Vascular endothelial cell growth factor activates CREB by signaling through the KDR receptor tyrosine kinase

Running Title: VEGF activation of CREB

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+Abbreviations: VEGF, vascular endothelial cell growth factor; PlGF, placental growth factor; HUVEC, human umbilical vein endothelial cells; CREB, CRE
binding protein; p-CREB, phosphorylated CREB; α-CREB, CREB antibody; ATP-1, activating transcription factor-1; α-ATF-1, ATF-1 antibody; MAPK, mitogen activated protein kinase; PKC, protein kinase C; electrophoretic mobility shift, EMSA; MOPS, [3-(N-Morpholino) propanesulfonic acid].

Vascular endothelial cell growth factor (VEGF) plays a crucial role in the development of the cardiovascular system and in promoting angiogenesis associated with physiological and pathological processes. While a great deal is known of the cytoplasmic signaling pathways activated by VEGF, much less is known of the mechanisms through which VEGF communicates with the nucleus and alters the activity of transcription factors. Binding of VEGF to the KDR/Flk1 receptor tyrosine kinase induces phosphorylation of the CREB transcription factor on serine-133 and increases CREB DNA binding and transactivation. p38 MAP kinase/MSK-1 and protein kinase C/p90 RSK pathways mediate CREB phosphorylation. Confocal microscopy shows that VEGF induced phosphorylation of nuclear CREB is blocked by pharmacological inhibition of protein kinase C and p38 mitogen-activated protein kinase signaling. Thus, KDR/Flk1 uses multiple pathways to transmit signals into the nucleus where CREB becomes activated. These results suggest that CREB may play a role in alterations of gene expression important to angiogenesis.
Angiogenesis plays an important role in embryonic development, wound healing and organ regeneration (1,2). Angiogenesis also contributes to pathologies dependent on neovascularization, among which are tumor growth, diabetes mellitus, ischemic ocular diseases, and rheumatoid arthritis. Vascular endothelial cell growth factor (VEGF)\(^1\) is an endothelial cell specific mitogen that promotes many other events necessary for angiogenesis (1,3-6). VEGF induces endothelial cell proliferation and movement, remodeling of the extracellular matrix, formation of capillary tubules, and vascular leakage. VEGF plays a role in the development of the cardiovascular system and in the physiology of normal vasculature (3,7-11). VEGF is produced by many types of tumor cells and, by promoting vascularization, plays a significant role in the progression of cancer (12-14).

VEGF exerts its actions by binding to two cell surface receptors, KDR (the human homolog of Flk1) and Flt1 (15-20), structurally similar to members of the platelet-derived growth factor receptor family (21). Flk1 and Flt1 are essential for fetal angiogenesis and mouse embryos null for either receptor die in utero between days 8.5 and 9.5 (22,23). Hematopoietic and endothelial cell
development are impaired in Flk1 null mutant mice (23), whereas there is overgrowth of endothelial cells and disorganized blood vessels in Flt1 null mutant mice (22). The phenotypes of Flk1 and Flt1 deficient embryos shows that each plays a distinct role in angiogenesis and suggests that KDR/Flk1 and Flt1 utilize distinct signaling cascades to elicit responses.

Flk1 is abundant on the proliferating endothelial cells of vascular sprouts of embryonic and early postnatal brain, but is reduced in adult brains in which endothelial cell proliferation has ceased (17). Experiments with mutant forms of VEGF that selectively bind one or the other VEGF receptor show that Flk1/KDR promotes endothelial cell proliferation and survival (24). The induction of chemotaxis and procoagulant activity by VEGF and placental growth factor (PlGF), a VEGF homolog that binds Flt1, suggests that these responses are mediated, at least in part, by Flt1 (25-27). Thus, the functions of VEGF are segregated between its two receptors.

In previous studies we demonstrated that VEGF promotes the tyrosine phosphorylation of signaling molecules that contain SH2 domains, and this process is associated with endothelial cell proliferation (28). By comparing signaling events induced by VEGF or PlGF, in the absence or presence of SU5416, a KDR/Flk1 antagonist (29), we identified signaling molecules that interact with and promote or inhibit KDR/Flk1 action (30). The present study finally identifies pathways through which cytoplasmic signaling pathways downstream of KDR/Flk1 transmit signals into the nucleus. VEGF promotes phosphorylation
and activation of the cyclic AMP responsive element binding protein by activation of p38 mitogen-activated protein kinase/MSK-1 and protein kinase C/p90RSK signaling pathways downstream of KDR. As CREB is a transcription factor that plays a important role in promoting proliferation and cellular adaptive responses (31), a mechanism has been defined through which VEGF/KDR may allow endothelial cells to respond to changes in their environment through alterations of gene expression.

EXPERIMENTAL PROCEDURES

Materials-Human recombinant VEGF was a gift from Genentech (South San Francisco, CA). SU5416 was a gift from SUGEN, Inc. (South San Francisco, CA). Placenta growth factor and VEGF-D were from R & D Laboratories (Minneapolis, MN). Antibodies to phosphorylated CREB/ATF-1, phosphorylated MSK-1, phosphorylated and non-phosphorylated p38 MAPK and MAPK were from Cell Signaling Technology (Beverly, MA). Anti-MAPKAP kinase 2 and recombinant ATF-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MAPKAP kinase 3 was from Upstate Biotechnology (Lake Placid, NY). PGAL4-CREB was from Stratagene (La Jolla, CA) and the GAL4 luciferase reporter construct was a kind gift of Dr. Lawrence Quilliam (Indiana University School of Medicine). GF109203X was from LC Laboratories (Boston, MA). All other inhibitors were from Calbiochem (San Diego, CA) or Sigma, Inc. (St. Louis, MO).

Cell Culture and Western blotting-HUVEC were grown on 0.2% gelatin-
coated tissue culture plates in endothelial cell growth medium (Clonetics, San Diego CA.) under 5% CO2 at 37°C. For VEGF stimulation, the cells were cultured in endothelial cell basal medium (Clonetics) that was supplemented with 1% bovine serum albumin. After 16 hours, the cells were treated with various inhibitors as described in the figure legends and then stimulated with 1 nM VEGF for 10 minutes. Cells were harvested into lysis buffer (50 mM Hepes pH 7.0, 150 mM NaCl, 10% glycerol, 1.2% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM sodium orthovanadate) that was supplemented with 1 mM PMSF, 0.15 units/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A and incubated on ice for 30 minutes, being vortexed every 10 min. Fifty micrograms from each sample was fractionated by SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked in 5% nonfat dried milk in PBST. Antibodies used to probe the Western blots were p-ERK, ERK, p38MAPK, MAPK, pCREB and CREB (Cell Signaling Tech., Beverly, MA). Proteins on the Western blots were detected using the enhanced chemiluminescent detection system.

*In vitro kinase assays*-Whole cell lysates were prepared from HUVEC treated with medium or VEGF for 10 min as described above. In independent experiments, MAPKAP kinase-2, MAPKAP kinase-2, or MSK-1 were immunoprecipitated from 500 µg of whole cell lysate (2 h incubations at 4°C using 2 µg of an antibody specific to each kinase). Protein A/G agarose was
added and incubation continued for 1 hour. After centrifugation, the pellets were washed with kinase assay buffer (20 mM MOPS, pH 7.2, 25 mM, \(\beta\)-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 75 mM MgCl\(_2\), 1 mM dithiothreitol) and this procedure was repeated twice. The washed pellet was incubated for 10 min at 37°C in 25 \(\mu\)l of kinase assay buffer modified to contain 50 \(\mu\)M ATP and 1 \(\mu\)g of recombinant ATF-1. Immunoprecipitated proteins were fractionated on 8% polyacrylamide gels and Western blots were probed with antibodies to p-CREB/ATF-1 or p-MSK-1.

Electrophoretic Mobility Shift and Gene Reporter Assays-Nuclear protein extracts were prepared using the NE-PER kit by cell lysis and centrifugation according to the directions of the manufacturer (Pierce, Inc.). The CRE and mutant CRE double stranded DNA oligonucleotides were purchased from Santa Cruz Biotechnology, Inc. The CRE oligonucleotide was end labeled with \(\gamma^{32}\)p using the T4 polynucleotide kinase and purified by passage through G25 spin column. To initiate formation of DNA-protein complexes six micrograms of each nuclear extract, binding buffer (10 mM Tris-HCl pH 7.5, 50 mM KCl, 50 mM NaCl, 1 mM MgCl, 1 mM EDTA, 5 mM DTT, and 5% glycerol), and 1\(\mu\)g poly dIdC in a final volume of 20 \(\mu\)l were incubated on ice for 10 min. and then 5 ng of end-labeled oligonucleotide was added to the reaction which was continued for 20 additional minutes. The reaction mixture was fractionated on a 5% native polyacrylamide gel (1.5 hours, 180 V). The gel was dried, and exposed to film.
For gene reporter assays, cells were transiently cotransfected with β-galactosidase and CMV-Gal4-CREB (a plasmid consisting of the DNA binding domain of Gal4 and the transactivation domain of CREB) and pGal4-Tk-Luc (an expression vector for the luciferase reporter gene driven by the enhancerless thymidine kinase promoter linked to four copies of the Gal4 regulatory sequence). After 24 h, luciferase activity was assayed in cell lysates and normalized to β-gal expression.

*Confocal microscopy*-HUVEC incubated with vehicle, SB 202190 or GF109203X for 1 h and then with VEGF for 10 minutes were fixed, permeated with 0.1% Triton X-100 and blocked with 2% bovine serum albumin. Anti-phosphoCREB was added followed by a Texas red conjugated sheep anti-rabbit secondary antibody. The nucleus was detected by staining with nuclear dye Syto 16. Excitation of the stains was performed on a Bio Rad MRC 1024 Krypton/Argon laser confocal imaging system.

**RESULTS**

Experiments were conducted to determine whether VEGF induces CREB phosphorylation and transactivation and if so to identify the receptor through which this effect is mediated. HUVEC were stimulated with VEGF and a Western blot prepared from cell lysates was probed with an antibody directed towards CREB phosphorylated on serine-133. VEGF induced CREB phosphorylation in a time-dependent manner (Fig. 1A). Due to the structural similarities between CREB and ATF-1 (31), the phospho-CREB antibody also
recognizes phosphorylated serine-63 of ATF-1. The phosphorylation kinetics of CREB and ATF-1 increased in parallel. To evaluate which VEGF receptor was responsible for inducing CREB and ATF-1 phosphorylation, HUVEC were stimulated with VEGF or PlGF, which only binds Flt1 (26,27). VEGF, but not PlGF, induced CREB phosphorylation, showing that KDR induced this event (Fig. 1B). Additionally, SU5416, a low molecular weight KDR antagonist (29), abrogated VEGF induced CREB and ATF-1 phosphorylation. To further exclude the possibility that Flt1 contributes to activation of CREB, VEGF-D, another member of the VEGF family does not bind Flt1 (32), was tested and found to induce phosphorylation of CREB and ATF-1 (Fig. 1C).

Phosphorylation of serine 133 in CREB does not alter the DNA binding of CREB to CRE, but increases its association with adapter proteins such as the CREB-binding protein, leading to activation of transcription. Therefore, the demonstration that treatment of HUVEC with VEGF leads to phosphorylation of CREB downstream of KDR next led us to evaluate whether CREB transcriptional activity was increased by VEGF. A gene reporter assay in which HUVEC were transfected with pGal4-CREB and a Gal4 luciferase reporter construct demonstrated that VEGF induces a 2.5-fold stimulation of CREB transcriptional activation (Fig. 1D).

Experiments were next conducted to identify the signaling pathways that mediated CREB phosphorylation. By probing Western blots of HUVEC with antibodies to activated MAPK (ERK1 and 2) and p38 MAPK, we found that
VEGF activates these each of these kinases, maximal activation occurring after 10 min (Figs. 2A and B). To test whether activation of MAPK family members was mediated by signaling through KDR, HUVEC were incubated in the absence or presence of SU5416 and then stimulated with VEGF or PlGF. The KDR antagonist abrogated VEGF activation of p38 MAPK (Figure 3A) and ERK1 and ERK2 (Figure 3B), indicating that the MAPKs are activated through KDR signaling. This result is consistent with the inability of PlGF to activate p38 MAPK or MAPK (Figs. 3A and B).

To determine whether MAPK, or p38 MAPK, plays a role in CREB phosphorylation, HUVEC were treated with PD98059, a MEK inhibitor which blocks MAPK activation (33), or SB202190, an inhibitor of p38 MAPK (34), and then with VEGF. CREB phosphorylation induced by VEGF was unaffected by inhibition of MAPK by PD98059. However, p38 MAPK inhibition partly suppressed CREB phosphorylation (Fig. 4A), showing that this member of the MAPK family plays a role in VEGF activation of CREB.

We next examined whether other kinases contribute to CREB activation by VEGF. HUVEC were treated with wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase), H89, an inhibitor of protein kinase A (PKA), K252A, an inhibitor of CaM kinase II, or GF109203X, an inhibitor of protein kinase C (PKC)(35). PD98059 and SB202190 were included in the experiment as positive and negative controls. LY294002, H89, and K252A did not impair CREB phosphorylation induced by VEGF, whereas GF109203X partly
inhibited phosphorylation (Fig. 4B). Treatment of cells with GF109203X and SB202190 showed that inhibition of PKC and p38 MAPK had no effect on basal CREB phosphorylation, but blocked VEGF-induced phosphorylation (Fig. 4C).

To identify the downstream kinase that mediated CREB phosphorylation induced by PKC, HUVEC were incubated with GF102203X or SB202190 and then with VEGF (Fig. 4D). A Western blot prepared from cell lysates was probed with an antibody to phosphorylated (active) p90 RSK, a CREB kinase downstream of PKC. VEGF promoted p90 RSK phosphorylation and this was inhibited by GF102203X, but not SB202190, suggesting that a PKC/p90 RSK pathway is a component of the mechanism through which VEGF induces CREB phosphorylation.

MAPKAP kinase 2, MAPKAP kinase 3 and MSK-1 are CREB and ATF-1 kinases activated by p38 MAPK (36-39). As antibodies to phosphorylated forms of each of these CREB kinases are not available, their activation was determined by an in vitro kinase assay in which ATF-1 was the substrate. HUVEC were stimulated with vehicle or VEGF and then MAPKAP kinase 2, MAPKAP kinase 3, or MSK-1 were immunoprecipitated from cell lysates and incubated with ATF-1. A Western blot was probed with an antibody to phosphorylated CREB/ATF-1 and, of the three kinases, VEGF activated only MSK1 (Fig. 4E). HUVEC were next incubated with GF102203X or SB202190, to inhibit PKC and p38 MAPK activation, respectively, and then stimulated with VEGF. MSK1 immunoprecipitated from cell lysates was incubated with ATF-1 and a Western
blot probed with antiphospho-CREB/ATF-1 and then with antiphospho-MSK-1. VEGF induced phosphorylation of ATF-1 and MSK-1 and this was blocked by inhibition of p38 MAPK but not PKC (Fig. 4F). This result suggests that activation of p38 MAPK/MSK1 is a component of a signaling cascade through which VEGF induces CREB phosphorylation.

Confocal microscopy was conducted to establish a bridge between phosphorylation of CREB on serine 133 and subsequent DNA binding of the transcription factor. HUVEC were stimulated with VEGF or treated with inhibitors of PKC and p38 MAPK and then with VEGF. Confocal microscopy established that nuclear phosphorylation of CREB was induced by VEGF and this was inhibited by pharmacological blockade of the p38 MAPK and PKC signaling cascades (Fig. 5).

CREB binds to the specific sequence, 5’-TGFCGTCA-3’, known as CRE. An electrophoretic mobility shift assay (EMSA) showed that stimulation of HUVEC with VEGF induced the formation of a protein-DNA complex. The protein-DNA complex was lost upon addition of control but not mutant CRE. Furthermore, supershifting with antibodies directed against CREB, c-fos and ATF2 showed that only CREB was a component of the retarded DNA-protein complex induced by VEGF (Fig. 6A). To establish that KDR was the receptor required for induction of CREB/DNA binding, HUVEC were treated with SU5416 before incubation with VEGF. CREB DNA binding was absent after exposure of HUVEC to SU5416, demonstrating that KDR is required for
activation of CREB (Fig. 6B).

DISCUSSION

Angiogenesis requires the coordinated expression of gene products that allow endothelial cells to alter their environment, proliferate, migrate and intubate (2). Physiological processes, such as wound healing, or pathological processes, such as tumor growth, require that the cells involved switch from a quiescent to an angiogenic phenotype (2). This process, called the angiogenic switch, is characterized by increased production of angiogenic mediators, such as VEGF and its receptors, decreased production of inhibitors of angiogenesis, such as thrombospondin, or both types of events (8,9,11,17,40-42). VEGF induced angiogenesis can be exploited to perfuse tissues isolated by blocked blood vessels, and inhibition of VEGF and its receptors blocks the progression of cancer (1,12,19,43,44).

Results from this and previous studies are summarized in the model in Figure 7, which shows that by signaling through KDR/Flk1, VEGF induces activation of MAPK, p38 MAPK, Akt, and tyrosine phosphorylation of phospholipase Cγ and focal adhesion kinase (30,45,46). FAK is associated with cell migration whereas PI 3-kinase/AKT and MAPK signaling promote cell survival (47). MAPK also promotes the proliferative response of endothelial cells to VEGF (30). KDR/Flk1 binds a novel adaptor protein, VRAP, which may shuttle proteins that contain SH2 domains to the receptor (48) and the SHP-1 protein tyrosine phosphatase (49). The level of SHP-1 associated with
KDR/Flk1 is augmented by tumor necrosis factor, indicating that the activity of this receptor and its association with signaling proteins is regulated by cytokines (49).

Stimulation of porcine aortic endothelial cells overexpressing KDR/Flk1 or Flt1 with VEGF induces association of the former, but not the latter, receptor with Shc, Grb2, Nck, SHP-1 and SHP-2 (50,51). MAPK activation, changes of cell morphology, actin reorganization, membrane ruffling, chemotaxis and cell proliferation are also induced by signaling through KDR/Flk1. These distinctions between KDR/Flk1 and Flt1 were not observed in studies with NIH 3T3 cells transfected with these receptors (52,53,57) or in experiments in which affigel-immobilized Flt1 was used to precipitate proteins from cell lysates (54). However, the weight of evidence from experiments with indicates that KDR/Flk1 is a pleiotropic inducer of VEGF action in the endothelium, whereas the role of Flt1 needs to be more clearly resolved. Thus, considerable insight has been gained into cytoplasmic events initiated by VEGF in support of its activities. Far less is known of how VEGF induces changes of gene expression that underlie angiogenesis.

CREB is a 43-kDa member of the CREB/CREM/ATF family of nuclear transcription factors that was first identified as a mediator of cyclic AMP-induced gene expression (55). Activation of CREB is mediated by phosphorylation of serine-133 (56), which augments CREB induced gene transcription (57). Peptide hormones, growth factors, neuronal activity, UV
irradiation and cross-linking of surface IgG activate CREB (38,57-61). This diversity of signal initiation, and the different signaling systems through which these cellular stressors act, indicates that serine-133 in CREB may be a substrate for a multiplicity of protein kinases. Mitogen-induced phosphorylation commences with stimulation of guanine nucleotide exchange factors, such as SOS, which activates Ras. Ras induces activation of the Raf serine-threonine kinase, an upstream activator of MAP kinase kinases (MEKs). Downstream targets of the MEKs are the MAP kinases ERK1 and ERK2, which in turn can play a role in activation of ribosomal S6 kinase pp90rsk, a CREB kinase (62). p38 MAPK and its downstream effectors MAPKAP-K2 and 3 are components of an alternative pathway leading to CREB activation (36-39). Other kinases that activate CREB are MSK1, a relative of p90RSK, which is activated by ERKs, calcium calmodulin-dependent kinases, and protein kinase C (61,63-65).

The present study has shown that VEGF increases phosphorylation of nuclear CREB on serine-133 and increases CREB DNA binding and transactivation. Thus, three independent lines of experimentation support the conclusion that VEGF activates CREB. The ability of VEGF and VEGF-D, but not PlGF, to induce CREB phosphorylation shows that it is by signaling through KDR that CREB activation is mediated. This conclusion is supported by the ability of SU5416 to block CREB activation by VEGF. Thus, only one of the two VEGF receptors plays a role in the induction of CREB activity.
ERK1 and ERK2, PI 3-kinase, PKA, and CAM kinase II were excluded as mediators of VEGF-induced CREB phosphorylation based on the inability of PD98059, LY294002, H89 and K252A, respectively, to inhibit VEGF-induced CREB phosphorylation. Inhibition of p38 MAPK by SB202190 partly inhibited VEGF-induced activation of CREB, as did GF109203X, an inhibitor of PKC. Complete blockade of CREB activation was observed when p38 MAPK and PKC were inhibited. These results show that p38 MAPK and PKC mediate CREB phosphorylation downstream of KDR.

CREB plays an important role in cell proliferation, differentiation, adaptive responses and metabolism (31,66). In mice, CREB is required for the generation of a normal repertoire of T cell lineages during development and the absence of CREB leads to dwarfism and cardiac myopathy in the adult (67-69). CREB family members are important for learning and memory and contribute to the neuronal adaptation to drugs of abuse (70,71). Our observations now suggest that by mediating cellular responses to VEGF, CREB is likely to play an important role in endothelial cell function and blood vessel development.

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Figure legends

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Fig. 1. **CREB phosphorylation and activation induced by VEGF is mediated by signaling through KDR.**  
A. HUVEC were incubated with 1 nM VEGF for various times and then a Western blot prepared from cell lysates was probed with anti-pCREB (top) or anti-CREB (bottom).  
B. HUVEC were incubated with medium or 1 μM SU5416 for 1 h and then with 1 nM VEGF or PlGF for 10 min. A Western blot was probed with anti-pCREB (top) or anti-CREB (bottom).  
C. HUVEC were incubated with medium 1 nM VEGF-D for 10 min. A Western blot was probed with anti-pCREB (top) or anti-CREB (bottom).  
D. CREB reporter activity in cells treated with vehicle or VEGF was assayed.

Fig. 2. **p38 MAPK and MAPK activation by VEGF.**  
HUVEC were treated with VEGF for various times and Western blots were prepared from cell lysates.  
A. A Western blot was probed with an anti-phospho-p38 MAPK (top) and then with anti-p38 MAPK (bottom).  
B. A Western blot was probed with anti-pMAPK (top) and then with anti-MAPK (bottom).

Fig. 3. **p38 MAPK and MAPK are activated by signaling through KDR.**  
HUVEC incubated in the absence or presence of 1 μM SU5416 were treated with VEGF.  
A. A Western blot was probed with anti-phospho-p38 MAPK (top) and then with anti-p38 MAPK (bottom).  
B. A Western blot was probed with anti-MAPK (top) and then with anti-MAPK (bottom).
Fig. 4. **Signaling pathways used by VEGF to promote CREB phosphorylation.** 

A. HUVEC were treated with PD98059 or SB202190 for 1 h and then incubated in the absence or presence of 1 nM VEGF for 10 min. CREB phosphorylation was assayed by Western blot analysis. 

B. HUVEC were treated with medium, 25 μM PD98059, 20 μM Ly294002, 25 μM SB202190, 20 μM H89, 100 nM K252A, 1 μM GF109203X or GF109203X and SB202190 and then with VEGF for 10 min. CREB phosphorylation was assayed by Western blot analysis. 

C. HUVEC were incubated with medium, SB202190, or GF109203X and then treated with medium or VEGF. CREB phosphorylation was assayed Western blotting. 

D. HUVEC were incubated with medium, SB202190 or GF109203X and then with medium or VEGF. p90 RSK phosphorylation was assayed by probing a Western blot with anti-phospho RSK. 

E. An *in vitro* kinase assay using ATF-1 as substrate was used to test for activation of MAPKAP kinase 2, MAPKAP kinase 3, and MSK-1. These kinases were immunoprecipitated from lysates of control or VEGF-treated HUVEC and a Western blot was probed with anti-phosphorylated CREB/ATF1. 

F. HUVEC were incubated with GF109203X or SB202190 and then with vehicle or VEGF. MSK1 was immunoprecipitated from cell lysates and a Western blot was probed with antibodies to phosphorylated CREB/ATF1 and phosphorylated MSK-1.

Fig. 5. **Confocal microscopy.** HUVEC were treated with vehicle or SB202190 together with GF109203X for 1 hour and then stimulated with VEGF. Confocal
microscopy was used to detect and localize phosphorylated CREB/ATF-1.

Fig. 6. **Binding of CREB to DNA.** *A.* Nuclear extracts of HUVEC treated with vehicle or VEGF were incubated with a 100-fold excess of control or mutant CRE before CREB DNA binding was assayed by EMSA. Supershifting with anti-CREB (αCREB), anti-fos (αfos), or anti-ATF2 (αATF2) established the component(s) of the retarded complex. *B.* HUVEC were treated with medium or SU5416 for 1 h and then with VEGF. Nuclear extracts were used for EMSA.

Fig. 7. **A model of KDR/Flk1 signaling.**
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