Angiotensin II Induces Expression of the Tie2 Receptor Ligand, Angiopoietin-2, in Bovine Retinal Endothelial Cells

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Recent studies have shown that angiopoietins (Angs) and their receptor, Tie2, play a role in vascular integrity and neovascularization. The renin-angiotensin system has been hypothesized to contribute to the development of diabetic retinopathy. In this study, we investigated the effect of angiotensin II (AII) on Ang1 and Ang2 expression in cultured bovine retinal endothelial cells (BRECs). AII stimulated Ang2 but not Ang1 mRNA expression in a dose- and time-dependent manner. This response was inhibited completely by angiotensin type 1 receptor (AT1) antagonist. AII increased the transcription of Ang2 mRNA, but did not change the half-life. Protein kinase C (PKC) inhibitor completely inhibited AII-induced Ang2 expression, and the mitogen-activated protein kinase (MAPK) inhibitor also inhibited it by 69.4 ± 15.6%. In addition, we confirmed the upregulation of Ang2 in an AII-induced in vivo rat corneal neovascularization model. These data suggest that AII stimulates Ang2 expression through AT1 receptor-mediated PKC and MAPK pathways in BREC, and AII may play a novel role in retinal neovascularization. ***Diabetes*** **50:867–875, 2001**

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). AII has been reported to regulate cell growth by inducing several growth factors (19–21). The growth-promoting effect of AII 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,

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AII, angiotensin II; Ang, angiopoietin; AT1, angiotensin type 1 receptor; BAEC, bovine aortic endothelial cell; BREC, bovine retinal endothelial cell; BRP, bovine retinal pericyte; DMEM, Dulbecco's modified Eagle's medium; MAPK, mitogen-activated protein kinase; PCR, polymerase chain reaction; PDHS, plasma-deprived horse serum; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RAS, renin-angiotensin system; RTK, receptor tyrosine kinase; VEGF, vascular endothelial growth factor.

we showed that AII potentiates VEGF-mediated angiogenic activities through upregulation of VEGF-R2 expression in bovine retinal endothelial cells (BRECs) 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 AII on Ang1 and Ang2 expression in BRECs.

RESEARCH DESIGN AND METHODS

Cell cultures. Primary BREC cultures were isolated by homogenization and a series of filtration steps, as previously described (29). Primary BRECs 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 (PDHS) (Wheaton, Pipersville, PA), 50 mg/l heparin, and 50 U/l 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% PDHS. The cells were cultured in 5% CO_2 at 37°C. Endothelial cell homogeneity was confirmed by immunoreactivity with anti-factor VIII antibodies analyzed by confocal microscopy. For AII receptor–antagonist studies, BRECs were pretreated with AT1 antagonist, losartan (Merck Research Laboratories, Rahway, NJ), or AT2 antagonist PD123319 (Research Biochemicals International, Natick, MA) for 15 min followed by stimulation with 10 nmol/l AII for 4 h. To determine the roles of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) on AII-stimulated Ang2 mRNA expression, BRECs were pretreated with 10 μmol/l GF109203X (GFX; Calbiochem-Novabiochem, La Jolla, CA) or 10 μmol/l PD98059 (Research Biochemicals International), respectively, followed by stimulation with 10 nmol/l AII or 160 nmol/l phorbol 12-myristate 13-acetate (PMA) (Sigma).

Northern blot analysis. Total RNA was isolated from individual tissue culture plates using guanidium thiocyanate. Northern blot analysis was performed on 20 μg total RNA after 1% agarose-2 mol/l formaldehyde gel electrophoresis and subsequent capillary transfer to Biodyne nylon membranes (Pall BioSupport, East Hills, NY), and ultraviolet crosslinking was performed using a FUNA-UV-LINKER (FS-1500; Funakoshi, Tokyo). Radioactive probes were generated using Amersham Megaprime labeling kits and ^{32}P-labeled dATP (Amersham, Arlington Heights, IL). Blots were prehybridized, hybridized, and washed in 0.5 × SSC, 5% SDS, at 65°C with four changes over 1 h in a rotating hybridization oven (TAITEC, Koshigaya, Japan). All signals were analyzed using a densitometer (BAS-2000II; Fuji Photo Film, Tokyo), and lane-loading differences were normalized using a 36B4 cDNA probe, which hybridizes to acidic ribosomal phosphoprotein PO (30). cDNA probes for human Ang1 and Ang2 were synthesized by reverse transcriptase–polymerase chain reaction (PCR) (31). For Ang1 and Ang2 cDNAs, a standard PCR was performed (PCR optimizer kit; Invitrogen) using 5′-AGA ACC ACA CGG CTA CCA TGC T-3′ (Ang1 sense primer corresponding to nucleotides 1671–1692), 5′-TGT GTC CAT CAG CTC CAG TTG C-3′ (Ang1antisense primer), 5′-AGC TGT GAT CTT GTC TTG GC-3′ (Ang2 sense primer corresponding to nucleotides 1377–1396), and 5′-GTT CAAGTC TCG TGG TCT GA-3′ (Ang2 antisense primer corresponding to nucleotides 1802–1821).

Analysis of Ang2 mRNA half-life. To determine whether the increase in Ang2 mRNA was caused by increased mRNA stability, BRECs were exposed to 4 μmol/l actinomycin-D (Wako, Osaka, Japan) after 4 h incubation with vehicle or 10 nmol/l AII. The total RNA was then extracted, and Northern blot analyses were performed.

Nuclear run-on analysis. BRECs were treated with vehicle or 10 nmol/l AII for 4 h, then the cells were lysed in solubilizing buffer (10 mmol/l Tris-HCl, 10 mmol/l NaCl, 3 mmol/l $MgCl_2$, and 0.5% NP-40), and the nuclei were isolated. ATP CTP, GTP (500 mmol/l each), and 3.7 MBq of ^{32}P-labeled UTP (110 Tbq/mmol; Amersham) were added to the nuclear suspension (100 μl) and incubated for 30 min. The samples were extracted with phenol/chloroform and precipitated. cDNA probes (Ang2 and 36B4, 10 μg each) were slot blotted onto nitrocellulose filters (Schleicher & Schuel, Keene, NH) and hybridized with the precipitated samples of equal counts per minute per milliliter in hybridization buffer at 45°C for 48 h. The filters were washed, and the radioactivity was measured using the densitometer (BAS-2000II; Fuji Photo Film).

Ang2 protein synthesis. BRECs were treated with 10 nmol/l AII, 160 nmol/l PMA, or vehicle for 5 h, and the culture media were replaced with labeling media (DMEM without methionine and cysteine: 100 μCi ^{35}S-labeled methionine and cysteine) supplemented with AII, PMA, or vehicle. After a 3-h incubation, the cells were lysed in solubilizing buffer (50 mmol/l Hepes, pH 7.4, 10 mmol/l EDTA, 100 mmol/l NaF, 10 mmol/l Na pyrophosphate, 1% Triton X-100, 10 mmol/l $NaVO_4$, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 2 mmol/l phenyl methylsulfonyl fluoride) at 4°C for 1 h. To clear the protein extract, protein A-Sepharose (20 μl of a 50% suspension; Pharmacia Biotech, Uppsala, Sweden) was added to the cell lysate and incubated for 1 h, followed by centrifugation and collection of the supernatant. A specific antibody to Ang2 (50 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA) was added to the protein samples (500 μg) and rocked at 4°C for 1.5 h; 10 μg protein A-Sepharose was then added, and the sample was rocked another 1.5 h at 4°C. Protein A-Sepharose antigen-antibody conjugates were separated by centrifugation, washed five times, and boiled for 3 min in Laemmli sample 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 AII in Ang2 expression in vivo, we made an AII-induced corneal angiogenesis model as previously reported (32). We made a slow-release formulation polyacrylamide gel containing AII 10 umol/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 scarifying 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 acetone 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-amino-9-ethylcarbazole) (DAKO, Carpenteria, 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

AII stimulates Ang2 but not Ang1 mRNA expression in BRECs. To investigate the effect of AII on Ang1 and Ang2 mRNA expression, BRECs were treated with 10 nmol/l AII for the indicated times, and Northern blot analysis was performed. From several independent experiments, the effect of AII 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. 1*A*). AII also stimulated Ang2 mRNA expression in a dose-dependent manner with an EC_{50} of ~3 nmol/l and a maximal 2.4 ± 0.2-fold ($P < 0.01$) increase at a concentration of 10 nmol/l (Fig. 1*B*). In both experiments, AII did not affect the expression of Ang1 mRNA (Fig. 1*A* 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 AII stimulation (Fig. 1*C*).

Role of AII receptor subtypes AT1 and AT2 in the AII stimulated Ang2 mRNA expression in BRECs. To characterize the AII 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 AII (Fig. 2). In contrast with PD123319, losartan inhibited AII-induced Ang2 mRNA expression (Fig. 2). The

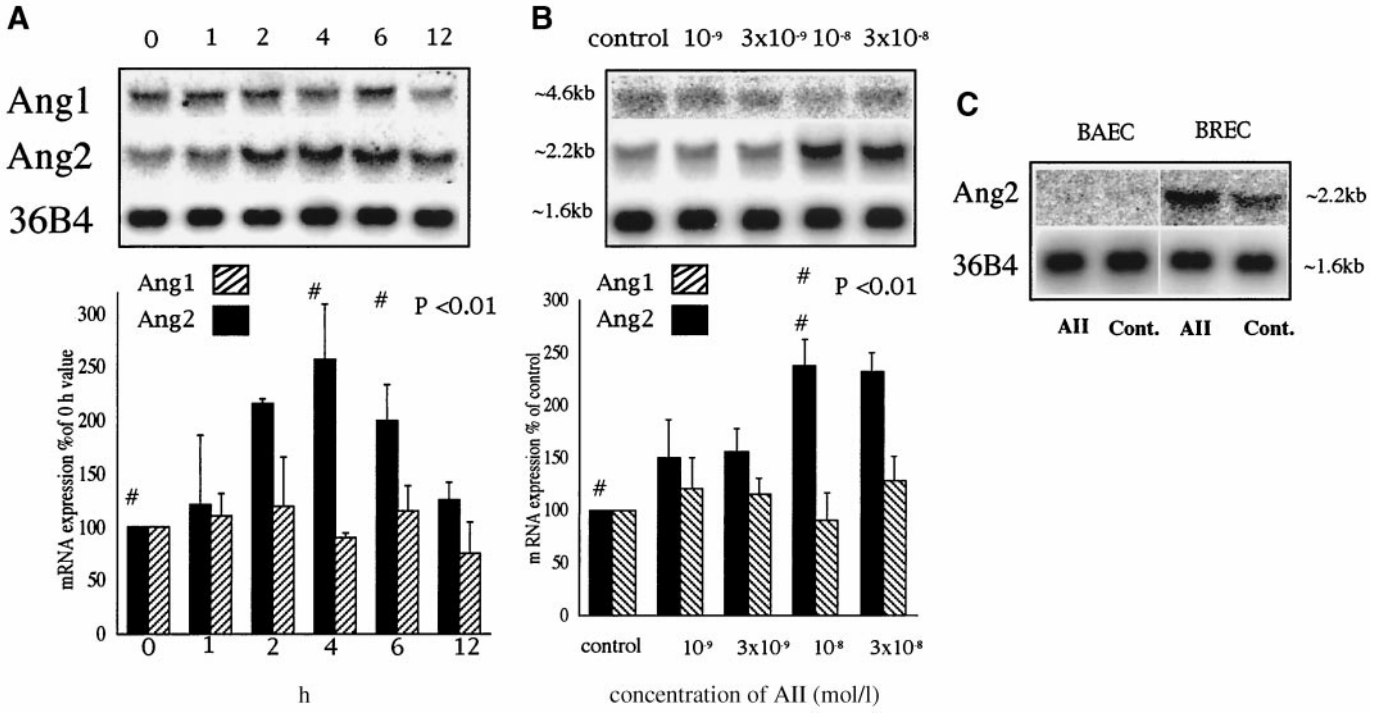

FIG. 1. Ang1 and Ang2 mRNA expression in BRECs and BAECs. AII stimulated Ang2 mRNA expression in a time- and dose-dependent manner. *A*: Time course study; BRECs were stimulated with 10 nmol/l AII for the indicated number of hours. Values are presented as percentages of the 0-h value ($n = 3$). Representative blots are shown (top panels). *B*: Dose-response study; BRECs were stimulated with the indicated doses of AII for 4 h. Representative blots are shown ($n = 3$) (top panel). Values are shown as percentages of the unstimulated controls. *C*: Ang2 mRNA expression in BRECs and BAECs; BAECs and BRECs were stimulated with 10 nmol/l AII for 4 h, and Northern blot analysis was performed. In contrast to BRECs, Ang2 mRNA expression in BAECs is very faint. Representative blots are shown ($n = 3$).

observed response suggests that AII stimulates Ang2 induction mainly through the AT1 receptor.

AII 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. 3*A*). 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. 3*B*). 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) was completely inhibited by 10 μmol/l GFX (Fig. 4*A*). The same concentration of GFX inhibited 10 nmol/l AII-induced Ang2 mRNA expression completely (Fig. 4*A*). Treatment with PD98059 reduced AII-induced Ang2 mRNA expression in a dose-dependent manner (0–50 μmol/l), and maximal inhibition (69.4 ± 15.6%) at 10 μmol/l was observed (Fig. 4*B*). PD98059 (10 μmol/l) also inhibited PMA (160 nmol/l)-induced Ang2 expression by 76.8 ± 8.1% (Fig. 5). These data suggest that AII upregulates Ang2 mRNA expression totally through the PKC-dependent pathway, and MAPK is also involved in the downstream PKC pathway.

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 ^{35}S-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. 7*A* 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. 7*D* and *E*). These data indicate that AII increased protein synthesis of Ang2 in BRECs.

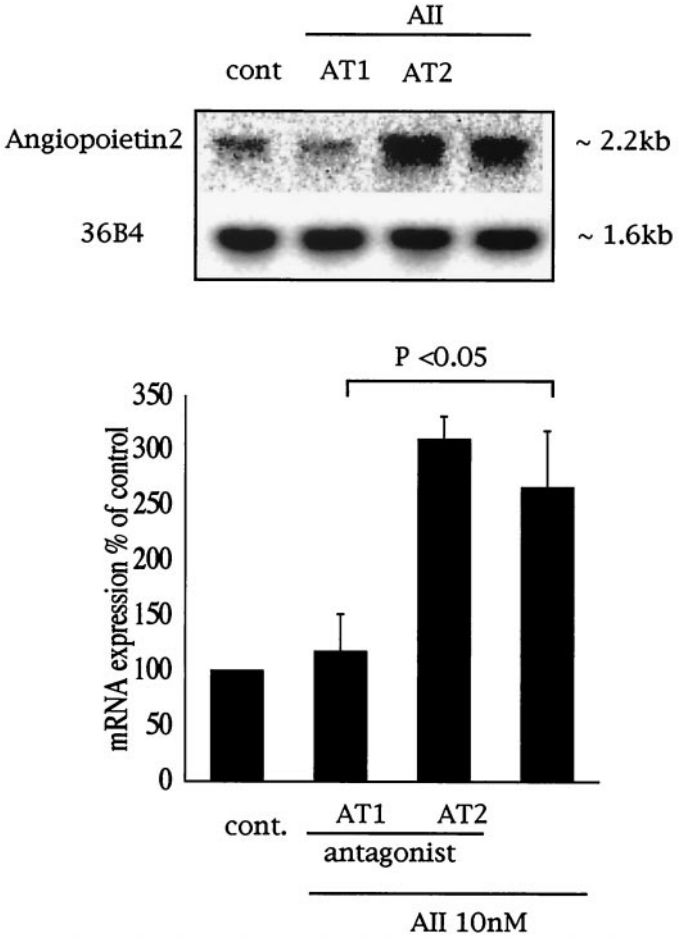

FIG. 2. Effect of AT1 and AT2 antagonists on AII-stimulated Ang2 mRNA expression. BRECs were pretreated with losartan or PD123319 for 15 min followed by stimulation of 10 nmol/l AII for 4 h. The AT1 antagonist completely blocked AII-induced Ang2 mRNA expression. Representative blots are shown (top panel). Results are expressed as a percentage of the uninhibited controls (n = 3) (bottom panel).

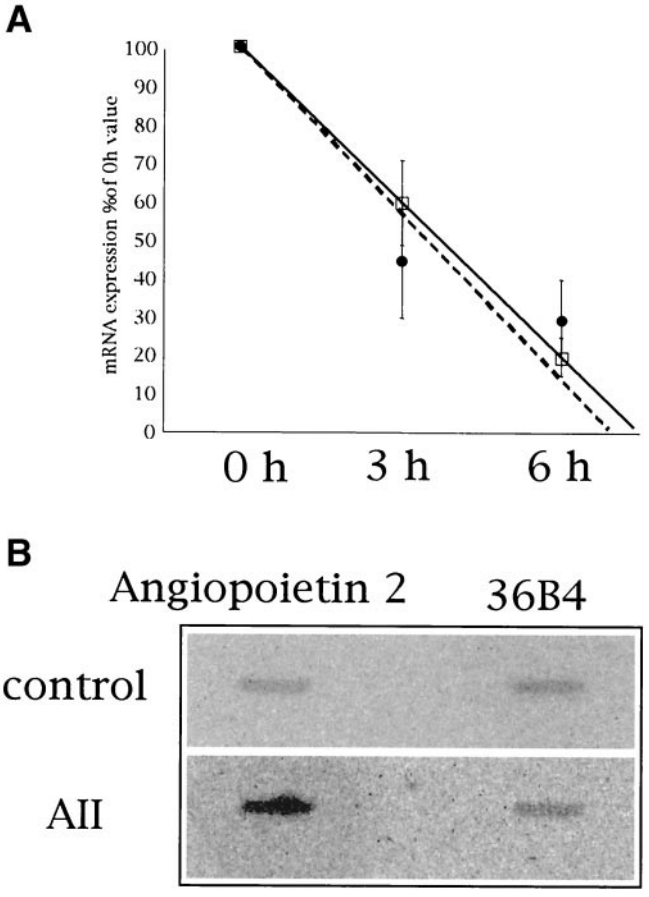

FIG. 3. To investigate whether the AII-induced increase of Ang2 mRNA is mediated through regulation of mRNA stability or transcription, we performed an actinomycin-D study and nuclear run-on analysis. *A*: The effect of actinomycin-D on AII-induced Ang2 mRNA expression. BRECs were exposed to either vehicle or 10 nmol/l AII for 4 h, and de novo mRNA transcription was inhibited by adding 4 μmol/l actinomycin-D. □, control cells; ●, AII-treated cells. Each plot is a percentage of the 0-h value in logarithmic scale. The dotted line and the solid line indicate the estimated decay line of Ang2 mRNA decay in AII-stimulated BRECs and nonstimulated BRECs, respectively. *B*: The effect of AII on the transcriptional rate of Ang2; the results of nuclear run-on analysis. BRECs were treated with 10 nmol/l AII or vehicle for 4 h. Equal amounts of ^{32}P-labeled RNA probes were hybridized to the nitrocellulose filters on which Ang2 and 36B4 cDNA had been blotted. AII did not change the stability of Ang2 mRNA, but it increased the rate of Ang2 gene transcription 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. 8*A*); in contrast, polyacrylamide gel alone failed to stimulate any significant new vessel formation (Fig. 8*B*). 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.

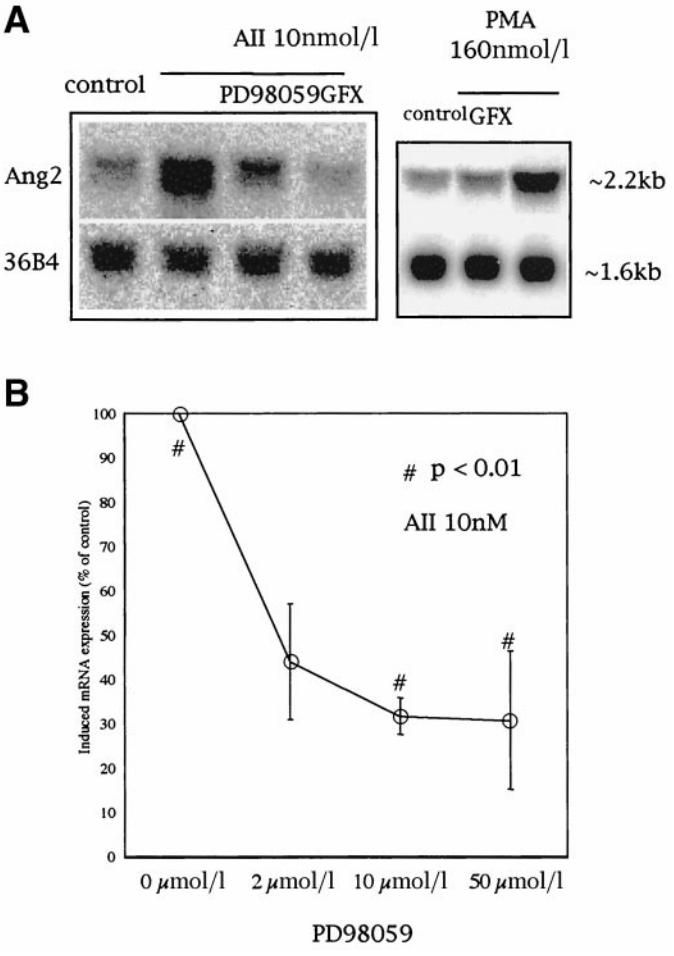

FIG. 4. The role of PKC and MAPK in AII- and PMA-induced Ang2 mRNA expression. *A*: BRECs were pretreated with GFX or PD98059 followed by stimulation with 10 nmol/l AII for 4 h (left panel), and BRECs were pretreated with GFX and stimulated with PMA 160 nmol/l for 4 h (right panel). PKC activation significantly 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 ($n = 3$).

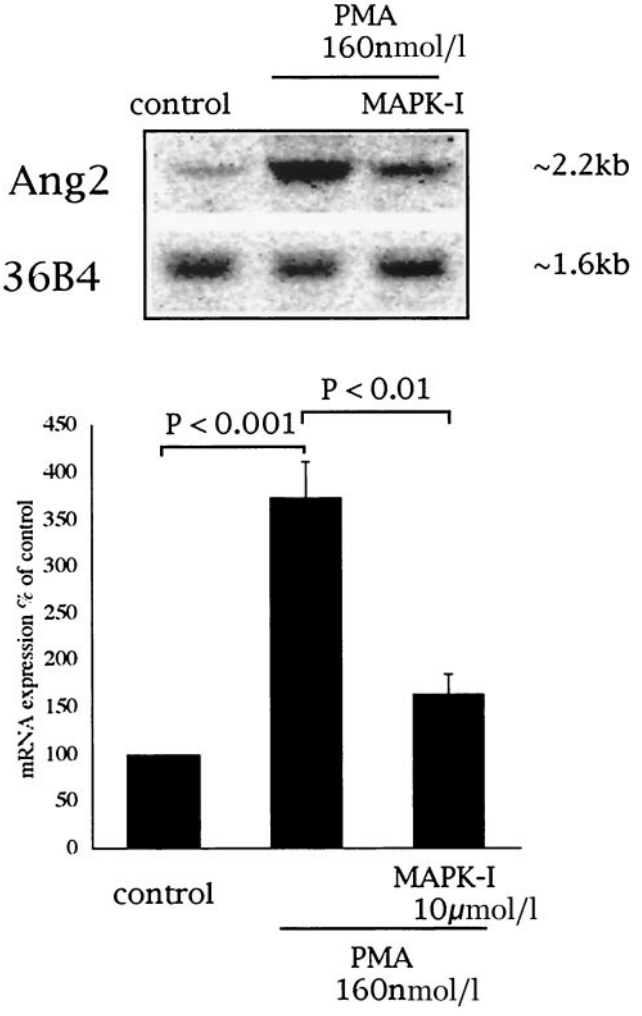

FIG. 5. Role of MAPK in PMA-induced Ang2 mRNA expression. BRECs were pretreated with PD98059 10 nmol/l for 15 min followed by stimulation with 160 nmol/l PMA for 4 h. The MAPK inhibitor inhibited PKC-induced Ang2 mRNA expression by 76.8 ± 8.1%. Results are expressed as percentages of the controls ($n = 3$) (bottom panel), and representative blots are shown (top panel).

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 β-1, 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.

Because VEGF regulates both angiopoietins in the similar way (31,40), one could imagine that VEGF possibly mediates the observed AII-dependent regulation of Ang1 and Ang2 mRNA expression. However, this is not the case, because AII did not alter the VEGF mRNA level in BRECs. 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 EC_{50} 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.

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

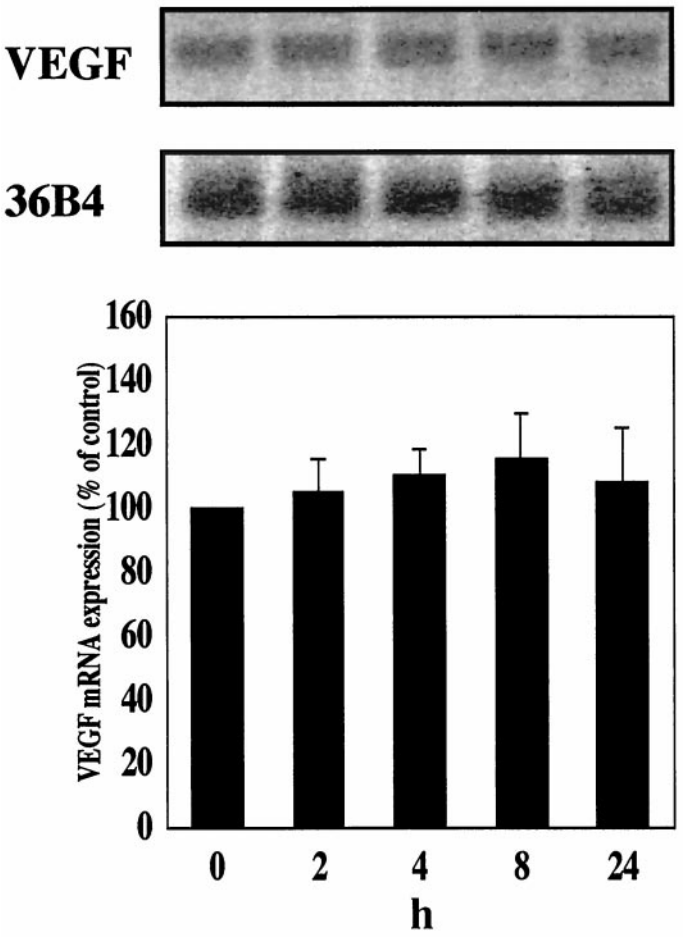

FIG. 6. AII does not stimulate VEGF mRNA expression in BRECs. BRECs were stimulated with 10 nmol/l AII for the indicated number of hours. Values are presented as percentages of the 0-h value (n = 3), and representative blots are shown (top panel). Northern blot analysis revealed that 10 nmol/l AII did 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 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. 4*A*). 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. 4*A*). MAPK inhibitor also blocked AII-induced Ang2 mRNA expression in a dose-dependent manner, with a maximal inhibition of 69.4 ± 15.6% (Fig. 4*B*). 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 ^{35}S-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

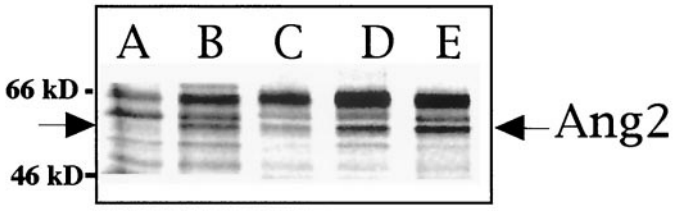

FIG. 7. Immunoprecipitation analysis of AII-stimulated and PMA-induced Ang2 protein synthesis. Starved BRECs were treated with 10 nmol/l AII, 160 nmol/l PMA, or vehicle for 5 h and labeled with ^{35}S-methionine, and SDS-PAGE was performed (lanes *C*, *D*, and *E*). Labeled proteins were visualized and 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).

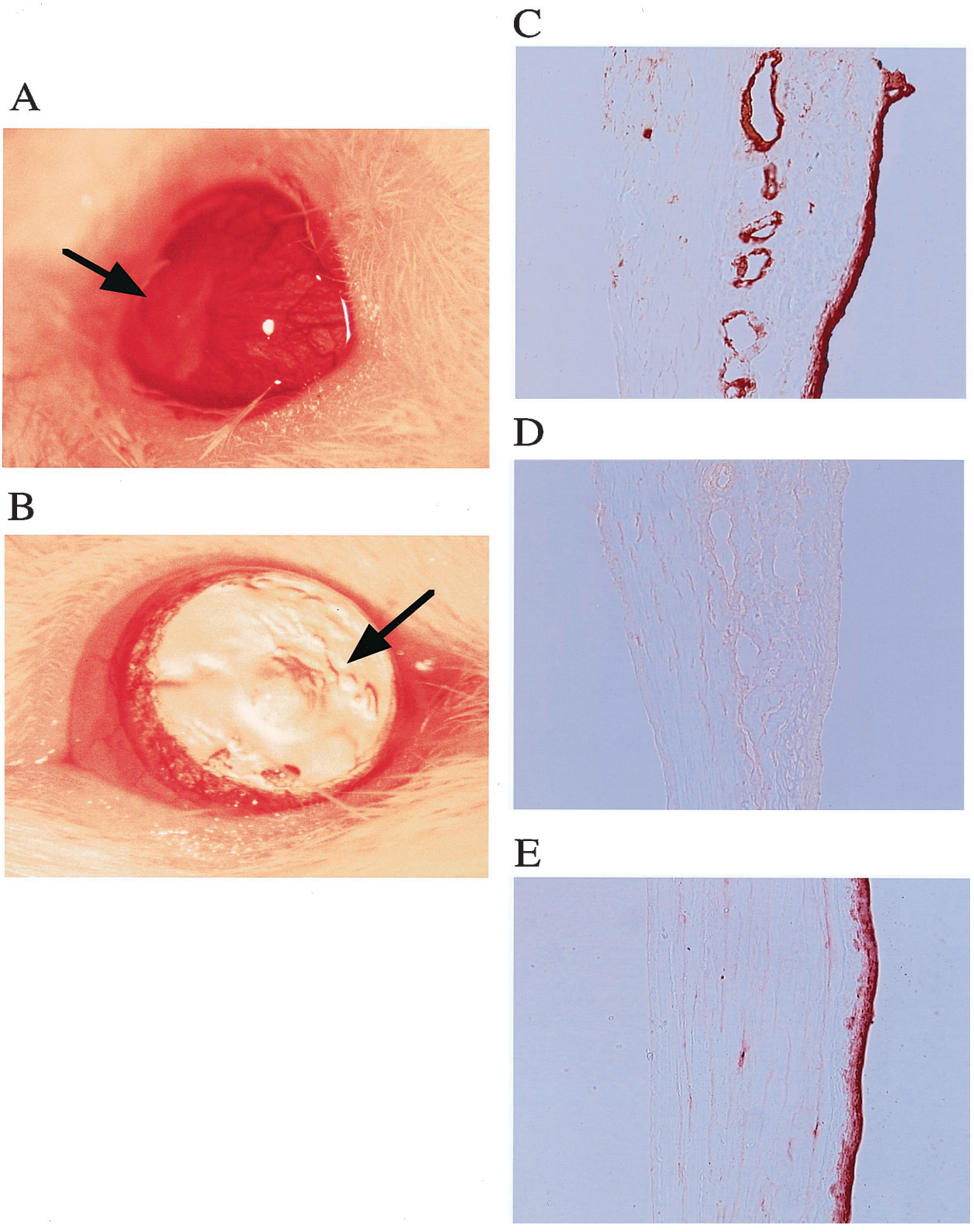

FIG. 8. In vivo analysis of AII-induced Ang2 expression. AII 10 μmol/l was incorporated in slow-release formulation polyacrylamide gel and implanted in a pocket made in the rat cornea. *A*: AII with polyacrylamide gel. *B*: Polyacrylamide gel alone (control). *C*: Immunohistochemical analysis with anti-Ang2 antibody in AII-stimulated cornea. *D*: Negative staining of AII-stimulated cornea; the primary antibody preincubated with the immunizing peptide (Santa Cruz Biotechnology) was used. *E*: Immunohistochemical analysis with Ang2 antibody in control cornea. Arrows indicate the implanted polyacrylamide gels.

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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REFERENCES

1. Risau W: Mechanisms of angiogenesis. *Nature* 386:671–674, 1997
2. Hanahan D: Signaling vascular morphogenesis and maintenance. *Science* 277:48–50, 1997
3. Millauer B, Wizigmann-Voos S, Schnurch H, Martinez R, Moller NP, Risau W, Ullrich A: High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 72:835–846, 1993
4. Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K: A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* 15:1751, 1996
5. de-Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT: The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 255:989–991, 1992
6. Sato TN, Qin Y, Kozak CA, Audus KL: Tie-1 and Tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. *Proc Natl Acad Sci U S A* 90:9355–9358, 1993
7. Puri MC, Rossant J, Alitalo K, Bernstein A, Partanen J: The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. *EMBO J* 14: 5884–5891,1995
8. Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W, Qin Y: Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 376:70–74, 1995
9. Dumont DJ, Gradwohl G, Fong GH, Puri MC, Gertsenstein M, Auerbach A, Breitman ML: Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev* 8:1897–1909, 1994
10. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC: Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376:62–66, 1995
11. Vikkula M, Boon LM, Carraway K3, Calvert JT, Diamonti AJ, Goumnerov B, Pasyk KA, Marchuk DA, Warman ML, Cantley LC, Mulliken JB, Olsen BR: Vascular dysmorphogenesis caused by an activating mutation in the receptor tyrosine kinase TIE2. *Cell* 87:1181–1190, 1996
12. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD: Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 277:55–60, 1997
13. Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD: Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* 87:1161–1169, 1996
14. Oh SJ, Jeltsch MM, Birkenhager R, McCarthy JE, Weich HA, Christ B, Alitalo K, Wilting J: VEGF and VEGF-C: specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. *Dev Biol* 188:96–109, 1997
15. Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD: Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 87:1171–1180, 1996
16. Wong AL, Haroon ZA, Werner S, Dewhirst MW, Greenberg CS, Peters KG: Tie2 expression and phosphorylation in angiogenic and quiescent adult tissues. *Circ Res* 81:567–574, 1997
17. Asahara T, Chen D, Takahashi T, Fujikawa K, Kearney M, Magner M, Yancopoulos GD, Isner JM: Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization. *Circ Res* 83:233–240, 1998
18. Dzau VJ: Molecular biology of angiotensin II biosynthesis and receptors. *Can J Cardiol* 11:21F–26F, 1995
19. Naftilan AJ, Pratt RE, Dzau VJ: Induction of platelet-derived growth factor A-chain and c-*myc* gene expressions by angiotensin II in cultured rat vascular smooth muscle cells. *J Clin Invest* 83:1419–1424, 1989
20. Delafontaine P, Lou H: Angiotensin II regulates insulin-like growth factor I gene expression in vascular smooth muscle cells. *J Biol Chem* 268:16866–16870, 1993
21. Tomita H, Egashira K, Ohara Y, Takemoto M, Koyanagi M, Katoh M, Yamamoto H, Tamaki K, Shimokawa H, Takeshita A: Early induction of transforming growth factor-beta via angiotensin II type 1 receptors contributes to cardiac fibrosis induced by long-term blockade of nitric oxide synthesis in rats. *Hypertension* 32:273–279, 1998
22. Powell JS, Clozel JP, Muller RK, Kuhn H, Hefti F, Hosang M, Baumgartner HR: Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. *Science* 245:186–188, 1989
23. Jeunemaitre X, Soubrier F, Kotelevtsev YV, Lifton RP, Williams CS, Charru A, Hunt SC, Hopkins PN, Williams RR, Lalouel JM, Carrol P: Molecular basis of human hypertension: role of angiotensinogen. *Cell* 71:169–180, 1992
24. Cambien F, Poirier O, Lecerf L, Evans A, Cambou JP, Arveiler D, Luc G, Bard JM, Bara L, Ricard S, Tiret L, Amouyel P, Alhene-Gelas F, Soubrier F: Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction. *Nature* 359:641–644, 1992
25. Chaturvedi N, Sjolie AK, Stephenson JM, Abrahamian H, Keipes M, Castellarin A, Rogulja Pepeonik Z, Fuller JH: Effect of lisinopril on progression of retinopathy in normotensive people with type 1 diabetes: the EUCLID Study Group. *Lancet* 351:28–31, 1998
26. Lewis EJ, Hunsicker LG, Bain RP, Rohde RD: The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy: the Collaborative Study Group. *N Engl J Med* 329:1456–1462, 1993
27. Otani A, Takagi H, Suzuma K, Honda Y: Angiotensin II potentiates vascular endothelial growth factor-induced angiogenic activity in retinal microcapillary endothelial cells. *Circ Res* 82:619–628, 1998
28. Otani A, Takagi H, Suzuma K, Oh H, Honda Y: Angiotensin II stimulates vascular endothelial growth factor (VEGF) expression in bovine retinal microcapillary pericytes. *Invest Ophthalmol Vis Sci* 41:1192–1199, 2000
29. King GL, Goodman AD, Buzney S, Moses A, Kahn CR: Receptors and growth-promoting effects of insulin and insulin-like growth factors on cells from bovine retinal capillaries and aorta. *J Clin Invest* 75:1028–1036, 1985
30. Liang P, Pardee AB: Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257:967–971, 1992
31. Oh H, Takagi H, Suzuma K, Otani A, Honda Y: Vascular endothelial growth factor (VEGF) and hypoxia regulate angiopoietin2 expression in retinal vascular endothelial cells. *J Biol Chem* 274:15732–15739, 1999
32. Fernandez LA, Twickler J, Mead A: Neovascularization produced by angiotensin II. *J Lab Clin Med* 105:141–145, 1985
33. Koblizek TI, Weiss C, Yancopoulos GD, Deutsch U, Risau W: Angiopoietin-1 induces sprouting angiogenesis in vitro. *Curr Biol* 8: 529–32,1998
34. Witzenbichler B, Maisonpierre PC, Jones P, Yancopoulos GD, Isner JM: Chemotactic properties of angiopoietin-1 and -2, ligands for the endothelial-specific receptor tyrosine kinase Tie2. *J Biol Chem* 273:18514–18521, 1998
35. Shweiki D, Itin A, Soffer D, Keshet E: Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359:843–845, 1992
36. Adamis AP, Miller JW, Bernal MT, D'Amico DJ, Folkman J, Yeo TK, Yeo KT: Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. *Am J Ophthalmol* 118:445–450, 1994
37. Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JE, Nguyen HV, Aiello LM, Ferrara N, King GL: Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med* 331:1480–1487, 1994
38. Tanaka S, Mori M, Sakamoto Y, Makuuchi M, Sugimachi K, Wands JR: Biologic significance of angiopoietin-2 expression in human hepatocellular carcinoma. *J Clin Invest* 103:341–345, 1999
39. Stratmann A, Risau W, Plate KH: Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis. *Am J Pathol* 153:1459–1466, 1998
40. Mandriota SJ, Pepper MS: Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. *Circ Res* 83:852–859, 1998
41. Danser AH, Derkx FH, Admiraal PJ, Deinum J, de-Jong PT, Schalekamp MA: Angiotensin levels in the eye. *Invest Ophthalmol Vis Sci* 35:1008–1018, 1994
42. Sramek SJ, Wallow IH, Tewksbury DA, Brandt CR, Poulsen GL: An ocular

renin-angiotensin system: immunohistochemistry of angiotensinogen. *Invest Ophthalmol Vis Sci* 33:1627–1632, 1992
43. Wagner J, Jan-Danser AH, Derkx FH, de-Jong TV, Paul M, Mullins JJ, Schalekamp MA, Ganten D: Demonstration of renin mRNA, angiotensinogen mRNA, and angiotensin converting enzyme mRNA expression in the human eye: evidence for an intraocular renin-angiotensin system. *Br J Ophthalmol* 80:159–163, 1996
44. Kohler K, Wheeler Schilling T, Jurklies B, Guenther E, Zrenner E: Angiotensin II in the rabbit retina. *Vis Neurosci* 14:63–71, 1997
45. Chiu AT, Herblin WF, McCall DE, Ardecky RJ, Carini DJ, Duncia JV, Pease LJ, Wong PC, Wexler RR, Johnson AL, Timmermans PBMWM: Identification of angiotensin II receptor subtypes. *Biochem Biophys Res Commun* 165:196–203, 1989
46. Whitebread S, Mele M, Kamber B, de-Gasparo M: Preliminary biochemical characterization of two angiotensin II receptor subtypes. *Biochem Biophys Res Commun* 163: 284–291,1989
47. Matsubara H: Pathophysiological role of angiotensin II type 2 receptor in cardiovascular and renal diseases. *Circ Res* 83:1182–1191, 1998
48. Akishita M, Ito M, Lehtonen JY, Daviet L, Dzau VJ, Horiuchi M: Expression of the AT2 receptor developmentally programs extracellular signal-regulated kinase activity and influences fetal vascular growth. *J Clin Invest* 103:63–71, 1999
49. Kim S, Izumi Y, Yano M, Hamaguchi A, Miura K, Yamanaka S, Miyazaki H, Iwao H: Angiotensin blockade inhibits activation of mitogen-activated protein kinases in rat balloon-injured artery. *Circulation* 97:1731–1737, 1998
50. Li X, Lee JW, Graves LM, Earp HS: Angiotensin II stimulates ERK via two pathways in epithelial cells: protein kinase C suppresses a G-protein coupled receptor-EGF receptor transactivation pathway. *EMBO J* 17: 2574–2583, 1998
51. Murasawa S, Mori Y, Nozawa Y, Gotoh N, Shibuya M, Masaki H, Maruyama K, Tsutsumi Y, Moriguchi Y, Shibazaki Y, Tanaka Y, Iwasaka T, Inada M, Matsubara H: Angiotensin II type 1 receptor-induced extracellular signal-regulated protein kinase activation is mediated by Ca^{2+}/calmodulin-dependent transactivation of epidermal growth factor receptor. *Circ Res* 82: 1338–1348, 1998
52. Cobb MH, Goldsmith EJ: How MAP kinases are regulated. *J Biol Chem* 270:14843–14846, 1995
53. Tian Y, Smith RD, Balla T, Catt KJ: Angiotensin II activates mitogen-activated protein kinase via protein kinase C and Ras/Raf-1 kinase in bovine adrenal glomerulosa cells. *Endocrinology* 139:1801–1809, 1998