Basic Fibroblast Growth Factor Induces Expression of VEGF Receptor KDR Through a Protein Kinase C and p44/p42 Mitogen-Activated Protein Kinase–Dependent Pathway

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Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are angiogenic molecules whose combined mitogenic activity is potently synergistic. However, the molecular mechanism underlying this synergy is incompletely understood. We examined whether VEGF and bFGF affect expression of each other or alter expression of the VEGF receptor KDR in retinal capillary endothelial cells. In addition, we investigated the intracellular signaling mechanisms involved in this response. VEGF-induced [^{3}H]thymidine uptake was tightly correlated with KDR mRNA and protein concentrations, suggesting that increased KDR expression might account for VEGF's synergistic activity in the presence of bFGF. bFGF (10 ng/ml) induced KDR mRNA expression within 4 h and attained a 4.0-fold increase after 24 h. KDR protein expression was increased 7.5-fold after 48 h. VEGF (=50 ng/ml) did not alter bFGF, VEGF, or KDR mRNA expression under serum-deprived conditions. In contrast, VEGF increased KDR mRNA expression 87% under growth conditions and 2.9-fold under serum-deprived conditions in the presence of bFGF. The protein kinase C (PKC) agonist phorbol myristate acetate (PMA) induced KDR mRNA expression 5.1-fold at 100 nmol/l. bFGF increased p44/p42 mitogen-activated protein kinase (MAPK) phosphorylation within 5 min, reaching a maximum within 15 min and remaining significantly elevated for >6 h. bFGF-induced MAPK phosphorylation and KDR mRNA expression were almost completely inhibited by 5 µmol/l GFX, a non–isoform-selective PKC inhibitor. MAPK inhibitor PD98059 reduced KDR mRNA expression 72% at concentrations that inhibited bFGF-induced MAPK phosphorylation 100%, suggesting that pathways in addition to MAPK might also be involved. Inhibitors of the β isoform of PKC (LY333531), protein kinase A (PKA) (H89), and phosphotidylinositol (PI) 3 kinase (wortmannin) had no significant effect. These data suggest that bFGF stimulates KDR expression through a PKC and p44/p42 MAPK–dependent pathway not primarily involving the β isoform of PKC, PKA, or PI-3 kinase. Since bFGF induces VEGF expression and since increased KDR expression potentiates VEGF action, resulting in additional KDR expression and marked mitogenic activity, these data provide a novel mechanistic explanation for the angiogenic synergy between VEGF and bFGF. *Diabetes* 48:1145–1155, 1999

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bFGF, basic fibroblast growth factor; BREC, bovine retinal capillary endothelial cells; BSA, bovine serum albumin; DMEM, Dulbecco's minimal essential medium; ECGF, endothelial cell growth factor; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase; MEK, MAPK or ERK kinase; PBS, phosphate-buffered saline; PDHS, plasma-derived horse serum; PI, phosphotidylinositol; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; VEGF, vascular endothelial growth factor.

Basic fibroblast growth factor (bFGF) (1) and vascular endothelial growth factor (VEGF) (2) are endothelial cell mitogens. VEGF is essentially an endothelial-cell–selective mitogen, whereas bFGF also stimulates fibroblasts, smooth muscle cells, and epithelial cells (3–5).VEGF, also known as vascular permeability factor (VPF) (6) and vasculotropin (7), is induced by hypoxia (8–10) and has potent vasopermeability activity (6,11). VEGF is thought to play a central role in mediating angiogenesis during fetal development (7), wound healing (12), collateral vessel formation (13), and tumorigenesis (14). In the eye, VEGF and bFGF are important mediators of pathologic intraocular neovascularization (15–26), which accounts for the visual loss associated with numerous ocular diseases such as diabetic retinopathy (15–19), retinopathy of prematurity (15,20), neovascular glaucoma (15,24), age-related macular degeneration (21,22,27), and central retinal vein occlusion (15,25). Several retinal cell types produce VEGF (8,20,28,29) and bFGF (30–33). Of the two cytokines, VEGF is currently believed to be primarily responsible for mediating intraocular neovascularization since, unlike bFGF (34), it is secreted and diffusible within the eye. VEGF is also increased up to 30-fold under hypoxic conditions (8,10,35), and intraocular concentrations are highly correlated with human ocular ischemic neovascular disorders (15,17,21, 22,36) and representative animal models (20,26,27). In animals, inhibition of VEGF activity can suppress ischemia-induced neovascularization of both the retina (37,38) and the iris (39).

bFGF is a heparin-binding protein usually tightly associated with the extracellular matrix (40,41) and known to be present in the retina (42). Although bFGF is also an endothelial cell growth factor, it is currently thought to play a more secondary/modulating role in ocular disease than does VEGF

since, unlike VEGF, it lacks a consensus signal peptide and is not secreted from the cell by classic mechanisms (34). In addition, no direct causal relationship with neovascularization has been confirmed, and examination of ocular and systemic fluids from individuals with proliferative diabetic retinopathy have yielded inconsistent results (16,43–46).

VEGF exerts its action through two high-affinity, tyrosine-phosphorylating, transmembrane receptors named KDR/flk-1 (47) and flt-1 (48). KDR/flk-1 expression is selective for endothelial cells in vivo (48), whereas flt-1 is expressed in both endothelial (48) and non-endothelial cells (49–51). Retinal endothelial cells predominantly express KDR in vitro (50,52). VEGF binding to either receptor can activate numerous intracellular signaling molecules including phosphotidylinositol (PI) 3 kinase, phospholipase C (PLC)-γ, and protein kinase C (PKC) α, β, and δ isoforms (53,54). Histologically, KDR knockout mice show no endothelial cell proliferation or blood island formation, in contrast to Flt-1 knockout mice, suggesting that signal transduction through Flt may be required for late-stage vasculogenesis, while signaling through KDR may be primarily required for endothelial cell growth (55,56). In addition, the expression of VEGF and bFGF have been colocalized in tumor tissues (57) and neovascular membranes (58) in vivo.

Interestingly, the mitogenic actions of bFGF and VEGF are potently synergistic both in vivo (59) and in vitro (60,61). Although bFGF can increase VEGF expression in human tumors and rabbit smooth muscle cells (62,63), the molecular mechanisms underlying this synergy have not been fully elucidated, especially in ocular tissues. Pepper and Mandriota (64) reported that VEGF induces KDR expression in bovine aortic endothelial cells; however, the mechanism underlying this response was not elucidated. In this study, we evaluated whether bFGF altered the expression of KDR or VEGF in retinal capillary endothelial cells and examined the intracellular signaling pathways involved in this response. We demonstrate that VEGF-stimulated endothelial cell growth is closely correlated with the extent of KDR expression. In addition, bFGF increases the expression of its receptor KDR through a PKC and p44/p42 mitogen-activated protein kinase (MAPK)–dependent mechanism. Finally, VEGF increases KDR expression under conditions with adequate basal KDR expression, but not under conditions, such as serum-deprivation, where this is not the case. These data provide a novel mechanistic explanation for the angiogenic synergy between VEGF and bFGF and suggest that VEGF-mediated actions may be effectively regulated by the extent of KDR expression.

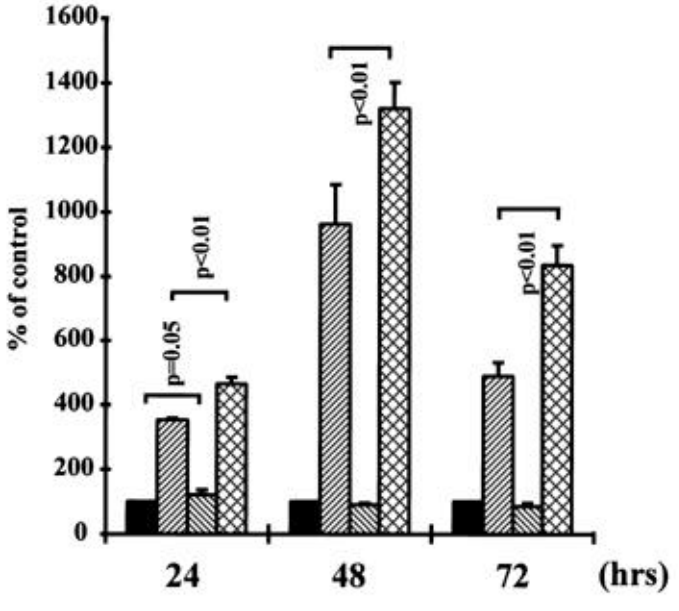

FIG. 1. VEGF and bFGF synergistically stimulate retinal endothelial cell growth even under conditions where VEGF alone is ineffective. BREC were seeded in 24-well plates at a density of 10^4 cells/well. After 24 h of starvation in 1% CS, cells were either untreated (■) or treated with 10 ng/ml bFGF (▨), 25 ng/ml VEGF (▧), or both (⊠) for 24, 48, or 72 h as indicated in the figure. [^{3}H]thymidine (0.25 µCi/well) was added to the cells 6 h before assaying for [^{3}H]thymidine incorporation by scintillation counting.

RESEARCH DESIGN AND METHODS

Cell culture. Primary cultures of bovine retinal capillary endothelial cells (BREC) were isolated from fresh slaughterhouse tissues by homogenization and a series of filtration steps as previously described (8). BREC were cultured in endothelial basal medium (Clonetics, San Diego, CA) with 10% plasma-derived horse serum (PDHS) (Wheaton, Millville, NJ), heparin (50 mg/l), and 50 µg/ml endothelial cell growth factor (ECGF) (Boehringer Mannheim, Chicago) in fibronectin-coated dishes. Within a week after initial isolation, BREC were trans-

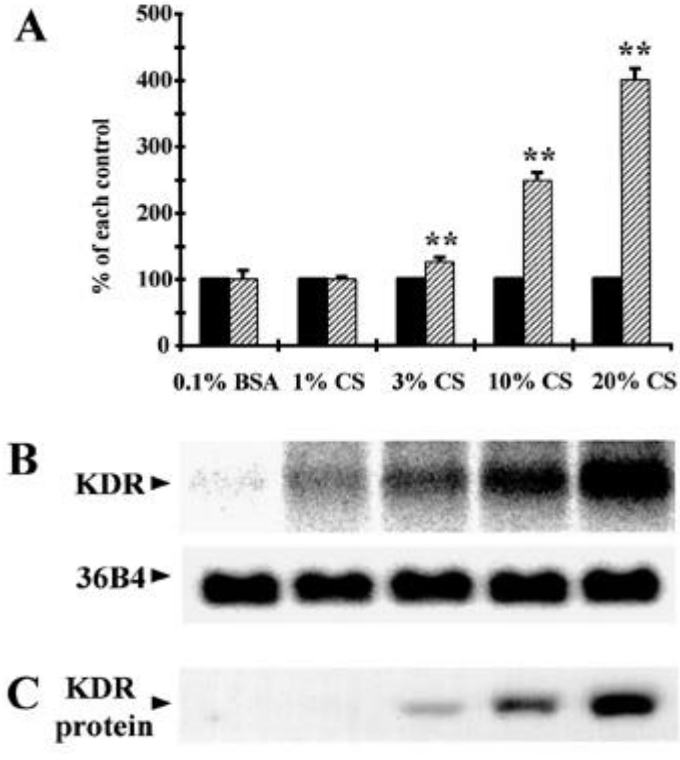

FIG. 2. VEGF's mitogenic activity is closely correlated with the extent of KDR expression. *A*: BREC were cultured under the conditions noted in the figure for 24 h before stimulation with 25 ng/ml VEGF for 24 h (▨). Control cells received no VEGF but were cultured in the same respective serum concentration (■). [^{3}H]thymidine incorporation was determined as described in Fig. 1. *B*: After 24 h in the indicated media, Northern blot analysis using human KDR cDNA was performed on 20 µg total RNA per lane. Hybridization of the same blots was also performed with 36B4 cDNA as an internal lane loading control. **$P < 0.001$. *C*: After 36 h in the indicated media, Western blot analysis of total cell lysate was performed using an anti-human KDR polyclonal antibody as described in METHODS. For all panels, each experiment was repeated at least three times, and representative results are shown.

ferred to new fibronectin-coated dishes using a cloning ring and Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY) and 50 µg/ml ECGF (growth medium). Cells were cultured at 37°C in 5% CO_2, 95% air, and media were changed every 2–3 days.

Endothelial growth assays. BREC were seeded into 24-well plates at a density of 10^4 cells/well in growth medium. The next day, the medium was changed to the indicated medium. After 24 h, cells were stimulated with bFGF and/or VEGF. To examine thymidine incorporation in BREC, [^{3}H]thymidine (NEN) was added to the cells (0.25 µCi/well) for the last 6 h of stimulation. After washing with phosphate-buffered saline (PBS) and fixation by 5% trichloroacetic acid, cells were lysed with 1 N NaOH, and [^{3}H]thymidine incorporation was determined by scintillation counting as previously described (65).

Northern blot analysis. Total RNA samples were isolated from BREC using TRIZOL reagent (Gibco BRL) and subjected to Northern blot analysis as previously described (66). Radioactive probes were generated using Amersham Multiprime labeling kits and [^{32}P]dCTP (NEN). Quantitation of Northern blots was performed using a computing PhosphorImager with ImageQuant software analysis (Molecular Dynamics, Sunnyvale, CA). Lane loading differences were normalized by rehybridization with radiolabeled 36B4 cDNA probe (66).

KDR protein detection. BREC were washed once in cold 1× PBS and lysed in urea buffer (6 mol/l urea, 10 mmol/l imidazole, 2 mmol/l $MgCl_2$, 0.1% Triton X-100, pH 7.4). Protease inhibitors (1 mmol/l phenylmethylsulfonyl fluoride [PMSF], 2 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mmol/l NaF, 0.5 mmol/l Na_3VO_4) were added to the buffer just before use. Protein concentrations were determined by the method of Bradford (67). Fifty micrograms of total cell lysate was subjected to SDS-PAGE under reducing conditions, and proteins were transferred to nitrocellulose filters (Bio-Rad, Hercules, CA). The blots were incubated with anti-human KDR polyclonal antibody (Santa-Cruz Biological, Santa Cruz, CA) followed by incubation with horseradish peroxidase–conjugated secondary antibody (Amersham, Piscataway, NJ). Visualization was performed using Amersham enhanced chemiluminescence (ECL) detection system per manufacturer's instructions.

p44/p42 MAPK phosphorylation. BREC were washed once in cold 1× PBS and lysed in 1× Laemmli buffer (50 mmol/l Tris, pH 6.8, 2% SDS, 10% glycerol) containing protease inhibitors (1 mmol/l PMSF, 2 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mmol/l NaF, 0.5 mmol/l Na_3VO_4). Cell lysates were heated to 95°C for 2 min, and equal volumes of lysate were subjected to SDS-PAGE under reducing conditions. The blots were incubated with anti-phosphospecific ERK1(p44)/ERK2(p42) antibody (New England BioLabs, Beverly, MA) followed by incubation with horseradish peroxidase–conjugated secondary antibody. Visualization was performed using Amersham ECL detection system as described above. Lane loading differences were normalized by reblotting with non–phosphorylation-specific anti-ERK1 antibody (Santa-Cruz Biological).

Statistical analysis. All experiments were repeated at least three times with similar findings, and results are expressed as mean ± SD. The unpaired t test was used for comparisons of groups with equal variance and normal distribution. A P value <0.05 was considered statistically significant.

RESULTS

bFGF potentiates VEGF-induced retinal endothelial cell growth even under conditions where VEGF alone is ineffective. To examine the effects of bFGF and/or VEGF on retinal endothelial cell growth, BREC were starved with 1% calf serum (CS) for 24 h and then stimulated with 10 ng/ml bFGF, 25 ng/ml VEGF, or both for 24, 48, and 72 h. As shown in Fig. 1, after this extent of starvation, VEGF could not effectively stimulate [^{3}H]thymidine uptake in BREC. In contrast, bFGF increased [^{3}H]thymidine uptake 3.5-, 9.6-, and 4.9-fold after 24, 48, and 72 h, respectively ($P <$ 0.001). [^{3}H]thymidine uptake was further increased at all time points when cells were stimulated simultaneously with both bFGF and VEGF. Combined stimulation resulted in increases of 20, 37, and 71% after 24, 48, and 72 h, respectively, compared with bFGF stimulation alone ($P < 0.003$). Thus, bFGF potentiates VEGF-stimulated [^{3}H]thymidine uptake in retinal endothelial cells even under conditions where VEGF stimulation alone is ineffective.

VEGF's mitogenic activity is closely correlated with the extent of KDR expression. Since VEGF signaling through KDR is thought to be required for VEGF to stimulate endothelial cell growth, we examined whether VEGF's inability to induce [^{3}H]thymidine uptake under starved conditions resulted from alterations in KDR expression. BREC were cultured under the conditions noted in Fig. 2 for 24 h before stimulation with 25 ng/ml VEGF for 24 h. As shown in Fig. 2*A*, VEGF could not effectively stimulate endothelial cell growth under conditions containing less than 3% CS. VEGF stimulated

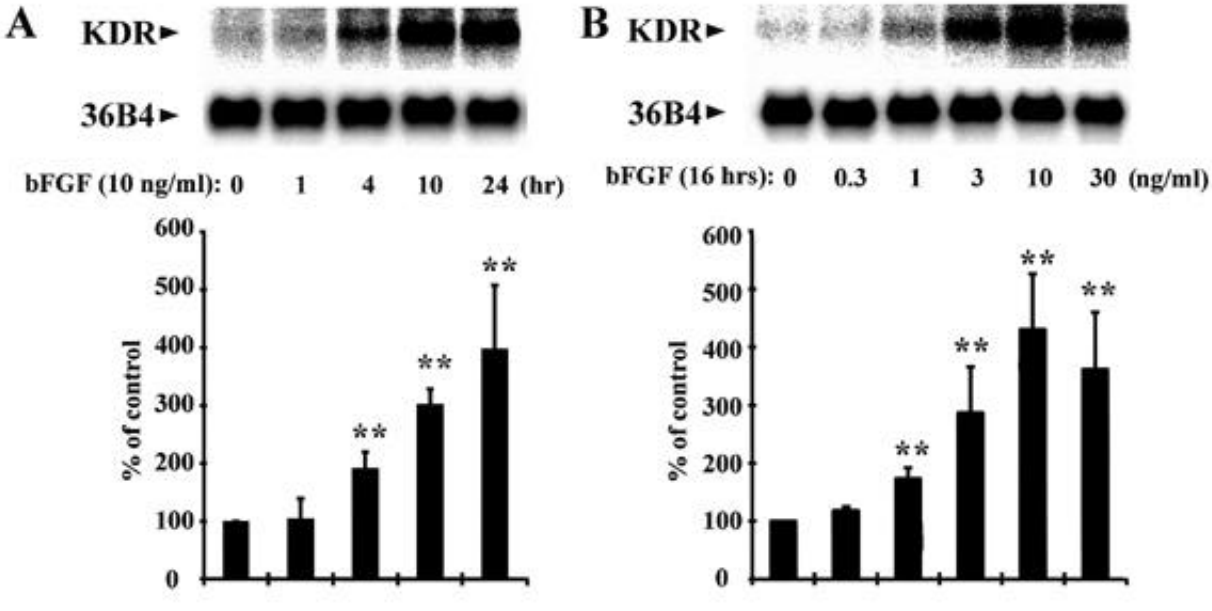

FIG. 3. bFGF increases KDR mRNA expression in BREC in a time- and dose-dependent manner. Total RNA was isolated and subjected to Northern blot analysis using radiolabeled KDR and 36B4 cDNA probes as described in Fig. 2*B*. Top panels show representative Northern blots, and bottom panels present quantitation derived from multiple experiments. For quantitation, all KDR signal intensities were normalized to those of 36B4 to account for differences in lane loading. *A*: BREC were starved in DMEM containing 1% CS for 24 h, followed by addition of 10 ng/ml bFGF for 1–24 h as indicated in the figure. *B*: Similarly starved BREC were exposed for 16 h to 0.3–30 ng/ml bFGF as indicated in the figure. **$P < 0.010$.

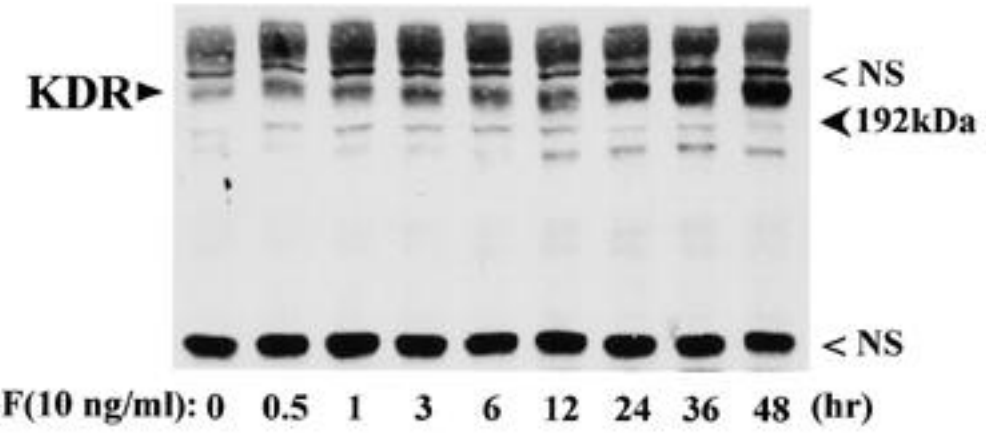

FIG. 4. bFGF increases KDR protein expression in BREC. BREC were cultured for 24 h in 1% CS and then stimulated with 10 ng/ml bFGF for 0.5–48 h as indicated in the figure. Cells were lysed in urea buffer as described in METHODS and subjected to 6% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blotted with anti-human KDR polyclonal antibody. Visualization was performed using the Amersham ECL detection system. NS, nonspecific.

[^{3}H]thymidine uptake 26, 148, and 300% in 3, 10, and 20% CS, respectively. KDR mRNA (Fig. 2*B*) and protein (Fig. 2*C*) concentrations correlated with VEGF's mitogenic effect. KDR mRNA concentrations were increased 1.5-, 2.8-, 3.0-, and 4.1-fold after 24 h in 1, 3, 10, and 20% CS, respectively, compared with 0.1% bovine serum albumin (BSA). KDR protein increased correspondingly. These results demonstrate that alterations in KDR mRNA and protein expression correlate closely with VEGF's mitogenic effect and suggest that the increase of VEGF activity observed in the presence of bFGF could theoretically be mediated by an increase in KDR expression.

bFGF increases KDR mRNA and protein expression in a time- and dose-dependent manner. To determine whether bFGF alters KDR gene expression, BREC were starved with 1% CS for 24 h and then stimulated with 10 ng/ml bFGF for 1–24 h. As demonstrated in Fig. 3*A*, 10 ng/ml bFGF stimulated KDR mRNA expression in a time-dependent manner that was initially evident after 4 h and attained a 4.0-fold increase after 24 h ($P < 0.010$). KDR mRNA expression was increased 5, 91, 202, and 299% after 1, 4, 10, and 24 h, respectively. This effect was also dose-dependent as shown in Fig. 3*B*, with statistically significant increases in KDR mRNA expression initially detectable at a concentration of 1 ng/ml bFGF for 16 h ($P < 0.050$) and a maximal stimulation of 4.3-fold achieved at a concentration of 10 ng/ml. KDR mRNA expression was increased 13, 72, 187, 330, and 262% by 0.3, 1, 3, 10, and 30 ng/ml bFGF, respectively.

The effect of bFGF on KDR protein expression was evaluated by Western blot analysis using BREC total cell lysates. After addition of 10 ng/ml bFGF, KDR protein increased most markedly between 12 and 24 h (Fig. 4). KDR protein concentrations increased 41–210% between 0.5 and 12 h after

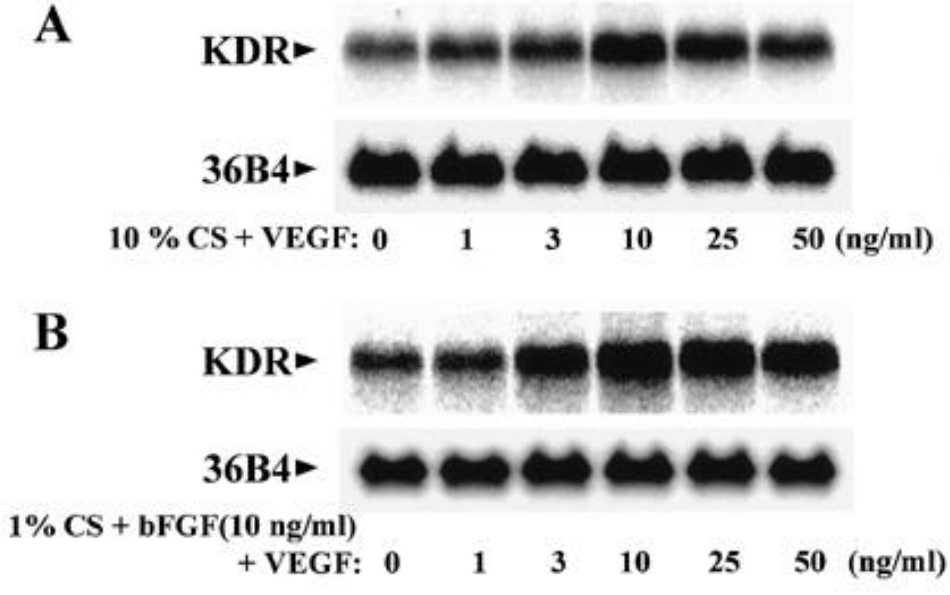

FIG. 5. VEGF-induced KDR mRNA expression is correlated with the extent of basal KDR expression. *A*: *BREC* were cultured for 24 h in 10% CS and then exposed to the indicated concentrations of VEGF for 16 h. Total RNA was isolated and subjected to Northern blot analyses using radiolabeled KDR and 36B4 cDNA probes. KDR expression was increased ($P < 0.05$) at VEGF concentrations ≥3 ng/ml. *B*: The experiment was performed exactly as described in *A* except that all cells were exposed to 1% CS and 10 ng/ml bFGF instead of 10% CS. KDR expression was increased ($P < 0.05$) at VEGF concentrations ≥3ng/ml.

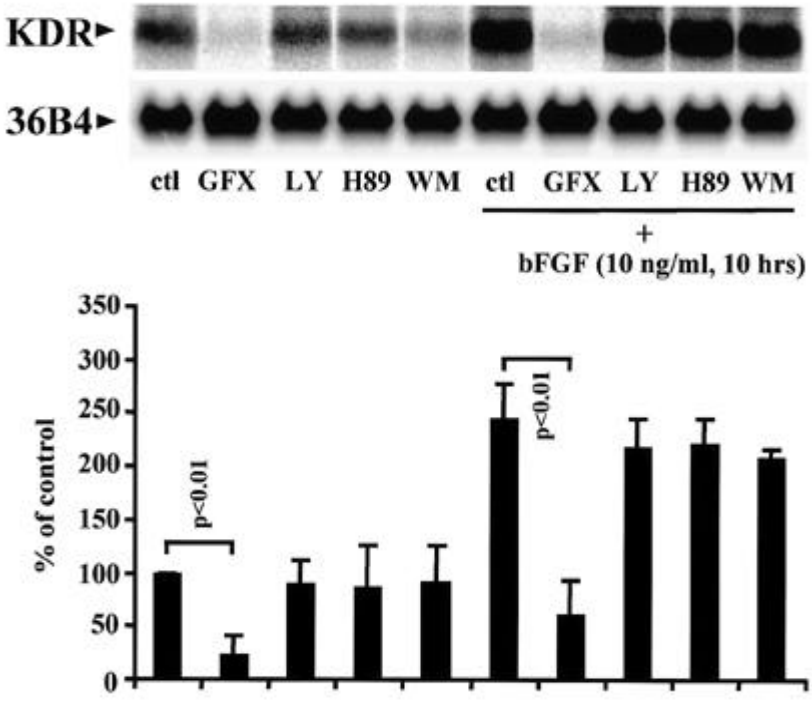

FIG. 6. Involvement of protein kinases in bFGF-induced KDR gene expression. Subconfluent BREC were starved with 1% CS for 24 h. Cells were either untreated or pretreated with GFX (5.0 µmol/l), LY333531 (20 nmol/l), H89 (3.0 µmol/l), or wortmannin (100 nmol/l) for 30 min before stimulation with 10 ng/ml bFGF for 10 h. Total RNA was isolated and subjected to Northern blot analysis for KDR mRNA expression, and membranes were stripped and rehybridized with 36B4 cDNA probe for normalization (*A*). Quantitation from multiple experiments after normalization to 36B4 is also presented (*B*).

bFGF stimulation, compared with 352, 430, and 650 ± 151% ($P < 0.01$) after 24, 36, and 48 h, respectively. These results demonstrate that physiologic concentrations of bFGF can induce KDR mRNA and protein expression.

VEGF-induced KDR mRNA expression is correlated with the extent of basal KDR expression. Unlike bFGF, VEGF did not alter KDR mRNA expression at concentrations of =50 ng/ml and exposures up to 24 h when cells were starved for 24 h in 1% CS (data not shown). This effect probably results from the low basal amount of KDR present under these conditions. However, after exposure to 10% CS for 24 h, which increases basal KDR expression 2.0-fold, VEGF increased KDR mRNA expression in a dose-dependent manner, with increased KDR expression initially detectable at 3 ng/ml VEGF and maximal stimulation of 1.9-fold observed at 25 ng/ml ($P < 0.017$) (Fig. 5*A*). When 1% CS starved cells were exposed to 10 ng/ml bFGF, which increased basal KDR expression 4.0-fold, VEGF was now capable of increasing KDR expression with initial effect noted at 3 ng/ml VEGF and maximal stimulation of 2.9-fold observed at 25 ng/ml ($P < 0.008$) (Fig. 5*B*). These results demonstrate that VEGF is not capable of stimulating KDR expression under conditions where basal KDR levels are low but can effectively increase KDR expression when basal KDR levels are elevated either by serum or addition of physiologic concentrations of bFGF.

Involvement of protein kinase C in bFGF-induced KDR gene expression. We examined the intracellular signal transduction mechanism underlying bFGF-induced KDR gene expression in BREC using the non–isoform-selective PKC inhibitor GFX (5 µmol/l), the PKC β isoform-selective inhibitor LY333531 (20 nmol/l), the PKA inhibitor H89 (3 µmol/l), and the PI 3 kinase inhibitor wortmannin (100 nmol/l). As shown in Fig. 6, only GFX significantly inhibited bFGF-induced KDR gene expression, whereas H89, wortmannin, and a PKC-β isoform selective dose of LY333531 had no significant effect. In addition, GFX also reduced basal KDR gene expression by 77% ($P < 0.003$) without affecting cellular morphology or attachment as assessed by light microscopy. Confirmation that PKC activation stimulated KDR gene expression in BREC was obtained using the PKC agonist PMA (Fig. 7). After 4 h exposure to PMA, KDR gene expression was increased 2.5-, 4.1-, 4.4-, 5.1-, and 4.8-fold

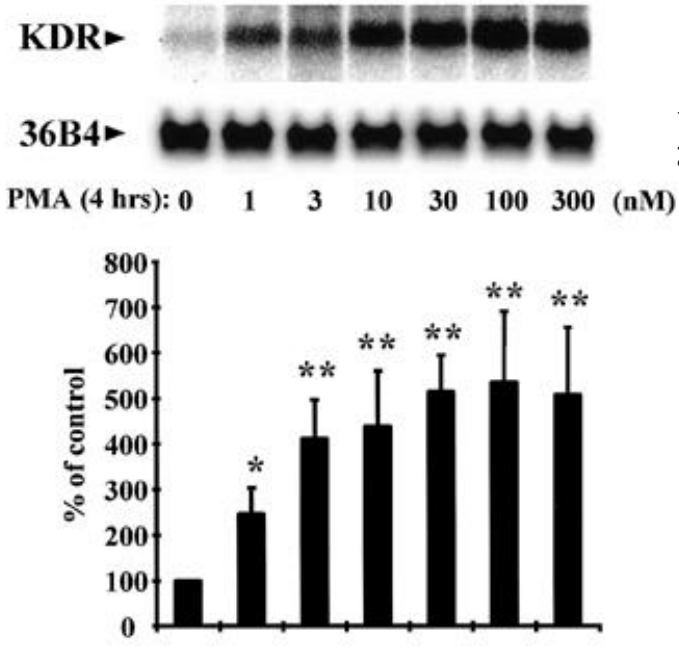

FIG. 7. PMA stimulates KDR gene expression in BREC. BREC were stimulated with the concentrations of PMA indicated in the figure for 4 h. Total RNA was subjected to Northern blot analysis as described above. A representative Northern blot (*A*) and quantitation from multiple experiments after normalization to 36B4 are presented (*B*). *$P < 0.050$; **$P < 0.010$.

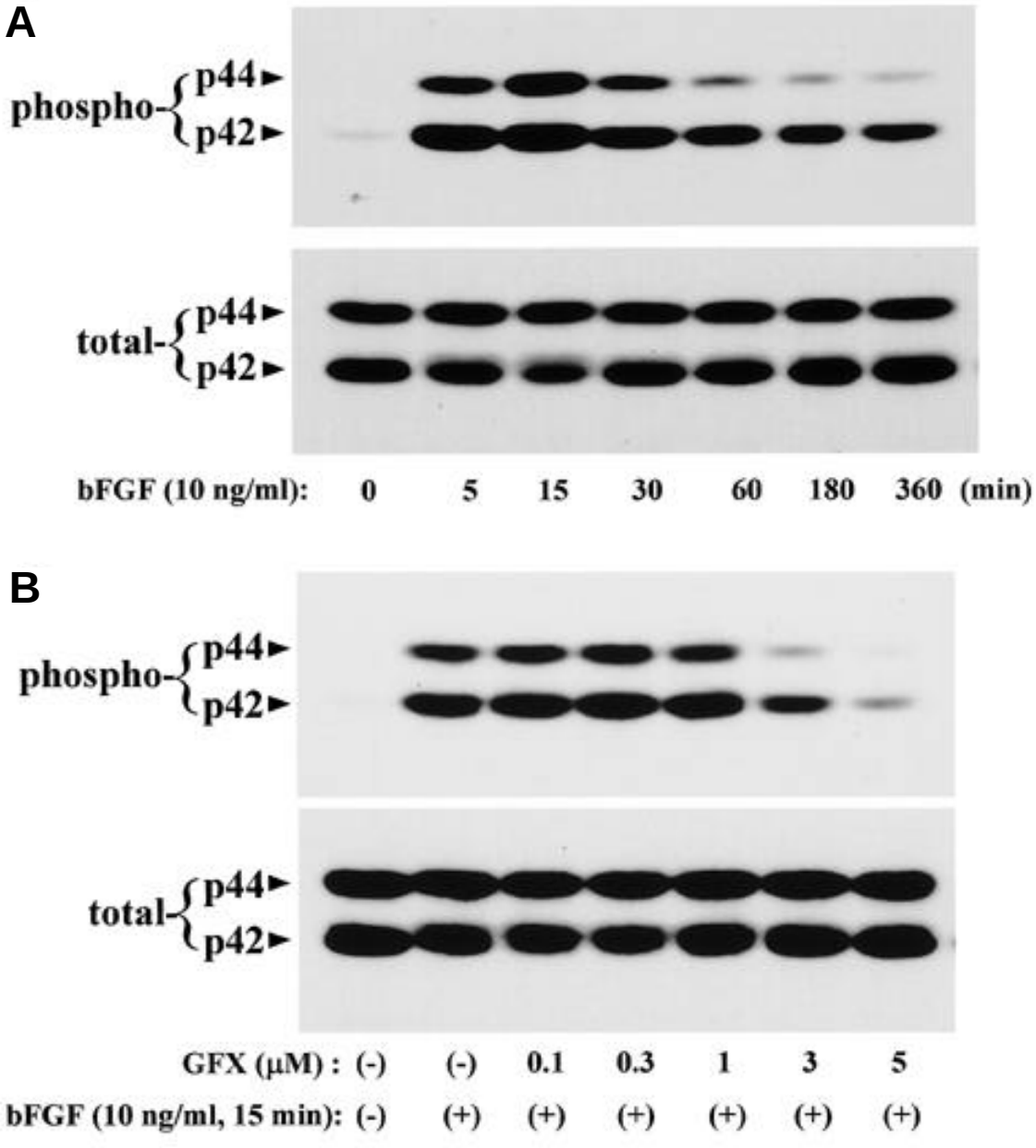

FIG. 8. bFGF induces p44/p42 MAPK phosphorylation through a PKC dependent pathway. *A*: Quiescent BREC, either untreated or treated with 10 ng/ml of bFGF for 5, 15, 30, 60, 180, or 360 min, were lysed in 1× Laemmli buffer, and the lysates were subjected to 10% SDS-PAGE followed by transfer to nitrocellulose membranes. The membranes were probed with an anti-phosphospecific p44/p42 MAPK antibody (top) and then stripped and reprobed with anti-ERK1 antibody (bottom). *B*: Cells received the concentrations of GFX indicated in the figure for 30 min before stimulation with 10 ng/ml bFGF for 15 min. Evaluation of p44/p42 MAPK phosphorylation was performed as described in *A*.

($P < 0.009$) at 1, 3, 10, 30, 100, and 300 nmol/l PMA, respectively. Maximal stimulation was observed at 100 nmol/l PMA.

bFGF stimulates MAPK phosphorylation in BREC through a PKC-dependent pathway. It has been reported that bFGF stimulates the growth of many cell types through p44/p42 MAPK activation (68–70). Thus, we examined whether bFGF-induced KDR gene expression is also mediated through activation of p44/p42 MAPK. As shown in Fig. 8*A*, bFGF dramatically stimulated p44/p42 MAPK phosphorylation within 5 min, with maximal effect achieved after 15 min. Significant activation was maintained for >6 h. Total p44/p42 MAPK concentrations remained unchanged.

To determine if PKC activation was required prior to bFGF-induced p44/p42 MAPK phosphorylation, BREC were pretreated with different concentrations of the non–isoform-selective PKC inhibitor GFX and then stimulated with 10 ng/ml bFGF for 15 min. bFGF-induced p44/p42 MAPK phosphorylation was inhibited in a dose-dependent manner, with 52 and 90% inhibition observed at 3 and 5 μmol/l GFX, respectively (Fig. 8*B*).

bFGF-induced KDR gene expression is partially mediated through p44/p42 MAPK activation. To determine the role of p44/p42 MAPK activation in bFGF-induced KDR expression, we investigated whether suppression of MAPK could

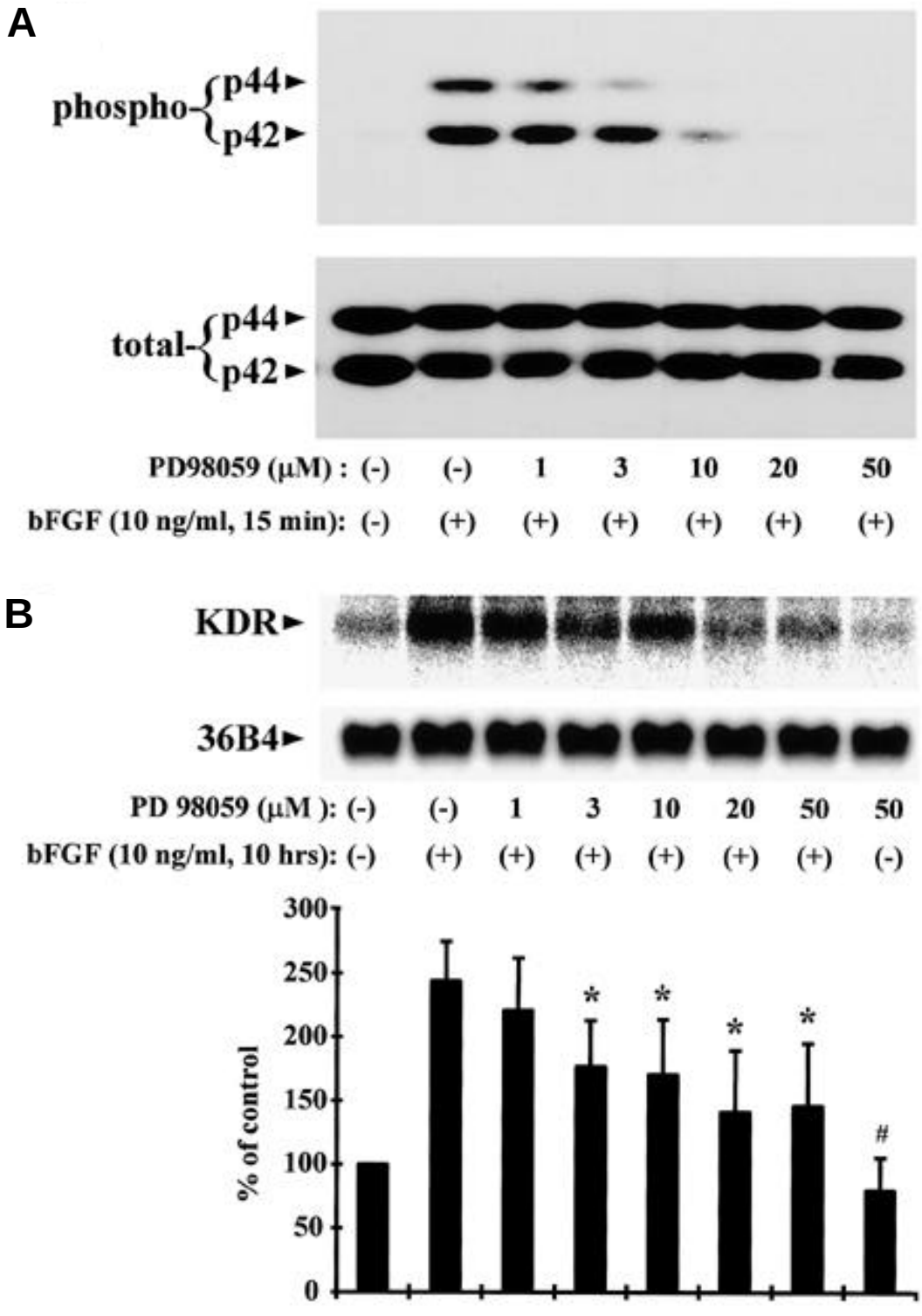

FIG. 9. Effect of MEK-1 inhibitor PD98059 on bFGF-induced KDR mRNA expression and p44/p42 MAPK phosphorylation. Subconfluent BREC were cultured for 24 h in 1% CS and then pretreated for 30 min with the concentrations of PD98059 indicated in the figure. Cells were subsequently stimulated with 10 ng/ml bFGF for 15 min for evaluation of MAPK phosphorylation (*A*) or 10 h for Northern blot analysis (*B*). Representative blots are shown, and quantitation from multiple experiments after normalization to 36B4 is presented in *B*. **P* < 0.050 compared with PD(−), bFGF(+); #*P* = not significant compared with PD(−), bFGF(−).

inhibit bFGF-induced KDR gene expression in BREC using the MAPK or ERK kinase (MEK) inhibitor PD98059. As expected, PD98059 inhibited bFGF-induced p44/p42 MAPK phosphorylation by 43% at 3 μmol/l and 100% at 20 μmol/l (Fig. 9*A*). bFGF-induced KDR expression was inhibited by concentrations of PD98059 >3 μmol/l (Fig. 9*B*). However, KDR gene expression was not completely inhibited even at high concentrations of the MEK inhibitor, with inhibition of

16, 47, 51, 72, and 68% observed at 1, 3, 10, 20, and 50 µmol/l PD98059, respectively.

bFGF stimulates VEGF gene expression in BREC. The effect of bFGF on VEGF gene expression in BREC was confirmed. bFGF (10 ng/ml) stimulated VEGF gene expression in a time-dependent manner, becoming initially evident after 4 h and demonstrating a 2.8-fold increase after 24 h ($P < 0.01$, data not shown). After 16-h exposure to bFGF, increased VEGF gene expression was observed at bFGF concentrations as low as 3 ng/ml, with maximal stimulation of 123% observed at 10 ng/ml ($P < 0.02$, data not shown).

DISCUSSION

In this study, we demonstrate that bFGF induces the expression of VEGF receptor KDR through a PKC and p44/p42 MAP kinase–dependent mechanism in retinal microvascular endothelial cells. KDR mRNA and protein expression was induced 4.0- and 6.6-fold, respectively, by physiologic concentrations of bFGF. PKC activity was required for bFGF-induced MAP phosphorylation. Furthermore, we confirm previous findings (62,63) that bFGF induces VEGF mRNA expression (2.8-fold) and, thus, demonstrate a potential mechanistic explanation for the synergistic activity of VEGF and bFGF.

The significant role of PKC in mediating this response is supported by several findings. Addition of the non–isoform-selective PKC inhibitor GFX abolished bFGF-stimulated KDR expression and reduced basal KDR expression by 77%. In addition, the PKC agonist PMA increased KDR expression 5.1-fold. Although it is suspected that PKC-β activation is involved in many aspects of diabetic vascular complications (11,54,71), the PKC β-isoform selective inhibitor LY333531 did not inhibit bFGF-induced KDR expression at isoform selective concentrations (54,71,72). This suggests that both the bFGF signaling pathway and the basal expression of KDR primarily involve PKC isoforms other than β. The lack of effect of inhibitors H89 and wortmannin suggests that PKA and PI 3 kinase do not play significant roles in either bFGF stimulation or basal expression of KDR. Finally, inhibition of PKC eliminated bFGF-induced p44/p42 MAPK phosphorylation, demonstrating that the activation of PKC lies upstream of p44/p42 MAPK phosphorylation in the bFGF signal transduction pathway.

p44/p42 MAPK phosphorylation was markedly increased by bFGF in <5 min, and activation was maintained for >6 h. Although MAPK phosphorylation could be inhibited 100% by =20 µmol/l MEK inhibitor PD98059, bFGF-induced KDR expression was only reduced ~70%, suggesting that although the majority of bFGF-induced KDR expression is mediated by p44/p42 MAPK, other pathways are also involved.

bFGF also stimulated VEGF expression in our studies (data not shown), consistent with previous reports in other cell types (62,63). Since bFGF induces expression of both KDR and VEGF, it is possible that a portion of the bFGF mitogenic response might be mediated through VEGF rather than through intrinsic bFGF activity. However, with BREC in culture this mechanism appears not to play a major role, since bFGF-induced [^{3}H]thymidine incorporation was not significantly inhibited by 10 µg/ml VEGF neutralizing antibody (R&D Systems) despite the ability of this antibody to inactivate 100% of the activity of 10 ng/ml VEGF at this concentration (data not shown). In vivo, the majority of VEGF acting on the endothelial cells is expected to be exogenous, since numerous non-endothelial retinal cell types have a more robust VEGF expression response (8,20). However, the possibility of autocrine activity due to localized expression cannot be eliminated. Furthermore, additional investigation is required to determine whether bFGF-induced VEGF production in other cell types is adequate to effect a paracrine augmentation of bFGF's intrinsic endothelial mitogenic activity.

VEGF had no significant effect on bFGF expression in BREC (data not shown). However, the effect of VEGF on KDR expression was dependent on the extent of serum content and the resultant expression of KDR itself. Under 1% CS conditions, KDR expression is minimal. KDR expression increases as serum increases, becoming 2.0-fold greater in 10% CS. Similarly, VEGF cannot increase KDR expression in 1% CS but increases KDR expression 1.9-fold in 10% CS. Likewise, under conditions of 1% CS in the presence of bFGF, which induces basal KDR expression, VEGF can induce KDR expression an additional 2.9-fold. These data suggest that the ability of retinal endothelial cells to respond to VEGF is at least partially dependent on the expression of KDR and that the receptor may be limiting under serum-deprived conditions. Stimulation by bFGF, however, does not require KDR to induce KDR expression. Once bFGF induces KDR expression, VEGF can further increase KDR expression through these bFGF-induced receptors, even under serum-deprived conditions. KDR upregulation may occur as a result of a variety of stimuli (not just bFGF), since MAPK pathway activation is involved in the mitogenic signaling pathways of numerous growth factors and our studies demonstrate that

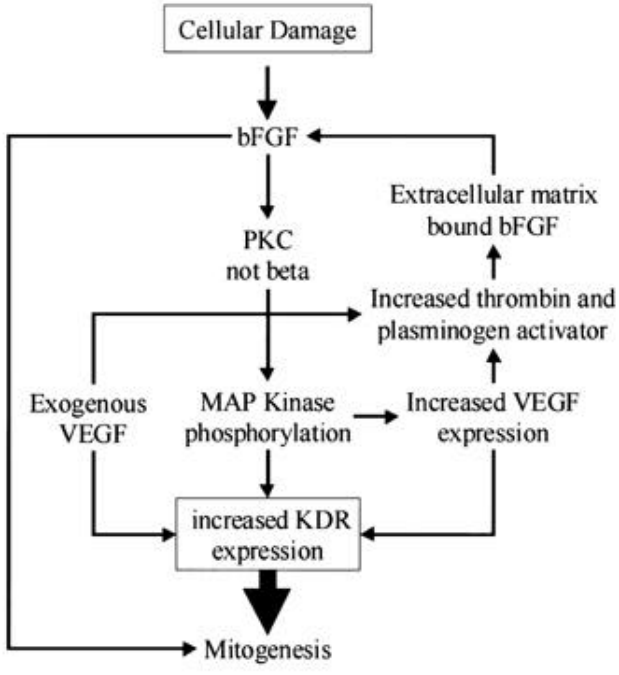

FIG. 10. Schematic diagram of postulated molecular mechanism underlying the synergistic activity of bFGF and VEGF. Exposure to bFGF results in activation of non-β isoforms of PKC, which increase p44/p42 MAPK phosphorylation, leading to increased KDR and VEGF (79) expression. Exogenous and newly induced VEGF can further increase KDR expression when sufficient KDR is expressed. VEGF also increases thrombin (80) and plasminogen activator (81) expression, which can release bioactive bFGF from extracellular matrix stores (75,78). The increase in KDR, VEGF and bFGF result in marked mitogenic synergy.

serum (which contains many growth factors) effectively increases KDR expression.

Overall, since VEGF action is closely correlated with mitogenic activity, these data also suggest that the extent of KDR expression might reflect the mitogenic activity of the endothelial cell. This hypothesis is consistent with the findings of Ortega et al. (73) that KDR is a functional marker of the angiogenic phenotype in endothelial cells. To determine whether this relationship remains true for other mitogenic stimuli will require further investigation.

To our knowledge, these data suggest a novel mechanistic explanation for the potent mitogenic synergy between bFGF and VEGF (Fig. 10). In ischemic retinopathies where bFGF and VEGF are thought to play significant roles, damaged and dying retinal cells release bFGF (74). In addition, bFGF stored in the extracellular matrix can be released as a bioactive bFGF-glycosaminoglycan complex (75–78). The bFGF activates PKC, probably an isoform other than β, which in turn induces p44/p42 MAPK phosphorylation. Both increased VEGF (79) and KDR expression result. Even under conditions where KDR concentrations might have been limiting, the increased KDR expression may permit both exogenous and bFGF-induced VEGF to augment mitogenesis. VEGF can also induce both thrombin (80) and plasminogen activator (81) expression, which releases bioactive bFGF from the extracellular matrix (75,78). Thus, bFGF and VEGF can initiate numerous pathways that would augment each other's mitogenic activity and account for their observed synergy.

In summary, these data clearly demonstrate that angiogenic cytokines can effect the expression of other angiogenic molecules and their receptors, resulting in synergistic activity. The extent of this phenomenon in other angiogenic processes remains to be determined and is the focus of future studies.

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