Angiogenisis II Potentiates Vascular Endothelial Growth Factor–Induced Angiogenic Activity in Retinal Microcapillary Endothelial Cells

Atsushi Otani, Hitoshi Takagi, Kiyoshi Suzuma, Yoshihito Honda

Abstract—Angiotensin II (Ang II) plays a role in the development of many vascular diseases. In the present study, we have investigated the effect of Ang II on vascular endothelial growth factor (VEGF) receptor expression and VEGF-induced angiogenic activity in bovine retinal microcapillary endothelial cells (BRECs). Ang II induced a significant increase of kinase domain–containing receptor/total liver kinase (KDR/Flk-1) mRNA in a time- and dose-dependent manner, with a maximal 4.3±0.8-fold increase after a 4-hour stimulation. Ang II increased the rate of KDR gene transcription by 5.4-fold, whereas the half-life of KDR mRNA was not increased significantly. The increase depended partially on new protein synthesis. The Ang II–induced KDR mRNA increase was inhibited by either [Sar², Ile⁸]angiotensin or angiotensin type 1 receptor antagonists but was not significantly altered by angiotensin type 2 receptor antagonists. The PKC inhibitor reduced Ang II–induced KDR mRNA expression by 70±15%. The tyrosine kinase inhibitor reduced the Ang II– and phorbol 12-myristate 13-acetate–induced KDR mRNA increases by 35% and 44%, respectively. Ang II increased by 3.1-fold the ³⁵S-labeled KDR/Flk-1 immunoprecipitated by a specific antibody to KDR/Flk-1. Scatchard analysis demonstrated that Ang II induced a significant increase of binding sites without changing binding affinity. Ang II enhanced VEGF-induced cell growth and tube formation. Ang II itself had no effect on cell growth, tube formation, or mRNA levels of VEGF and Flt-1, whereas Ang II and other autocrine growth factors increased VEGF-induced angiogenic activity in BRECs. These findings suggest that Ang II might potentiate VEGF-induced angiogenic activity through an increase of the VEGF receptor KDR/Flk-1. (Circ Res. 1998;82:619-628.)

Key Words: diabetic retinopathy  vascular endothelial growth factor  KDR/Flk-1  angiotensin II  angiogenesis

Diabetic retinopathy is one of the major complications of diabetes mellitus and often results in catastrophic loss of vision. Although the pathogenesis of this complication is not fully understood, emerging evidence strongly implicates VEGF, not only in the ischemic retinal neovascularization observed in proliferative retinopathy but also in early stages of diabetic retinopathy. VEGF is a potent angiogenic factor and vasopromotability factor whose expression is increased by hypoxia, which is one of the primary stimuli for ocular neovascularization. VEGF mediates its effects through endothelial cell–specific high-affinity phosphotyrosine kinase receptors: Flt-1 (VEGFR1) and KDR/Flk-1 (VEGFR2). These two receptors have been demonstrated to be different in function. KDR–expressing cells show changes in morphology, chemotaxis, and mitogenicity on VEGF stimulation, whereas Flt-1–expressing cells lack such a response. Gene-knockout experiments also suggest differences of these receptor functions in the development of the vascular system. VEGF does not bind Flt-4 (VEGFR3), but a ligand for VEGFR3 has recently been cloned as VEGF-C, which binds KDR/Flk-1 as well.

Ang II is well known to be a key factor in cardiovascular homeostasis and to exert many actions, such as controlling vascular tone, hormone secretion, and neuronal effects on the heart, vascular system, kidneys, adrenal glands, and central nervous system. From experimental data and clinical evidence, the RAS is thought to play an important role in many cardiovascular disorders. Ang II has been reported to regulate cell growth of vascular SMCs and to stimulate the induction of PDGF, basic FGF, IGF, and other autocrine growth factors in SMCs and the induction of ET-1 in endothelial cells. These effects have been linked to myocardial infarction, myocardial proliferation after vascular injury, essential hypertension, and diabetic nephropathy, and ACE inhibitors have been reported to be beneficial in these diseases.

In diabetic retinopathy, intraocular and serum levels of ACE, prorenin, and Ang II have been reported to be correlated with the severity of retinopathy. ACE inhibitors have been reported to improve the blood-retina barrier in diabetic patients and to have a favorable effect on diabetic retinopathy. These reports suggest that Ang II may play a role in the development of diabetic retinopathy. How RAS is involved in the pathogenesis of diabetic retinopathy, however, has not been investigated in detail.

In the present study, we report that Ang II is a potent stimulant of VEGF-induced proliferation and tube formation.

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in retinal microvascular endothelial cells through the induction of the VEGF receptor KDR/Flik-1. This Ang II–induced KDR upregulation is transcriptionally regulated through AT1 receptors, with subsequent activation of both the PKC–dependent and tyrosine kinase–dependent signaling pathways.

Materials and Methods

Cell Cultures

Primary cultures of BRECs were isolated by homogenization and a series of filtration steps as previously described. Primary BRECs were grown on fibronectin (Sigma Chemical Co)–coated dishes (Iwaki Biodyne nylon membranes (Pall BioSupport) and ultraviolet cross-linking using a FUNA-UV-LINKER (FS-1500, Funakoshi Inc). Radioactive probes were generated using Amersham Megaprime labeling kits and [32P]dATP (Amersham). Blots were prehybridized, hybridized, and washed in 0.5× SSC and 5% SDS at 65°C with four changes over 1 hour in a rotating hybridization oven (TAITEC). All signals were analyzed using a densitometer (BAS-2000II, Fuji Photo Film), and lane loading differences were normalized using a 36B4 cDNA probe, which hybridizes to acidic ribosomal phosphoprotein P0. Human KDR cDNA was used as a probe (generously provided by Dr Loyd P. Aiello, Boston, Mass).

Analysis of KDR mRNA Half-Life

To determine whether the increase in KDR mRNA was caused by an increase in transcription, BRECs were exposed to actinomycin D (4 μmol/L, Wako) after 4 hours of incubation with vehicle or Ang II (10 nmol/L). The total RNA was then extracted, and Northern blot analyses were performed.

Nuclear Run-on Analysis

Confluent BRECs were serum-deprived for 18 hours in 0.1% BSA DMEM, followed by treatment with vehicle or Ang II (10 nmol/L) for 4 hours. 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. ATP, CTP, and GTP (500 mmol/mL each) and 3.7 MBq of [3P]labeled UTP (Amersham) were added to the nuclear suspension (100 μL) and incubated for 30 minutes. The samples were extracted with phenol/chloroform and precipitated. cDNA probes (KDR and 36B4, 10 ng) were then slot-blotted onto nitrocellulose filters (Schleicher & Schuell, Inc) and hybridized with the precipitated samples of equal counts per minute per milliliter in hybridization buffer at 45°C for 48 hours. The filters were washed, and the radioactivity was measured using the densitometer (BAS-2000II, Fuji Photo Film). The levels of KDR mRNA were normalized to 36B4 mRNA expression.

Immunoprecipitation Analysis of KDR/Flik-1

Confluent BRECs were serum-deprived for 24 hours and treated with 10 nmol/L Ang II or vehicle for 24 hours. The cells were then incubated with [35S]methionine (100 μCi/mL, Amersham) in methionine-free DMEM (Dainippon Pharmaceutical Co) for 4 hours and lysed in solubilizing buffer (50 mmol/L HEPES, pH 7.4, 10 nmol/mL EDTA, 100 nmol/mL NaF, 10 nmol/mL sodium pyrophosphate, 1% Triton X-100, 10 mmol/mL NaVO4, 20 μmol/L leupeptin, 1.5 μmol/L aprotinin, and 2 mmol/L phenylmethylsulfonyl fluoride) at 4°C for 1 hour. To clear the protein extract, protein A Sepharose (20 μL of 50% suspension, Pharmacia Biotech) was added to the cell lysate and incubated for 1 hour, followed by centrifugation and collection of the supernatant. Protein concentrations were measured by a protein assay (BCA, Pierce). Specific antibody to Flk-1 (50 ng/mL, Santa Cruz Biotechnology Inc) was added to the protein samples (500 μg) and rocked at 4°C for 1.5 hours, and then 1 μg protein A Sepharose was added and rocked another 1.5 hours at 4°C. Protein A Sepharose antigen-antibody conjugates were separated by centrifugation, washed five 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), and the gel was vacuum-dried. Results were visualized and quantified by a BAS-2000II densitometer (Fuji Photo Film).

VEGF Binding Analysis

Monolayers of confluent BRECs grown in 12-well dishes (Iwaki Glass) were incubated with Ang II for 12 to 48 hours and then placed on ice and washed three times with ice-cold PBS containing calcium and magnesium. 125I-labeled VEGF was added, along with increasing amounts of unlabeled VEGF, and binding was carried out by rocking for 4 hours at 4°C. Binding was terminated by washing each well three times with ice-cold PBS containing 0.1% BSA. The cells were lysed in 1 mL of 0.1% SDS and counted in a gamma counter (ARC-600, Aloka).
[³H]Thymidine Incorporation Assay
Subconfluent BRECs (grown on a 24-well dish) that were serum-deprived for 24 hours in DMEM with 0.1% BSA were pretreated with Ang II (Sigma) at 1, 10, or 100 nmol/L or with vehicle for 12 hours before the addition of 0.6 nmol/L (25 ng/mL) VEGF (recombinant human VEGF, Genzyme) or vehicle. The cells were treated with vehicle or VEGF for 18 hours and then labeled with 1 μCi/mL of [³H]thymidine (Amersham) for 4 hours. The labeled cells were washed with ice-cold PBS, fixed in ice-cold 10% trichloroacetic acid, and then lysed with 0.5N NaOH. The incorporated [³H]thymidine was extracted by filtration (Whatman GF/C filter) and measured in a liquid scintillation counter (Aloka).

Tube Formation Assay
Vitrogen 100 (Celtrix), 0.2N NaOH, and 200 mmol/L HEPES (8:1:1 [vol/vol/vol]) and 10× RPMI medium (GIBCO BRL) were made to 400 μL and added to 24-well plates. After polymerization of the gels, 1.0×10⁴ BRECs were seeded and incubated with DMEM containing 20% FBS and 20% PDHS for 24 hours at 37°C. The medium was removed, and additional collagen gel was introduced on the cell layer. Ang II (10 nmol/L) or vehicle was added in the medium and incubated for 24 hours, and then the cells were stimulated with 0.6 nmol/L (25 ng/mL) VEGF. Five days later, five different fields (×10 objective) were chosen, and total tubelike structures were measured using Adobe Photoshop (Adobe Systems Inc).

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*<.05.

Results
Ang II stimulates KDR/Flk-1 mRNA Expression in BRECs and BAECs
To investigate the effect of Ang II on VEGF receptor expression, BRECs were treated with 10 nmol/L Ang II for the indicated times, and Northern blot analysis was performed on 20 μg/lane of total RNA (Fig 1A). After 1 hour of stimulation with 10 nmol/L Ang II, an increase in KDR mRNA was observed. Furthermore, this increase was time dependent, with a maximal 4.3±0.8-fold (*P*<.05) increase at 4 hours (Fig 1A). To investigate dose dependence of the Ang II effect, BRECs were stimulated with various concentrations of Ang II for 4 hours, and KDR mRNA expression was examined by Northern blot analysis (Fig 1B). We found that Ang II stimulates KDR mRNA expression in a dose-dependent fashion, with an EC₅₀ of ≈3 nmol/L and a maximal 4.4±1.1-fold (*P*<.05) increase at 10 nmol/L (Fig 1B). These data suggest that Ang II increases the expression of mRNA in BRECs for the VEGF receptor KDR/Flk-1 in a time- and dose-dependent manner.

To determine whether a similar response occurred in macrovascular endothelial cells, BAECs were stimulated with 10 nmol/L Ang II for 4 hours. Again, Ang II increased mRNA levels of KDR by 2.5±0.7-fold in BAECs (Fig 1B).

Although KDR, rather than Flt-1, was predominantly expressed in BRECs, we also examined Ang II effect on expression of the other VEGF receptor, Flt-1. Expression of Flt-1 was not observed in either Ang II–stimulated or non-stimulated BRECs using similar Northern blot analysis (data not shown). Thus, subsequent studies evaluated only KDR/Flk-1 expression in BRECs.

Ang II Does Not Increase the Half-Life of KDR/Flk-1 mRNA
We investigated whether the Ang II–induced increase of KDR mRNA level is mediated through regulation of transcription or mRNA stability. To determine whether Ang II affects the half-life of KDR mRNA, we examined the effect of inhibition of de novo gene transcription. Northern blot analyses were performed after the administration of actinomycin D.
(4 μmol/L) with or without 10 nmol/L Ang II. The half-life of KDR mRNA was 1.3 hours in unstimulated controls and 1.5 hours after treatment with 10 nmol/L Ang II. No significantly difference was observed (Fig 2).

Ang II Increases the Rate of Transcription of KDR mRNA in BRECs

To determine whether Ang II affects the transcriptional rate of KDR, we performed nuclear run-on analysis in the presence or absence of Ang II. To correct for differences in loading of the RNA probe, the rate of KDR mRNA transcription was compared with that of 36B4 mRNA, which was constitutively expressed. Treatment with 10 nmol/L Ang II increased the rate of KDR gene transcription 5.4-fold compared with that of control (Fig 3). These data clearly demonstrate that increased expression of KDR mRNA induced by Ang II was not through mRNA stability but through an increase in transcriptional rate.

Role of Ang II Receptor Subtypes AT1 and AT2 in the Ang II–Stimulated KDR/Flk-1 mRNA Expression in BRECs

To characterize the Ang II receptor subtype that is responsible for KDR induction, Northern blot analyses were performed using total RNA from BRECs pretreated with the non–subtype-specific Ang II antagonist saralasin, the AT1 antagonist DuP735, or the AT2 antagonist PD123319 for 15 minutes before Ang II stimulation. Saralasin inhibited the KDR mRNA expression to the control level by 98.6 ± 1.6% (Fig 4), and the AT1 antagonist inhibited the Ang II–induced KDR mRNA expression significantly by 92.7 ± 3.6% (P < .05). In contrast, the AT2 antagonist inhibited the Ang II–induced KDR mRNA expression by only 20.3 ± 10.3% (Fig 4). These data suggest that Ang II–induced KDR expression is mediated mainly through the AT1 receptor. We also examined the role of AT1 and AT2 in VEGF-induced BRECs growth by thymidine incorporation. When pretreated with the AT1 antagonist before the addition of Ang II, VEGF-induced BRECs growth was inhibited significantly (P < .05), by 46 ± 26%, whereas the AT2 antagonist had no inhibitory effects (Fig 8).
Role of PKC and Tyrosine Kinase in Ang II–Induced KDR mRNA Expression

Previous reports have shown that PKC and tyrosine kinase have a role in Ang II–stimulated signaling pathways. To determine the role of PKC and tyrosine kinase in Ang II–induced KDR mRNA expression, BRECs were pretreated with a highly selective PKC inhibitor, bisindolylmaleimide (GFX), or a specific tyrosine kinase inhibitor, genistein, followed by treatment with Ang II and PMA, a direct PKC stimulator. PMA increased the expression of KDR mRNA 1.9 ± 0.2-fold after 2 hours of stimulation compared with unstimulated control (data not shown), and the effect was completely inhibited by 10 μmol/L GFX (Fig 5). The same concentration of GFX reduced Ang II–induced KDR mRNA expression by 70 ± 15% (Fig 5). The role of tyrosine phosphorylation in Ang II–stimulated and PMA-stimulated KDR expression was also examined. Treatment with 20 μmol/L genistein reduced the Ang II–induced and PMA-induced KDR mRNA expression by 35 ± 8% and 44 ± 26%, respectively (Fig 5). When both inhibitors were applied simultaneously, KDR increase was inhibited by 90 ± 10%, which was more than when each component was applied separately (Fig 5). The 0.1% (vol/vol) dimethyl sulfoxide carrier used to solubilize these inhibitors did not significantly alter KDR mRNA expression (data not shown). These data indicate that PKC has a predominant role in the pathway of Ang II–stimulated KDR mRNA expression and that tyrosine phosphorylation may contribute to both the PKC-dependent and -independent mechanisms of Ang II–stimulated KDR mRNA expression in BRECs.

Ang II Increases KDR Protein Synthesis and Cell Surface Binding Sites

To determine whether the increase in KDR mRNA expression was accompanied by an increase of new protein synthesis, we precipitated the 35S-labeled cell lysates of BRECs with anti-KDR antibody. A single band at 195 kD was detected by immunoprecipitation of a rabbit anti-human KDR antibody, and the level was increased by 3.1-fold with Ang II stimulation at 10 nmol/L (Fig 6).

To determine whether the number or affinity of VEGF binding sites at the cell surface was changed in BRECs by Ang II stimulation, we performed 125I-VEGF binding analysis. Time-course study indicated that specific VEGF binding increased after 12 hours, and maximal increase was observed after 24 hours (data not shown). Scatchard analysis was performed after 24 hours and demonstrated a significant increase of binding sites (5.8 ± 0.56 10^4/cell to 9.9 ± 0.26 10^4/cell, P < .004) without changes of binding affinity (0.27 ± 0.02 to 0.29 ± 0.03 nmol/L, P < .5) after 24 hours of stimulation (Fig 7).

These results suggest that Ang II increased KDR protein synthesis and cell surface VEGF binding sites in BRECs.

Ang II Accelerates the VEGF-Induced Cell Growth in BRECs

To investigate the effect of Ang II on VEGF-induced angiogenesis, we measured [3H]thymidine incorporation in BRECs. Stimulation with 0.6 nmol/L (25 ng/mL) VEGF increased thymidine incorporation 1.3 ± 0.1-fold (P < .01) compared with the unstimulated control, and treatment with 10 nmol/L of Ang II alone did not affect thymidine incorporation in BRECs (Fig 8). When BRECs were pretreated with Ang II (10 nmol/L) followed by VEGF stimulation, thymidine incorporation was enhanced in a dose-dependent manner, with a maximum 2.1 ± 0.1-fold (P < .01) increase at 10 nmol/L (Fig 8). These data show that Ang II enhances the effect of VEGF on cell proliferation of BRECs.
Ang II Enhances VEGF-Induced Tube Formation in BRECs

BRECs were seeded in three-dimensional gels and pretreated with Ang II (10 nmol/L) or vehicle for 24 hours, followed by treatment with VEGF to evaluate tube formation. The lengths of five different fields per well were measured and compared. From three independent experiments, Ang II alone had little effect on tube formation of BRECs (Fig 9). VEGF, however, induced tube formation that was 7.5 ± 0.5-fold (P < .01) greater than that seen in controls (Fig 9), and pretreatment with Ang II further promoted the VEGF-induced tube formation in BRECs by 1.6 ± 0.1-fold compared with VEGF stimulation alone (P < .01, Fig 9). These data suggest that Ang II potentiates VEGF-induced angiogenic activity in retinal microvascular endothelial cells and that Ang II alone has no significant effect on retinal endothelial tube formation and cell growth.

Discussion

In diabetic retinopathy, development of retinal vascular non-perfusion followed by pathological angiogenesis often leads to vision loss. VEGF has been suggested to mediate such an ischemia-induced retinal neovascularization. Indeed, suppression of VEGF has been shown to inhibit neovascularization in animal models of retinal ischemia, and VEGF levels are elevated in patients with proliferative retinopathy and decrease.
after successful laser treatment. In addition, VEGF itself is sufficient to produce many of the vascular abnormalities common to diabetic retinopathy, and an increase of VEGF expression is observed in the retinas of diabetic patients with little or no retinopathy, suggesting a role of VEGF in the initiation and early stages of diabetic retinopathy. From this evidence, VEGF-induced angiogenesis in retinal microvascular cells might be a predominant pathological change in the development of diabetic retinopathy.

RAS has also been suggested to play a role in the development of diabetic retinopathy, indicated by the correlation of increased levels of serum and intraocular RAS with the clinical progression of retinopathy and by the favorable results produced by the ACE inhibitor on diabetic retinopathy. However, it remains unclear what mechanism underlies the interaction of RAS and the development of diabetic retinopathy. In the present study, we investigated whether Ang II affects VEGF/VEGF receptor expression and VEGF-induced angiogenic activity, which is thought to be a major pathological change in retinal microvascular cells in diabetic retinopathy. We have demonstrated that Ang II increases the expression of the VEGF receptor KDR/Flk-1 and potentiates VEGF-dependent cell growth (Fig 8) and tube formation (Fig 9) in cultured BRECs. In addition, Ang II itself had no effect on cell growth, tube formation, or mRNA levels of VEGF and Flk-1 in BRECs. These data suggest that Ang II potentiates VEGF-induced angiogenic activity probably results from an Ang II–induced increase in the expression of the VEGF receptor KDR/Flk-1 in BRECs.

Since VEGF exerts its biological effects through binding to two high-affinity tyrosine kinase receptors, KDR/Flk-1 and Flk-1, we sought to determine whether Ang II upregulates VEGF receptor expression as a mechanism of potentiating angiogenic effects. We performed Northern blot analysis and showed that Ang II stimulates KDR mRNA expression in BRECs in a dose- and time-dependent manner (Fig 1). In contrast, as previously reported, we could not detect Flk-1 mRNA in nonstimulated BRECs, and Flk-1 mRNA was not observed even in Ang II–stimulated BRECs studied by similar Northern blot analysis using total RNA. Although quantitative polymerase chain reaction analysis or RNA protection assay might detect the upregulation of Flk-1 mRNA, the effect of VEGF is probably negligible in BRECs. Thus, we focused on KDR expression for further analysis. The dose–response study demonstrated an EC50 of ~3 nmol/L and a maximal 4.4 ± 1.1-fold increase at 10 nmol/L Ang II stimulation. Although the concentrations are considerably higher than those in the plasma and vitreous fluid of diabetic patients, they are similar to or lower than others that have been reported. Moreover, it is likely that local concentrations of Ang II in retinal microvasculature are much higher than serum and vitreous levels, since an autocrine paracrine production system of Ang II is present in ocular tissues. Two major angiotensin receptor subtypes have been defined: AT1 and AT2. Most of the actions of angiotensin are mediated by the AT1 receptor, whereas actions of the AT2 receptor are not well understood. The expression of AT2 receptors is reported to be regulated by cell types and the developmental stage of tissues and is speculated to be involved in tissue growth and differentiation. The growth–promoting effect of Ang II in SMCs has been reported to be through the AT1 receptor, and recently, the AT2 receptor was reported to mediate antitumor effects on AT2-overexpressed vascular SMCs and coronary endothelial cells. In our experiments using AT1- and AT2–specific receptor antagonists, the receptor subtypes involved in both the regulation of KDR and the potentiation of VEGF mitogenic effects in Ang II–stimulated BRECs indicated that most of these effects were via AT1 receptors (Figs 4 and 8). The observed responses are well correlated with the concept that AT1 mediates proliferative effects and AT2 elicits antiproliferative responses. AT1 blockade did not completely block the effects Ang II on VEGF-induced cell growth, whereas it blocked KDR induction almost completely. There is a possibility that Ang II might affect VEGF–elicited signal transduction or posttranscriptional regulation of KDR. Stoll et al have reported that in coronary endothelial cells, AT1 mediates antiproliferative effects and offsets the growth–promoting effects mediated by AT1, whereas Ang II stimulates the growth of quiescent vascular SMCs that express only AT1. In our experiments, Ang II alone had no significant effects on cell proliferation, suggesting that both AT1 and AT2 receptors probably exist in BRECs, similar to coronary endothelial cells. AT2 inhibition did not affect VEGF-induced growth nor KDR expression significantly, suggesting that AT2 receptor mediation is probably not involved in the regulation of such cell responses in BRECs (Fig 8).

We made further analyses to delineate the signal transduction pathway responsible for the effect of Ang II on increases of the KDR gene. The AT1 receptor is a G protein–coupled receptor and activates phospholipase C, which is known to induce the hydrolysis of phosphoinositols and the activation of PKC. Ang II–induced increases of several growth factors, such as PDGF A–chain, TGF-β, basic FGF, IGF I, and ET-1, are reported to be mediated through AT1 with activation of PKC. In BRECs, KDR induction by Ang II appeared to be mediated predominantly by a PKCdependent pathway. Tyrosine phosphorylation has also been reported to be elicited by Ang II and PMA in vascular SMCs, glomerular mesangial cells, and microvessel endothelial cells. Experiments using genistein revealed that tyrosine phosphorylation is required in both Ang II–stimulated and PMA–stimulated expression of KDR. Feener et al reported that Ang II–induced and PMA–induced plasminogen activator inhibitor–2 mRNA expression is inhibited by >70% with the same concentration of genistein in rat epididymal fat–derived microvascular endothelial cells. Since the inhibitory effect of genistein in BRECs is not so potent, the tyrosine phosphorylation pathway is probably not so much involved in the induction of the KDR gene in BRECs as it is in plasminogen activator inhibitor–2 expression in rat microvascular endothelial cells. The PKC–independent pathway also contributed to Ang II–induced KDR mRNA expression, which is defined as the Ang II–induced increase in the KDR mRNA level in the presence of the same concentration of GFX that could completely reverse PMA–induced change. This pathway accounts for ~30% of the total effect of Ang II. The inhibition of this component by genistein
suggests that this pathway also involves tyrosine phosphorylation.

The time-course study demonstrated that the Ang II–induced increase of KDR mRNA was rapid and peaked at 4 hours (Fig 1A). Nuclear run-on assays and experiments using actinomycin D to inhibit RNA synthesis indicate that the effect of Ang II is primarily to increase transcription of the KDR gene. Moreover, new protein synthesis was necessary for complete upregulation of KDR mRNA by Ang II (data not shown). These data suggest that transcriptional regulation of this gene is mediated through a transacting transcription factor. Similar transcriptional regulation was observed in tumor necrosis factor-α–induced downregulation of KDR expression.44 Recent analyses demonstrated that the 5′ flanking region of KDR or the Flk-1 gene contains several potential binding sites for a transacting transcription factor, such as activator protein–2, nuclear factor-κB, or stimulatory protein–1.55 The PKC–dependent signaling pathway might stimulate transcription of the KDR gene through some transcriptional factor, such as nuclear factor-κB, which is activated by the PKC–dependent pathway. Further studies are to be performed to elucidate the detailed mechanism of transcriptional regulation of the KDR gene by Ang II.

The increase of KDR mRNA expression was accompanied by new protein synthesis of the receptor. Scatchard analyses further demonstrated an increase of cell surface binding sites for VEGF. We detected no significant effect of Ang II on the affinity of the receptor. Ang II has been reported to regulate several receptors, such as those for IGF I, ET-1, and low density lipoprotein, but regulation of receptor affinities has not been reported.56–58 The magnitudes of the Ang II–induced increases in KDR/Flk-1 mRNA, protein synthesis, and VEGF binding sites correlated well with each other. Although we have not investigated posttranscriptional regulation of KDR by Ang II, such as recycling of the receptor, the observed upregulation of the cell surface receptors probably results from an increase of KDR gene expression induced by Ang II.

Ang II has been reported to regulate cell growth through induction of several autocrine growth factors, such as PDGF A-chain, TGF-β, basic FGF, and IGF I in vascular SMCs,18,19,46,53 and through ET-1 in cardiomyocytes.40 In vascular SMCs, Ang II is reported to increase the gene expression of VEGF.54 We examined VEGF mRNA induction by Ang II to determine whether Ang II induces the endogenous production of VEGF in BRECs. We did not find any increase in VEGF mRNA levels (data not shown). Ang II alone had no significant effect on cell proliferation or tube formation in retinal microvascular endothelial cells (Figs 8 and 9). These findings suggest that Ang II potentiation of VEGF–induced angiogenic activity is probably not through induction of autocrine growth factors but through the induction of KDR/Flk-1. However, we cannot exclude the possibility that a growth factor such as FGF, which has synergism with VEGF in angiogenic effect,57 might be induced by Ang II but that its effects are suppressed by antiproliferative growth factor (such as TGF-β) induction.53 On VEGF stimulation, such growth factors might stimulate BREC more potently than does Ang II stimulation alone.

Regulation of blood pressure and ocular blood flow by Ang II and regulation of Na+,K+-ATPase by ACE have been suggested as functions of the RAS in the progression of diabetic retinopathy.59,60 Our results suggest a new hypothesis: Ang II potentiates the progression of diabetic retinopathy by stimulating VEGF-induced retinal neovascularization through an increase of VEGF receptor KDR/Flk-1. Besides angiogenic effects, VEGF has been reported to increase vasopermeability and to generate a procoagulant state by the induction of von Willebrand factor and tissue factor.61,62 The upregulation of KDR/Flk-1 expression probably potentiates these functions of VEGF as well as the angiogenic effect in retinal microvascular cells, which might worsen the retinal vascular embolization, exudation, and macular edema in diabetic retinopathy. In the macrovascular milieu, we have demonstrated that Ang II upregulates KDR mRNA expression in BAECS. In addition, Williams et al14 have reported that Ang II increases VEGF mRNA expression in vascular SMCs. These observations suggest upregulation of the VEGF paracrine system in large vessel walls, which probably plays a prominent role in vascular injury such as atherosclerosis.

From a clinical standpoint, our data suggest that inhibition of RAS is beneficial for the treatment of diabetic retinopathy. An ACE inhibitor, captopril, was recently reported to suppress neovascularization by directly inhibiting migration of vascular endothelial cells.53 Such drugs and an AT1 blocker might be proven to be effective in the prevention of diabetic retinopathy.

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References


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