

Specificity, diversity, and convergence in VEGF and TNF- α signaling events leading to tissue factor up-regulation via EGR-1 in endothelial cells

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ABSTRACT Tissue factor (TF) has been shown to be up-regulated in endothelial cells by the inflammatory cytokine tumor necrosis factor α (TNF- α) as well as by the main angiogenic factor VEGF. Since both stimuli induce the transcription factor EGR-1, which is critically involved in TF gene regulation, we used EGR-1-dependent TF induction as a model to identify potential cross-talks between the various signal transduction cascades initiated by VEGF and TNF- α . The data show that at the MAP kinase level, VEGF mainly activates ERK1/2 and p38 MAP kinases in human endothelial cells. TNF- α is able to activate all three MAP kinase cascades as well as the classical inflammatory I κ B/NF κ B pathway. Furthermore, the MEK/ERK module of MAP kinases appears to act as the convergence point of VEGF- and TNF- α -initiated signaling cascades, which lead to the activation of EGR-1 and subsequent TF expression, whereas the upstream signals are distinct. We found that induction of TF by VEGF via EGR-1 is strongly PKC dependent. The TNF- α -initiated MEK/ERK cascade connected to EGR-1 and TF expression is clearly less sensitive to PKC inhibition. TNF- α -mediated activation of MEK/ERK and EGR-1 can be blocked by adenoviral expression of a dominant negative mutant of IKK2, whereas the VEGF signaling pathway is unaffected. Thus, our data demonstrate a new link between the classical inflammatory IKK/I κ B and the MEK/ERK cascades triggered by TNF- α . The additional finding that EGF induces ERK and EGR-1 in a PKC-independent manner and that this signal is not sufficient to up-regulate TF emphasizes the importance of a VEGF-specific signaling pattern for the induction of TF.—Mechtcheriakova, D., Schabbauer, G., Lucerna, M., Clauss, M., de Martin, R., Binder, B. R., Hofer, E. Specificity, diversity, and convergence in VEGF and TNF- α signaling events leading to tissue factor up-regulation via EGR-1 in endothelial cells. *FASEB J.* 15, 230–242 (2001)

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UNTIL RECENTLY, THE main established role of tissue factor (TF) was the primary cellular trigger of the blood coagulation cascade (1, 2). Interest in this protein has

gained additional impetus with the identification of novel properties/functions such as transmission of intracellular signals and a significant participation in embryonic blood vessel development as well as tumor-associated angiogenesis (3–7). Another characteristic feature of TF is that its expression and functional activity can be induced by both the classical inflammatory stimulus tumor necrosis factor α (TNF- α) and the main angiogenic growth factor, vascular endothelial growth factor (VEGF) (8–11). Unlike growth factor receptors, which have large cytoplasmic domains with intrinsic protein tyrosine kinase activity (12), or some cytokine receptors, which are directly coupled to JAK tyrosine kinase (13), the TNF- α receptors themselves seem to lack identifiable catalytic activity (14). Binding of TNF- α to TNFR1 or TNFR2 induces receptor oligomerization and recruitment of several downstream adaptor and signaling proteins to their cytoplasmic domains (15, 16). Nevertheless, both classes of receptors can activate distinct and overlapping sets of genes and biological responses in target cells (14, 17). Among the classical transcription factors activated by TNF- α , nuclear factor κ B (NF κ B) plays the major specific role in regulation of inflammatory response genes (18, 19) and does not seem to be activated by any of the growth factors including VEGF (11). NF κ B is regulated primarily by phosphorylation of inhibitory proteins, the I κ Bs, which retain it in the cytoplasm of unstimulated cells (20). Upon cell stimulation, a signaling cascade leads to phosphorylation of I κ B via a cytokine-activated protein kinase complex called I κ B kinase (IKK) (21, 22). In addition, TNF- α can induce the transcription factor EGR-1 (early growth response-1), which belongs to the immediate-early (IE) gene products. Regulation of the *egr-1* gene by TNF- α has so far been analyzed only in human fibroblasts (23). The precise biological role of this activation as well as the protein kinases involved in the pathway have not been described to our knowledge.

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On the other side, our recent data demonstrate that EGR-1 is rapidly induced by VEGF in endothelial cells, thereby leading to TF up-regulation (11, 24). These findings led to the intriguing hypothesis that there might be cross-talk between the induction programs of gene expression responsible for angiogenic and inflammatory responses of endothelial cells, possibly starting at the level of activated kinases and leading further to certain transcription factors, and the spectrum of induced genes. Thus, the activation of TF via EGR-1 provides a model to 1) reconstitute a detailed picture of downstream signaling events initiated by VEGF and TNF- α , 2) identify the possible interactions between the various signal transduction components, and 3) link an immediate-early gene response to a subsequent secondary gene expression.

Mitogen-activated protein (MAP) kinases are common participants in signal transduction pathways initiated by growth factors, cytokines, stress stimuli, and various pharmacological compounds (25, 26). The mammalian MAP kinase family members identified so far form three major types of MAP kinase modules with a generally high degree of specificity and functional separation: ERK1/2, SAPK/JNK, and p38 kinase. Each module contains at least three protein kinases that work sequentially and can be activated by dual phosphorylation on tyrosine and threonine residues. How a primary signaling pathway is selected to achieve the induction of a certain immediate-early gene by a given ligand is far from being understood.

In the present study, we have focused on those of the multiple signaling cascades triggered by VEGF and TNF- α receptors that are linked to EGR-1 activation and followed by TF expression on endothelial cells. We used an approach with small-molecule inhibitors in combination with inactive mutants of kinases for the selective blockade of signal transduction pathways. We could demonstrate that induction of TF by VEGF via EGR-1 is protein kinase C (PKC) dependent and mediated directly by ERK. Furthermore, our data suggest that the MEK/ERK module of MAP kinases functions as the convergence point of VEGF and TNF- α signaling that leads to the activation of EGR-1, whereas the upstream signals are distinct. We discovered a new link between the classical inflammatory IKK/I κ B and the MEK/ERK cascades induced by TNF- α . Although the MEK/ERK cascade can be activated by a broad spectrum of growth factors, in contrast to VEGF, epidermal growth factor (EGF) does not trigger TF induction. Our data show that EGF initiates a pathway from MEK/ERK to EGR-1 in a PKC-independent manner, which suggests specificity in VEGF vs. EGF signaling.

MATERIALS AND METHODS

Cell culture and materials

Human umbilical vein endothelial cells (HUVEC) were cultured at 37°C and 5% CO₂ in medium 199 supplemented with 20% SCS (HyClone, Logan, Utah), 1 U/ml heparin, 50

μ g/ml ECGS, 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were used for experiments up to passage number 5. Serum-starved HUVEC were obtained by starving with 1% SCS for 5 h. Recombinant human VEGF₁₆₅ was obtained from PromoCell (Heidelberg, Germany) or was prepared as described (27). Recombinant human EGF was purchased from PromoCell (Heidelberg, Germany), TNF- α from Genzyme Inc. (Cambridge, Mass.), phorbol 12-myristate 13-acetate (PMA), wortmannin, citrated plasma, and thromboplastin from Sigma Chemicals (St. Louis, Mo.). The MEK inhibitor PD098059, the p38 kinase inhibitor SB203580 and the PKC inhibitor bis-indolylmaleimide I (synonyms: GF109203X and Gö6850) were obtained from Calbiochem (La Jolla, Calif.). Phospho-specific MEK1/2, ERK 1/2, p38 kinase polyclonal antibodies, nonphospho ERK1/2 antibodies, and LumiGLO chemiluminescent reagent were from New England BioLabs (Beverly, Mass.); polyclonal EGR-1 antibody, NF κ B p65, and I κ B α were from Santa Cruz Biotechnology (Santa Cruz, Calif.). Polyclonal TF antibodies were a gift from Dr. W. Ruf (Scripps Research Institute, La Jolla, Calif.). Peroxidase-conjugated donkey anti-rabbit immunoglobulin G (IgG) and sheep anti-mouse IgG were purchased from Amersham Life Science (Amersham Place, England), fluorescein isothiocyanate-labeled goat anti-rabbit IgG from Accurate Scientific (Westbury, N.Y.). Immobilon-P transfer membranes were products of Millipore (Bedford, Mass.).

Clotting assay

Cells were seeded in 6-well plates at 80–90% confluency and grown overnight. Cells were scraped from the plates and analyzed for TF activity as described (10, 28). Briefly, after induction for 4 h with VEGF, TNF- α , PMA, or EGF, cells were washed twice and scraped in 1 ml clotting buffer (12 mM sodium acetate, 7 mM diethylbarbitate, and 130 mM sodium chloride; pH 7.4); 100 μ l of resuspended cells was mixed with 100 μ l of citrated plasma and clotting times were measured after recalcification with 100 μ l of 20 mM CaCl₂ solution at 37°C. TF equivalents were determined by using a standard curve obtained from rabbit brain thromboplastin.

Western blot analysis

After various treatments, the cells were washed twice with cold phosphate-buffered saline (PBS), lysed in 100 μ l of Laemmli buffer, scraped, and heated for 5 min at 95°C. Total cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P membrane. The membrane was blocked for 30 min with PBS containing 0.1% Tween-20 and 3% skim milk and incubated for 1 h at room temperature with a primary antibody diluted in blocking buffer. Then the membrane was washed three times for 5 min with PBS containing 0.1% Tween-20 and incubated with peroxidase-conjugated secondary antibody for 1 h at room temperature. After a washing step, the membrane was incubated for 1 min with ECL reagent and exposed to film as required. For reprobing with another antibody, the membrane was washed twice in PBS, stripped for 30 min at 55°C with stripping buffer (62.5 mM Tris-HCL, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol), and washed three times for 5 min with PBS at room temperature. The membrane was stored wet wrapped in Saran Wrap at 4°C after each immunodetection.

Recombinant adenoviral constructs and infection

HUVEC grown in 6-well plates were washed twice with PBS and incubated at a multiplicity of infection of 100 with

recombinant adenovirus that express dominant negative mutant of IKK2 (K44A) (AdIKK2dn) (W. Oitzinger et al., unpublished results) or control adenovirus (AdGFP) (29) in PBS for 30 min. Thereafter, cells were washed with PBS and then cultured in normal HUVEC medium. Twenty-four hours postinfection, cells were short-starved in 1% SCS medium for 5 h, followed by appropriate stimulation. For immunofluorescence assay, cells were grown and infected in LabTek tissue culture chamber slides.

Immunofluorescence

Immunofluorescence assay was performed mainly as described previously (30). Briefly, cells were grown in LabTek tissue culture chamber slides for at least 24 h prior to fixation. After appropriate stimulation, cells were washed twice with PBS, fixed for 10 min at room temperature with 3.7% formaldehyde, 2% sucrose in PBS, and permeabilized for 5 min with 0.5% Triton X-100 in PBS. Primary antibodies were diluted in PBS, 1% bovine serum albumin and incubated with the cells for 1 h at room temperature. Cells were washed in PBS and incubated with Texas Red-labeled goat anti-rabbit IgG for 1 h at room temperature. To visualize cell nuclei, a fluorescent groove binding probe for DNA, 4',6-diamidino-2-phenylindole (DAPI; Sigma), was added to the secondary antibody solution at 100 ng/ml. Slides were washed in PBS and mounted with mounting fluid. The immunofluorescence results were analyzed with a Bio-Rad MRC 600 confocal laser scanning microscope.

Statistical analysis

The results obtained were analyzed by one-way analysis of variance and the Student's paired *t* test.

RESULTS

Induction of EGR-1 and TF expression by VEGF and TNF- α in endothelial cells

We recently demonstrated that VEGF treatment of endothelial cells resulted in rapid induction of the transcription factor EGR-1, thereby activating TF gene expression (11). To examine whether EGR-1 can also be induced in endothelial cells by the inflammatory stimulus TNF- α , HUVEC were treated for various time periods with either TNF- α (100 U/ml) or VEGF (1.25 nM). As shown previously, at these concentrations the two agents caused maximal induction of TF procoagulant activity when monitored by a one-stage clotting assay (10, 11). Cell lysates were prepared and subjected to Western blot analysis. A low level of endogenous EGR-1 was detectable in short-starved unstimulated endothelial cells, whereas a transient induction of EGR-1 was observed upon treatment of these cells with VEGF as well as TNF- α (Fig. 1). The maximum induction of EGR-1 expression by both agents was at ~60 min after treatment of the cells and dropped to basal levels within 4 h. To determine the level of TF up-regulation in these cells, the membranes were reprobed with anti-TF antibodies. TF was almost undetectable in unstimulated HUVEC and, as expected, was strongly induced by VEGF and TNF- α , reaching maximal expres-

sion at 4 h. Thus, our data clearly demonstrate that EGR-1 expression is induced in endothelial cells by the inflammatory stimulus TNF- α to an extent comparable to the angiogenic growth factor VEGF.

VEGF- and TNF- α -induced MAP kinase 'signature' in endothelial cells

Next we tested whether there is a convergence point of VEGF- and TNF- α -initiated signaling pathways leading to induction of EGR-1. Stimulation of all three MAP kinase cascades by VEGF and TNF- α in human endothelial cells was measured under the same conditions and compared (Fig. 2). Since members of the MAP kinase families are known to be activated by dual phosphorylation on Tyr/Thr (25), we examined kinase activation using a Western blot technique with phospho-specific antibodies. These reagents selectively recognize only active forms of the kinases and do not cross-react with other related family members. Compared to unstimulated cells, VEGF strongly activated ERK1/2, with a peak at 10 min and values still above the basal level by 60 min (Fig. 2). The much weaker and more transient activation of p38 kinase observed after VEGF treatment returned to baseline by 20 min, whereas no activation of JNK by VEGF was detectable in HUVEC. In agreement with our previous data (11), I κ B levels were not affected by VEGF treatment. Antibodies to the nonphosphorylated form of ERK1/2 were used to monitor sample loading. In the case of TNF- α -treated cells, activation of ERK1/2 was transient, with a maximum at 10–20 min and decreasing to the baseline at 30 min, whereas strong activation of p38 kinase was sustained up to 60 min. Activation of JNK was transient, with maximum levels detected at 10 min. As expected, TNF- α treatment of cells resulted in rapid degradation of I κ B within 10 min, followed by resynthesis of the protein starting after 30 min. These results show that in endothelial cells, VEGF mainly activates ERK1/2 and p38, but no detectable levels of JNK. TNF- α is able to

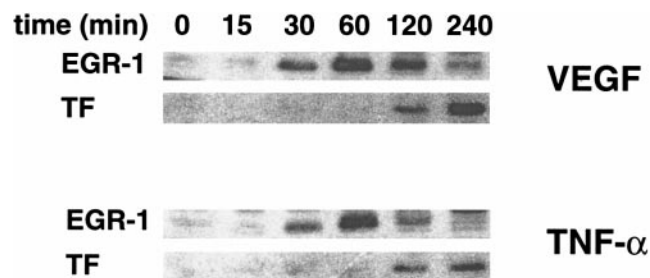


Figure 1. Both VEGF and TNF- α induce EGR-1 and TF in HUVEC. Western blot analysis of total cell extracts from short-starved unstimulated cells ('0' time point) and cells exposed for 15–240 min to VEGF (1.25 nM) or TNF- α (100 U/ml). After treatment, samples were harvested and protein lysates were separated by SDS-PAGE and subjected to Western blot analysis. Membranes were probed with anti-EGR-1 antibodies, followed by reprobing with anti-TF antibodies. Representative results from two separate experiments are shown.

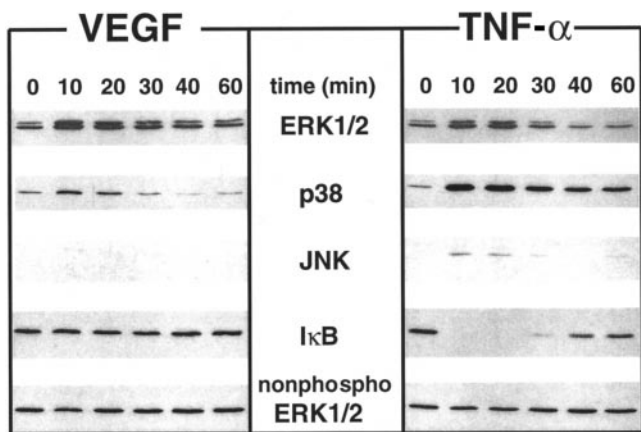


Figure 2. Activation kinetics of MAP kinases by VEGF and TNF- α in endothelial cells. Short-starved HUVEC were exposed to VEGF (1.25 nM) and TNF- α (100 U/ml) for 10–60 min. After treatment, cells were harvested and protein lysates separated by SDS-PAGE and subjected to Western blot analysis. Phospho-specific antibodies were used to selectively recognize the active forms of ERK1/2, JNK, and p38 kinase. To monitor the activation of the NF κ B pathway, blots were reprobed with anti-I κ B antibodies. Antibodies to the non-phosphorylated form of ERK were used to ensure equal loading of samples. Results are representative of two separate experiments.

activate all three MAP kinase pathways in addition to the cascade leading to I κ B degradation.

A specific inhibitor of MEK blocks VEGF- and TNF- α -induced EGR-1 expression

To determine which of the MAP kinase cascades, if any, mediates the induction of EGR-1 by VEGF and TNF- α , a specific inhibitor of MEK1/2 activity, PD098059 (31), and a specific inhibitor of p38 kinase, SB203580 (32), were used. Cells were preincubated with the inhibitors and then induced for 10 min (for maximal kinase activation) or 60 min (for maximal EGR-1 expression) (Fig. 3). The activation of ERK1/2 by VEGF and TNF- α was prevented by PD098059, but not by SB203580, as predicted, since to date ERK1/2 are the only known substrates for MEK1/2. Furthermore, induction of EGR-1 by both stimuli was also inhibited by PD098059. In contrast, the p38 kinase inhibitor did not block VEGF- or TNF- α -induced EGR-1 expression. Moreover, in the presence of SB203580 the activation of ERK1/2 by VEGF and TNF- α was slightly enhanced, showed prolonged kinetics, and was followed by increased expression of EGR-1. Thus, the data obtained suggest that the MEK/ERK cascade is required for EGR-1 induction in response to both VEGF and TNF- α . At the same time, reprobing with anti-I κ B antibodies clearly demonstrated that the MEK inhibitor PD098059 had no effect on TNF- α -induced I κ B degradation (Fig. 3, I κ B) and p65 nuclear translocation analyzed by immunofluorescence (data not shown). These data indicate that PD098059 selectively blocks the ERK pathway triggered by VEGF or TNF- α , but is not inhibitory in

general to other kinase cascades induced by TNF- α such as the NIK/IKK or p38 kinase cascades. Furthermore, these data linked the MEK/ERK module of MAP kinases to EGR-1 induction whether VEGF or TNF- α initiated this cascade.

VEGF and TNF- α regulate TF activity by mechanisms involving EGR-1 in a PD098059-sensitive fashion

As we reported earlier (11, 24), EGR-1 plays a significant role in TF activation by VEGF. In the case of inflammatory stimuli, induction of the TF promoter was previously analyzed in monocytic cells and porcine aortic endothelial cells and found to be mediated mainly by NF κ B in combination with AP-1 (8, 10, 24, 33). At the same time, it was not determined whether TNF- α -induced EGR-1 plays a significant role in TF up-regulation in human endothelial cells. Based on the above results, we next tested to what extent inhibition of EGR-1 expression by PD098059 would lead to TF inhibition. Cells were preincubated with the inhibitor and then induced by TNF- α or VEGF for 4 h. Stimulated TF activity was measured in a one-stage clotting assay and TF expression was analyzed in parallel by Western blots (Fig. 4). VEGF-induced TF activity was strongly suppressed in a dose-dependent manner by the MEK inhibitor PD098059, showing 80% inhibition at 25 μ M (Fig. 4). TNF- α -stimulated TF activity was clearly less affected, but was still reduced by 50–60% at 25 μ M. These results were confirmed at the level of protein expression by Western blot analysis (Fig. 4, insert), illustrating good correlation between the data obtained by both methods. To test whether the residual activities

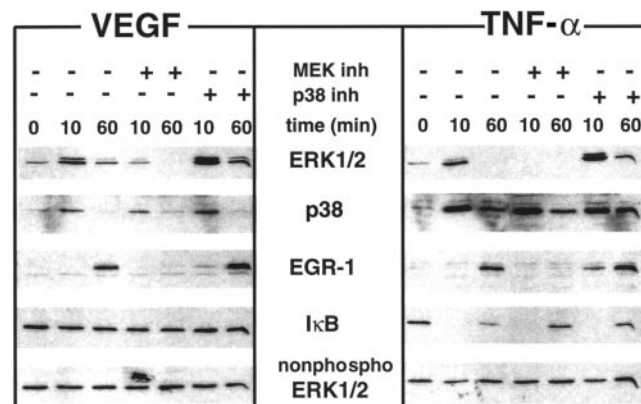


Figure 3. Effect of MEK and p38 kinase inhibitors on VEGF- and TNF- α -induced EGR-1 expression. HUVEC were pre-treated for 30 min with the MEK inhibitor PD098059 (25 μ M) or the p38 kinase inhibitor SB203580 (10 μ M) and exposed to VEGF or TNF- α for 10 and 60 min. SB; 203580 acts by inhibiting p38 kinase activity through competition with ATP and does not prevent p38 phosphorylation (74). Total cell lysates were subjected to Western blot analysis with phospho-specific antibodies to the active forms of ERK1/2, p38 kinase as well as anti-EGR-1 and anti-I κ B antibodies. As previously, antibodies detecting the nonphosphorylated form of ERK were used to estimate sample variations in loading. Representative results from three separate experiments are shown.

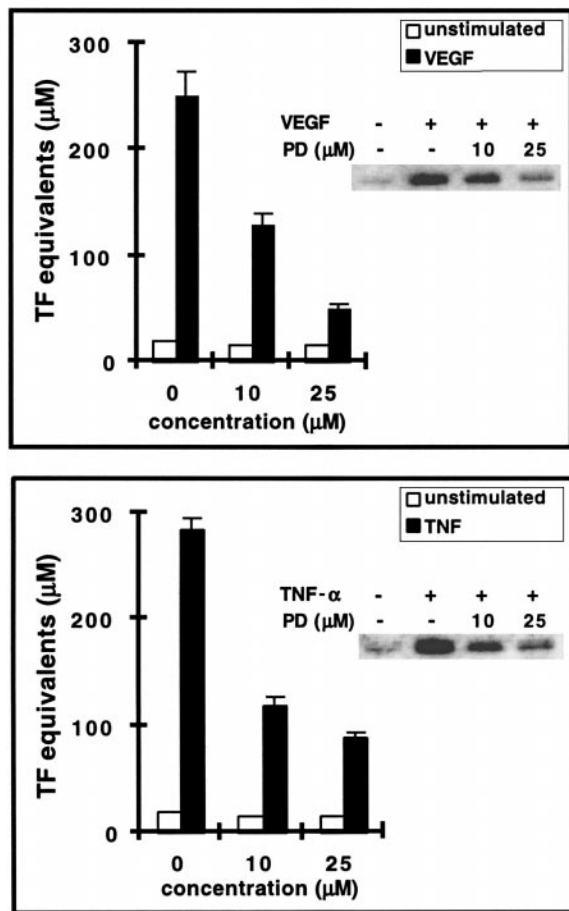


Figure 4. Effect of the MEK inhibitor PD098059 on VEGF- and TNF- α -stimulated TF activity/expression. Equivalents of TF activity were determined in a one-stage clotting assay in parallel to Western blot analysis of TF expression (see insert). HUVEC were preincubated for 30 min with increasing concentrations of PD098059 before stimulation with VEGF or TNF- α for 4 h. DMSO alone at the appropriate concentration was used as a control and showed no effect (data not shown). Each value in a one-stage clotting assay is the mean \pm SD of triplicate in one experiment representative of three performed with similar results.

detected were in part due to an incomplete inhibitory potency of PD098059, another potent MEK inhibitor, U0126 (34), was used at 50 μ M and gave similar results (data not shown). These data show that induction of the MEK/ERK/EGR-1 cascade is required to a large degree for TF activation by VEGF, but also plays a significant role in TNF- α -triggered TF.

Effect of a PKC inhibitor on EGR-1 and TF induced by PMA, VEGF, or TNF- α

The mechanisms involved in the activation events for MEK and ERK have been studied extensively. In both cases, two phosphorylations within the activation loop of the kinases are required (35, 36). In contrast to these activation mechanisms, the regulation of Raf, the immediate upstream kinase of MEK, is substantially more complex. Among the mechanisms involved, both PKC-

dependent and -independent pathways of Raf activation in response to different agonists may occur (37). We used a broad spectrum inhibitor of PKC isozymes, bis-indolylmaleimide I (also known as GF109203X or Gö6850), to assay the contribution of PKC in the signaling chain leading to EGR-1 activation by VEGF and TNF- α . Bis-indolylmaleimide I is known to inhibit membrane and cytosolic PKC with similar potencies. IC₅₀ values of inhibition increase from conventional to novel and atypical PKC isoforms (38, 39).

Since the biological properties of phorbol esters are based on their ability to mimic the responses of diacylglycerol, allowing direct activation of PKC (40), we used PMA as a positive control to activate the PKC-dependent MEK/ERK module. The data in Fig. 5A show that PMA treatment resulted in strong induction of EGR-1. As expected, the PKC inhibitor at 5 μ M inhibited over 90% of EGR-1 induction, and at 20 μ M the expression of EGR-1 was blocked completely (data not shown). Among the properties of phorbol esters is the PKC-mediated activation of NF κ B (41). As shown in Fig. 5A, PMA treatment resulted in I κ B degradation, but with slower kinetics when compared to TNF- α . Moreover, bis-indolylmaleimide I at 5 μ M completely abolished PMA-induced I κ B degradation, thus clearly demonstrating the potency of the inhibitor used.

VEGF-initiated signaling events leading to EGR-1 were very similar to PMA with respect to sensitivity to the PKC inhibitor. Pretreatment of the cells with bis-indolylmaleimide I before VEGF stimulation repressed expression of EGR-1 (Fig. 5A). Taken together, our findings indicate that induction of EGR-1 by VEGF is mediated by a signaling cascade involving PKC and ERK.

TNF- α -induced EGR-1 expression was not altered or only slightly affected in some experiments by 5 μ M of bis-indolylmaleimide I (Fig. 5A) and was partially inhibited by 20 μ M of the compound (data not shown). Thus, the TNF- α signaling chain leading to EGR-1 induction is less sensitive to alterations in PKC activity than the cascades induced by PMA or VEGF. bis-Indolylmaleimide I did not inhibit TNF- α -mediated activation of p38 kinase (data not shown) and, in contrast to the results with PMA described above, did not alter TNF- α -initiated I κ B degradation (Fig. 5A). This illustrates the specific action of the PKC inhibitor as well as the important differences in the multiple signaling pathways leading to NF κ B activation.

Our next step was to evaluate whether the alterations in signaling observed in the presence of the PKC inhibitor would affect induction of TF activity. From three stimuli used, PMA caused the strongest up-regulation of TF activity whereas VEGF and TNF- α induced TF to a comparable extent (Fig. 5B). Bis-indolylmaleimide I caused a dose-dependent inhibition of TF activity induced by all three stimuli. In PMA- and VEGF-stimulated cells, the level of TF activity was repressed over 90% by 5 μ M bis-indolylmaleimide I, whereas only 20% inhibition was observed in TNF- α -stimulated cells. Thus, the TNF- α -initiated signaling

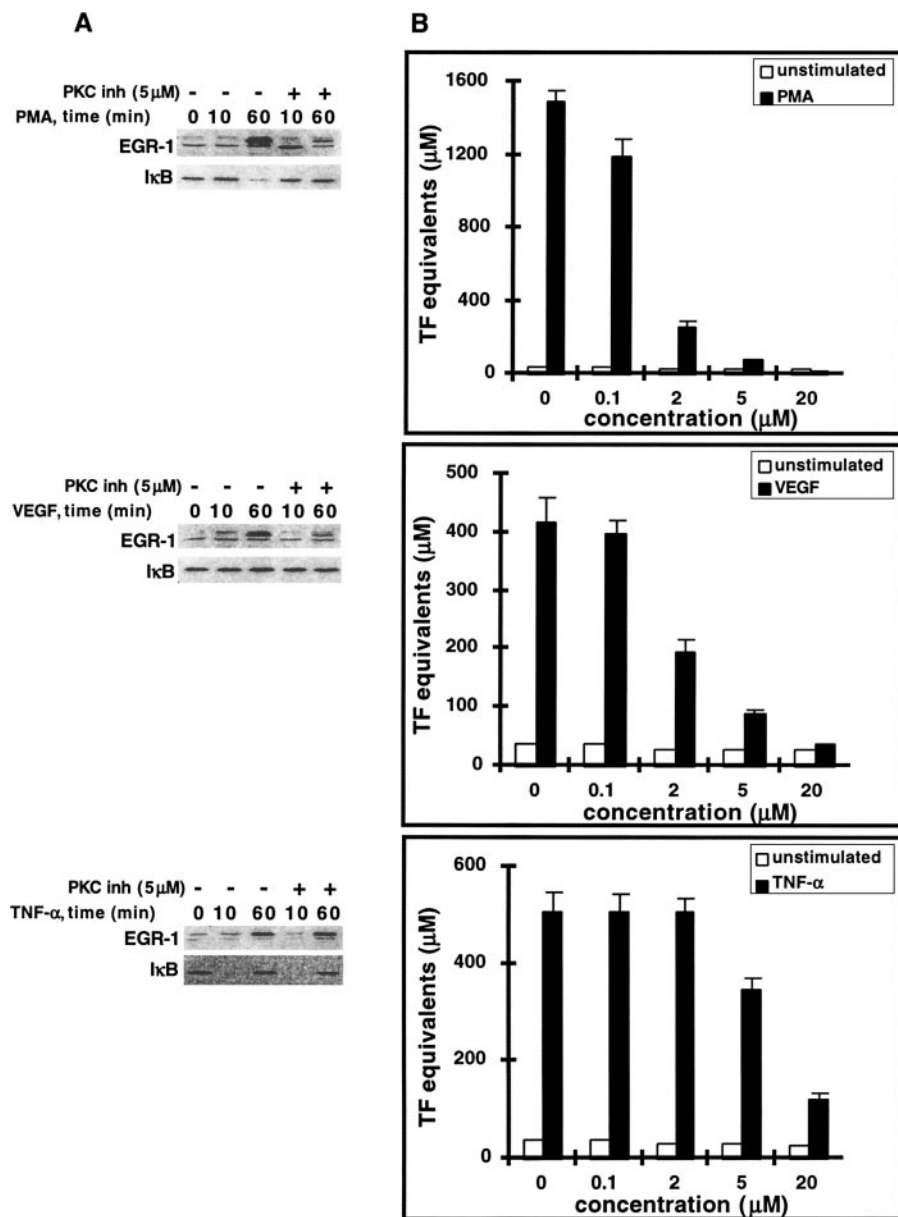


Figure 5. Analysis of the PKC dependence of PMA-, VEGF- or TNF- α -activated pathways leading to EGR-1 and TF. *A*) Western blot analysis of EGR-1 expression in the presence of PKC inhibitor bis-indolylmaleimide I. HUVEC were treated with PMA (10 nM), VEGF (1.25 nM), or TNF- α (100 U/ml) in the presence or absence of 5 μ M bis-indolylmaleimide I (30 min preincubation). Lysates were subjected to Western blot analysis. Blots were probed with anti-EGR-1 antibodies and reprobated with anti-I κ B antibodies. Representative results from two independent experiments are shown. *B*) TF activity equivalents were determined in a one-stage clotting assay. HUVEC were preincubated for 30 min with increasing concentrations of bis-indolylmaleimide I before stimulation with PMA, VEGF or TNF- α for 4 h. Each value is the mean \pm SD of triplicate in one experiment representative of three performed with similar results.

cascade connected to TF expression was clearly less sensitive to the PKC inhibitor.

A dominant negative I κ B kinase 2 (IKK2) mutant selectively blocks the induction of EGR-1 by TNF- α

Our findings clearly show that TNF- α -induced EGR-1 expression in endothelial cells is MEK/ERK dependent. On the other hand, it is well established that induction of the major specific transcriptional programs by TNF- α is primarily mediated by the activation of NF κ B via the NIK/IKK pathway. To test whether this NF κ B-activating cascade interferes with a MAP kinase-mediated induction of EGR-1, we used a recombinant adenovirus expressing a dominant negative IKK2 mutant (AdIKK2dn). This mutant has been described to block TNF- α - and interleukin 1 (IL-1)-induced NF κ B activation (21, 42). In parallel, a GFP-expressing virus

(AdGFP) was used as a negative control. HUVEC infected with virus were stimulated with VEGF or TNF- α for 1 h. Immunofluorescence analysis showed that the IKK2 mutant, as expected, inhibited p65 nuclear translocation in TNF- α -stimulated cells and had no effect on the subcellular localization of p65 in unstimulated HUVEC (Fig. 6A). We have previously analyzed the subcellular localization and expression of EGR-1 protein in unstimulated and VEGF-induced endothelial cells and demonstrated a rapid accumulation of EGR-1 in the nucleus on induction (11). HUVEC infected with control AdGFP showed a diffuse cytoplasmic staining for EGR-1 in unstimulated cells and predominant nuclear localization on stimulation with VEGF or TNF- α (Fig. 6B). The IKK2 mutant blocked TNF- α -induced nuclear accumulation of EGR-1. In contrast, VEGF-triggered EGR-1 induction was almost unaffected (Fig. 6B).

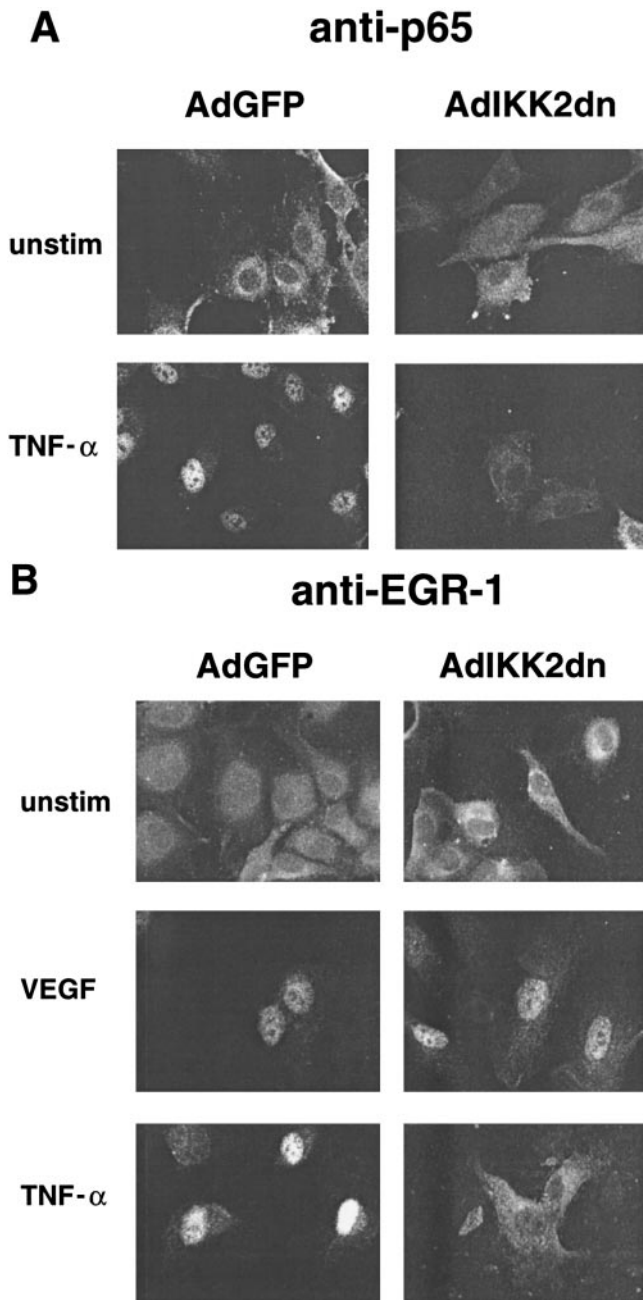


Figure 6. A dominant negative mutant of IKK2 selectively blocks TNF- α -mediated EGR-1 induction. HUVEC were infected with the recombinant adenoviruses AdGFP (control) or AdIKK2dn. 24 h postinfection, short-starved HUVEC were exposed to TNF- α or VEGF for 1 h before fixation. *A*) Immunofluorescence staining of the p65 NF κ B subunit in unstimulated and TNF- α -treated HUVEC. *B*) Immunofluorescence staining of EGR-1 in unstimulated, VEGF-, and TNF- α -treated cells. Similar results were obtained in additional two independent experiments.

We next tested whether the dominant negative IKK2 mutant interferes with the TNF- α -initiated activation of the MEK/ERK cascade. Cells were infected with AdIKK2dn or AdGFP, stimulated by VEGF or TNF- α for 10 and 30 min, and subjected to Western blot analysis. Staining with phospho-specific MAP kinase antibodies showed that the activation of MEK, ERK, and p38

kinase typically observed in VEGF- or TNF- α -treated HUVEC (compare Fig. 2) was unaltered in the AdGFP-infected cells (Fig. 7). In marked contrast, TNF- α -induced MEK as well as ERK activation was inhibited by AdIKK2dn, whereas activation of the p38 kinase was not affected. In comparison, VEGF-initiated signals were not altered significantly. Reprobing with anti-I κ B antibodies was used to demonstrate the inhibitory potency of the dominant negative IKK2 mutant on the NF κ B pathway. Indeed, overexpression of the IKK2 mutant completely prevented TNF- α -induced I κ B degradation (Fig. 7; compare 10 min time points for AdGFP and AdIKK2dn). Taken together, these data indicate that the IKK2 mutant interferes specifically with the TNF- α -induced pathway connected to NF κ B activation as well as the TNF- α -induced MEK/ERK cascade leading to EGR-1 induction. These results lend further support to our hypothesis that from the multiple pathways triggered by TNF- α and VEGF receptors, those that lead to EGR-1 induction converge on the level of MEK, whereas the initial upstream events are distinct.

TF is activated by VEGF but not EGF in endothelial cells

Since the MEK/ERK module of MAP kinases is triggered by a wide range of growth factors, one would expect that TF expression should be generally induced by all growth factors for which receptors are present on endothelial cells. However, in contrast to VEGF, treatment of endothelial cells with EGF did not increase TF expression and procoagulant activity, whereas EGR-1 was strongly induced (Fig. 8A, insert). On the other hand, though by itself noninducing, EGF was able to

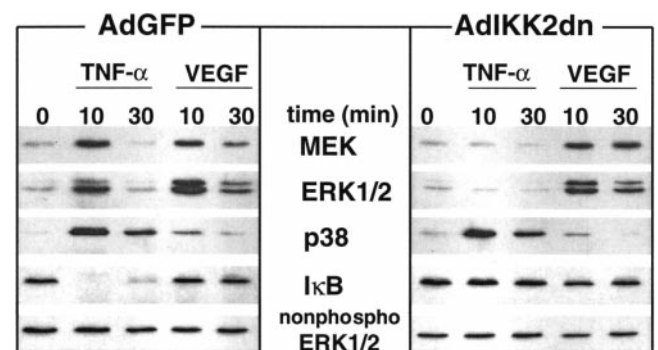


Figure 7. A dominant negative mutant of IKK2 specifically interferes with the TNF- α -induced MEK/ERK cascade. HUVEC were infected with the recombinant adenoviruses AdGFP (control) or AdIKK2dn. 24 h postinfection, short-starved HUVEC were induced for 10 and 30 min by TNF- α or VEGF. After treatment, samples were harvested; protein lysates were separated by SDS-PAGE and subjected to Western blot analysis. Phospho-specific antibodies to the active forms of MEK, ERK, and p38 kinase were used. To control the inhibitory potency of the dominant negative IKK2 mutant on the NF κ B activation pathway, blots were reprobbed with anti-I κ B antibodies. Antibodies to nonphosphorylated form of ERK were used to ensure equal loading of samples. Results are representative of two separate experiments.

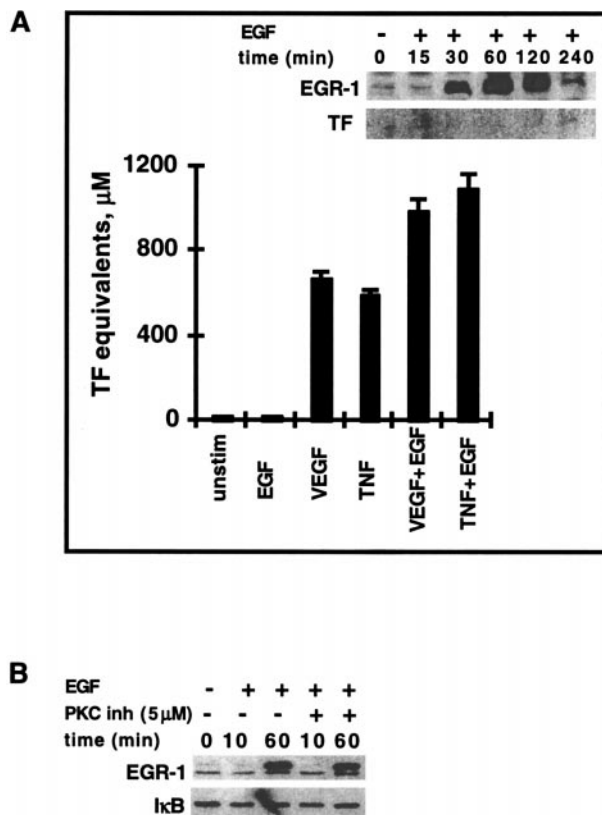


Figure 8. EGF does not induce expression of TF in endothelial cells. *A*) Western blot analysis of total cell extracts from short-starved HUVEC treated with EGF (10 ng/ml) for 15–240 min. Membranes were probed with anti-EGR-1 antibodies, followed by reprobing with anti-TF antibodies (shown as insert). TF activity equivalents were determined in a one-stage clotting assay. HUVEC were stimulated for 4 h with EGF, VEGF \pm EGF, and TNF- α \pm EGF. Each value is the mean \pm SD of triplicate in one experiment. Representative results from two separate experiments are shown. *B*) The PKC inhibitor bis-indolylmaleimide I does not prevent induction of EGR-1 by EGF. Short-starved HUVEC were pretreated with 5 μM of bis-indolylmaleimide I, followed by stimulation with EGF (10 ng/ml) for 10 and 60 min. Lysates were subjected to Western blot analysis. Blots were probed with anti-EGR-1 antibodies and reprobbed with anti-I κ B antibodies to test for sample variations. Representative results from three independent experiments are shown.

enhance TF activity induced by VEGF and TNF- α (Fig. 8A). These data suggest that EGF does not display an inhibitory potency, but rather an important step in the signaling cascade necessary for TF induction is missing. Therefore, we compared the signaling events triggered by VEGF and EGF. The EGF-induced pattern of the different MAP kinase modules was similar to VEGF and the ERK cascade was predominantly induced. Furthermore, addition of EGF to cells led to a sustained high level of phospho-ERK; thus, no principal differences were detected in the kinetics of ERK activation by VEGF and EGF. In addition, EGF stimulation of EGR-1 could be blocked by the MEK inhibitor PD98059 but not by the p38 kinase inhibitor (data not shown), similar to the effects seen in VEGF-treated cells. Despite of these clear similarities in signaling events induced by these

growth factors, one principal difference was observed: the PKC inhibitor did not prevent EGR-1 activation by EGF (Fig. 8B), which suggests that the signaling pathway leading from the EGF receptor to EGR-1 is MEK/ERK mediated but, in contrast to VEGF, PKC independent.

DISCUSSION

EGR-1 and TF induction: dependence on MEK/ERK

A single cytokine or growth factor can activate a characteristic network of multiple signal transduction pathways. However, there is considerable overlap between the unique signaling ‘fingerprints’ of different factors. Whereas the broad classes of stimuli elicit signaling responses resulting in MAP kinase activation and IE gene transcription, the precise quantitative ratios between the three major MAP kinase cascades and the individual IE genes appear to be characteristic for each agent and cell type. It determines to a large extent the specific pattern of secondary genes up-regulated and the unique profile of biological responses induced by a given factor.

In this study, we have used the induction of EGR-1 and subsequent TF expression in endothelial cells to 1) characterize the MAP kinase profiles induced by VEGF and TNF- α and 2) define common routes that mediate similar cellular responses to these agents. The findings of the present study demonstrate that VEGF activates mainly ERK1/2 and p38 kinase, but no or only trace amounts of JNK. TNF- α was able to strongly activate all three MAP kinases in addition to the classical inflammatory cascade leading to I κ B degradation and NF κ B activation. The ERK cascade plays the major role in controlling EGR-1 by both agents.

In principle, activation of the *egr-1* gene, similar to other IE genes, is a complex process and can receive input from several pathways. Different MAP kinase subtypes may lead to phosphorylation and subsequent activation of transcription factors, such as Elk-1 and the related ternary complex factor, serum response factor, and CREB. From the current work, it is now clear that a single MAP kinase cascade, MEK/ERK, is absolutely required for EGR-1 induction by the inflammatory TNF- α and the angiogenic VEGF. This is in contrast to stress-induced EGR-1, which has been shown to be mediated by p38 kinase and JNK (43).

Furthermore, it appears that VEGF and TNF- α signaling converges at the level of MEK, whereas initial upstream events leading to MEK activation are distinct. In the case of TNF- α signaling, a new link was identified between IKK2 and MEK activation. When overexpressed, a dominant negative IKK2 mutant, as expected, inhibited TNF- α -initiated I κ B degradation and p65 nuclear translocation, confirming the critical contribution of IKK2 to NF κ B activation. The kinase inactive mutant of IKK2 also blocked TNF- α -triggered MEK phosphorylation/activation and EGR-1 induction. One

possible explanation would be that the two pathways use common upstream signal transduction component(s). Several lines of evidence suggest that MEKK1 (MAPK kinase kinase 1) could be an appropriate candidate for coordinated regulation of both NF κ B and MAP kinase pathways. MEKK1 was shown to be able to induce I κ B phosphorylation/NF κ B activation and the JNK pathway (44, 45). At the same time, previous studies clearly indicate that MEKK1 can regulate two different MAP kinase modules: ERK and JNK (46, 47). However, these data do not explain why a dominant negative IKK2 mutant would block the activity of an upstream kinase for MEK such as MEKK1. Recently, researchers described IKK as one of the essential kinases in the multiprotein signaling complex, called signalsome, that regulates NF κ B activation in response to proinflammatory cytokines. MEKK1 has also been identified to be a component of the same signalsome (21). Thus, it seems possible that incorporating an inactive kinase in the complex may potentially lead to an inactive state of the signalsome as a whole. Indeed, data demonstrating that dominant negative MEKK1 inhibited TNF- α -induced NF κ B activation (44, 48) would be consistent with this interpretation. However, the potential contribution of MEKK1 to the activation of MEK/ERK by TNF- α reflects only part of the possibilities, since a number of different stimuli can use alternative pathways of activation, such as Raf family members, which also regulate MEK/ERK (49).

The results of the present study show that in contrast to the TNF- α -mediated MEK/ERK/EGR-1 cascade, the VEGF-initiated MEK/ERK/EGR-1 pathway was not significantly altered by the dominant negative mutant of IKK2. Selective or preferential activation of either MEKK1 or Raf by the two different stimuli is therefore one possible interpretation of these data. There exists an alternative, nonexclusive explanation for the TNF- α specific effect of the IKK2 mutant. Like MEK, IKK contains a canonical MAP kinase kinase (MAPKK) activation loop motif (Ser-X-X-X-Ser, where X is any amino acid: SLCTS FVGT for IKK2 vs. SMANS FVGT for MEK). Thus, according to the current model of sequential kinase cascade organization, MEK and IKK belong to the same level of hierarchy within the MAP kinase families. It is tempting to speculate that a hypothetical, not-yet-identified scaffold protein could coordinate these two signal transducers in signalsomes triggered by TNF- α . Furthermore, the fact that the catalytically inactive mutant of IKK2 did not block MEK/ERK activation triggered by VEGF may point to the existence of signalsome complexes specific for the individual receptors and containing signal-determined components.

The demonstrated activation of common cascades by TNF- α and VEGF could account for the ability to elicit in part similar responses from their target cells in certain instances. One example of common responses of these factors based on MEK/ERK activation in endothelial cells is the induction of TF.

Among additional described common bioactivities elicited by TNF- α and VEGF are the release of soluble tie-1 from endothelial cells (50) and the ability to increase vascular permeability (28, 51, 52). The exact pathways leading to these responses have not been defined yet.

EGR-1 and TF induction: dependence on PKC

The transcriptional induction of EGR-1 has previously been described to be mediated by PKC-dependent and -independent mechanisms. In fact, the PKC pathway can connect extracellular stimuli to EGR-1 induction as it can be shown that direct activation of PKCs by PMA induces EGR-1 (53) (see Fig. 5). A PKC-dependent EGR-1 induction has been shown in cells activated by hypoxia (54). On the other hand, pathways independent of PKC can also play a role in EGR-1 induction. In fibroblasts deficient in PKC signaling, EGR-1 was strongly induced by serum and EGF (53). The results presented in this study demonstrate that, in the case of VEGF stimulation of endothelial cells, PKC activation is an essential step in the chain of events connecting the VEGF receptor (VEGFR) with TF activation via EGR-1. The fact that, in comparison to the other stimuli used, PMA treatment of the cells resulted in the strongest induction of TF further emphasizes that PKC can play an important role for TF expression. In line with the findings concerning TF expression inhibitors of PKC have been reported to inhibit angiogenesis induced by VEGF (55). Taking into consideration the fact that the amino acid sequence of EGR-1 includes several potential PKC phosphorylation sites (D. Mechtcheriakova et al., unpublished data), it is tempting to speculate that PKC is involved not only in the activation of MEK/ERK/EGR cascade, but also in additional direct phosphorylation of EGR-1 to modulate and/or specify its transcriptional activity.

In contrast to PMA or VEGF, the TNF- α -initiated signaling cascade connected to TF expression was clearly less sensitive to the PKC inhibitor. This may be due to only a partial involvement of this pathway or a difference in PKC isozymes. Indeed, previous studies have demonstrated that TNF- α is a potent activator of the atypical PKCs (56). The important contribution of these isoforms in the activation of the MEK/ERK pathway (57) as well as in an NF κ B activation through the IKK pathway (58) has been described. On the other side, in contrast to conventional PKCs, much higher concentrations of bis-indolylmaleimide I have to be used to block the activity of atypical PKCs (38).

EGR-1 and TF induction: VEGF vs. EGF

In several instances, treatment of a single cell type with different factors can activate similar signaling pathways but result in different biological outcomes. In this respect it is an open question to what extent the VEGF signals are distinct from other growth

factors with growth-promoting activity on endothelial cells and lead to a VEGF characteristic response pattern. One classical example of differential responses to growth factors are the responses of PC12 cells to NGF and EGF. In these cells EGF promotes mitogenesis, but NGF promotes differentiation (59). A possible explanation is that the initial signals triggered by the stimuli can be diverted to different outcomes by differences in the strength or kinetics of the signals, or both (59, 60). EGF induces transient activation of ERK and stimulates proliferation of PC12 cells, whereas NGF stimulates prolonged ERK activation and induces cellular differentiation. An alternative possibility is that all growth factors may induce ERK, but differences exist in the upstream signals that couple receptor tyrosine kinases to ERK. Indeed, supporting data for this possibility have been published recently showing that PKC δ is required for the activation of MEK/ERK by differentiating factors in neuronal cells, but not by the growth-promoting EGF (61).

The data presented here demonstrate a differential response of endothelial cells to EGF and VEGF. In contrast to VEGF, EGF treatment of the cells did not lead to up-regulation of TF, although both growth factors triggered sustained ERK activation resulting in EGR-1 induction. One significant difference is that despite the dominant role of the MEK/ERK cascade in EGF- and VEGF-induced EGR-1, the EGF activation seems to be PKC independent. This suggests that distinct upstream signals initiated by VEGF and EGF lead to MEK activation. We believe that this could result in a qualitative difference of EGF- vs. VEGF-induced EGR-1 such as a differential phosphorylation/dephosphorylation and a different response of the TF gene.

Alternatively, it is possible that a second factor(s) is important for the full response of the TF promoter that may not be active in EGF-treated cells. The transcription factor NFAT could be an appropriate candidate since, in addition to EGR-1 (11), NFAT has also been implicated in transcriptional activation of the TF gene by VEGF (62). It is noteworthy that the NFAT binding site within the TF promoter overlaps with the previously identified NF κ B-like site participating in TF activation in response to lipopolysaccharides or TNF- α (10, 33). Therefore, it is possible that full transcriptional activity of the TF gene would require the presence and cooperation of both EGR-1 and NF κ B for TNF- α responsiveness or EGR-1 and NFAT, occupying the overlapping NF κ B/NFAT binding site, for VEGF responsiveness.

EGR-1 and TF induction: correlation with VEGFR-2 activation

VEGF is considered to be a main mediator of angiogenesis. Numerous proteins closely related in primary structure to VEGF have been grouped in the VEGF family. The angiogenic activity of various family mem-

bers is mediated in turn by several VEGF receptors (63). The VEGF-A₁₆₅ isoform binds with high affinity to both VEGFR-1 (flt-1) and VEGFR-2 (flk-1), whereas VEGF-A₁₂₁ acts solely via VEGFR-2 (64). Recent data demonstrate that VEGF-E, a novel member of the VEGF family, possesses an angiogenic activity as potent as VEGF-A. In contrast to VEGF-A, VEGF-E binds selectively to VEGFR-2 but not to VEGFR-1 (65). This finding is in accordance with previous studies suggesting that the angiogenic signal by VEGF-A itself may be predominantly mediated by VEGFR-2 (66, 67). These data indicate that VEGFR-2 activation can mediate angiogenesis efficiently without concomitant VEGFR-1 activation. In addition, placental growth factor (PlGF) binds to VEGFR-1, but not to VEGFR-2, and its role in angiogenesis is currently not well understood. The different VEGFs also have different capacities to modulate TF activity on endothelial cells. Both isoforms of VEGF-A, VEGF₁₆₅ and VEGF₁₂₁, were equipotent to induce TF (11). VEGF-E was found to have a similar bioactivity to VEGF-A with respect to TF induction (65). In contrast, TF was only slightly induced by PlGF (twofold) in comparison to a significantly higher up-regulation by similar concentrations of VEGF-A (25- to 80-fold) (9, 11). These findings apparently reflect clear parallels between VEGFR-2 activation and TF expression and thereby suggest that activation of VEGFR-2 seems to be sufficient for TF induction in endothelial cells.

The biological relevance of VEGF-induced TF expression in the complex process of new vessel formation is still unresolved. Since in the present study we did not investigate this aspect directly, it is only possible to speculate based on two experimental observations. First, it appears that there are clear differences in the abilities of growth factors to induce TF expression: VEGF is a strong stimulus whereas EGF does not activate TF in endothelial cells. Second, VEGF-triggered signaling via VEGFR-2 seems to be sufficient to induce TF. It thus appears that TF induction parallels the property of VEGF as a strong inducer of angiogenesis via VEGFR-2. It is widely accepted that active endothelial cell proliferation, migration, and matrix remodeling are all essential components of angiogenesis, and it is conceivable that a small amount of fibrin deposition is necessary for angiogenesis to proceed. For the process of vasculogenesis during development, one of the initial characteristic steps involves differentiation of the mesoderm-derived angioblasts into endothelial cells. It is not completely clear to what extent the proposed recruitment of endothelial precursor cells from the circulation may also play a role in angiogenesis in parallel to endothelial cell migration and sprouting from preexisting mature endothelial cells. It is possible that the pathway delineated here, which connects MEK/ERK activation to EGR-1, will also be important for the process of endothelial cell differentiation. The involvement of this route in differentiation processes has been described for other cell

types. The sustained ERK activation, followed by EGR-1 induction, which is required for NGF-induced differentiation of PC12 cells (68, 69), is one example. As with VEGF induction, this process is PKC dependent (61). Other examples are the granulocyte-macrophage colony-stimulating factor (GM-CSF) inducing the differentiated functions of mature granulocytes and monocytes/macrophages (70) and IL-3 resulting in proliferation and maturation of early bone marrow progenitor cells (71). Pathways involving MEK/ERK are activated by both factors (72). Furthermore, signals from the GM-CSF and IL-3 receptors lead to rapid induction of the egr-1 gene (73). Given the close relationship of endothelial cell precursors with the hemopoietic lineage, a common theme in the induction of differentiated functions seems possible. **[FJ]**

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