Angiogenesis, the growth of new vessels, is a physiologic process that occurs under normal conditions, such as during embryonic growth and wound healing. During these processes, angiogenesis is well regulated by a balance of positive and negative factors. However, in various disease states, such as tumor progression, inflammation, and diabetic retinopathy, deregulated overactive angiogenesis contributes to disease progression (1). Recent reports suggest that receptor tyrosine kinases (RTKs) of endothelial cells play a major role in both physiological and pathological angiogenesis (1,2). Two distinct RTK subfamilies are characterized by their abundant expression of endothelium. One subfamily consists of vascular endothelial growth factor (VEGF) receptors Flt-1/VEGF-R1, Flk-1/VEGF-R2, and Flt-4/VEGF-R3 (3–5). VEGF, also known as vascular permeability factor, is an endothelial cell-specific mitogen that induces angiogenesis and increases vasopermeability (1). The other endothelium-specific RTK subfamily is the Tie receptor family, consisting of Tie1 and Tie2 (6). Tie1-null mice die in utero with defects that may implicate the hemodynamics of transcapillary fluid exchange (7,8). Similarly, Tie2-knockout mice die from day 9.5 to 10.5, because of immature vessels and lack of microvessel formation (8,9). Unlike the VEGF receptor–knockout mouse (10), the number of endothelial cells was normal, and tubular formation was detected in Tie2-knockout mice. A mutation in Tie2 in humans was reported to cause venous malformations, which are typically an imbalance of endothelial cells and smooth muscle cells (11). These findings suggest that the Tie2 system has a role in endothelial-stromal cell communication and in maturation and stabilization of vascular structures.

Ligands for the Tie2 receptor have been identified as angiopoietin (Ang)-1 and Ang2 (12,13) and, more recently, Ang3 and Ang4 (14). Ang1 phosphorylates Tie2 in cultured endothelial cells (13), whereas Ang2 does not induce phosphorylation of Tie2, but rather inhibits the Ang1-induced phosphorylation of Tie2 in vascular endothelial cells (12). The activities of Ang3 and Ang4 have not yet been identified. Ang2-overexpressing transgenic mice die with vascular defects similar to Tie2- or Ang1-knockout mice (8,15). These observations suggest that Ang2 acts as a natural antagonist of Tie2 by blocking receptor activation by Ang1 (12). Recently, wide expression of Tie2 in the quiescent vasculature of adult tissues was reported (16). A study using a corneal angiogenesis model revealed that Ang1 and Ang2 facilitates VEGF-induced neovascularization; Ang1 promotes vascular network maturation, whereas Ang2 initiates neovascularization (17). These data support the idea that angiopoietins/Tie2 may have a role not only in embryonic angiogenesis, but also in postnatal angiogenesis.

The renin-angiotensin system (RAS) is known to be a key factor in the cardiovascular homeostasis that regulates blood pressure and fluid electrolyte balance (18). AngII has been reported to regulate cell growth by inducing several growth factors (19–21). The growth-promoting effect of AngII has been shown to be involved in remodeling the heart and vessels after myocardial infarction, injury, and chronic hypertension (22–24). RAS abnormalities have also been reported to play a role in the progression of diabetic nephropathy and retinopathy (25,26). Previously,
we showed that ALL potentiates VEGF-mediated angiogenic activities through upregulation of VEGF-R2 expression in bovine retinal endothelial cells (BREC)s) and upregulation of VEGF in bovine retinal pericytes (BRPs), which suggests a major role for RAS in the retinal angiogenic abnormalities associated with diabetes (27,28). To further investigate the role of RAS in diabetic retinopathy, we studied the effects of ALL on Ang1 and Ang2 expression in BREC.s.

**RESEARCH DESIGN AND METHODS**

**Cell cultures.** Primary BREC cultures were isolated by homogenization and a series of filtration steps, as previously described (29). Primary BREC.s were grown on fibronectin (Sigma, St. Louis, MO)-coated dishes (Iwaki Glass, Tokyo) containing Dulbecco’s modified Eagle’s medium (DMEM) with 5.5 mmol/l glucose, 10% plasma-deprived horse serum (PDSI) (Wheaton, Pipersville, PA), 50 mg/l heparin, and 50 U1 endothelial cell growth factor (Boehringer Mannheim, Indianapolis, IN). Bovine aortic endothelial cells (BAECs) were also isolated from bovine aorta and cultured in DMEM containing 5% calf serum and 10% PDSI. The cells were cultured in 5% CO2 at 37°C. Endothelial cell homogeneity was confirmed by immunoreactivity with anti-factor VIII antibodies analyzed by confocal microscopy. For ALL receptor-antagonist studies, BREC.s were pretreated with AT1 antagonist, losartan (Merck Research Laboratories, Rahway, NJ), or AT2 antagonist PD123319 (Research Biochemicals International, Natick, MA) for 15 min following stimulation with ALL. To determine the time course of effects, BREC.s were cultured for 4 h, then the cells were lysed in solubilizing buffer (10 mmol/l Tris-HCl, 10 mmol/l NaCl, 3 mmol/l MgCl2, and 0.5% NP-40), and the nuclei were isolated. For 4 h, then the cells were lysed in solubilizing buffer for denaturation. The samples were separated by 7.5% SDS-polyacrylamide gel (Bio-Rad Laboratories, Richmond, CA), and the gel was vacuum dried. Results were visualized and quantified by a densitometer (Fuji Photo Film). To obtain specificity of the signals, we made a control in which antibodies had been preincubated for 1 h with a fivefold molar excess of the respective blocking peptides (Santa Cruz Biotechnology).

In vivo corneal angiogenesis model. To identify the effect of ALL on Ang2 expression in vivo, we made an ALL-induced corneal angiogenesis model as previously reported (32). We made a slow-release formulation polyacrylamide gel containing ALL 10 mmol/l and implanted it in a pocket made in the corneal stroma of pigmented Long-Evans rats. For controls, polyacrylamide gel alone was implanted. Neovascularization was assessed on day 14 by direct examination with a surgical microscope. The eyes were enucleated after sacrificing the animal. The eyes were fixed in 4% paraformaldehyde solution. A frozen block of the specimen was made using Tissue Tek (Sakura, Torrance, CA), and serial frozen sections were cut from the specimens in a cryostat (Leica-Reichert, Vienna, Austria) at −20°C and mounted on slides. The sections were fixed in aceton at 4°C for 10 min, and immunohistochemical staining was performed according to the manufacturers protocol (Elite ABC kit; Vector Laboratories, Burlingame, CA). The specimens were incubated overnight at 4°C with goat polyclonal anti–angiopoietin-2, 1:500 dilution (Santa Cruz Biotechnology, Santa Cruz, CA), and then a standard indirect immunoperoxidase procedure using the ABC kit was performed with AEC (3-amin-9-ethylcarbazole) (DAKO, Carpinteria, CA) as the substrate. For the negative control, the primary antibody preincubated with the immunizing peptide (Santa Cruz Biotechnology) was used. Other staining procedures were the same as previously described. Statistical analysis. Determinations were performed in triplicate, and experiments were performed at least three times. Results are expressed as means ± SE, unless otherwise indicated. For multiple treatment groups, a factorial analysis of variance followed by Fisher’s least-significant difference test was performed. Results were considered significant when P was <0.05.

**RESULTS**

ALL stimulates Ang2 but not Ang1 mRNA expression in BREC.s. To investigate the effect of ALL on Ang1 and Ang2 mRNA expression, BREC.s were treated with 10 nmol/l ALL for the indicated times, and Northern blot analysis was performed. From several independent experiments, the effect of ALL on Ang2 mRNA expression was time-dependent, with a maximal 2.6 ± 0.5-fold increase at 4 h (P < 0.01) that progressively diminished up to 12 h (Fig. 1A). ALL also stimulated Ang2 mRNA expression in a dose-dependent manner with an EC50 of ~3 nmol/l and a maximal 2.4 ± 0.2-fold (P < 0.01) increase at a concentration of 10 nmol/l (Fig. 1B). In both experiments, ALL did not affect the expression of Ang1 mRNA (Fig. 1A and B). We also examined the Ang1 and Ang2 mRNA expression in BAECs. The expression of Ang2 mRNA in BAECs was very faint, and no changes could be detected after ALL stimulation (Fig. 1C).

**Role of ALL receptor subtypes AT1 and AT2 in the ALL stimulated Ang2 mRNA expression in BREC.s.** To characterize the ALL receptor subtype that is responsible for Ang2 mRNA induction, Northern blot analyses were performed using the AT1 antagonist losartan or the AT2 antagonist PD123319 for 15 min before stimulation with 10 nmol/l ALL (Fig. 2). In contrast with PD123319, losartan inhibited ALL-induced Ang2 mRNA expression (Fig. 2). The
observed response suggests that AII stimulates Ang2 induction mainly through the AT1 receptor.

**All does not increase the half-life, but increases the rate of transcription of Ang2 mRNA in BRECs.** We investigated whether the AII-induced increase of the Ang2 mRNA level is mediated through regulation of mRNA stability or transcription. To determine whether AII affects the half-life of Ang2 mRNA, we performed Northern blot analysis using a de novo gene transcription inhibitor, 4 μmol/l actinomycin-D with or without 10 nmol/l AII. AII did not change the stability and half-life of Ang2 mRNA (Fig. 3A). However, 10 nmol/l AII increased the rate of Ang2 gene transcription by 2.1-fold in BRECs that were examined by nuclear run-on analysis (Fig. 3B). These data suggest that AII stimulated the increase of the Ang2 mRNA level mainly through an increase of the transcriptional rate.

**The role of PKC and MAPK in AII-induced Ang2 mRNA expression.** To determine the role of PKC and MAPK in AII-induced Ang2 mRNA expression, BRECs were pretreated with a highly selective PKC inhibitor, bisindolylmaleimide (GFX), or a specific MAPK inhibitor (PD98059) followed by treatment with 10 nmol/l AII and PMA, a direct PKC stimulator. PMA (0–500 nmol/l) increased the expression of Ang2 mRNA in a dose-dependent manner (data not shown), and the effect (160 nmol/l AII did not alter the mRNA level of Ang2 mRNA (Fig. 3A). However, 10 nmol/l AII increased the rate of Ang2 gene transcription by 2.1-fold in BRECs that were examined by nuclear run-on analysis (Fig. 3B). These data suggest that AII stimulated the increase of the Ang2 mRNA level mainly through an increase of the transcriptional rate.

AII does not stimulate VEGF mRNA expression in BRECs. Because VEGF upregulates Ang2 expression without affecting Ang1 expression in the same cell type (31), VEGF induction by AII might be involved in the observed AII-dependent regulation of Ang1 and Ang2. To investigate this possibility, the effect of AII on VEGF expression was determined. Northern blot analysis revealed that 10 nmol/l AII did not alter the mRNA level of VEGF during 2–24 h in BRECs. The data suggest that VEGF induction is not involved in the Ang1 and Ang2 mRNA expression (Fig. 6).

**AII increases Ang2 protein synthesis.** To determine whether the increase in Ang2 mRNA expression was accompanied by an increase in new protein synthesis, we precipitated the 35S-labeled cell lysates of BRECs with anti-Ang2 antibody and analyzed them by SDS-PAGE. The molecular mass of Ang2 protein was reported to range from 55 to 70 kDa because of glycosylation (13). In our experiments, several bands that agree with the molecular mass were detected by immunoprecipitation of a rabbit anti-human Ang2 antibody (Fig. 7). Specificity controls were made with nonstarved cells and performed by incubating the antibodies with an excess of the blocking peptide before detection (Fig. 7A and B), and a band ~55 kDa was detected as Ang2 protein. The level of the band was increased by adding AII as well as PMA (Fig. 7D and E). These data indicate that AII increased protein synthesis of Ang2 in BRECs.
AII increases Ang2 in corneal angiogenesis models.

To determine whether the AII-induced Ang2 expression is involved in neovascularization in vivo, we made a rat corneal angiogenesis model. AII significantly stimulates new vessel formation (Fig. 8A); in contrast, polyacrylamide gel alone failed to stimulate any significant new vessel formation (Fig. 8B). Immunohistochemical study revealed that Ang2 was strongly positive in these AII-induced vessels in the corneal stroma, whereas no staining was observed in the control cornea. The data suggest that AII induces Ang2 in vivo.

DISCUSSION

Recent in vitro studies demonstrated that Ang1 induces potent chemotaxis, weak but positive mitogenesis, and capillary sprouts in endothelial cells, which confirm the critical role of Ang1 in angiogenesis (33). Although Ang2 has been shown to be a natural antagonist to Ang1 in developmental angiogenesis and cultured endothelial cells (12,34), when co-administered with VEGF in vivo, Ang2 can potentiate VEGF-induced angiogenesis, probably by enhancing the initiation of neovascularization, whereas Ang1 may potentiate vascular network maturation (17). In the present study, we demonstrated that AII stimulates Ang2 expression, but has no significant effect on Ang1 expression in retinal microvascular endothelial cells. This finding might implicate a substantial effect of AII in promoting pathologic angiogenesis in diabetic retinopathy and other ischemic neovascular diseases in which VEGF-dependent angiogenesis is a predominant pathologic change (35–37).

Previous studies have implicated RAS as a key factor in the development of diabetic retinopathy. The severity of retinopathy correlates with RAS activity, and the treatment of diabetic patients with an ACE inhibitor reduces both the rate of progression of nonproliferative retinopathy and the development of proliferative changes (25). However, it remains unclear what mechanism underlies the interaction of RAS and the development of diabetic retinopathy. The present findings might implicate a novel role of AII in the development of diabetic retinopathy, i.e., AII may enhance pathological angiogenesis by a selective induction of Ang2 in diabetic retinopathy.
Ang2 expression has recently been demonstrated in a subset of angiogenic vessels in glioblastoma and hypervascularized specimens of hepatocellular carcinoma, suggesting its biologic importance in tumor-associated angiogenesis (38,39). Furthermore, recent studies also demonstrated that Ang2 is upregulated by angiogenic stimuli, such as hypoxia, VEGF, and basic fibroblast growth factor, and is downregulated by transforming growth factor-b1, which is associated with vessel stabilization (31,40). These data suggest that Ang2 has a prominent role in pathologic angiogenesis. The present data showing selective upregulation of Ang2 by AII agrees with previously published studies and may strongly define the role of Ang2 in retinal angiogenesis in diabetic retinopathy.

Two major angiotensin receptor subtypes have been defined: the AT1 and AT2 receptors (45,46). Most of the actions of AII, including its growth-promoting effect, are mediated by the AT1 receptor (18). The actions of the AT2 receptor have not been well defined (18), but recent studies suggest that it is involved in both apoptosis and

![Image](image-url)

**FIG. 4.** The role of PKC and MAPK in AII- and PMA-induced Ang2 mRNA expression. A: BRECs were pretreated with GFX or PD98059 followed by stimulation with 10 nmol/l AII for 4 h (left panel), and BRECs were pretreated with GFX and stimulated with PMA 160 nmol/l for 4 h (right panel). PKC activation significantly induced Ang2 mRNA expression (P < 0.05), and the PKC inhibitor completely inhibited AII-induced Ang2 expression. Representative blots are shown (n = 3). B: Dose-response study of MAPK inhibitor in AII-induced Ang2 mRNA expression. BRECs were pretreated with the indicated concentrations of PD98059 for 15 min, followed by stimulation with 10 nmol/l AII for 4 h. The MAPK inhibitor also inhibited AII-induced Ang2 expression, but not completely. Results are expressed as a percentage of uninhibited controls (n = 3).

Furthermore, the time-course study demonstrated that Ang2 induction by AII started at 2 h and peaked at 4 h and returned to the basal level at 12 h. The observed prompt induction of Ang2 suggests that VEGF production is not associated with the AII-stimulated Ang2 induction.

The dose-response study demonstrated an EC50 of ~3 nmol/l and a maximal increase at 10 nmol/l AII stimulation. The AII concentrations used in our experiments were considerably higher than those found in the plasma and vitreous of diabetic patients (41). However, the presence of angiotensinogen, renin, and ACE has been reported in ocular tissues (42,43), and the AII retinal concentration in ocular tissues is much higher than that in plasma (44). These data support the presence of intraocular RAS. AII may act as an autocrine or paracrine factor in retinal tissues. 

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**FIG. 5.** Role of MAPK in PMA-induced Ang2 mRNA expression. BRECs were pretreated with PD98059 10 nmol/l for 15 min followed by stimulation with 160 nmol/l PMA for 4 h. The MAPK inhibitor inhibited PKC-induced Ang2 mRNA expression by 76.8 ± 8.1%. Results are expressed as percentages of controls (n = 3) (bottom panel), and representative blots are shown (top panel).
Ang2 mRNA expression in a dose-dependent manner (the maximal effect was observed at 160 nmol/l), and this effect was completely inhibited by adding 10 μmol/l GFX (Fig. 4A). The same GFX concentration completely inhibited AII-induced Ang2 mRNA expression, suggesting that the AII-induced increase of Ang2 mRNA is mediated totally by a PKC-dependent signaling pathway (Fig. 4A). MAPK inhibitor also blocked AII-induced Ang2 mRNA expression in a dose-dependent manner, with a maximal inhibition of 69.4 ± 15.6% (Fig. 4B). MAPK inhibitor blocked PMA-induced Ang2 mRNA expression by 76.8 ± 8.1% (Fig. 5); this inhibitory effect was well correlated with that in AII-induced Ang2 mRNA expression. These data suggest that MAPK is also involved in the downstream pathway of PKC; most AII-induced Ang2 expression is PKC-activated and MAPK-dependent, and the remainder is PKC-dependent without MAPK activation.

The effect of AII on Ang2 protein synthesis was studied by immunoprecipitation of 35S-labeled cell lysates of BRECs. In our experiments, a rabbit anti-human Ang2 antibody detected several bands from 55 to 70 kDa. Because a band at 55 kDa was reduced when antibodies with an excess of the blocking peptide were used before detection, it can be concluded that that band represents Ang2 protein and the other bands probably result from cross-reactivity of the antibody. This is further confirmed by the finding that the signal of the band is increased by PMA stimulation, as previously reported in bovine microvascular endothelial cells (40). The signal of the band is increased by AII stimulation, which indicates that AII stimulates Ang2 protein synthesis. The AII effect on Ang2 protein synthesis was also confirmed in vivo by immunohistochemistry in a model of corneal angiogenesis.

In our experiments, Ang2 expression in BAECs was undetectable using a similar Northern blot analysis. Because the expression of Tie2 was similar in both cell types (data not shown), the difference in Ang2 expression might suggest that Ang2 regulation is more important in retinal microvasculature than in large vasculature. However, further investigations, including histologic studies and tests of other cell types, are needed to confirm this theory.

In addition to our previous findings that AII promotes VEGF-dependent angiogenesis (27,28), the data presented here demonstrate that RAS upregulates both the VEGF/VEGF receptor system and Ang2 expression in retinal

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**FIG. 6.** AII does not stimulate VEGF mRNA expression in BRECs. BRECs were stimulated with 10 nmol/l AII for the indicated number of hours. Values are presented as percentages of the 0-h value (n = 3), and representative blots are shown (top panel). Northern blot analysis revealed that 10 nmol/l AII did not alter the mRNA level of VEGF during 2–24 h in BRECs.

Regeneration (47,48). In our study, AII-induced Ang2 mRNA expression was mediated through the AT1 receptor, and the AT2 receptor was not involved.

Nuclear run-on assays and experiments using actinomycin-D indicated that the primary effect of AII is to increase transcription of the Ang2 gene. These data might suggest that transcriptional regulation of this gene is mediated through a transacting transcription factor. Further studies are necessary to elucidate in detail the mechanism of transcriptional regulation of Ang2, including cis- and trans-activation.

The major signaling events activated by AII through the AT1 receptor include production of diacylglycerol and inositol 1,4,5-triphosphate by phospholipase C-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (18). Diacylglycerol activates PKC. AII also activates MAPK, which is thought to play a critical role in cellular proliferation (49–51). In many receptor types, including RTK and G protein-coupled receptors, MAPK activation is induced through PKC activation (52). In adrenal glomerulosa cells, AII was reported to act as a mitogen in its primary culture through the G protein-coupled AT1 receptor, and it activates MAPK via PKC and Ras/Raf-1 kinase (53). In the present study, we examined the involvement of PKC and MAPK in AII-stimulated Ang2 expression. PMA induced

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**FIG. 7.** Immunoprecipitation analysis of AII-stimulated and PMA-induced Ang2 protein synthesis. Starved BRECs were treated with 10 nmol/l AII, 160 nmol/l PMA, or vehicle for 5 h and labeled with 35S-methionine, and SDS-PAGE was performed (lanes C, D, and E). Labeled proteins were visualized and analyzed using a densitometer. Specificity controls were made with nonstarved cells and performed by incubating the antibodies with an excess of the blocking peptide before detection (lanes A and B). A: Blocking peptide used in nonstarved cells. B: Nonstarved cells. C: Unstimulated control. D: PMA 160 nmol/l stimulation. E: AII 10 nmol/l stimulation. Arrows point to specific bands that likely represent Ang2 protein. The level was increased with PMA or AII stimulation. Representative data are shown (n = 3).

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microvascular cells. Such upregulation of an angiogenesis-promoting environment most likely contributes to the development of VEGF-dependent angiogenesis in ischemic neovascular diseases, such as diabetic retinopathy. AII-induced upregulation of these molecules is similarly mediated through the AT1 receptor. AT1 blockade, as well as
the already proven ACE inhibitor, may thus be an effective modality to prevent the development of diabetic retinopathy.

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