Vascular Endothelial Growth Factor Induces Expression of Connective Tissue Growth Factor via KDR, Flt1, and Phosphatidylinositol 3-Kinase-Akt-dependent Pathways in Retinal Vascular Cells

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters.

Citation
Suzuma, K., K. Naruse, I. Suzuma, N. Takahara, K. Ueki, L. P. Aiello, and G. L. King. 2000. Vascular Endothelial Growth Factor Induces Expression of Connective Tissue Growth Factor via KDR, Flt1, and Phosphatidylinositol 3-Kinase-Akt-Dependent Pathways in Retinal Vascular Cells. Journal of Biological Chemistry 275, no. 52: 40725–40731. doi:10.1074/jbc.m006509200.

Published Version
doi:10.1074/jbc.M006509200

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:33776262

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
Vascular Endothelial Growth Factor Induces Expression of Connective Tissue Growth Factor via KDR, Flt1, and Phosphatidylinositol 3-Kinase-Akt-dependent Pathways in Retinal Vascular Cells*

Received for publication, July 21, 2000, and in revised form, September 28, 2000
Published, JBC Papers in Press, October 3, 2000, DOI 10.1074/jbc.M006509200

Kiyoshi Suzuma‡§, Keiko Naruse‡, Izumi Suzuma‡, Noriko Takahara‡, Kohjiro Ueki‡,
Lloyd P. Aiello‡¶, and George L. King‡**
From the ‡Research Division and §Beetham Eye Institute, Joslin Diabetes Center, and the ¶Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts 02215

Fibroblastic proliferation accompanies many angiogenesis-related retinal and systemic diseases. Since connective tissue growth factor (CTGF) is a potent mitogen for fibrosis, extracellular matrix production, and angiogenesis, we have studied the effects and mechanism by which vascular endothelial growth factor (VEGF) regulates CTGF gene expression in retinal capillary cells. In our study, VEGF increased CTGF mRNA levels in a time- and concentration-dependent manner in bovine retinal endothelial cells and pericytes, without the need of new protein synthesis and without altering mRNA stability. VEGF activated the tyrosine receptor phosphorylation of KDR and Flt1 and increased the binding of phosphatidylinositol 3-kinase (PI3-kinase) p85 subunit to KDR and Flt1, both of which could mediate CTGF gene induction. VEGF-induced CTGF expression was mediated primarily by PI3-kinase activation, whereas PKC and ERK pathways made only minimal contributions. Furthermore, overexpression of constitutive active Akt was sufficient to induce CTGF gene expression, and inhibition of Akt activation by overexpressing dominant negative mutant of Akt abolished the VEGF-induced CTGF expression. These data suggest that VEGF can increase CTGF gene expression in bovine retinal capillary cells via KDR or Flt receptors and the activation of PI3-kinase-Akt pathway independently of PKC or Ras-ERK pathway, possibly inducing the fibrosis observed in retinal neovascular diseases.

Angiogenesis and fibrosis are key components in development, growth, wound healing, and regeneration (1). In addition, these processes commonly occur together in many disease states where neovascularization is believed to initiate the pathological cascade. Some of these diseases are proliferative diabetic retinopathy (2), rheumatoid arthritis (3), and age-related macular degeneration (4). Thus, it is possible that the factors that regulate angiogenesis may also induce factors that stimulate extracellular matrix production and fibrosis. Accordingly, we have studied the ability of vascular endothelial growth factor (VEGF), an established angiogenic factor, to regulate the expression of connective tissue growth factor (CTGF), a growth factor with known actions on fibroblast proliferation, matrix production, and associated with fibrotic disorders.

VEGF is expressed as a family of peptides of 121, 145, 165, 189, and 206 amino acid residues (5). Its expression is induced by hypoxia (6) and is essential in the vasculogenesis process during development (7). Several receptors have been shown to mediate the action of VEGF, and most of them belong to the tyrosine kinase receptor family (8). Upon the binding of VEGF to its receptors, multiple signaling cascades are activated, including the tyrosine phosphorylation of phospholipase Cγ, elevation of intracellular calcium and diacylglycerol, activation of protein kinase C (PKC), and extracellular signal-regulated kinase (MAPK/ERK) for endothelial cell proliferation (9–12). In addition, VEGF also stimulates activation of phosphatidylinositol (PI) 3-kinase leading to Akt/PI3K activation and possibly enhancing endothelial cell survival (13–15). However, in non-endothelial cells such as capillary pericytes that predominantly express Flt1 receptor, the action of VEGF is poorly understood.

Connective tissue growth factor (CTGF), a member of CCN family (CYR61, CTGF, and NOV) (16, 17), is a potent and ubiquitously expressed growth factor that has been shown to play a unique role in fibroblast proliferation, cell adhesion, and the stimulation of extracellular matrix production (18, 19). The 38-kDa protein was originally identified in conditioned medium from human umbilical vein endothelial cells (20), and the expression was shown to be selectively stimulated by transforming growth factor-β (TGF-β) in cultured fibroblasts (21). Due to its mitogenic action on fibroblasts and its ability to induce the expression of the extracellular matrix molecules, collagen type I, fibronectin, and integrin α1 (18), CTGF is supposed to play an important role in connective tissue cell proliferation and extracellular matrix deposition as one of the mediators of TGF-β.

* This work was supported in part by National Institutes of Health Grants EY5110 (to G. L. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Recipient of the Mary K. Iacono Fellowship.
§ To whom correspondence should be addressed: Research Division, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215. Tel.: 617-732-2622; Fax: 617-732-2637; E-mail: George.King@joslin.harvard.edu.

1 The abbreviations used are: VEGF, vascular endothelial growth factor; CTGF, connective tissue growth factor; Flt, fms-like tyrosine kinase; KDR/Flk1, fetal liver kinase/kinase domain-containing receptor; VEGFR, vascular endothelial growth factor receptor; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; kb, kilobase pair; PKC, protein kinase C; PI, phosphatidylinositol; TGF-β, transforming growth factor-β; BREC, bovine retinal endothelial cells; PIGF, placenta growth factor; BRPC, bovine retinal pericytes; PCR, polymerase chain reaction; CAAX, constitutive active Akt; DNAkt, dominant negative Akt; DNRas, dominant negative K-Ras; DNERK, dominant negative extracellular signal-regulated kinase; DNPKCζ, dominant negative PKCζ.
(22), CTGF also seems to be an important player in the pathogenesis of various fibrotic disorders, since it was shown to be overexpressed in scleroderma, keloids, and other fibrotic skin disorders (23), as well as in stromal rich mammary tumors (24), and in advanced atherosclerotic lesions (25). Recently, the integrin α6β1 has been reported to serve as a receptor on endothelial cells for CTGF-mediated endothelial cell adhesion, migration, and angiogenesis (26, 27).

Besides TGF-β, the expression of CTGF is reported to be regulated by dexamethasone in BALB/c 3T3 cells (28), high glucose in human mesangial cells (29), kinin in human embryonic fibroblasts (30), factor VIIa, and thrombin in WI-38 fibroblasts (31), tumor necrosis factor α in human skin fibroblast (32), and cAMP in bovine endothelial cells (33). Since many of these cytokines are known to induce VEGF, it is possible that increased VEGF expression can regulate the expression of CTGF. In the present study, we have investigated the regulation of CTGF by VEGF in retinal endothelial cells and pericytes via the PI3-kinase and several other signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Materials**—Endothelial cell basal medium was purchased from Clonetics (San Diego, CA). Endothelial cell growth factor was purchased from Roche Molecular Biochemicals. Dulbecco’s modified Eagle’s medium and fetal bovine serum were obtained from Life Technologies, Inc. VEGF, placenta growth factor (PIGF), TGF-β, and anti-CTGF antibody were ordered from R & D Systems (Minneapolis, MN). Anti-KDR (Flk1) and anti-Fit1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protein A-Sepharose was purchased from Amersham Pharmacia Biotech. Anti-phospho-ERK, anti-ERK, anti-phospho-Akt, and anti-Akt were purchased from New England Biolabs (Beverly, MA). Anti-p85 and anti-phosphotyrosine were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Phosphatidylinositol (PI) was purchased from Avanti (Alabaster, AL), and PD98059, wortmannin, and GF 109203X were obtained from Calbiochem. All other materials were ordered from Fisher and Sigma.

**Cell Culture**—Primary cultures of bovine retinal endothelial cells (BREC) and pericytes (BRPC) were isolated by homogenization and a series of filtration steps as described previously (34). BREC were subsequently cultured with endothelial cell basal medium supplemented with 10% plasma-derived horse serum, 50 mg/liter heparin, and 50 μg/ml endothelial cell growth factor. BRPC were cultured in Dulbecco’s modified Eagle’s medium with 5.5 mm glucose and 20% fetal bovine serum. Cells were cultured in 5% CO2 at 37° C, and media were changed every 3 days. Cells were characterized for their homogeneity by immunoreactivity with anti-factor VIII antibody for BREC and with monoclonal antibody 3G5 for BRPC (35). Cells remained morphologically unchanged under these conditions, as confirmed by light microscopy. Only cells growing in monolayer culture were used for experiments.

**Recombinant Adenoviruses**—cDNA of constitutive active Akt (CAkt, Gag protein fused to N-terminal of wild type Akt) was constructed as described (36). cDNA of dominant negative Akt (DNAkt, substituted Thr-308 to Ala and Ser-473 to Ala) was constructed as described (37). cDNA of dominant negative K-Ras (DNKras, substituted Ser-17 to Asn) was kindly provided by Dr. Takai (Osaka University) (38). cDNA of dominant negative extracellular signal-regulated kinase (DNERK, substituted Lys-52 to Arg in ATP-binding site) was constructed as described (39). cDNA of Δp85 was kindly provided by Dr. Kasauf (Kobe University) (40). cDNA of PKCζ was kindly provided by Dr. Douglas Ways (Lilly). cDNA of dominant negative PKCζ (DNPKCζ, substituted Lys-273 to Trp in ATP-binding site) was constructed as described (41). The recombinant adenoviruses were constructed by homologous recombination between the parental virus genome and the expression cosmID cassette or shuttle vector as described (42, 43). The adenoviruses were applied at a concentration of 1×106 plaque-forming units/ml, and adenoviruses with the same parental genome carrying the lacZ gene or enhanced green fluorescent protein gene (CLONTECH, Palo Alto, CA) were used as controls. Expression of each recombinant protein was determined by Western blot analysis at least about 10-fold compared with cells infected with the control adenovirus.

**Immunoprecipitation**—Cells were washed three times with cold phosphate-buffered saline and solubilized in 200 μl of lysis buffer (1% Triton X-100, 50 mmol/liter HEPES, 10 mmol/liter EDTA, 10 mmol/liter sodium pyrophosphate, 100 mmol/liter sodium fluoride, 1 mmol/liter sodium orthovanadate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 2 mmol/liter phenylmethylsulfonyl fluoride). After centrifugation at 12,000 rpm for 10 min, 10 μg of protein was subjected to immunoprecipitation. To clear the protein extract, protein A-Sepharose (20 μl of a 50% suspension) was added to the cell lysates, after which they were incubated for 1 h, followed by centrifugation and collection of the supernatant. A specific rabbit anti-KDR or Fit1 antibody was added and rocked at 4 °C for 2 h; 20 μl of protein A-Sepharose was then added, and the sample was rocked for another 2 h at 4 °C. For denaturation, protein A-Sepharose antigen-antibody conjugates were separated by centrifugation, washed five times, and boiled for 3 min in Laemmli sample buffer.

**Northern Blot Analysis**—Immunoprecipitated proteins or 30 μg of total cell lysates were subjected to SDS-gel electrophoresis and electrotransferred to nitrocellulose membrane (Bio-Rad). The membrane was soaked in blocking buffer (phosphate-buffered saline containing 0.1% Tween 20 and 5% bovine serum albumin) for 1 h at room temperature and incubated with primary antibody overnight at 4 °C followed by incubation with horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech). Visualization was performed using the enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech) per the manufacturer’s instructions.

**PI3-Kinase Assay**—PI3-kinase activities were measured by the in vitro phosphorylation of P1 (10). Cells were lysed in ice-cold lysis buffer containing 50 mmol/liter HEPES, pH 7.5, 137 mmol/liter NaCl, 1 mmol/liter MgCl2, 1 mmol/liter KCl, 5 mmol/liter Na2VO4, 0.1% Triton X-100, 10% glycerol, 1 mmol/liter phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 μg/ml pepstatin. Insoluble material was removed by centrifugation at 15,000 g for 10 min at 4 °C. PI3-kinase was immunoprecipitated from aliquots of the supernatant with antiphosphotyrosine antibodies. After successive washings, the pellets were resuspended in 50 μl of 10 mmol/liter Tris, pH 7.5, 100 mmol/liter NaCl, and 1 mmol/liter EDTA, 10 μl of 100 mmol/liter MgCl2, and 10 μl of P1 (2 μg/ml) sonicated in 10 mmol/liter Tris, pH 7.5, with 1 mmol/liter EDTA was added to each pellet. The PI3-kinase reaction was initiated by the addition of 5 μl of 0.5 mmol/liter ATP containing 30 μCi of (γ-32P)ATP. After 10 min at room temperature with constant shaking, the reaction was stopped by the addition of 20 μl of 8 N HCl and 160 μl of chloroform/methanol (1:1). The samples were centrifuged, and the organic phase was removed and applied to silica gel TLC plates developing in CHCl3/CH3OH/H2O/NH4OH (60:47:11:2). The radioactivity in spots was quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Amplification of Human CTGF cDNA Using Reverse Transcriptase-Polymerase Chain Reaction (PCR)**—cDNA templates for PCR were synthesized by reverse transcriptase (First Strand cDNA Synthesis Kit, Ambion, Austin, TX). First strand cDNA was synthesized by reverse transcriptase (First Strand cDNA Synthesis Kit, Invitrogen) using 5 μg of total RNA with 250 μmol/liter oligo (dT)12-18 primer and 200 μmol/liter dNTPs. PCR was then subcloned into a vector (pCRII, Invitrogen) and sequenced in the method recommended by the manufacturer. A standard PCR was performed (PCR optimizer kit, Invitrogen, Carlsbad, CA) using 5′-AGGGCTCTTCTTGACTTGGC-3′ (sense primer) and 5′-TCATGCTGTC- TCCGTACATCT-3′ (antisense primer) (20). The PCR products were then subcloned into a vector (pCRII, Invitrogen) and sequenced in their entirety, and comparison with the published human sequences revealed a complete sequence identity. This cDNA probe was used for hybridization.

**Northern Blot Analysis**—Total RNA was isolated using acid-guanidinium thiocyanate, and Northern blot analysis was performed as described previously (44). Total RNA (20 μg) was electrophoresed through 1% formaldehyde-agarose gels and then transferred to a nylon membrane. 32P-Labeled cDNA probes were generated by use of labeling kits (Megaprima DNA labeling systems, Amersham Pharmacia Biotech). After ultraviolet cross-linking using a UV cross-linker (Stratagene, La Jolla, CA), blots were pre-hybridized, hybridized, and washed in 0.5× SSC, 5% SDS at 65 °C with 4 changes over 1 h. All signals were analyzed using a PhosphorImager, and lane loading differences were normalized using the 36B4 cDNA probe (45).

**Analysis of CTGF mRNA Half-life**—CTGF mRNA half-life experiments were carried out using BREC and BRPC. The cells were exposed to vehicle or VEGF (25 ng/ml) for the indicated periods prior to mRNA stability measurements. Transcription was inhibited by the addition of actinomycin D (5 μg/ml). For inhibition of protein synthesis, cells were treated with cycloheximide (10 μg/ml) for the times indicated.

**Statistical Analysis**—Determinations were performed in triplicate, and each experiment was repeated at least three times. Results are expressed as the mean ± S.D., unless otherwise indicated. Statistical analysis employed Student’s t test or analysis of variance to compare quantitative data populations with normal distributions and equal variance. Data were analyzed using the Mann-Whitney rank sum test or the Kruskal-Wallis test for populations with non-normal distributions or unequal variance. A p value of <0.05 was considered statistically significant.

**Vascular Endothelial Growth Factor Induces CTGF Gene Expression**
RESULTS

CTGF mRNA Expression by VEGF and PlGF—The effects of VEGF on the expression of CTGF mRNA were studied by Northern blot analysis in BREC and BRPC. As shown in Fig. 1, A and B, 25 ng/ml VEGF increased CTGF mRNA (2.4 kb) levels in a time-dependent manner, reaching a maximum after 6 h in BREC (3.1 ± 0.70-fold, p < 0.001, Fig. 1A) and after 9 h in BRPC (2.0 ± 0.22-fold, p < 0.01, Fig. 1B).

The dose response to VEGF-induced CTGF mRNA expression was studied after 6 h of VEGF stimulation. As shown in Fig. 1, C and D, the expression of CTGF mRNA was up-regulated in a dose-dependent manner, with significant increases observed at concentrations as low as 0.25 ng/ml in both BREC (Fig. 1C) and BRPC (Fig. 1D). Maximal increases were observed at VEGF concentrations of 25 ng/ml in both BREC and BRPC.

Since BREC and BRPC may express both KDR and Flt1, we examined the effects of PIGF, a Flt1-specific ligand (46–48), on the induction of CTGF gene expression in vascular cells. As shown in Fig. 1E, CTGF mRNA levels were not affected after stimulation of 25 ng/ml of PIGF in BREC. In contrast, PIGF increased CTGF mRNA after 3 h of stimulation, which peaked after 9 h in BRPC (1.9 ± 0.30-fold, p < 0.01, Fig. 1F), suggesting that VEGF-induced CTGF gene expression was mediated primarily by KDR in BREC and Flt1 in BRPC.

VEGF Induction of CTGF Protein Production—To determine if the effects of VEGF on CTGF mRNA were correlated with its protein level, CTGF protein expression was assessed by Western blot analysis using anti-human CTGF antibody. As shown in Fig. 2A, the detected size of CTGF protein was ~38 kDa in both BREC and BRPC. VEGF (25 ng/ml) increased the level of CTGF protein after 10 h in both BREC and BRPC. Comparative studies were performed on the effects of VEGF (25 ng/ml) and TGF-β1 (10 ng/ml) on the expression of CTGF mRNA and protein. As shown in Fig. 2B, VEGF and TGF-β1 increased CTGF protein expression by a similar amount (2.5 ± 0.4- and 2.8 ± 0.8-fold, respectively, in BREC). CTGF mRNA levels were also increased a similar extent (3.0 ± 0.3- and 3.3 ± 0.5-fold, respectively).

Effects of VEGF on the Half-life of CTGF mRNA—The effects of VEGF on the stability of CTGF mRNA were examined. Northern blot analyses were performed with addition of actinomycin D (5 μg/ml) after 6 h of VEGF (25 ng/ml) stimulation. In BREC (Fig. 3A) and BRPC (Fig. 3B), the half-life of CTGF mRNA was 1.7 and 3.6 h, respectively. There was no significant difference between VEGF-treated and -untreated cells.

Effects of Cycloheximide on CTGF mRNA Regulation—In order to examine the possibility that VEGF regulates CTGF mRNA expression through new protein synthesis of cytokines or transcription factors, cells were treated for 6 h with VEGF (25 ng/ml) and a protein synthesis inhibitor, cycloheximide (10 μg/ml). Fig. 3, C and D, shows that cycloheximide did not prevent the increase of CTGF mRNA. Addition of both VEGF and cycloheximide increased CTGF mRNA 2.4 ± 0.41-fold in BREC (Fig. 3C) and 2.5 ± 0.40-fold in BRPC (Fig. 3D) after 6 h as compared with cycloheximide alone (p < 0.01). These data suggest that the stimulation of CTGF mRNA expression by VEGF was not induced by increased synthesis of a regulatory protein.

Involvement of ERK and PI3-Kinase-Akt in VEGF Signal-
**VEGF Induces CTGF Gene Expression**

**FIG. 3.** A and B, decay of CTGF mRNA in the presence of actinomycin D. C and D, effects of cycloheximide on CTGF mRNA regulation. A (BREC) and B (BRPC), cells were incubated with or without 25 ng/ml VEGF for 6 h, and de novo mRNA transcription was inhibited by addition of actinomycin D (5 \(\mu\)g/ml). Total RNA was extracted at 2 and 4 h after administration of actinomycin D. The values in the graph indicate the percentage of initial CTGF mRNA signal remaining in the specific conditions. C (BREC) and D (BRPC), cells were incubated with or without VEGF (25 ng/ml) and cycloheximide (CHX, 10 \(\mu\)g/ml) for 6 h, and total RNA was analyzed. Representative Northern blots and quantitation of three experiments are shown (bottom). Asterisks indicate significant differences at \(p < 0.05\) (*) and \(p < 0.01\) (**).

**FIG. 4.** VEGF effects on the ERK and PI3-kinase-Akt. A (BREC) and B (BRPC), cells were incubated with VEGF (25 ng/ml) or PIGF (25 ng/ml) for 5 min. Proteins immunoprecipitated (IP) with KDR (A) or Flt1 (B) antibodies were fractionated by SDS-polyacrylamide gel electrophoresis. Immunoblots were probed with an antibody to phosphotyrosine (pY, top) and reprobed with antibody to p85 (bottom) and KDR (A, middle) or Flt1 (B, middle). C (BREC) and D (BRPC), cells were incubated with VEGF (25 ng/ml) for 15 min. Immunoblots were probed with an antibody to phosphorylated Akt (top) and reprobed with antibody to total Akt (middle) or phosphorylated ERK (bottom). WB, Western blot.

**FIG. 5.** Effects of VEGF on PI3-kinase activation in BRPC. BRPC were incubated with VEGF (25 ng/ml) for the indicated times and harvested. Equal amounts of lysates were immunoprecipitated with anti-phosphotyrosine antibody, and immunocomplexes were assayed for their ability to phosphorylate PI to phosphatidylinositol phosphate (PIP). Representative autoradiogram (right) and quantitation of three experiments in the percentage of intensity of control (left) are shown. Asterisks indicate significant differences at \(p < 0.05\) (*) and \(p < 0.01\) (**).

---

Since ERK and PI3-kinase-Akt pathways have been reported to play central roles in VEGF signaling and biological actions (9–15), we investigated whether or not VEGF can activate ERK and PI3-kinase-Akt pathways equally in BREC and BRPC. As shown in Fig. 4A, immunoblot analysis of immunoprecipitates of KDR from BREC stimulated with VEGF or PIGF using an antibody to phosphotyrosine and PI3-kinase p85 subunit demonstrated that VEGF, but not PIGF, promoted the tyrosine phosphorylation of KDR and interactions of KDR and p85 subunit of PI3-kinase. In contrast, as shown in Fig. 4B, immunoblot analysis of immunoprecipitates of Flt1 from BRPC stimulated with VEGF or PIGF demonstrated that both VEGF and PIGF increased the tyrosine phosphorylation of Flt1 and interactions of Flt1 and p85 subunit of PI3-kinase. These data suggest that VEGF can activate the receptor tyrosine phosphorylation and interaction with PI3-kinase p85 subunit in both KDR and Flt1.

To investigate the activation of Akt and ERK, we next performed immunoblot analysis with anti-phosphorylated Akt or anti-phosphorylated ERK antibodies using total cell lysates from BREC or BRPC stimulated with VEGF. As shown in Fig. 4C and D, VEGF induced phosphorylation of both Akt and ERK in BREC by 3.1- and 5.8-fold (Fig. 4C), but only induced phosphorylation of Akt in BRPC by 2.6-fold (Fig. 4D). No effect on ERK phosphorylation was observed in BRPC. These data suggest that VEGF activated both ERK and PI3-kinase-Akt pathways in BREC, but stimulated only PI3-kinase-Akt pathway in BRPC.

Since the activation of PI3-kinase by VEGF has not been reported in BRPC, we studied the effects of VEGF on PI3-kinase activity in BRPC. As shown in Fig. 5, the addition of VEGF (25 ng/ml) increased PI3-kinase activity in a time-dependent manner by 2.1 ± 0.27-fold \((p < 0.01)\) after 5 min and by 1.6 ± 0.17-fold \((p < 0.05)\) after 10 min in BRPC.

**Effects of PKC, ERK, and PI3-Kinase Inhibition on VEGF-induced CTGF Expression**—To investigate the signaling pathways involved in VEGF-induced CTGF expression, the effects of inhibition of PKC, ERK, and PI3-kinase were determined. Cells were treated with 25 ng/ml VEGF for 6 h after pretreatment with the kinase inhibitor GF 109203X, a classical and novel PKC-specific inhibitor (1 \(\mu\)M) (49, 50); PD98059, a MAPK/ERK kinase inhibitor (20 \(\mu\)M); or wortmannin, a PI3-kinase inhibitor (100 \(\mu\)M) (6, A and B). Neither GF 109203X nor PD98059 had significant effects on VEGF-induced CTGF mRNA expression, but wortmannin inhibited the effects of VEGF by \(88 \pm 6.5\%\) \((p < 0.01)\) in BREC (Fig. 6A) and \(78 \pm 22\%\) \((p < 0.01)\) in BRPC (Fig. 6B). To confirm further the involvement of PI3-kinase in VEGF-induced CTGF expression, we used recombinant adeno-viruses encoding DNRas, DNERK, or \(\Delta p 85\) of PI3-kinase. BREC were transduced with each adenoviral vector, followed by stimulation with 25 ng/ml VEGF for 6 h. Neither DNRas nor DNERK had significant effects on VEGF-induced increase in CTGF mRNA, but \(\Delta p 85\) of PI3-kinase completely inhibited VEGF-induced CTGF expression \((p < 0.001, \text{Fig. 6C})\).

**Role of PKC\(_i\) and Akt/PKB in VEGF-induced CTGF Expression**—Since it has been reported that atypical PKC (51–55) and
Akt/PKB (13, 14, 56) have significant roles as signaling molecules downstream of PI3-kinase, we examined the involvement of PKCζ and Akt in this process. BREC were infected with each adenoviral vector, followed by stimulation with 25 ng/ml VEGF for 6 h. Neither wild type PKCζ nor DNPKCζ had significant effects on VEGF-induced increase in CTGF mRNA (Fig. 7A). In contrast, as shown in Fig. 7B, infection with CAAkt increased CTGF mRNA expression 2.1 ± 0.21-fold (p < 0.01) without VEGF and 2.5 ± 0.40-fold with VEGF. Overexpression with adenoviral vector containing DNAkt inhibited VEGF-induced CTGF expression by 85 ± 13% (p < 0.01).

DISCUSSION

In this study, we have shown that VEGF can increase the mRNA expression of CTGF in a time- and concentration-dependent manner in both microvascular endothelial cells and contractile cells (capillary pericytes) possibly indicating that the effects of VEGF on CTGF expression may occur in all cells with VEGF receptors. This possibility is supported by the results showing that both Flt1 (VEGFR1) and KDR/Flk1 (VEGFR2) can mediate the increases in CTGF mRNA expression. The ability of Flt1 to induce increases in CTGF mRNA levels is demonstrated in the pericytes that have predominantly Flt1 receptors and where the expression of KDR/Flk1 receptors were not significantly high enough to be determined by Northern blot analysis as reported in a previous publication (57). In addition, PlGF, a Flt1 receptor-specific ligand (48), was able to induce CTGF mRNA levels in BRPC but not in BREC, again supporting the postulate that VEGF can induce CTGF mRNA by activating through Flt1 in pericytes. The KDR/Flk1 receptors in the endothelial cells can also induce CTGF gene expression since KDR/Flk1 receptors are the predominant VEGF receptors in endothelial cells (58), and PlGF was not effective in inducing CTGF mRNA expression in endothelial cells. Further studies will be necessary to determine whether other types of VEGF receptors, such as Flt4 and neuropilin-1 (60), which are present in endothelial cells, can also induce CTGF expression.

The VEGF dose-response curves for CTGF in both BRPC and BREC are similar and suggest that the VEGF binds to high affinity receptors, consistent with the known K_d values of Flt1 and KDR/Flk1 at 10–100 pM (58, 61). VEGF-induced CTGF mRNA is most likely due to an induction of transcription rather than altering the half-life of CTGF mRNA since the addition of VEGF failed to change the degradation rates of CTGF mRNA. The time course of the action of VEGF on CTGF (which required 6–9 h) suggests this is potentially a chronic action of VEGF. In addition, the time needed to achieve maximum effect
is also consistent with the calculated mRNA half-life of CTGF mRNA of 2–4 h.

From a biological perspective, the effects of VEGF on CTGF mRNA could potentially have important physiological impact for several reasons. First is that the increase in CTGF mRNA results in increased protein levels. Second, the VEGF concentration that was minimally active (0.25 ng/ml) can easily bind and activate a significant percentage of the VEGFR-1, -2 receptors (58, 61). Third, this low level of VEGF may exist even in non-pathological states, suggesting that low levels of VEGF may have physiological actions on maintaining extracellular matrix production via the induction of CTGF. At 2.5–25 ng/ml VEGF which are encountered in hypoxic and angiogenic states (62), the induction of CTGF expression by VEGF could potentially induce the fibrosis that frequently accompanies neovascularization. This possibility is supported further by the demonstration that the protein levels of CTGF expression were increased 10 h after the addition of VEGF that was consistent with the maximum increase in the mRNA levels at 6–9 h. In addition, the potency of VEGF on CTGF expression appeared to be similar to TGF-β1, suggesting that both of them could induce fibrosis associated with neovascularization.

The activation of the endogenous tyrosine kinases of KDR/Fk1s can stimulate multiple signaling pathways, including Ras-ERK (63), PI3-kinase-Akt (13–15), and phospholipase C pathway (64, 65), the induction of CTGF expression by VEGF could potentially induce the fibrosis that frequently accompanies neovascularization. This possibility is supported further by the demonstration that the protein levels of CTGF expression were increased 10 h after the addition of VEGF that was consistent with the maximum increase in the mRNA levels at 6–9 h. In addition, the potency of VEGF on CTGF expression appeared to be similar to TGF-β1, suggesting that both of them could induce fibrosis associated with neovascularization.

However, Pendurthi et al. (31) reported that factor VII and thrombin induced CTGF gene expression through a PI3-kinase-dependent pathway. TGF-β has been reported to increase the transduction rates of CTGF. A promoter element of CTGF, which is responsive to TGF-β stimulation, has been reported to be present between –162 and –128 nucleotides in the 5’ region (65). However, it is unlikely that the effects of VEGF on CTGF mRNA levels are mediated via the expression of TGF-β since the addition of cycloheximide did not change these effects.

In summary, these results have provided the first evidence that VEGF can induce the expression of CTGF via both Flt1 and KDR/Fk1 by the selectively activated PI3-kinase-Akt pathway but independent of the Ras-ERK pathway. In addition, the spectrum of signaling pathways may be different between Flt1 and KDR/Fk1, possibly reflecting their physiological roles. Biologically, these results support the conclusion that VEGF, through its effects on CTGF expression, may have physiological roles such as the maintenance of capillary strength and wound healing via the extracellular matrix production. In disease states, VEGF-induced CTGF may cause the proliferation of fibrocellular components in retinal neovascular diseases such as proliferative diabetic retinopathy and age-related macular degeneration.

Acknowledgment—We thank Dr. Edward P. Feener for suggestions during the preparation of this manuscript.

REFERENCES

1. Klagsbrun, M., and D’Amore, P. A. (1991) Annu. Rev. Physiol. 53, 217–239
2. Akiyama, H., Itoh, T., Nakamura, Y., and Imai, T. (1999) J. Biol. Chem. 274, 19858–19865
3. Firestein, G. S. (1999) J. Clin. Invest. 103, 3–4
4. Lopez, F. P., Sippky, B. D., Lambert, H. M., Thach, A. B., and Hinton, D. R. (1999) Invest. Ophthalmol. Vis. Sci. 40, 883–888
5. Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. (1999) FASEB J. 13, 9–22
6. Shimosato, D., Itin, A., Soffer, D., and Keshet, E. (1992) Nature 359, 843–845
7. Ferrara, N., Carver-Keen, M., Chen, H., Dowd, M., Lu, L., O’Shea, K. S., Powell-Broxton, L., Hillan, K. J., and Moore, W. M. (1996) Nature 380, 429–442
8. Petrova, T. V., Makinen, T., and Alitalo, K. (1999) Cell 98, 1273–1276
9. Wu, L. W., Mayo, L. D., Dunbar, D. J., Kessler, K. M., Baerwald, M. R., Jaffe, E. A., Wang, D., Warren, R. S., and Donner, D. B. (2000) J. Biol. Chem. 275, 5996–5993
10. Xia, P., Aiello, L. P., Ishii, H., Jiang, Z. Y., Park, D. J., Robinson, G. S., Takagi, H., Newsome, W. P., Jirousek, R. M., and King, G. L. (1996) J. Clin. Invest. 98, 2018–2026
11. Takahashi, T., Ueno, H., and Shibuya, M. (1999) Oncogene 18, 2221–2230
12. He, H., Venema, V. J., Gu, X., Venema, R. C., Marrero, M. B., and Caldwell, R. B. (1999) J. Biol. Chem. 274, 25120–25125
13. Fujio, Y., and Walsh, R. (1999) J. Biol. Chem. 274, 16349–16354
14. Gerber, H. P., McGurty, A., Kowalski, J., Yan, M., Keyt, B. A., Dicz, V., and Ferrara, N. (1998) J. Biol. Chem. 273, 30336–30343
15. Thakker, G. D., Haji-Douk, P. W., Muller, A. W., and Rosengart, T. K. (1999) J. Biol. Chem. 274, 10002–10007
16. Bork, P. (1993) FEBS Lett. 327, 125–130
17. Lau, L. F., and Lam, S. C. (1999) Exp. Cell Res. 248, 44–57
18. Frazier, K., Williams, S., Klappper, H., and Grotendorst, G. R. (1999) J. Invest. Dermatol. 107, 404–411
19. Kireeva, M. L., Latinkin, V. B., Kolesnikova, T. V., Chen, C. C., Yang, G. P., Abler, A. S., and Lau, L. F. (1997) Exp. Cell Res. 233, 63–77
20. Bradham, D. M., Igarashi, A., Potter, R. L., and Grotendorst, G. R. (1993) J. Biol. Chem. 268, 637–645
21. Igarashi, A., Okochi, H., Bradham, D. M., and Grotendorst, G. R. (1999) Mol. Cell. Biol. 4, 637–645
22. Kothapalli, D., Frazier, K. S., Welply, A., Segarini, P. R., and Grotendorst, G. R. (1997) Cell Growth Differ. 8, 61–68
23. Igarashi, A., Nashiro, K., Kikuchi, K., Sato, S., Ihn, H., Fujimoto, M., Grotendorst, G. R., and Takekara, K. (1996) J. Invest. Dermatol. 106, 729–733
24. Frazier, K. S., and Grotendorst, G. R. (1997) Int. J. Biochem. Cell Biol. 29, 153–161
25. Oemar, B. S., Werner, A., Garnier, J. M., Do, D. G., Godoy, N., Nauck, M., Marx, W., Rupp, J., Peck, M., and Lascher, T. F. (1997) Circulation 95, 821–839
26. Babic, A. M., Chen, C. C., and Lau, L. F. (1999) Mol. Cell. Biol. 19, 2958–2966
27. Shimo, T., Nakashima, T., Nishida, T., Asano, M., Kanyama, M., Kuboki, T., Tamatani, T., Tezuka, M., Kikuma, K., Matsumura, T., and Takigawa, M. (1999) J. Biochem. (Tokyo) 126, 137–145
28. Dammeyer, J., Beer, H. D., Brauchle, M., and Werner, S. (1998) J. Biol. Chem. 273, 18185–18190
29. Murphy, M., Godson, C., Cannon, S., Kato, S., Mackenzie, H. S., Martin, F., and Brady, H. R. (1999) J. Biol. Chem. 274, 5830–5834
30. Ricupero, D. A., Romero, J. R., Rishikof, D. C., and Goldstein, R. H. (2000) J. Biol. Chem. 275, 14632–14641
31. Abraham, D. J., Shiwen, X., Black, C. M., Sa, S., Xu, Y., and Leask, A. (2000) J. Biol. Chem.
VEGF Induces CTGF Gene Expression

40731

J. Biol. Chem. 275, 15220–15225
33. Boes, M., Duke, B. L., Booth, B. A., Erondu, N. E., Oh, Y., Hwu, V., Rosenfeld, R., and Bar, R. S. (1999) Endocrinology 140, 1575–1580
34. King, G. L., Goodman, A. D., Dake, B. L., Booth, B. A., Erondu, N. E., Oh, Y., Hwu, V., Rosenfeld, R., and Bar, R. S. (1999) J. Clin. Invest. 75, 1028–1036
35. Nayan, R. C., Berman, A. B., George, K. L., Eisenbarth, G. S., and King, G. L. (1985) Nature 316, 1003–1015
36. Burgering, B. M., and Coffer, P. J. (1995) Nature 376, 599–602
37. Kitamura, T., Ogawa, W., Sakaue, H., Hino, Y., Kuroda, S., Takata, M., Matsumoto, M., Maeda, T., Kojima, H., Kikikawa, U., and Kasuga, M. (1998) Mol. Cell. Biol. 18, 3708–3717
38. Ueki, K., Yamamoto-Honda, R., Kaburagi, Y., Yamauchi, T., Toke, K., Burgering, B. M., Coffer, P. J., Komuro, I., Akanuma, Y., Yazaki, Y., and Kadowaki, T. (1998) J. Biol. Chem. 273, 5315–5322
39. Her, J. H., Lakhanle, S., Zu, K., Vila, J., Dent, P., Sturgill, T. W., and Weber, M. J. (1993) Biochem. J. 296, 25–31
40. Hara, K., Yonezawa, K., Sakaue, H., Ando, A., Kotani, K., Kitamura, T., Kitamura, Y., Ueda, H., Stephens, L., Jackson, T. R., Hawkins, P. T., Dhand, R., Clark, A. E., Holman, G. D., Waterfield, M. D., and Kasuga, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7415–7419
41. Uberall, F., Hellbert, K., Kamber, S., Maly, V., Villinger, A., Spitalet, M., Mwanjewe, J., Baier-Bitterlich, G., Bainer, G., and Gruit, H. H. (1999) J. Cell Biol. 144, 413–425
42. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2509–2514
43. Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., Takamori, K., Tokuda, C., and Saito, I. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1320–1324
44. Kuboki, K., Jiang, Z. Y., Takahara, N., Ha, S. W., Igarashi, M., Yamauchi, T., Fu, Y. Z., Hiebert, J. T., Rhodes, C. J., and King, G. L. (2000) Circulation 101, 676–681
45. Liang, P., and Pardee, A. B. (1992) Science 257, 967–971
46. Claus, M., Weich, H., Breier, G., Knie, U., Rockl, W., Waltenberger, J., and Risau, W. (1996) J. Biol. Chem. 271, 17629–17634
47. Sawano, A., Takahashi, T., Yamaguchi, S., Asonuma, M., and Shibuya, M. (1996) Cell Growth Differ. 7, 213–221
48. Pak, J. E., Chou, T., Housh, K. A., and Ferrara, N. (1994) J. Biol. Chem. 269, 2546–2554
49. Park, J. Y., Takahara, N., Gabriele, A., Chou, E., Naruse, K., Suzuma, K., Yamauchi, T., Ha, S. W., Meier, M., Rhodes, C. J., and King, G. L. (2000) Diabetes 49, 1239–1244
50. Toullec, D., Pianti, P., Caste, H., Bellivergues, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boisson, P., Bourier, E., Loriole, F., Dunhanel, L., Chardon, D., and Kirilovsky, J. (1991) J. Biol. Chem. 266, 15771–15781
51. Kotani, K., Ogawa, W., Matsumoto, M., Kitamura, T., Sakaue, H., Hino, Y., Miyake, K., Sano, W., Akimoto, K., Ohno, S., and Kasuga, M. (1998) Mol. Cell. Biol. 18, 6971–6982
52. Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P., and Parker, P. J. (1998) Science 281, 2542–2545
53. Standaert, M. L., Galloway, L., Cams, P., Bandypadhyaid, G., Moscat, J., and Farese, R. V. (1997) J. Biol. Chem. 272, 30075–30082
54. Standaert, M. L., Bandypadhyaid, G., Perez, L., Price, D., Galloway, L., Poklepovic, A., Sajaj, M. P., Cenni, V., Sirri, A., Moscat, J., Toker, A., and Farese, R. V. (1999) J. Biol. Chem. 274, 25308–25316
55. Wellmer, M., Maasch, C., Kuprinn, C., Lindschau, C., Lu, F. C., and Haller, H. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 178–185
56. Jiang, B. H., Zheng, J. Z., Aoki, M., and Vogt, P. K. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1749–1753
57. Takagi, H., King, G. L., and Aiello, L. P. (1996) Diabetes 45, 1016–1023
58. Millauer, I., Wizigmann-Voss, S., Schnurch, H., Martinez, R., Moller, N. P., Risau, W., and Ullrich, A. (1993) Cell 72, 835–846
59. Joukov, V., Pausolika, K., Kapiainen, A., Chilov, D., Lahtinen, I., Kukk, E., Saksela, O., Kulkkinen, N., and Aitalo, K. (1996) EMBO J. 15, 290–298
60. Soder, S., Takashima, S., Miao, H. Q., Neufeld, G., and Klagsbrun, M. (1998) Cell 92, 735–745
61. de-Vries, C., Escobedo, J. A., Ueno, H., Housc, K., Ferrara, N., and Williams, L. T. (1992) Science 255, 989–991
62. Aiello, L. P., Avery, R. L., Arrigg, P. G., Keyt, B. A., Jampel, H. D., Shah, S. T., Pasquale, L. R., Thiene, H., Iwamoto, M. A., Park, J. E., Nguyen, H. V., Aiello, L. M., Ferrara, N., and King, G. L. (1994) N. Engl. J. Med. 331, 1480–1487
63. Guo, D., Jia, Q., Song, H. Y., Warren, R. S., and Donner, D. B. (1995) J. Biol. Chem. 270, 6729–6733
64. Kanno, S., Oda, N., Abe, M., Terai, Y., Ito, M., Shibata, K., Shibuya, M., and Sato, Y. (2000) Oncogene 19, 2138–2146
65. Grotendorst, G. R., Okochi, H., and Hayashi, N. (1996) Cell Growth Differ. 7, 469–480
Vascular Endothelial Growth Factor Induces Expression of Connective Tissue Growth Factor via KDR, Flt1, and Phosphatidylinositol 3-Kinase-Akt-dependent Pathways in Retinal Vascular Cells
Kiyoshi Suzuma, Keiko Naruse, Izumi Suzuma, Noriko Takahara, Kohjiro Ueki, Lloyd P. Aiello and George L. King

J. Biol. Chem. 2000, 275:40725-40731.
doi: 10.1074/jbc.M006509200 originally published online October 3, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M006509200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 65 references, 42 of which can be accessed free at
http://www.jbc.org/content/275/52/40725.full.html#ref-list-1