Angiotensin II–Stimulated Vascular Endothelial Growth Factor Expression in Bovine Retinal Pericytes

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PURPOSE. Angiotensin II (AII) has been shown to play a role in many vascular diseases. In the study described, the effect of AII on vascular endothelial growth factor (VEGF) expression and related intracellular signaling mechanism was investigated in bovine retinal microcapillary pericytes.

METHODS. Cultured bovine retinal microvascular endothelial cells and pericytes were prepared. VEGF expression was determined by Northern blot analysis and immunoprecipitation assay. Cell proliferation was assessed by DNA content growth assay. Reporter gene studies were performed to identify the AII responsible transcription-activating region of VEGF gene.

RESULTS. Angiotensin II induced a significant increase in VEGF mRNA in a time- and dose-dependent manner. Angiotensin II type I receptor antagonist inhibited this effect. Angiotensin II activates the transcription of VEGF gene without changing the mRNA half-life, and the AII responsible region was found in the 5′-flanking region of the VEGF gene. Angiotensin II also increased the expression of c-fos and c-Jun mRNA, and antisense oligonucleotides against c-Fos blocked the AII-induced VEGF mRNA expression. The conditioned media of AII-stimulated pericycle cultures had a growth-promoting effect on endothelial cells, and this effect was inhibited almost completely by VEGF neutralizing antibody.

CONCLUSIONS. These findings suggest that AII might induce angiogenic activity through a paracrine function of VEGF in retinal microvascular cells. (Invest Ophthalmol Vis Sci. 2000;41:1192–1199)

R etinopathy is a major complication of diabetes mellitus and is one of the leading causes of vision loss in developed countries.1 Recent studies have shown that vascular endothelial growth factor (VEGF) plays a major role in the initiation and development of this particular form of retinopathy. VEGF is a potent angiogenic factor2–4 and vasopermeability factor5 and has been reported to generate a procoagulant state by induction of von Willebrand factor and a tissue factor.5,6 VEGF per se is sufficient to produce many of the vascular abnormalities common to diabetic retinopathy,7 and an increase in VEGF expression is seen in retinas of diabetic patients with little or no retinopathy.8 Furthermore, VEGF expression is increased by ischemia,9 and suppression of VEGF has been shown to inhibit neovascularization in animal models of retinal ischemia.10,11 VEGF levels are elevated also in patients with proliferative retinopathy and decrease after successful laser treatment,12,13 suggesting its importance in the early and proliferative stage of retinopathy.

Angiotensin II (AII) is known to be a key factor in cardiovascular homeostasis, and one that has many functions.14 Angiotensin II also has a growth-promoting effect and has been reported to regulate the growth of vascular smooth muscle cells (SMCs)15 and to stimulate the induction of many growth factors.16–19 Based on these experimental data and clinical evidence, the renin-angiotensin system (RAS) is thought to play an important role in many cardiovascular disorders. Recent studies suggest that abnormalities in the RAS play a role also in the progression of diabetic nephropathy and retinopathy.20–24 In diabetic retinopathy, angiotensin-converting enzyme (ACE) inhibitors have been reported to improve the blood–retina barrier and to have favorable effects on patients with diabetic retinopathy.24,25 Furthermore, intraocular and serum levels of AII, prorenin, and ACE have been reported to be correlated with the severity of retinopathy.20–22

VEGF mediates its effects through endothelial cell–specific, high affinity phosphotyrosine kinase receptors: Flt-1 (VEGFR1)26 and KDR/Fk-1 (VEGFR2).26–27 Recently VEGFs have been found to appear to interact with a neuronal cell–guidance receptor, neuropilin-1 (NP-1).28 Previously, we reported that AII potentiates VEGF-mediated angiogenic activities of bovine retinal endothelial cells (BRECs) through upregulation of VEGFR2 expression, which suggests a substantial role for RAS in the pathogenesis of diabetic retinopathy.29 In that study, we found no stimulatory effect of AII on VEGF expression in BRECs. In contrast, a stimulatory effect of AII on VEGF expression has recently been reported in human vascular SMCs30 and rat heart endothelial cells.31

To further investigate how the RAS is involved in the pathogenesis of diabetic retinopathy, we determined the effect of AII on VEGF expression in bovine retinal microcapillary pericytes (BRPs), which are the other component cells of retinal microvasculature.

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METHODS

Cell Cultures

Primary cultures of BRPs and BRECs were isolated as previously described. Briefly, bovine retinas were homogenized and the homogenate was passed over 210-, 88-, and 53-μm nylon meshes (Nippon Kikagaku Kikai, Tokyo, Japan). The materials caught up by the 53- and 88-μm meshes were plated on the fibronectin-coated dishes (Iwaki, Tokyo, Japan). BRPs (fraction from 53-μm mesh) were grown with Dulbecco’s modified Eagle’s medium (DMEM) with 15% fetal bovine serum (FBS; Wheaton, Pipersville, PA). BRECs (fractions from both 88- and 53-μm mesh) were cultured in DMEM with 5.5 mM glucose, 10% plasma-derived horse serum (PDHS; Wheaton), 50 mg/l heparin, and 50 U/l endothelial cell growth factor (Boehringer Mannheim, Indianapolis, IN). To keep homogeneity of the cells, contaminated cells were excluded by a weeding procedure. When the cells reached subconfluence, BRPs were passaged after trypsinization, and cells from the 2nd and 3rd passages were used for the experiments after serum starvation with 0.5% PDHS for BRECs and 0.1% bovine serum albumin (BSA) for BRPs. For all receptor antagonist studies, we used 1 μM of all type 1 receptor (AT₁)–specific antagonist DuP735 (Merck Research Laboratories, Rahway, NJ), a nonpeptide imidazole derivative or nonpeptide All type 2 receptor (AT₂) antagonist, PD123319 (Research Biochemicals International, Natick, MA) for 15 minutes, followed by stimulation with AII for 3 hours. In all experiments we used vehicle (DMEM containing 0.1% BSA) as control.

Pericyte and Endothelial Cell Identification

Endothelial cell homogeneity was confirmed by immunoreactivity with anti-factor VIII antibodies (Dako, Glostrup, Denmark) analyzed by confocal microscopy. Pericyte homogeneity were confirmed by its characteristic features and immunoreactivity with 3G5 monoclonal antibodies (a generous gift from George L. King, Joslin Diabetes Center, Boston, MA) that was negative for SMCs. To avoid contamination of endothelial cells and glial cells, we confirmed the negative immunoreactivities for anti-factor VIII antibodies or anti–glial fibrillary acidic protein, respectively, by confocal microscopy.

Northern Blot Analysis

Total RNA was isolated from individual tissue culture plates using guanidine thiocyanate. Northern blot analysis was performed on 15 μg total RNA after 1% agarose–2 M formaldehyde gel electrophoresis and subsequent capillary transfer to Biodyne nylon membranes (Pall BioSupport, East Hills, NY) and ultraviolet cross-linking using a FUNA-UV-LINKER (model FS-1500; Funakoshi, Tokyo, Japan). Radioactive probes were generated using Amersham Megaprime labeling kits and [32P]-dCTP (DuPont, Wilmington, DE). Blots were prehybridized, hybridized, and washed in 0.5× SSC, 5% sodium dodecyl sulfate (SDS) at 65°C with 4 changes over 1 hour in a rotating hybridization oven (TAITEC, Koshigaya, Japan). All signals were analyzed using a densitometer (model BAS-2000II; Fuji Photograph Film, Tokyo, Japan), and lane loading differences were normalized using a 36B4 cDNA probe, which hybridizes to acidic ribosomal phosphoprotein PO. Human VEGF cDNA (generously provided by Loyd P. Aiello, Boston, MA) and c-fos DNA (Takara shuzo, Shiga, Japan) and c-jun cDNA (Calbiochem, La Jolla, CA) were used as probes.

Analysis of VEGF mRNA Half-Life

To determine whether the increase in VEGF mRNA was caused by an increase in transcription, BRPs were exposed to 5 μg/ml actinomycin D (Wako, Osaka, Japan) after 3 hours of incubation with vehicle or AII (10 nM). The total RNA was then extracted, and Northern blot analysis was performed.

VEGF Protein Synthesis

Subconfluent cultures of BRPs were treated with 10 nM All or vehicle for 3 hours. The culture media were then replaced with labeling media (DMEM minus methionine and cysteine, 100 μCi 3S–methionine and cysteine) supplemented with All or vehicle, as described above. After 2 hours’ incubation, the medium was removed and the cells were lyzed in solubilizing buffer (50 mM HEPES, pH 7.4, 10 mM EDTA, 100 mM NaF, 10 mM Na pyrophosphate, 1% Triton X-100, 10 mM NaVO₄, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride) at 4°C for 1 hour. Protein concentrations were measured by the Pierce BCA procedure (BCA protein assay; Pierce, Rockford, IL). Specific antibody to VEGF (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 hours, and then 10 μg protein A Sepharose was added and rocked for another 1.5 hours at 4°C. Protein A Sepharose antigen antibody conjugates were separated by centrifugation, washed 5 times, and boiled for 3 minutes in Laemmli sample buffer to denature. 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 BAS-2000II densitometer (Fuji Photograph Film).

Reporter Gene Studies

A series of plasmid constructs were made from a genomic DNA clone of the human VEGF gene, which contained approximately 2.5 kb of the 5’-flanking region with the putative promoter and 1 kb of the 5’-untranslated region that was generously provided by Scios (Sunnyvale, CA). These constructs have a series of deletion constructs of a region from 80 bp up to 3.2 kb upstream of the translation start site of the VEGF gene and subcloned upstream of the luciferase gene in the promotorless luciferase reporter vector pGL2-basic vector (Promega, Madison, WI), as shown Figure 3. As a control plasmid, we used renilla luciferase pRL-SV40 vector (Toyo Ink, Tokyo, Japan). Plasmids were transfected into BRPs by LipofectAMINE reagent (Life Technologies, Gaithersburg, MD). BRPs were seeded in 35-mm-diameter culture dishes (Iwaki) and incubated until the cells became subconfluent. A total of 1.5 μg test plasmid and 0.05 μg control plasmid was mixed with LipofectAMINE and added to the cells. After 5 hours’ incubation, the mixture was replaced by normal growth medium and incubated an additional 20 hours. The cells were serum-deprived for 24 hours and then stimulated with 10 nM All or vehicle for 18 hours. Cell extracts were then prepared by Lysis Buffer (Toyo Ink), and luciferase and renilla luciferase activity were measured by Luminoskan (Labsystems, Helsinki, Finland) with a Luciferase Dual Assay System (Toyo Ink). To standardize the transfection efficiency, luciferase activity was divided by renilla luciferase activity, and the degree of induction by All for each test plasmid was determined as the ratio of standardized luciferase activity in All-treated cells to that in vehicle-treated cells.
Antisense Oligonucleotide Inhibition

Antisense oligonucleotides to the 5' ends of c-fos (5'-TGCGTGTTGAAGCCCGAGAA-3') and SP-1 (5'-CACCACAGCTGTCATTTCATCCATGG-3') and the corresponding sense oligonucleotides that were purified by high-performance liquid column chromatography were prepared. As described before, these oligonucleotides were transfected into the cells without any treatment. After cells were incubated with DMEM containing 1% FBS for 40 hours, 5mM oligonucleotides were added and incubated an additional 8 hours. The cells were then washed with serum-free DMEM and incubated with or without 10 nM AII for 4 hours.

BREC Growth Assay

Serum-deprived BRPs were treated with AII (10 nM) or vehicle for 24 hours, and the conditioned medium was prepared. BRECs were plated in 24-well plates (Iwaki) at a density of 3 x 10^3 cells/well in DMEM containing 10% calf serum (GIBCO, Grand Island, NY). After 24 hours at 37°C, the medium was replaced with the conditioned medium. After 4 days' incubation, the cells were lysed and DNA concentrations in each well were measured by DyNA Quant 200 (Hofer, San Francisco, CA).

Statistical Analysis

Determinations were performed in triplicate, and experiments were performed at least three times. Results were expressed as mean ± SE, unless otherwise indicated. For multiple treatment groups, a factorial ANOVA followed by Fisher's least significant difference test was performed. Statistical significance was accepted at P < 0.05.

RESULTS

Effects of AII on VEGF mRNA Expression in BRPs

From results of several independent experiments it was clear that the effect of AII (10 nM) was time-dependent, with a maximal 6.5 ± 0.4-fold increase at 4 hours (P < 0.01), which diminished progressively up to 12 hours (Fig. 1A). To define the concentration dependency of AII-induced VEGF mRNA expression in BRPs, we used the 3-hour time point. In this experiment, AII, 3 to 100 nM, significantly stimulated the induction of VEGF mRNA with an EC50 of approximately 3 nM and a maximal 3.1 ± 0.7-fold (P < 0.01) increase at 10 nM (Fig. 1B). AT1 antagonist but not AT2 antagonist inhibited the AII-induced VEGF mRNA expression (P < 0.05; Fig. 1C).

Effect of AII on Half-Life of VEGF mRNA

Cells were treated with actinomycin D (5mM), a de novo gene transcription inhibitor, and Northern blot analysis was performed to measure VEGF mRNA levels. The half-life of VEGF mRNA without AII was 2.1 hours, and after AII exposure the half-life had not changed significantly (Fig. 2).

AII-Responsible Region in 5'-Flanking Region of VEGF Gene

Induced luciferase activity by AII was observed in cells transfected with KpnI–NarI (23317 to 281), SpeI–Nhel (2848 to −984), and SacI–Nhel (2218 to −984) sites; however, no induction was observed in BanLI–Nhel (1925 to −984), PstI–Nhel (1828 to −984), or ApaI–Nhel (1169 to −984) fragment–transfected cells (Fig. 3). Two plates were transfected for each test plasmid at the same time, and three independent experiments were performed. From these data it appears that
AII-responsible region is located in the SacI–BanI fragment (293 bp, 2221 to 2192, indicated by the solid underline in Fig. 3).

AII-Stimulated c-fos and c-jun mRNA Expression in Pericytes

The AII-responsible fragment of the VEGF gene that we found in this study contains potential binding sites for transcription factors SP-1, AP-1, and HIF-1. To investigate the role of AP-1 in AII-induced VEGF expression, we performed Northern blot analysis. By stimulation with 10 nM AII, 12.5 ± 0.7-fold (P < 0.001, at 1 hour) and 4.4 ± 0.1-fold (P < 0.001, at 2 hours) increases in c-fos and c-jun mRNA expression were observed, respectively (Fig. 4).

Inhibition of VEGF mRNA Production by Antisense Oligonucleotides Against c-Fos

To further examine the transcriptional factors involved in AII-induced VEGF expression, we assessed the effects of antisense oligonucleotides targeted against c-Fos and SP-1. BRPs were pretreated with 5 μM oligonucleotides for 8 hours before

**FIGURE 2.** Effect of actinomycin D (ACD) on VEGF mRNA expression in response to AII in BRPs. BRPs were exposed to either vehicle or AII (10 nM) for 3 hours, and de novo mRNA transcription was inhibited by the addition of ACD 5 μM. Total RNA was extracted at 1 hour and 3 hours, and Northern blot analysis was performed to detect VEGF mRNA level. The y-axis represents VEGF mRNA level and x-axis represents time after treatment. Each plot is a percentage of 0 hour value in logarithmic scale. The half-lives are indicated by drawing a line at the 50% point. Data from three independent experiments (n = 3) are shown.

**FIGURE 3.** VEGF-luciferase deletion constructs and degrees of induction by AII stimulation. (A) Linear map of the 5′-flanking and 5′-untranslated regions of the human VEGF gene. Nucleotides were numbered from the translation start site, and the transcription start site is indicated with an arrow. (B) VEGF-luciferase deletion constructs and degrees of induction by AII. Data from three independent experiments (n = 3) are shown.

**FIGURE 4.** All-stimulated c-fos and c-jun mRNA expression in BRPs. Total RNA was isolated at the indicated times after being stimulated with 10 nM AII. Northern blot analysis was performed with cDNA probes for c-fos and c-jun. Representative blots from three experiments (n = 3, *P < 0.0001, †P < 0.0001) are shown (top).
stimulation with AII. Antisense oligonucleotides against c-Fos blocked the AII-induced VEGF mRNA expression by 81.5% (P < 0.01, Fig. 5). c-Fos sense and SP-1 antisense oligonucleotides did not affect AII-induced VEGF mRNA induction, and c–fos antisense oligonucleotides did not affect the basal VEGF synthesis (data not shown).

Increase of VEGF Protein Synthesis by AII
Bands at approximately 23 and 21 kDa were detected by immunoprecipitation with a rabbit anti-human VEGF antibody, and these are related to VEGF isoforms 165 and 121, respectively. The major band, which represents VEGF 165, was increased 3.7 ± 0.6-fold by AII stimulation at 10 nM (Fig. 6).

Growth Effect of Conditioned Medium from All-Treated BRPs on BRECs
From five independent assays, conditioned media of the All-treated BRPs increased the proliferation of BRECs 1.5 ± 0.1-fold above control media levels (P < 0.01), and this effect was inhibited almost completely by adding VEGF neutralizing antibody (R&D Systems, Minneapolis, MN; Fig. 7).

**DISCUSSION**

Pericytes are intramural cells that surround endothelial cells in capillaries and postcapillary venules and have multiple physiologic functions, including regulation of vascular tone, vascular permeability, and endothelial growth and differentiation in retinal microvessels. Pericytes are the cells most proximal to endothelial cells and are in intimate contact via adhesion plaques, gap junctions, and pericytic processes. The ratio of pericytes to endothelial cells is higher in the retina than in other tissues, suggesting that interaction between pericytes and endothelial cells is important in the retinal microcirculation. To the best of our knowledge, this study is the first to demonstrate that AII induces VEGF in retinal microcapillary
pericytes and that VEGF released by the pericytes stimulates retinal endothelial growth in a paracrine manner. We have demonstrated that all significantly increases the level of VEGF mRNA in a time- and dose-dependent manner. We also detected the new protein synthesis of VEGF protein by immunoprecipitation assay (Fig. 6). In contrast to our results, a lack of significant effect of AII on VEGF expression was recently reported in bovine retinal pericytes.50 In that report, the authors derived conclusions from the experiments using a single dose of 1 μM AII. Our dose-dependent study revealed that peak response was observed at 10 nM, and the higher concentrations had less stimulatory effect on VEGF expression. The discrepancy probably results from the lack of dose-dependent experiments in that study. The significant response was observed at concentrations higher than normal circulating levels (1 nM). It is likely that local concentrations of AII in retinal microvasculature are much higher than serum and vitreous levels, because an autocrine–paracrine production system for VEGF mRNA is 2.1 hours, which is in concordance with previous reports.51

Angiotensin II has two major receptor subtypes, AT1 and AT2.52 Most of the actions of AII are mediated by AT1, but actions of the AT2 are not well understood.11 In the present study, the effect of AII on VEGF expression was completely inhibited by the AT1 receptor antagonist but not by the AT2 antagonist, suggesting that AII-induced VEGF expression is mediated by AT1 receptors. Although not to a significant degree, the average VEGF expression was increased by AT2 receptor blockade. This might suggest that AT2 receptors mediate an inhibitory effect on VEGF induction, which is in agreement with previous reports.52,53 However, further study is needed on the distribution of AII receptors and the changes in the AII effect on retinal microvascular systems.

The AII-induced increase in VEGF mRNA was rapid and peaked at 4 hours (Fig. 1A). Experiments in which actinomycin D was used to inhibit RNA synthesis indicate that the half-life of VEGF mRNA is 2.1 hours, which is in concordance with previous reports in SMCs,54 and AII did not primarily change the mRNA stability of VEGF (Fig. 2). This suggests that AII-induced VEGF mRNA induction is most likely through transcriptional regulation. To further investigate transcriptional regulation of the VEGF gene, we performed transient transfection reporter assay using a series of deletion constructs of the 5‘-flanking region of the human VEGF gene.42,43 As expected, reporter gene activities were upregulated by AII. In addition, we found that a 293-bp fragment (SacI–BamHI) of the VEGF gene has a responsible element for AII stimulation (Fig. 3). Angiotensin II is reported to stimulate the expression of c-fos and c-jun and their respective proteins, c-Fos and c-Jun, which constitute the heterodimer complex called AP-1, which transactivates many genes that have a TPA responsive element (TRE) in their promoter region.55 Because the AII-responsive region we found contains potential binding sites for AP-1, we further investigated a role for AP-1 in the induction of VEGF in BRPs. Northern blot analysis revealed rapid and marked c-jun and c-fos induction by AII in BRPs (Fig. 4), and pretreatment with c-fos antisense oligonucleotides blocked the AII-induced VEGF mRNA expression (Fig. 5). SP-1 antisense and c-fos sense oligonucleotides did not affect AII-induced VEGF expression. These data might suggest a predominant role of AP-1 and its TRE activation in AII induction of VEGF in BRPs.

To investigate AII effects on the retinal pericyte–endothelial cell paracrine system, we determined growth-promoting effects of conditioned media from AII-treated pericyte cultures on BRECts. Conditioned media from AII-treated BRPs had a significantly greater stimulatory effect on BREC proliferation than did the media from unstimulated BRPs. As we reported previously, AII itself had no significant effect on BREC proliferation.29 These data suggest that AII induces a paracrine molecule in BRPs, which activates endothelial cell proliferation. Angiotensin II has been reported to regulate the induction of several autocrine growth factors, such as platelet-derived growth factor (PDGF) A–chain, transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF), and insulin-like growth factor I (IGF I).14–17 We did not examine the effect of AII on the regulation of these growth factors in BRPs; however, the addition of VEGF neutralizing antibody almost completely abolished growth stimulation capacity of the conditioned media (Fig. 7). This observation suggests that VEGF is probably a predominant factor that mediates paracrine activation of endothelial cell growth.

It has been suggested that the contact between pericytes and endothelial cells caused inhibition of endothelial cell growth.48 However, our observation that VEGF, which was produced in pericytes, induced endothelial cell growth in a paracrine manner indicates a proliferative effect of pericytes. In vivo, pericytes may have both effects, the balance of which is important for controlling endothelial cell growth. Under normal conditions, pericytes suppress endothelial cell growth by contact with endothelial cells, but in the later stage of diabetic retinopathy, thickening of the basement membrane suppresses the contact, the inhibitory effect is overcome by its stimulatory effects. Our data suggest that RAS might be one of the important factors regulating this growth-promoting effect. Because pericyte loss is very advanced in the later stages of diabetic retinopathy, pericytes might contribute little to VEGF activity. However, VEGF has been shown to have a possible role in the early stages of retinopathy. This paracrine action of VEGF produced by pericytes might be more important in the early stages.

Together with our previous finding that all potentiates VEGF-induced angiogenic activity through upregulation of VEGF receptor in retinal endothelial cells,29 the present study further clarifies the role of RAS in the development of diabetic retinopathy. Angiotensin II has a prominent stimulatory effect not only on VEGF receptor expression in endothelial cells but also on VEGF production in pericytes in the retinal microcirculation. Further studies, including an in vivo study to see the effect of AT1 antagonist, will strengthen this hypothesis.

In therapeutic aspects, inhibition of RAS is thought to be beneficial for the treatment of diabetic retinopathy. Indeed, the beneficial effects of ACE inhibition in patients with diabetic retinopathy have recently been shown in the EUCLID study and other studies.23,24,56 Our studies revealed that AT1 receptor mediation is predominant for the AII-induced responses. An AT1 blocker and ACE inhibitors might effectively prevent diabetic retinopathy.

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