

Vascular Endothelial Growth Factor Activates STAT Proteins in Aortic Endothelial Cells*

Received for publication, May 10, 2000, and in revised form, August 17, 2000
Published, JBC Papers in Press, August 28, 2000,
DOI 10.1074/jbc.C000318200

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Vascular endothelial growth factor (VEGF) intracellular signaling in endothelial cells is initiated by the activation of distinct tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (Flk-1/KDR). Because the tyrosine kinase-dependent transcription factors known as STAT (signal transducers and activators of transcription) proteins are important modulators of cell growth responses induced by other growth factor receptors, we have determined the effects VEGF of on STAT activation in BAEC (bovine aortic endothelial cells). Here, we show that VEGF induces tyrosine phosphorylation and nuclear translocation of STAT1 and STAT6. VEGF also stimulates STAT3 tyrosine phosphorylation, but nuclear translocation does not occur. We found that placenta growth factor, which selectively activates VEGFR1, has no effect on the STATs. However, upon VEGF stimulation, STAT1 associates with the VEGFR2 in a tyrosine kinase-dependent manner, indicating that VEGF-induced STAT1 activation is mediated primarily by VEGFR2. Thus, our study shows for the first time that VEGF activates the STAT pathway through VEGFR2. Because the growth-promoting activity of VEGF depends upon VEGFR2 activation, these findings suggest a role for the STATs in the regulation of gene expression associated with the angiogenic effects of VEGF.

VEGF¹ is a potent, multifunctional and endothelial cell spe-

* This work was supported in part by National Institutes of Health Grants EY04618 and EY11766 and by an unrestricted grant from Research to Prevent Blindness to the Department of Ophthalmology at the Medical College of Georgia. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; rhVEGF, recombinant human VEGF; PDGF, platelet-derived growth factor; SH2, Src homology 2; STAT, signal transducers and activators of transcription; BAEC, bovine aortic endothelial cells; PIGF, placenta growth factor; PMSF, phenylmethylsulfonyl fluoride; IFN, interferon; IL, interleukin; PI3 kinase, phosphatidylinositol 3-kinase; JAK, Janus kinase.

cific growth factor. It stimulates vasodilation and cell proliferation, increases permeability and migration, and promotes endothelial cell survival (1–4). Two high-affinity VEGF receptors, VEGFR1 (Flt-1) (5, 6) and VEGFR2 (Flk-1/KDR) (7, 8), have been identified in endothelial cells. VEGF receptors are tyrosine kinases that share in common with other growth factor receptors the ability to trans-phosphorylate and in turn phosphorylate on specific tyrosine residues SH2 domain-containing signaling molecules (13, 14).

Characterization of these receptors suggests that VEGFR1 mediates cell migration and differentiation, whereas VEGFR2 mediates cell proliferation and survival (6, 9–12). These functional differences correspond to diverse signal transduction properties. VEGFR2 undergoes strong ligand-dependent tyrosine phosphorylation, whereas VEGFR1 shows a weak response (6, 13). Furthermore, although both receptors activate the serine/threonine kinase mitogen-activated protein kinase, VEGFR1 is not able to mediate the mitogenic effect of VEGF in endothelial cells (14). This suggests that the tyrosine kinase pathway predominates in the mitogenic actions of VEGF.

A new class of SH2 domain-containing signaling molecules has been found recently to be involved in mediating the growth-promoting activity of other growth factors such as PDGF and EGF (epidermal growth factor) (15–17). These signaling molecules belong to a family of latent cytoplasmic transcription factors known as signal transducers and activators of transcription (STAT). To date, seven mammalian STATs (STAT1, -2, -3, -4, -5A/B, -6) have been identified. Biochemical and molecular analysis have indicated that STAT activity is regulated predominantly by phosphorylation on specific tyrosine residues, which causes the STATs to dimerize. STAT dimerization is usually followed by translocation into the nucleus (18). Within the nucleus, STATs recognize and bind to consensus DNA binding sites that represent enhancer sequences for a variety of genes, including the immediate early growth response genes. STATs are the only known phosphotyrosine-dependent transcription factors.

VEGF receptors share functional and structural homologies with other intrinsic tyrosine kinase receptors, such as PDGF receptor, which have been shown to activate STAT proteins (1, 19). Moreover, activation of VEGF receptors induces transcriptional activation of immediate early growth response genes, such as *c-fos*, without requiring *de novo* synthesis of new transcription factors (20, 21). Because PDGF receptor activation has been shown to cause STAT activation (16), we hypothesized that VEGF signaling also involves the STAT signaling pathway. To test this hypothesis, we treated cultured BAECs with recombinant human VEGF165 (rhVEGF165). Tyrosine-phosphorylated STATs were then detected in cellular and nuclear fraction extracts by immunoprecipitation and Western analysis. Confocal immunocytochemistry was conducted to monitor the intracellular mobilization of STATs following VEGF stimulation.

EXPERIMENTAL PROCEDURES

Materials—Genistein was from Calbiochem-Novabiochem Corp. rhVEGF165 and placenta growth factor (PIGF) were from R & D Systems (Minneapolis, MN). Monoclonal antibodies against STAT1, STAT3, STAT5, STAT6, JAK1, Tyk2, and phosphotyrosine (PY20) were from Transduction Laboratories (Lexington, KY). Anti-JAK2 antibody was from Upstate Biotechnology (Lake Placid, NY), protein A/G-agarose beads, anti-phosphotyrosine PY99, and a polyclonal antibody

against VEGFR2 (Flk-1/KDR) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibodies against tyrosine-phosphorylated STAT1, STAT3, and STAT6 were from New England Biolabs, Inc. (Beverly, MA). Propidium iodide and Oregon Green-conjugated anti-mouse and anti-rabbit secondary antibodies were from Molecular Probes (Eugene, OR).

Cell Culture—Endothelial cell primary cultures were prepared as described (22). The cells were used between passages 2 and 6.

Immunoprecipitation and Immunoblotting—Eighty-five percent confluent BAEC cultures were switched to serum-free medium for 16–18 h and then treated with rhVEGF165 for different times. A dose dependence curve established the effective dose of VEGF for STAT activation as 10 ng/ml.

Incubations were terminated by washing the cells twice in ice-cold phosphate-buffered saline containing 2 mM Na_3VO_4 and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were obtained using lysis buffer (Tris-HCl 20 mM (pH 7.4), EDTA 2.5 mM, NaF 50 mM, $\text{Na}_4\text{P}_2\text{O}_7$ 10 mM, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 1 mM PMSF, and 2 mM Na_3VO_4). Immunoprecipitation and Western analysis were done as described (23–26). Co-immunoprecipitation of STAT proteins and Flk-1/KDR was carried out.

Nuclear Fractionation—STAT proteins in nuclear fractions were detected by Western blotting analysis. Purified nuclei were prepared using a 350 mM sucrose cushion, and nuclear extracts were obtained as described (27). Nuclear extracts (25 μg protein/well) were then subjected to SDS-10% polyacrylamide gel electrophoresis and immunoblotted using anti-STAT antibodies.

Confocal Immunocytochemistry—Intracellular mobilization of STATs in response to VEGF treatment was also analyzed by confocal immunocytochemistry following a procedure described previously (27).

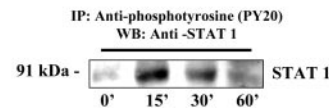
RESULTS AND DISCUSSION

STAT Expression Pattern in BAECs—The expression of STAT proteins differs in different cell types. Western blotting analysis of cell lysates from untreated BAECs showed that these cells express only four STAT family members: STAT1, STAT3, STAT5, and STAT6. Therefore, we examined the activation pattern of these STATs in response to stimulation with rhVEGF165 by following their tyrosine phosphorylation and translocation into the nuclear compartment. VEGF165 is the predominant molecular species and has been shown to have potent angiogenic activity in BAECs (28).

VEGF Stimulates STAT1 Tyrosine Phosphorylation and Nuclear Translocation—Tyrosine phosphorylation of STAT1 was detected in VEGF-treated BAEC using immunoprecipitation and immunoblotting techniques. Fig. 1A shows that VEGF stimulation resulted in a rapid and transient STAT1 tyrosine phosphorylation. Maximal tyrosine phosphorylation was detected at 15 and 30 min, returning to basal levels at 60 min. Fig. 1B shows that STAT1 was present in nuclear extracts at 30 and 40 min after VEGF stimulation. Confocal microscope immunocytochemistry confirmed these results. Fig. 2 shows that STAT1 immunoreactivity was undetectable in unstimulated BAECs (*panel A*) but became evident perinuclearly and within the nuclear compartment 30 min after VEGF stimulation (*panel B*). This rapid onset in STAT1 immunoreactivity with VEGF stimulation probably represents increased protein affinity and/or accessibility to the antibody consequent to STAT1 mobilization and dimerization because significant increases in STAT1 protein levels are unlikely to occur during the 15- and 30-min treatment times. Increases in STAT1 immunoreactivity have also been observed in human umbilical vein endothelial cells following STAT1 activation by urokinase (29).

Our data show that VEGF stimulation of BAECs results in STAT1 activation. STAT1 was first identified as a critical component of interferon (IFN)-stimulated transcription factor activation. Gene targeting studies also suggested a prominent role of STAT1 in modulating IFN-dependent natural immunity (30). However, growth-promoting agents such as PDGF and angiotensin II also activate STAT1 (17, 23). Mutational analysis of STAT members along with expression studies of gain-

A. Tyrosine phosphorylation



B. Nuclear translocation

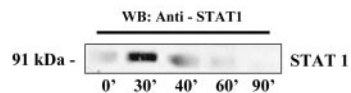


FIG. 1. VEGF-induced STAT1 activation. BAECs were treated with rhVEGF165 for the indicated times. *A*, cell lysates were immunoprecipitated (IP) with anti-phosphotyrosine antibody (PY20) and probed with anti-STAT1. The same results were obtained when immunoprecipitation was done using anti-STAT1 and the blots (WB, Western blot) probed with PY20. *B*, purified nuclear extracts were immunoblotted with anti-STAT1 antibody to monitor STAT1 translocation in the nuclear fraction of activated BAECs. These results are representative of three separate experiments.

and loss-of-function alleles have indicated a role for the STATs in cell growth and survival responses (31). STAT1 constitutive activation has been associated with uncontrolled growth in several malignancies. Moreover, the chemotherapeutic drug fludarabine has been found to exert its anti-tumor activity by inhibiting STAT1 expression (32). Therefore, STAT1 activation in endothelial cells is likely to play a role in controlling endothelial cell growth and survival.

VEGF Stimulates STAT6 Tyrosine Phosphorylation and Nuclear Translocation—The next experiments determined the tyrosine phosphorylation pattern of STAT6 in response to VEGF stimulation. As is shown in Fig. 3A, immunoreactivity corresponding to tyrosine-phosphorylated STAT6 was evident 30 and 60 min after VEGF stimulation. STAT6 was localized in the nuclear fraction 60 min after VEGF stimulation (Fig. 3B). Confocal images also revealed STAT6 immunolocalization within the nuclei of BAEC after 60 min of VEGF stimulation (Fig. 2D), whereas the nuclei of unstimulated control cells were negative (Fig. 2C).

STAT6 was first identified as a key player in the interleukin (IL)-4 signaling pathway in lymphoid cells (33). Targeted disruption of the *STAT6* gene showed that this transcription factor plays a role in cell responses to IL-12, IL-4, and IL-13 (30). Recent evidence indicates that STAT6 also plays a role in regulating cellular responses stimulated by PDGF (34). STAT6 has been indirectly implicated in regulating angiogenic responses because it binds to enhancer sequences found in the prolactin gene (18). Members of the prolactin/growth hormone family have been shown to possess pro-angiogenic activity. Interestingly, the 16-kDa N-terminal fragments of the latter proteins have been shown to possess anti-angiogenic activity (35). Thus, STAT6 may play a role in regulating endothelial cell growth responses because of its effects on prolactin family proteins. Further work is needed to test this hypothesis. In our experiments, STAT1 and STAT6 displayed different activation time courses in response to VEGF stimulation, with STAT1 activation occurring before that of STAT6. It will be of interest to evaluate whether this difference corresponds to diverse activation mechanisms and whether VEGF-induced STAT6 activation in aortic endothelial cells depends upon STAT1 activity.

VEGF Stimulates STAT3 Tyrosine Phosphorylation—The VEGF-induced STAT3 tyrosine phosphorylation pattern is represented in Fig. 4. Immunoreactivity specific for phosphotyrosine-STAT3 is detected in immunoblotted cell lysates after

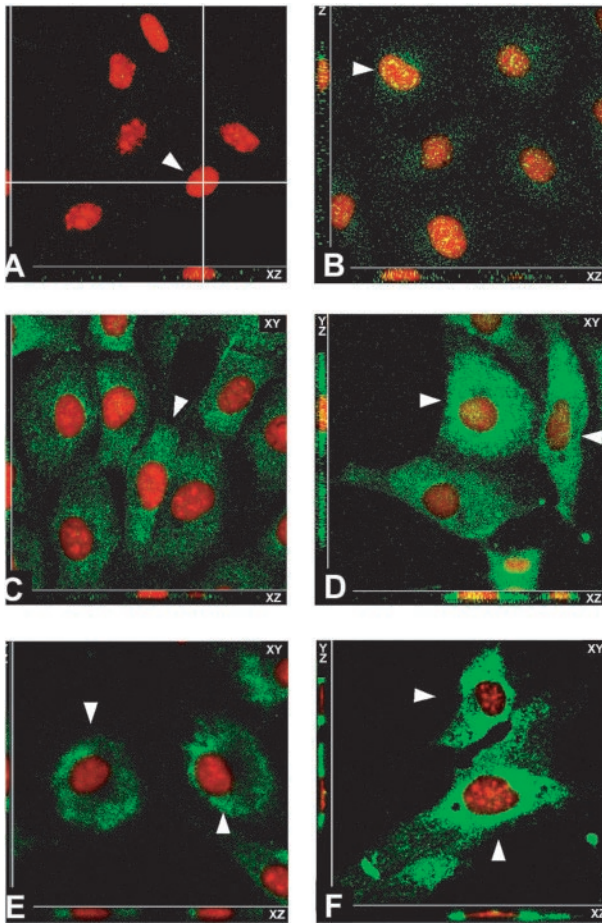


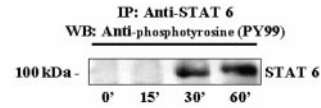
FIG. 2. VEGF-induced intracellular mobilization of STATs. BAEC were treated with rhVEGF165 for 0 min (A, C, and E), 30 min (B), 60 min (D), or 90 min (E) and double-labeled with antibodies against STAT1 (FITC, green, in A and B), STAT6 (FITC, green, in C and D), or STAT3 (FITC, green, in E and F) in combination with propidium iodide (red) to stain the nuclei. Confocal microscope techniques were used to generate optical sections through the center of the nucleus (arrowheads) in three planes (x-y, x-z, y-z). This analysis showed that the nuclei of untreated control cells (A, C, and E) contain no labeling for the STAT proteins. However, after VEGF treatment, the nuclei contain large and small clusters of immunoreactivity (yellow) for STAT1 (B) and STAT6 (D), whereas STAT3 remains localized in the perinuclear space (F). 400 \times .

only 60 and 90 min of VEGF stimulation. Western blotting of BAEC nuclear extracts showed occasional weak activity, but no significant nuclear translocation was observed. Confocal immunocytochemistry failed to detect immunoreactivity specific for STAT3 in the nuclear compartment of VEGF-stimulated BAEC. After 90 min of VEGF induction, the confocal images showed STAT3 immunoreactivity only in the perinuclear region (Fig. 2F). Neither longer nor shorter exposures to VEGF resulted in STAT3 nuclear translocation. Therefore, it appears that VEGF does not induce the STAT3 nuclear translocation that is required for its transcriptional activity.

Tyrosine phosphorylation of STATs has been shown to be necessary but not sufficient for their transcriptional ability, which also requires their nuclear translocation. This latter event is an active process for which the specific mechanisms remain unclear. Because STAT proteins lack a nuclear localization signal, it has been suggested that chaperone proteins are required to assist their active translocation into the nuclear compartment (36). Thus, differences between STAT protein interactions with chaperone proteins may contribute to differences in their intracellular compartmentalization.

Previous work has indicated that STATs can have other

A. Tyrosine phosphorylation



B. Nuclear translocation

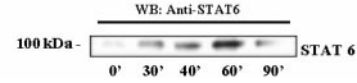


FIG. 3. VEGF-induced STAT6 activation. BAEC cultures were treated as indicated in Fig. 2. A, cell lysates were immunoprecipitated (IP) with anti-STAT6 antibody and probed with anti-phosphotyrosine antibody (PY99). The same results were also obtained when immunoprecipitation was done using PY99 and the blots (WB, Western blot) probed with anti-STAT6. B, purified nuclear extracts were blotted and probed with anti-STAT6 antibody to identify STAT6 translocation in the nuclear fractions. The results are representative of three separate experiments.

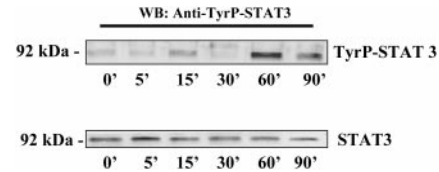
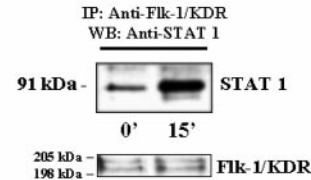


FIG. 4. VEGF-induced STAT3 tyrosine phosphorylation. BAECs were treated with rhVEGF165 for the indicated times. Cell lysates were subjected to immunoblot using anti-phosphotyrosine-STAT3 antibody. The same blots (WB, Western blot) were then striped and reprobbed with anti-STAT3 antibody to verify equal loading (lower panel). The results shown are representative of three separate experiments.

A.



B.

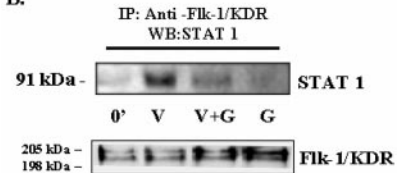


FIG. 5. VEGFR2 (Flk-1/KDR) interaction with STAT1. BAEC cultures were treated for 15 min with rhVEGF165 (V). A, cell lysates were immunoprecipitated (IP) with VEGFR2 (anti-Flk-1/KDR) antibody and probed with anti-STAT1 antibody. The same results were obtained using anti-STAT1 as the precipitating antibody and anti-Flk-1/KDR as the probing antibody. WB, Western blot. B, the experiments shown in A were repeated after pre-treating the cells with the tyrosine inhibitor genistein (G) (25 μ g/ml for 30 min). The blots were then striped and reprobbed with anti-Flk-1/KDR to verify equal loading. Data shown are representative of three separate experiments.

functions in addition to their actions as transcription factors within the nucleus. For example, STAT3 has been found to serve as an adaptor protein to couple phosphatidylinositol 3-kinase (PI3 kinase) to the IFNAR1 chain of the type I IFN receptor (37). Activation of the PI3 kinase pathway is known to

be involved in the survival-promoting function of VEGF. Thus, molecular interactions between STAT3 and PI3 kinase may be important in the regulation of endothelial cell survival. Experiments are underway to test this possibility. Experiments in progress show that STAT3 co-precipitates with PI3 kinase following VEGF stimulation, suggesting a role for STAT3 in coupling PI3 kinase to the VEGF signaling pathway.

VEGF and STAT5—When we analyzed STAT5 tyrosine phosphorylation, we found that unlike STAT1, -3, and -6, STAT5 was not tyrosine-phosphorylated or nuclear translocated in response to VEGF stimulation.

VEGF Induces the Association of STAT1 with VEGFR2—As has been accentuated by extensive experimental evidence, VEGFR1 and -2 possess distinct biological activities. To identify which receptor is involved in STAT activation, we next examined the activation pattern of the different STATs in BAEC treated with 25 ng/ml PIGF. PIGF at this concentration is able to bind exclusively to the VEGF receptor, VEGFR1. Under our experimental conditions, PIGF failed to activate the phosphorylation of STAT1, STAT3, or STAT6, suggesting that activation of the STAT pathway is specifically mediated by VEGFR2.

The STAT signaling pathway is classically known as the JAK/STAT pathway because STAT activation is often associated with and dependent upon the tyrosine phosphorylation of nonreceptor tyrosine kinases belonging to the Janus kinase (JAK) family. JAK1, JAK2, Tyk2, and JAK3 have been shown to phosphorylate STAT proteins (38). Our results show that BAEC only express JAK1 and Tyk2 proteins and that these kinases are not tyrosine-phosphorylated in response to VEGF. Recent reports have shown that Src kinases and the intrinsic tyrosine kinase activity of growth factor receptors can also phosphorylate the tyrosine residues of the various STAT proteins (39, 40). Because VEGFRs possess intrinsic tyrosine kinase activity, we conducted immunoprecipitation experiments to see whether STAT1 also interacts with VEGFR2. We focused our attention on STAT1 because its phosphorylation time course corresponded closely with that of VEGFR2. To assay for VEGFR2 mediation of STAT1 activation, we performed co-immunoprecipitation studies using precipitating antibodies directed against VEGFR2 and immunoblotting the precipitated proteins with anti-STAT1 antibodies. As shown in Fig. 5A, after 15 min of VEGF treatment, STAT1 co-precipitated with VEGFR2. These results suggest that, upon interaction with its ligand, VEGFR2 associates with STAT1. Furthermore, pretreatment of BAEC with the tyrosine kinase inhibitor genistein inhibited STAT1 association with VEGFR2 (Fig. 5B), suggesting that VEGFR2-STAT1 complex formation requires tyrosine phosphorylation. These results, along with our observations that JAK1 and Tyk2 are not activated in response to VEGF stimulation of BAEC, suggest that the intrinsic tyrosine kinase activity of VEGFR2 might be involved in STAT1 tyrosine phosphorylation. Given the well established role of VEGFR2 in signaling the effects of VEGF in promoting endothelial cell growth and survival, these data support a role for STAT1 activation in these processes.

A recent report provides additional evidence that the activity of endothelial cell-specific tyrosine kinase receptors is associated with the STAT pathway (41). The authors found that over-expression of VEGFR1, -2, and -3, as well as Tie-1 and Tie-2 in 293T cells, results in the activation of co-expressed STAT5 and STAT3 but not STAT1. STAT1 activation was seen only for a mutant form of the Tie-2 receptor (MTie-2), which has been associated with venous malformation. Similar results

were obtained analyzing STAT transcriptional activity in VEGF-stimulated human umbilical vein endothelial cells. In contrast, our results indicate that STAT1 and STAT6 are activated in response to VEGF in primary cultures of aortic endothelial cells. It is likely that the different experimental approaches and different cell types involve different regulatory mechanisms associated with the VEGF signaling pathway.

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