Signaling and regulation of endothelial cell survival by angiopoietin-2

Rania Harfouche and Sabah N. A. Hussain

Critical Care and Respiratory Divisions, McGill University Health Centre, Meakins-Christie Laboratories, McGill University, Montreal, Quebec, Canada

Submitted 14 December 2005; accepted in final form 10 May 2006

Harfouche, Rania, and Sabah N. A. Hussain. Signaling and regulation of endothelial cell survival by angiopoietin-2. Am J Physiol Heart Circ Physiol 291: H1635–H1645, 2006. First published May 19, 2006; doi:10.1152/ajpheart.01318.2005.—Angiopoietins are ligands for endothelial cell-specific Tie-2 receptors. Whereas angiopoietin-1 (Ang-1) activates these receptors and promotes cell survival, migration, and sprouting, little information is available regarding how Ang-2 influences these cells. In this study, we evaluated signaling pathways and biological effects of physiological concentrations of Ang-2 in cultured human umbilical vein endothelial cells. Ang-2 at 150 and 300 ng/ml elicited a transient (reaching peak values within 15 min of exposure) increase in the phosphorylation of Tie-2 receptors, protein kinase B (Akt), ERK1/2, and p38 members of the mitogen-activated protein kinases. However, unlike Ang-1, Ang-2 significantly inhibited JNK/SAPK phosphorylation. When vascular endothelial growth factor (VEGF) was present along with Ang-2, ERK1/2 phosphorylation was inhibited, whereas augmentation of Ang-1-induced ERK1/2 phosphorylation was triggered by VEGF. Ang-2 treatment had no effect on cell migration and in vitro wound healing but significantly attenuated serum deprivation-induced apoptosis and promoted survival. These effects were completely reversed by phosphatidylinositol 3 (PI3)-kinase and ERK1/2 inhibitors but were augmented by an inhibitor of the p38 pathway. These results suggest that Ang-2 promotes endothelial cell survival through the ERK1/2 and PI3-kinase pathways and that this angiopoietin is not a strong promoter of endothelial cell migration. We also conclude that the nature of interactions in terms of ERK1/2 activation between Ang-2 and VEGF is different from that of Ang-1 and VEGF.

ANGIPOIETINS (Ang-1, -2, -3, and -4) comprise a family of novel angiogenic modulators that all bind endothelial cell (EC)-specific Tie-2 receptors with equal affinity (5, 31, 38, 43). Several studies have revealed that Ang-1 and Ang-2 can be expressed in a variety of nonvascular and vascular cells, including ECs in vitro and endocardium in vivo (6, 9, 40, 45), whereas the cellular origins of Ang-3 and Ang-4 remain to be determined. Ligand-binding experiments revealed that Ang-1 and Ang-2 bind Tie-2 receptors with a similar affinity; however, only Ang-1 evokes strong autophosphorylation of Tie-2 receptors in ECs (31). Many studies have confirmed that the Ang-1/Tie-2 pathway plays important roles in embryonic development, physiological angiogenesis, and maintenance of vascular integrity, inflammation, and tumor growth (18, 38, 46). In mature vasculature or cultured ECs, Ang-1 inhibits apoptosis and promotes differentiation, sprouting, and migration (12, 21, 34, 35, 45). In addition, Ang-1 mobilizes endothelial cell adhesion molecule-1 to paracellular junctions, inhibits phosphorylation of vascular endothelial cadherin, and tightens EC junctions (10). Moreover, Ang-1 attenuates TNF-α- and VEGF-induced adhesion molecule expression, reduces leukocyte adhesion to ECs (10, 23), and lowers TNF-induced tissue factor expression (24).

The exact pathways through which Ang-1 exerts these effects remain under investigation. It has, however, been well established that, on ligation of Ang-1, Tie-2 receptors dimerize, autophosphorylate specific tyrosine residues, and recruit the adaptor proteins Grb2 and Grb14, the p85 subunit of the PI3-kinase protein complex, and a novel docking molecule, Dok-R (17–19, 26). As a result of these signaling events, both the protein kinase B/Akt pathway (an important antiapoptotic pathway) and the p21-activated protein kinase (PAK, a major promoter of cell migration) are activated and mediate effects such as inhibition of apoptosis and promotion of migration. Finally, our group has recently reported that the ERK1/ERK2 and p38 members of MAPKs are also activated by Ang-1 in ECs and that, whereas the ERK1/ERK2 pathway promotes EC survival, activation of the p38 pathway leads to apoptosis (13).

Unlike a general agreement about the influence of Ang-1 on EC biology, there are conflicting results regarding the physiological roles of Ang-2 in angiogenesis during embryonic developments and in mature vasculature. For instance, early animal experiments in which Ang-2 was overexpressed in mice using endothelial Tie-2 promoter revealed phenotypical changes similar to those found in Ang-1−/− and Tie-2−/− mice, including embryonic lethality and massive apoptosis of ECs (31). These observations led many investigators to conclude that Ang-2, by blocking Ang-1-induced Tie-2 phosphorylation during embryonic vascular development, causes EC apoptosis and poor angiogenesis and vasculogenesis. However, more recent observations suggest that Ang-2 may, under certain circumstances, activate Tie-2 receptors and promote EC survival. Indeed, Teichert-Kuliszewska et al. (41) have reported that 24-h pretreatment with Ang-2, followed by brief reexposure, produced Tie-2 phosphorylation in ECs comparable to that produced by Ang-1 and that Ang-2 is capable of inducing strong EC differentiation similar to that elicited by Ang-1. These results suggest that Ang-2 may promote EC survival and angiogenesis by itself independently of its antagonistic activity of Ang-1-induced Tie-2 phosphorylation (41). This notion is supported by the finding that genetic deletion of Ang-2 results in major defects in lymphatic development and angiogenic remodeling (9) and by the fact that systemic overexpression of Ang-2 interacts in a positive manner, just like Ang-1, in promoting TNF-α-induced corneal angiogenesis, indicating that Ang-2 may promote EC survival (4). Despite these recent observations confirming the importance of Ang-2...
as possible endogenous agonist for Tie-2 receptors, little is known about the signaling pathways triggered by physiological concentrations of Ang-2, including those activated by Ang-1, such as members of the MAPKs, Akt, and PAK-1. Also not known is whether physiological concentrations (≤300 ng/ml) of Ang-2 promote EC migration, apoptosis, and wound healing and whether Ang-2 interacts with other important angiogenesis factors, such as VEGF, in a fashion similar to Ang-1. Accordingly, we evaluated in this study the following aspects of Ang-2 biology in ECs at concentrations between 50 and 300 ng/ml: 1) modulation of Tie-2 receptor phosphorylation; 2) activation of various members of MAPKs and Akt; 3) the interactions between Ang-2 and VEGF in terms of signaling; and 4) modulation of EC survival, apoptosis, migration, and wound healing by Ang-2 and the roles of MAPKs and Akt in these biological effects.

**MATERIALS AND METHODS**

**Materials.** Polyclonal or monoclonal antibodies specific for cleaved caspase-3, -9,-8, Akt, and phospho-Akt (Ser473) as well as the total and phosphorylated forms of ERK1/2 and p38 were obtained from New England Biolabs (Beverly, MA). Propidium iodide and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). Ang-2 protein was purchased from R & D Systems (Minneapolis, MN) and was also a gift from G. Yancopoulos (Regeneron, Tarrytown, NY). Wortmannin (selective inhibitor of PI3-kinase) was purchased from Sigma, and 300 ng/ml Ang-2 (300 ng/ml). Cells were lysed as described above, and Tie-2 receptors were immunoprecipitated with a monoclonal antibody (Calbiochem) as described previously (31). The immunocomplexes were then separated on SDS-PAGE and immunoblotted with an anti-phosphotyrosine antibody (4G10, Upstate Biotechnology) and a polyclonal anti-Tie-2 antibody (Santa Cruz Biotechnology). To evaluate whether Ang-2 at 300 ng/ml is capable of inducing Tie-2 receptor tyrosine phosphorylation in other types of ECs, we used an identical approach to the one described above in mouse endothelium cell line, bEnd.3.

**Role of JNK/SAPK pathway.** To assess the influence of JNK/SAPK pathways on Ang-2-induced ERK1/2 and p38 phosphorylation, we used HUVECs that were stably transduced with a dominant negative JNK/SAPK (HUVEC-MSCV-JNK-APF) or mock (HUVEC-MSCV) retroviruses as previously described (20, 33). HUVECs were transduced with a dominant negative JNK/SAPK in which the phosphorylation site Thr-Pro-Tyr is mutated to Ala-Pro-Phe (JNK–APF). HUVEC-MSCV and HUVEC-MSCV-JNK-APF were constructed by retroviral transduction using MSCV- pac (empty vector) or MSCV-pac containing JNK-APF cDNA, respectively. HUVECs were transduced as previously described, except for few modifications indicated hereafter. Transient transfections of the Amphi Phoenix packaging cell line were performed using Fugene 6 transfection reagent (Boehringer Mannheim). Viral supernatants from transfected packaging cell lines were used to transduce the ECs in multiple rounds of infection. HUVECs were selected in puromycin. Expression of JNK–APF was confirmed by immunoblotting using an antibody for total JNK (Santa Cruz). These cells were maintained in the same conditions as HUVECs, except penicillin/streptomycin was also added to the media. Cells were serum deprived overnight and then stimulated with 300 ng/ml Ang-2 for 15 min. Cells were then lysed, and proteins then underwent immunoblotting and were probed with phospho- and total ERK1/2 and p38 antibodies as described above.

**MTT viability assay.** Subconfluent HUVECs in 24-well plates were incubated in serum deprivation medium supplemented with d-glutamine in the presence or absence of Ang-2 (300 ng/ml) for 6, 12, 18, and 24 h. Viable cells were then quantified with the tetrazolium salt MTT. MTT is reduced by mitochondrial dehydrogenases of live cells into purple formazan crystals that, once solubilized, can be read spectrophotometrically. Briefly, the cells were washed with PBS and incubated with 1 mg/ml of MTT for 4 h at 37°C, and the formazan crystals were solubilized with isopropanol. Absorbance (corresponds to the proportion of live cells) was then measured at 550 nm with a background subtraction of 660 nm in plate reader (Packard/Canberra, Meriden, CT).
Apoptosis detection. We used two protocols to evaluate the level of EC apoptosis, as described previously (14). In the first protocol, we used DNA profiling with propidium iodide (a DNA intercalative binding dye). Subconfluent cells were washed twice with PBS and then serum starved for 6 or 24 h in the absence and presence of Ang-2 (300 ng/ml). Selected plates were pretreated for 1 h with 10 μM SB-203580, 30 μM PD-9805, 10 μM UO-126, and 100 nM wortmannin before Ang-2 treatment. Floating cells were collected, the plates were washed twice with PBS, and the adherent cells were trypsinized, incubated at 37°C for 3 min, harvested, and fixed in ice-cold ethanol (70%) at 20°C for at least 1 h. The cell pellets were stained with 50 μg/ml propidium iodide (Sigma) at 4°C overnight. The DNA content was analyzed with an EPICS 750 series flow cytometer (Coulter, Hialeah, FL) from 50,000 events (cells) per group, using an argon excitation wavelength of 280 nm. The fluorescence was detected using the FL3 emission parameter. The results were analyzed using FCS EXPRESS software (Thornhill, ON, Canada), with the apoptotic cell population expressed as a percentage of the total events. In the second protocol, we monitored activation of caspase-3, -9, and -8 using immunoblotting with specific antibodies for the cleaved forms of these caspases as described previously (14).

Wound healing assay. HUVEC monolayers were maintained in a 24-well plate and were mechanically wounded with a sterile 200-μl pipette tip. Cells were then washed in basal media to remove debris. The wounded cells were maintained in basal media containing 2% FBS and stimulated for 12 h with 300 ng/ml of Ang-1, Ang-2, or BSA solvent (control samples). Wound healing was visualized with an inverted brightfield microscope and ×4 objective and was quantified by measuring the wounded area (Image Pro-Plus software) and reported as percentage wound healing with the following equation: % wound healing = [1 − (wound area at t12/6 wound area at t0)] × 100, where t0 is the time immediately after wounding.

Migration assays. Migration was assessed using a modified Boyden chamber. Transwell polycarbonate inserts (8.0 μm; Corning Costar, Mississauga, ON, Canada) were placed in a 24-well plate and coated for a minimum of 2 h with 50 μg/ml fibronectin, followed by 0.1% gelatin (Sigma-Aldrich, Oakville, ON, Canada). HUVECs (10^5 cells) were then seeded on the inserts (apical side) and Ang-1 or Ang-2 (300 ng/ml) or 0.1% BSA solvent (vehicle) was added in the lower chamber (control experiment), and the cells were allowed to migrate for 5 h. At the end of the migration period, the upper side of the insert was subsequently scraped with cotton swabs to remove nonmigrating cells. Migrating cells were fixed in 10% neutral buffered formalin, stained with Giemsa for 30 min, and visualized under an inverted phase-contrast microscope (Olympus IX70). Migration was quantitated using Image Pro-Plus software by counting cells in five random fields (×100) per well, and the mean number of migrated cells in response to Ang-1 or Ang-2 was expressed as percentage of control (BSA).

Data analysis. Six independent apoptosis or viability measurements were made within each group for migration, wound healing, MT T, and propidium iodide assays. For quantification of Tie-2 receptor, Akt, and MAPK phosphorylation, as well as caspase activation, three independent measurements were performed within each group. Differences in the number of apoptotic cells and differences in the optical densities between various groups were compared using two-way analysis of variance. A probability (P) value of <0.05 was considered significant.

RESULTS

Tie-2, Akt, and MAPK phosphorylation. Exposure of HUVECs to 50 to 300 ng/ml of Ang-2 elicited a dose-dependent increase in Tie-2 receptor tyrosine phosphorylation, as detected with an antibody selective to phosphorylated Tyr1101 residue of human Tie-2 receptor (Fig. 1A). Ang-2-dependent Tie-2 phosphorylation peaked after 15 min of exposure and declined thereafter (Fig. 1B). An antibody selective to phosphorylated Tyr1112 of human Tie-2 receptor also detected positive phosphorylation of this residue after 60 min of Ang-2 treatment (Fig. 1B), suggesting that both Tyr1101 and Tyr1112 of Fig. 1. A: representative and mean values of Tie-2 receptor tyrosine phosphorylation intensity response to increasing concentrations of angiopoietin-2 (Ang-2). Cell lysates were probed with a specific phospho (p)-Tie-2 receptor antibody that recognizes phosphorylated Tyr1101 of human Tie-2 receptors. *,** P < 0.05 and 0.01 compared with vehicle. B: representative and mean values of Tie-2 receptor tyrosine phosphorylation detected in response to Ang-2 (300 ng/ml). ** P < 0.01 compared with vehicle. C: representative example of tyrosine phosphorylation of Tie-2 receptors in response to vehicle (control), or Ang-2 (300 ng/ml of each). D: representative example of tyrosine phosphorylation of Tie-2 receptors detected by immunoprecipitation with an anti-Tie-2 antibody, followed by immunoblotting with anti-phosphotyrosine antibody (top) or anti-Tie-2 receptor antibody (bottom). Means ± SE of optical densities (OD) of phosphorylated Tie-2 receptors detected in response to Ang-1 or Ang-2. ** P < 0.05 and 0.01 compared with vehicle (control), respectively. D: representative example of tyrosine phosphorylation of Tie-2 receptors in response to vehicle (control), or Ang-2 (300 ng/ml) in mouse endothelium cell line (bEnd.3). Tyrosine phosphorylation of Tie-2 receptors was detected by immunoprecipitation with an anti-Tie-2 antibody, followed by immunoblotting with anti-phosphotyrosine antibody (top) or anti-Tie-2 receptor antibody (bottom). Arb, arbitrary.
Human Tie-2 receptors become phosphorylated in response to Ang-2. Measurements of Tie-2 phosphorylation using the conventional immunoprecipitation approach with an anti-Tie-2 antibody followed by anti-phosphotyrosine immunoblotting confirmed that Ang-2 (300 ng/ml) elicits a significant increase in tyrosine phosphorylation of Tie-2 in HUVECs; however, the degree of this phosphorylation was lower than that elicited by Ang-1 at the same concentration (Fig. 1C). The increase in Tie-2 tyrosine phosphorylation elicited by a 15-min exposure to Ang-2 (300 ng/ml) was also observed in mouse ECs (bEnd.3; Fig. 1D), indicating that this effect of Ang-2 on Tie-2 receptor phosphorylation is not limited to HUVECs.

Figure 2, A and B, illustrates that Ang-2 at 300 ng/ml elicited a time-dependent increase in Akt phosphorylation, which peaked after 15 min of exposure. Significant Akt phosphorylation was evident at 150 ng/ml Ang-2, with no further rise in phosphorylation with 300 ng/ml of Ang-2 (Fig. 2C).

Ang-2 at 300 ng/ml also evoked a time-dependent increase in ERK1/2 phosphorylation, which, like Akt, peaked within 15 min of exposure (Fig. 3A). Ang-2-induced ERK1/2 phosphorylation was even evident at 150 ng/ml but not at 50 ng/ml (Fig. 3C). Activation of ERK1/2 by Ang-2 was relatively weaker than that elicited by Ang-1 (Fig. 3, B and D). Interestingly, an additive effect was noticed in terms of ERK1/2 phosphorylation, when Ang-1 (150 ng/ml) and VEGF (40 ng/ml) were combined or when Ang-1 (150 ng/ml) and Ang-2 (300 ng/ml) were combined (Fig. 3, B and D). By comparison, when Ang-2 and VEGF were combined, a reduction of
ERK1/2 phosphorylation to levels lower than those elicited by individual factors was observed (Fig. 3, B and D).

Ang-2 at 300 ng/ml also evoked significant phosphorylation of p38 MAPK, which peaked within 15 min of exposure and declined back to baseline level after 60 min of exposure (Fig. 4A). Increased p38 MAPK phosphorylation was evident at 150 ng/ml but not at 50 ng/ml of Ang-2 (Fig. 4B). An additive increase in p38 phosphorylation was evident when Ang-2 was combined with VEGF (Fig. 4, C and D). A similar observation was found when Ang-1 was combined with VEGF.

Unlike the rise in ERK1/2 and p38 phosphorylation, Ang-2 (50–300 ng/ml) elicited as significant decline in phosphorylation of the 54- and 46-kDa JNK/SAPK proteins (Fig. 5, A and B). This effect is opposite of that elicited by Ang-1, where a significant increase in JNK/SAPK phosphorylation has recently been described (12). To assess the influence of the

Fig. 4. A: representative example of phosphorylated p38 after 5, 15, 30, and 60 min of Ang-2 (300 ng/ml) or vehicle. B: means ± SE of OD of phosphorylated p38 after 15 min of vehicle or 50, 150, or 300 ng/ml of Ang-2. *P < 0.05 compared with vehicle. C and D: representative and mean ± SE values of OD of phosphorylated p38 after 15 min of VEGF, Ang-1, Ang-2, or combination of Ang-1 + VEGF or Ang-2 + VEGF. *P < 0.05 compared with control (vehicle); #P < 0.05 compared with Ang-1 alone or Ang-2 alone.

Fig. 5. A and B: representative and mean ± SE values of OD of phosphorylated p54 and p46 isoforms of JNK/SAPK proteins in response to vehicle or increasing concentrations of Ang-2. *P < 0.05 compared with control vehicle. C: regulation of ERK1/2 by JNK/SAPK. D: regulation of p38 phosphorylation by JNK/SAPK. HUVECs infected with empty retroviruses (MSCV) or retroviruses expressing a dominant negative form of JNK1/SAPK (MSCV-JNK-APF) were exposed for 15 min to either Ang-1 or Ang-2. Intensities of total and phosphorylated ERK1/2 and p38 were then measured.
JNK/SAPK on ERK1/2 and p38 phosphorylation in the context of Ang-1 and Ang-2 exposure, we used HUVECs, which are transduced with an empty retrovirus (MSCV), and HUVECs transduced with retroviruses expressing a dominant negative form of JNK1/SAPK (MSCV-JNK-APF). Ang-1-induced but not Ang-2-induced ERK1/2 phosphorylation was substantially augmented in MSCV-JNK-APF cells compared with MSCV cells (Fig. 5C). By comparison, both Ang-1-induced and Ang-2-induced p38 phosphorylation were attenuated in MSCV-JNK-APF cells compared with MSCV cells. These results indicate that the JNK/SAPK pathway exerts a negative influence on ERK1/2 phosphorylation particularly in the presence of Ang-1 and a positive influence on p38 phosphorylation in the presence of either Ang-1 or Ang-2.

Apoptosis and viability. Representative and mean data of the percentage of apoptotic cells (evaluated by propidium iodide DNA profiling) was measured in response to serum deprivation in the absence and presence of Ang-2. The percentage of apoptotic cells more than tripled after 6 and 24 h of serum deprivation (Fig. 6, A and B). The presence of Ang-2 significantly attenuated but did not completely reverse serum deprivation-induced apoptosis. We measured the proportion of live cells remaining after 6 and 12, 18, and 24 h of serum deprivation in the presence and absence of Ang-2 (300 ng/ml) using the MTT assay. Ang-2 significantly increased EC survival after 6 and 12 h of serum deprivation (Fig. 6C). However, Ang-2 did not improve cell survival when cells were maintained for 18 and 24 h in serum deprivation medium (results not shown). Prominent cleaved caspase-3 protein (17 kDa) was detected in serum-deprived cells (Fig. 7A). The presence of Ang-2 (300 ng/ml) in the culture medium for 24 h reduced cleaved caspase-3 protein intensity significantly (Fig. 7, A and B).

Similarly, cleaved caspase-9 protein (37 kDa) was detectable after 2 and 6 h of serum deprivation, and the intensity of this cleaved caspase was significantly attenuated by 150 and 300 ng/ml of Ang-2 (Fig. 7, B and C). We also monitored the effect of Ang-2 on caspase-8 protein (58 kDa) and cleaved caspase-8 intermediate (43 kDa) and found no clear effect of Ang-2 on this pathway (Fig. 7C). Figure 8 shows the effects of selective inhibitors of the ERK1/2, p38, and the PI3-kinase pathway on the antiapoptotic effects of Ang-2. The degree of apoptosis measured after 24 h of serum deprivation in the presence of 300 ng/ml of Ang-2 was designated as 100%. When the culture medium contained Ang-2 in addition to MEK1/2 and ERK1/2 inhibitors (PD98059 and UO126) or the PI3-kinase inhibitor (wortmannin), the degree of apoptosis was augmented to levels that were significantly greater than those measured with Ang-2 alone (Fig. 8A). By comparison, the presence of SB-203580 (p38 MAPK inhibitor) along with Ang-2 resulted in significantly lower levels of apoptosis than those measured with Ang-2 alone (Fig. 8A). Similarly, the inclusion of PD-98059 or wortmannin along with Ang-2 increased caspase-3 cleavage by 9- and 3-fold, respectively, as compared with Ang-2 alone (Fig. 8, B and C). In contrast, SB-203580 augmented the inhibitory effects of Ang-2 on caspase-3 cleavage.

Wound healing and migration. Figure 9, A and B, shows representative examples and mean values of wound healing of cells incubated for 12 h with BSA (control), Ang-1 (300 ng/ml), or Ang-2 (300 ng/ml). Significant enhancement in wound healing was observed in response to Ang-1 but not Ang-2 (Fig. 9, A and B). Similarly, whereas Ang-1 (300 ng/ml) significantly enhanced cell migration after 5 h, Ang-2 failed to provoke an increase in cell migration (Fig. 9C).
DISCUSSION

Our study revealed the following novel observations in HUVECs: 1) Ang-2 at 50 to 300 ng/ml concentrations elicited Tie-2 receptor phosphorylation and activation of the Akt, ERK1/2, and p38 MAPK pathways while significantly inhibiting the JNK/SAPK pathway. Ang-2-induced phosphorylation of Tie-2 receptors was also observed in mouse ECs. 2) The presence of Ang-2 significantly attenuated VEGF-induced ERK1/2 phosphorylation; however, no inhibitory interactions between Ang-2 and VEGF were found in terms of p38 activation. 3) Ang-2 at 300 ng/ml had no effect on wound healing or migration but significantly attenuated serum deprivation-induced apoptosis. This antiapoptotic effect of Ang-2 is mediated through the PI3-kinase and ERK1/2 pathways.

Tie-2 receptor phosphorylation. Our results demonstrate that, at doses comparable to those of Ang-1 (50 to 300 ng/ml), Ang-2 is capable of enhancing Tie-2 receptor phosphorylation both in HUVECs and in mouse ECs (Fig. 1). When Ang-2 was first cloned in 1997, it was proposed as an endogenous antagonist of Ang-1 because its presence reduced Ang-1-induced Tie-2 phosphorylation (31). It was also reported that, at these concentrations, Ang-2 elicits robust Tie-2 phosphorylation in nonendothelial cells or tumorogenic ECs, leading to the revised concept that activation of Tie-2 receptors is cell specific and context dependent (7, 16). There are, however, recent studies that challenge the concept that Ang-2 is not capable of activating Tie-2 receptors at relatively low concentrations. Indeed, Lemieux et al. (27) have described a significant Tie-2 phosphorylation in response to 66 ng/ml of Ang-2 in HUVECs after 7.5 min of exposure. Similar results have recently been observed in bovine aortic ECs by the same research group (32). Another study found that 300 ng/ml of Ang-2 induces Tie-2 phosphorylation in ECs after only 1 h of exposure (41). We propose that a major cause of these discrepancies is related to methodological differences, such as our use of phospho-tyrosine-specific Tie-2 antibodies, which provide better sensitivity for the detection of phosphorylated Tie-2 receptors than the conventional Tie-2 receptor immunoprecipitation followed by probing with phosphotyrosine antibodies. Another confounding factor, which may explain the differences in the sensitivity of Tie-2 receptor phosphorylation in response to Ang-2, is the composition of culture medium (presence and absence of FBS) and the presence of different extracellular matrices (3, 8). Culturing ECs on certain matrix components stimulates integrin binding and consequently titrates some of the ligand away from Tie-2 receptors (3). Clearly, more studies are needed to uncover various factors determining the degree of Tie-2 phosphorylation in response to various angiopoietins.

Antiapoptotic effects of Ang-2. An important finding in our study is that Ang-2 rescued ECs from serum deprivation-induced apoptosis, an effect that is evident even after 24 h of serum deprivation. We should point out that MTT measurement...
ments revealed that increased viability in the presence of Ang-2 did not persist after 18 h of exposure, whereas the propidium iodide assay detected significant attenuation of apoptosis by Ang-2 at up to 24 h. We attribute this difference in the effect of Ang-2 to the fact that the MTT assay is a less sensitive and specific indicator of apoptosis than DNA profiling with propidium iodide, as it was initially designed for proliferating cells.

The observations that Ang-2 at 300 ng/ml promoted EC survival through the PI3-kinase and ERK1/2 pathways suggest that Ang-2 exerts qualitatively similar antiapoptotic effects to those elicited by Ang-1 (13, 14) and are in accordance with similar observations reported at relatively high concentrations of Ang-2 (4, 22). Despite the similarity in ERK1/2 activation, there is a distinct difference between the interaction between Ang-1 and Ang-2 with VEGF in terms of ERK1/2 phosphorylation. The presence of Ang-2 resulted in attenuation rather than a further increase in VEGF-induced ERK1/2 phosphorylation, whereas an additive effect on ERK1/2 phosphorylation was evident when Ang-1 and VEGF were present simultaneously or when Ang-1 and Ang-2 were combined (Fig. 3). The mechanism of this inhibitory interaction between VEGF and Ang-2 in terms of ERK1/2 phosphorylation is unclear. It is possible that the molecular partners of Tie-2 receptors after Ang-1 or Ang-2 treatment are not the same, and hence the interactions between Tie-2 receptors and VEGF receptors will be qualitatively different. One such partner would be Tie-1 receptors. Recent studies revealed that Ang-1, but not Ang-2, directly promote Tie-1 phosphorylation, and this effect is amplified by Tie-2-induced transphosphorylation of Tie-1 (37). Tie-1 is an orphan receptor predominantly expressed in ECs and is believed to regulate apoptosis by modulating Tie-2 and Akt signaling in ECs (25). Whether Tie-1 is responsible for the different potencies between Ang-1 and Ang-2 in mediating apoptosis and signaling remains to be investigated.

We should emphasize that the functional significance of a different interaction between Ang-2 and VEGF in terms of ERK1/2 phosphorylation as compared with Ang-1- and VEGF-treated ECs remains unclear, although previous studies have uncovered distinct differences between how Ang-1 interacts in vivo with VEGF as compared with that of Ang-2. In a cornea micropocket assay of neovascularization, for instance, Asahara et al. (1) have reported that Ang-2/VEGF promoted significantly longer and more circumferential neovascularity than...
that elicited by Ang-1 + VEGF. Others, including Holash et al. (15) and Lobov et al. (29) have demonstrated that Ang-2 is a proangiogenic agent in the presence of VEGF, whereas, in the absence of VEGF, Ang-2 promotes vascular regression. In the presence of VEGF, Ang-2 promotes angiogenesis by destabilizing the vasculature, thus rendering ECs more responsive to VEGF-mediated sprouting signals. The fact that combining Ang-2 and VEGF shifted the signaling patterns toward p38 MAPK activation in our study favors this model.

We report here that Ang-2 evokes p38 phosphorylation as strongly as that elicited by Ang-1. The role of the p38 MAPK in regulating EC apoptosis remains under investigation. In non-ECs, activation of this pathway evokes both anti- or proapoptotic effects, depending on the duration and intensity of activation and the cell-type, as well as cellular context. In this study, activation of the p38 MAPK pathway by Ang-2 appears to promote EC apoptosis because selective inhibition of this pathway improved EC survival and attenuated caspase-3 and -9 activation as compared with Ang-2 treatment alone. It should be pointed out that the fact that Ang-2 elicited relatively weaker ERK1/2 phosphorylation but similar level of p38 phosphorylation to those evoked by Ang-1 might explain why the antiapoptotic effect of Ang-2 is not as potent as that of Ang-1. It is interesting that, despite activation of the p38 MAPK, Ang-2 still inhibits serum deprivation-induced apoptosis, suggesting that the effects of antiangiogenic pathways (PI3-kinase/Akt and ERK1/2) predominate over the proangiogenic p38 MAP kinases, an effect that has already been documented in the case of VEGF and Ang-1 exposure in ECs (11, 13, 47). This predominance of antiangiogenic pathways could be mediated at different levels, including inhibition of upstream activators of the p38 MAPK and/or downstream regulators of apoptosis, such as the release of cytochrome c from the mitochondria and caspase activation. Gratton et al. (11) recently reported that in the presence of VEGF, the PI3-kinase pathway actively attenuates the activity of the p38 MAPK through selective inhibition of an upstream kinase, MEKK3 (MAPK/ERK kinase kinase 3). Similarly, we recently reported in ECs that both the ERK and the PI3-kinase pathways act like a switch in the presence of Ang-1, inhibiting the activation of the p38 MAPK pathway (28).

Role of the JNK/SAPK pathway. Our study shows, for the first time, a fundamental difference in JNK/SAPK activation by Ang-1 and Ang-2 in that, whereas Ang-1 increases, Ang-2 inhibits the phosphorylation of this pathway. We have no clear evidence explaining the divergence in these effects, but we speculate that the p21-activated kinase-1 (PAK-1) may be involved. PAK-1 is a promigratory kinase upstream of JNK/SAPK, which is activated in response to Ang-1 (12). We have conducted several experiments in which we evaluated whether Ang-2 (300 ng/ml) activates PAK-1 in HUVECs by using phospho-specific PAK-1 antibodies. These experiments (results not shown) revealed no significant increase in PAK-1 phosphorylation in response to 300 ng/ml. PAK-1 is activated downstream of Rac1 and cdc42, two GTPases that preferentially activate the JNK/SAPK and p38 MAPK pathways (42). The well-known role of PAK-1 in cell motility and the differential activation of PAK-1 by Ang-1, but not Ang-2, could explain our current observation that Ang-1, but not Ang-2, promotes EC migration and wound healing. However, more comprehensive evaluation of the effects of Ang-2 on PAK-1 activation, using various Ang-2 concentrations and different exposure times, is needed to confirm the differential effect of angiopoietins on PAK-1 activation.

There is increasing evidence that complex interactions occur between various members of MAPKs. The nature of these interactions is highly dependent on many variables, including the type of cell, nature of stimulus and the type of receptors involved. For instance, p38-dependent activation of ERKs have been observed in a variety of mammalian cells in response to arsinite treatment (30), whereas a direct inhibition of both ERK1 and ERK2 by p38 MAPK was described in HeLa and HEK-293 cells (48). In HUVECs, we recently reported that the ERK1/2 pathway, which is activated by Ang-1, exerts an inhibitory influence on p38 MAPK pathway activation, whereas the p38 pathway did not influence the degree of ERK1/2 activation in the presence of Ang-1 (13). Our current study revealed that the JNK/SAPK pathway inhibits the activation of the prosurvival ERK1/2 pathway while promoting the activation of the proapoptotic p38 pathway. These effects of the JNK/SAPK pathway have been previously described in COS cells and in hepatoma cells when these cells were activated with TNF-α and transforming growth factor-β, respectively (36, 39). The exact mechanisms responsible for these interactions between MAPKs are not clear, but investigators have proposed that mixed lineage kinases, MEKKs, JNK/SAPK, or downstream factors such as c-Jun might directly inhibit ERK1/2 proteins and render them insensitive to activation by growth factors (39). Others proposed that the interactions between the ERK1/2 and the JNK/SAPK may reside at the level of MEKK1 (44). It should be noted that the lack of effect of JNK/SAPK inhibition on ERK1/2 phosphorylation in the presence of Ang-2 is likely be due to inhibition of the JNK/SAPK by Ang-2. By comparison, the fact that we observed inhibition of p38 MAPK phosphorylation in dominant negative JNK/SAPK expressing cells stimulated with Ang-2 indicates the presence of basal positive influence of JNK/SAPK on p38 phosphorylation irrespective of the presence of growth factors.

In summary, our study indicates that Ang-2 at 50 to 300 ng/ml activates the Tie-2 receptors and enhances phosphorylation of the Akt, ERK1/2, and p38 MAPK pathways while significantly inhibiting the JNK/SAPK pathway. Moreover, we report here that a negative interaction occurs between VEGF and Ang-2 with respect to ERK1/2, but not p38, phosphorylation. Finally, our results reveal that Ang-2 at 300 ng/ml had no effect on wound healing or migration but significantly attenuates serum deprivation-induced apoptosis and promotes EC survival. Hence our results help explain the dichotomy reported concerning signaling activated by Ang-2 and the biological roles attributed to this angiopoietin.

ACKNOWLEDGMENTS

The authors are grateful to L. Franchi for technical assistance. S.N.A. Hussain is a National Scholar of the Fonds de la Recherche en Sante du Québec.

GRANTS

This study is funded by grants from the Heart and Stroke Foundation of Québec. R. Harfouche is funded by a Studentship from the Fonds de la Recherche en Sante du Québec.
REFERENCES


