNK, Western blot analysis with phospho-JNK-specific antibodies was performed, and these data confirmed that H$_2$O$_2$ increases JNK phosphorylation in both a time- and dose-dependent manner (Fig. 3A and B). PRECs were treated with 0.25 mM H$_2$O$_2$ for the indicated times (Fig. 3A), and an increase in phosphorylation of JNK was found to be time-dependent, with a maximal 3.4-fold increase at 60 min. Additionally, PRECs were stimulated with the indicated concentrations of H$_2$O$_2$ for 60 min, and H$_2$O$_2$ was also shown to increase the phosphorylation of JNK in a dose-dependent manner (Fig. 3B).

Based on these results, we then examined the effects of Ang1 on H$_2$O$_2$-induced JNK phosphorylation. PRECs were treated with 0.25 mM H$_2$O$_2$ for 60 min in the presence or absence of 100 ng/ml Ang1 pretreatment. JNK phosphorylation, induced by H$_2$O$_2$, was found to be 3.4 ± 0.4-fold greater than the control (p < 0.001), but pretreatment with Ang1 resulted in a marked inhibition of H$_2$O$_2$-induced JNK phosphorylation (p < 0.001), without altering basal JNK phosphorylation (Fig. 3C).

Ang1 Phosphorylates Tie2 and Akt through PI 3-Kinase—To investigate the signal transduction pathways through which Ang1 inhibits JNK phosphorylation, we analyzed the downstream signaling molecules of the Tie2 receptor. Previous studies have reported that Ang1 stimulation exerts its effects through intracellular signaling via Tie2 autophosphorylation, and to confirm this, we tested whether Tie2 phosphorylation is increased by Ang1. PRECs were treated with 100 ng/ml Ang1 for the indicated incubation times (Fig. 4A), and immunoprecipitations were performed with anti-Tie2 antibodies. Subsequent immunoblotting analysis with anti-phosphotyrosine antibodies indeed showed an increase in Tie2 phosphorylation by Ang1, which was time-dependent (Fig. 4A). Among the signal transduction molecules known to be recruited and activated by Tie2 (21, 34, 35), we further investigated those associated with the PI 3-kinase/Akt pathway. We determined that Akt phosphorylation induced by Ang1 was increased 6.6-fold (p < 0.001), whereas LY294002 and wortmannin significantly decreased Akt phosphorylation, by 100 ng/ml Ang1, in a time- and dose-dependent manner (data not shown). Additionally, the effects of the PI 3-kinase inhibitors LY294002 (50 µM) and wortmannin (100 µM) on Akt phosphorylation induced by Ang1 was analyzed. Induction of Akt phosphorylation by Ang1 was increased 6.6 ± 0.7-fold (p < 0.001), whereas LY294002 and wortmannin significantly decreased Akt phosphorylation to 0.90 ± 0.37-fold (p < 0.001) and 0.74 ± 0.19-fold (p < 0.001), respectively (Fig. 4B). This result suggests that Ang1 induces Akt phosphorylation through PI 3-kinase.
The Inhibitory Effects of Ang1 on JNK Phosphorylation Are Mediated via the PI 3-Kinase/Akt Pathway—Because our findings indicated that Ang1 attenuates H2O2-induced JNK phosphorylation and activates the PI 3-kinase/Akt pathway, we investigated whether suppression of JNK phosphorylation by Ang1 is also mediated through this pathway. Western blotting analysis using inhibitors of PI 3-kinase revealed that Ang1 suppresses H2O2-induced JNK phosphorylation to almost basal levels, whereas in the presence of LY294002 and wortmannin (Wo), phospho-Akt and Akt were detected by Western blot analysis. A representative Western blot is shown (top panel) as is quantitation of multiple experiments (bottom panel). Data are represented by the mean ± S.D.

FIG. 4. Ang1 phosphorylates Tie2 and Akt through PI 3-kinase. A, PRECs were treated with 100 ng/ml Ang1 for the indicated times. Proteins immunoprecipitated (IP) with Tie2 antibodies were resolved by SDS-PAGE, and immunoblots (IB) were performed with antibodies specific for either phosphotyrosine (PY) or Tie2. Each experiment was repeated twice with similar results. B, PRECs were treated with 0.25 mM H2O2, 100 ng/ml Ang1 and PI 3-kinase inhibitors, 50 μM LY294002 (LY), or 100 μM wortmannin (Wo). Phospho-Akt and Akt were detected by Western blot analysis. A representative Western blot is shown (top panel) as is quantitation of multiple experiments (bottom panel). Data are represented by the mean ± S.D.

FIG. 5. Both inhibitors of PI 3-kinase and expression of DN-Akt reverse the inhibitory effects of Ang1. A, PRECs were treated with 0.25 mM H2O2 and 100 ng/ml Ang1 in the presence or absence of the PI 3-kinase inhibitors, 50 μM LY294002 (LY), or 100 μM wortmannin (Wo). Phospho-JNK1 and JNK1 were detected by Western blot analysis. B, PRECs transfected with adenoviruses encoding DN-Akt (DN), CA-Akt (CA), or a β-galactosidase control (ctl) were treated with 0.25 mM H2O2 in the presence or absence of 100 ng/ml Ang1, and Western blot analysis was performed. Representative Western blots are shown (top panels) as is quantitation of multiple experiments (bottom panels). Data are represented by the mean ± S.D.
inhibit H₂O₂-induced JNK phosphorylation and to demonstrate that Ang1 attenuates H₂O₂-induced JNK phosphorylation through the PI 3-kinase/Akt pathway.

Angiopoietin-1 Attenuates H₂O₂- and TNF-induced SEK1 Phosphorylation at Thr²⁶¹ through the PI 3-kinase/Akt Pathway—Our findings in these studies further prompted us to investigate whether H₂O₂-induced phosphorylation of SEK1, an upstream regulator of JNK, is also attenuated by Ang1. SEK1 has several phosphorylation sites, and its activation occurs primarily through phosphorylation of serine and threonine residues at positions 257 and 261, respectively (36). To confirm the effects of H₂O₂ on SEK1 phosphorylation at Thr²⁶¹, PRECs were treated with 0.25 mM H₂O₂ over the indicated time course, followed by Western blot analysis with phospho-SEK1 (Thr²⁶¹) antibodies (Fig. 6A). H₂O₂ increased SEK1 phosphorylation in a time-dependent manner, with a maximal increase measured at 20 min (Fig. 6A). Based upon this result, the effects of Ang1 on H₂O₂-induced SEK1 phosphorylation were determined by treatment of PRECs with 0.25 mM H₂O₂ for 20 min following pretreatment of these cells with 100 ng/ml Ang1. H₂O₂ increased SEK1 phosphorylation by 2.9 ± 0.1-fold compared with control cells (p < 0.001), whereas pretreatment of PRECs with Ang1 resulted in a significant reduction in SEK1 phosphorylation (1.2 ± 0.2-fold, p < 0.001) (Fig. 6B).

This result led us to speculate whether the inhibitory effects of Ang1 on H₂O₂-induced SEK1 phosphorylation at Thr²⁶¹ are mediated through the PI 3-kinase/Akt pathway, as in the case of JNK phosphorylation. To determine this, experiments with the inhibitors of PI 3-kinase, LY294002 and wortmannin, were again undertaken, and it was found that these inhibitors significantly reversed the effects of Ang1 on H₂O₂-induced SEK1 phosphorylation at Thr²⁶¹, which measured 3.4 ± 0.6-fold (p < 0.001) and 3.1 ± 0.5-fold (p < 0.001) over control levels, respectively (Fig. 7A). This indicated that Ang1 does inhibit H₂O₂-induced
SEK1 phosphorylation through PI 3-kinase, and we therefore decided to test whether this inhibition is also mediated through Akt, the results of which are shown in Fig. 7B. In PRECs transfected with control vector, H$_2$O$_2$ induced SEK1 phosphorylation at Thr$^{380}$ by 3.2 ± 0.4-fold, which was attenuated by Ang1 (1.2 ± 0.4-fold compared with the control). DN-Akt reversed the effects of Ang1 significantly (3.0 ± 0.3-fold, p < 0.001), whereas CA-Akt expression, in the absence of Ang1, caused significant reductions in H$_2$O$_2$-induced SEK1 phosphorylation, compared with the control vector (0.97 ± 0.31-fold, p < 0.001) (Fig. 7B). These results demonstrate that the attenuation of H$_2$O$_2$-induced SEK1 phosphorylation by Ang1 is primarily transduced via Akt and indicate that Ang1 attenuates H$_2$O$_2$-induced SEK1/JNK phosphorylation through the PI 3-kinase/Akt pathway.

Because TNFα has been shown to induce SEK1/JNK phosphorylation (37), we investigated the effects of Ang1 on TNFα-induced SEK1 phosphorylation to confirm whether the inhibitory effects of Ang1 were specific or not. TNFα (10 ng/ml) was found to increase SEK1 phosphorylation at 20 min, which was attenuated by pretreatment with Ang1 (Fig. 7C). The inhibitors of PI 3-kinase, LY294002 and wortmannin (Fig. 7C), and DN-Akt reversed this inhibitory effect, whereas CA-Akt inhibited SEK1 phosphorylation without Ang1 treatment (Fig. 7D), as in the case of H$_2$O$_2$. These results indicate that Ang1 also inhibits TNFα-induced SEK1 phosphorylation through the PI 3-kinase/Akt pathway.

Ang1 Induces Phosphorylation of SEK1 at Ser$^{380}$ via the PI 3-Kinase/Akt Pathway—Recent reports have shown that Akt suppresses JNK through several pathways, and SEK1 has been shown to be phosphorylated at Ser$^{380}$ by Akt, resulting in its inactivation (38). We thus investigated whether Ang1 increases SEK1 phosphorylation at Ser$^{380}$ through the PI 3-kinase/Akt pathway. First, experiments with the LY294002 and wortmannin were performed and showed that SEK1 phosphorylation at Ser$^{380}$ was slightly increased by treatment with 0.25 mM H$_2$O$_2$ for 20 min (p = 0.49), whereas in the presence of pretreatment with 100 ng/ml Ang1 caused SEK1 phosphorylation to be significantly enhanced to 4.6 ± 0.4-fold (p < 0.001) (Fig. 8A). LY294002 and wortmannin significantly decreased SEK1 phosphorylation by both Ang1 and H$_2$O$_2$ to 1.5 ± 0.5-fold (p < 0.001) and 1.5 ± 0.5-fold (p < 0.001) levels, respectively (Fig. 8A). This result indicates that Ang1 induces SEK1 phosphorylation at Ser$^{380}$ through the PI 3-kinase/Akt pathway, and we next analyzed whether this phosphorylation occurs via activation of Akt. In PRECs transfected with control vector, treatment by both Ang1 and H$_2$O$_2$ induced SEK1 phosphorylation by 4.3 ± 0.5-fold (p < 0.001), which was significantly decreased by DN-Akt (1.7 ± 0.4-fold, p < 0.001) (Fig. 8B). Furthermore, in PRECs transfected with CA-Akt, SEK1 phosphorylation was induced significantly (5.2 ± 0.7-fold, p < 0.001) (Fig. 8B). Additional experiments using gene silencing by siRNA showed that in HUVECs transfected with control RNA duplex, SEK1 phosphorylation was increased by treatment with both Ang1 and H$_2$O$_2$ (4.4 ± 0.4-fold, p < 0.001), which was

**Fig. 8. Ang1 induces SEK1 phosphorylation at Ser$^{380}$ through the PI 3-kinase/Akt pathway.** A, PRECs were treated with Ang1 and H$_2$O$_2$, either with or without inhibitors of PI 3-kinase, LY294002 (LY), or wortmannin (Wo), and SEK1 phosphorylation at Ser$^{380}$ (pS80) was quantified by phospho-specific antibodies. B, PRECs transfected with adenoviruses encoding DN-Akt (DN), CA-Akt (CA), or a β-galactosidase control (ctl) were treated with Ang1 and H$_2$O$_2$. C, HUVECs were transfected with siRNA against Akt1, Akt2, both Akt1 and Akt2 (Akt1/2), or scramble siRNA (ctl). 48 h after transfection, the treatment with Ang1 and H$_2$O$_2$ was performed. Representative Western blots of SEK1 phosphorylation at Ser80 (pS80) are shown (top panels) and quantitation of multiple experiments of phospho-Ser80 (pS80) and total SEK1, total Akt, and β-actin are shown (bottom panels).
reversed by the gene silencing against both Akt1 and Akt2 (1.3 ± 0.3-fold, p < 0.001) (Fig. 8C). The knockdown of Akt1 reduced the SEK1 phosphorylation significantly (p < 0.001), whereas gene silencing of Akt2 decreased the phosphorylation slightly but not significantly (p = 0.053) (Fig. 8C). These results indicated that Ang1 induced SEK1 phosphorylation at Ser257 through the PI 3-kinase/Akt pathway and suggest that SEK1 phosphorylation at Ser261 and inactivation by Ang1 could be one of the potent pathways through which Ang1 suppresses H2O2-induced JNK phosphorylation.

**DISCUSSION**

In this study, we investigated the effect of Ang1 on H2O2-induced signal transduction pathways and the resulting apoptotic response, because oxidative stress in vascular endothelial cells plays an important role in the pathogenesis of both diabetes and atherosclerosis, whereas the prosurvival effects of Ang1 on oxidative damage have not been elucidated. First, we confirmed that in PRECs oxidative stress increases the number of apoptotic cells, which is consistent with previous findings (3). We then showed that Ang1 decreases the apoptotic cell number following oxidative stress, as is the case for other cellular stress inducers (25). Because caspase-3 is one of the key effectors of apoptosis and induces the proteolytic cleavage and activation of many apoptotic proteins, we elucidated that Ang1 also decreases the caspase-3 response to oxidative stress.

Oxidative stress activates various kinds of apoptotic signaling pathways, among which we focused particularly on JNK, as a number of recent reports have shown that JNK activation, following oxidative stress, induces apoptosis via activation of c-Jun (3), via the phosphorylation and inactivation of the myeloid cell leukemia (Mcl)-1 protein (39) in vascular endothelial cells, and through phosphorylation of Bcl-2 and Bax in other cell types. We performed experiments using recombinant adenosviruses or gene silencing by siRNA and showed that the increases in TUNEL-positive cells and caspase-3 activity levels, induced by H2O2, are mediated by JNK activation. Several apoptotic downstream effectors of JNK have now been reported, such as c-Jun and Bcl-2, and it remains to be determined which effectors are the principal mediators between JNK and caspase-3 activation in our experiments.

Based on these results, we further examined the signal transduction pathways that are activated by Ang1 and inhibit apoptosis via JNK-mediated pathways, and we demonstrated that Ang1 attenuates H2O2-induced JNK phosphorylation. Several studies have reported previously that Ang1 increases the phosphorylation of extracellular signal-regulated kinases 1/2 and p38 MAPK (40), but this is the first report to show that Ang1 inhibits H2O2-induced JNK phosphorylation. We investigated the signal transduction pathway through which Ang1 inhibits JNK phosphorylation, and we predicted that the most probable candidate was the PI 3-kinase/Akt pathway, as Akt is a potent survival factor and its activation inhibits JNK phosphorylation in other cell types (38, 41–43). By using inhibitors of PI 3-kinase and DN-Akt and CA-Akt constructs, we demonstrated that Ang1 indeed inhibits JNK phosphorylation via PI 3-kinase and that Akt activation by Ang1 is both necessary and sufficient for Ang1 to attenuate JNK phosphorylation. These data are the first to show that Akt activation by Ang1 suppresses JNK phosphorylation in vascular endothelial cells.

Our findings prompted us to further analyze through which pathways Akt activation by Ang1 inhibits JNK phosphorylation. Because SEK1 is an upstream regulator that is phosphorylated at both Ser257 and Thr261 and activated by various cellular stress signals (36, 44), we investigated the effects of Ang1 on SEK1 phosphorylation at Thr261. H2O2-induced phosphorylation of SEK1 at Thr261 was found to be abrogated by Ang1, mainly via the PI 3-kinase/Akt pathway, as in the case of JNK. Furthermore, because TNFα is thought to play an important role in diabetic complications and atherosclerosis (45) and also induces SEK1/JNK phosphorylation (37), we examined and demonstrated that TNFα-induced SEK1 phosphorylation at Thr261 was also inhibited by Ang1-dependent activation of the PI 3-kinase/Akt pathway. These results indicate that both H2O2 and TNFα can activate the same signal transduction pathways, which are attenuated by Ang1, and that the upstream regulators that are common to these pathways might be candidates as direct substrates of Akt. Akt has been reported to inhibit JNK phosphorylation via several pathways, an example of which is its phosphorylation and inhibition of apoptosis signal-regulating kinase 1 (a member of the MAPKKKs) at Ser257, followed by the inhibition of JNK phosphorylation (41). Ang1 has also been shown to interact with JIP1, a JNK-associated scaffolding protein, and prevent JNK activation in neurons (42). Mixed lineage kinase3 (MLK3), the MAPKKK of JNK, is also inhibited by Akt and down-regulates JNK activation (43). These molecules are therefore good candidates as upstream regulators of SEK1/JNK activation and as direct substrates of Akt, and further studies will be needed to determine the intracellular molecules that are directly phosphorylated and inactivated by Akt in PRECs.

Because SEK1 inactivation occurs via phosphorylation at Ser261 by Akt, which leads to the suppression of JNK phosphorylation (38), we further examined the effects of Ang1 on SEK1 phosphorylation. We found that Ang1 also phosphorylates SEK1 at Ser261 through the activation of the PI 3-kinase/Akt pathway, and the specific knockdown experiments using siRNA showed that the phosphorylation is mediated mainly through Akt1 rather than Akt2 in vascular endothelial cells. These results suggest one more pathway through which Ang1 suppresses H2O2-induced JNK phosphorylation.

In conclusion, we have further elucidated the intracellular mechanism underlying the prosurvival effects of Ang1 upon endothelial cell apoptosis, induced by oxidative stress. These data are the first to demonstrate that Ang1 attenuates both JNK phosphorylation and the apoptotic response of PRECs to oxidative stress, induced by H2O2. This is also the first report to show that Ang1 inhibits SEK1/JNK phosphorylation via PI 3-kinase/Akt activation. These findings suggest that Ang1 may have therapeutic benefits by ameliorating the well-documented exacerbation of retinal vascular injury by oxidative stress in various vascular diseases, including diabetic retinopathy.

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