Induction of Hypoxia-inducible Factor 1α Gene Expression by Vascular Endothelial Growth Factor*

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Transcriptional regulation of vascular endothelial growth factor (VEGF) is critically dependent on hypoxia-inducible factor 1 (HIF-1). However, not only hypoxia, but selected growth factors can induce HIF-1. High levels of both VEGF and HIF-1 coexist in certain conditions, e.g. tumors. Nonetheless, the possibility that the stimulatory relationship between HIF-1 and VEGF may be bi-directional has not been addressed up to date. The present study in endothelial cells analyzed whether HIF-1 is regulated by a product of its own transcriptionally activated genes, namely, VEGF. As a main finding, VEGF-A165 induced the increase of HIF-1α mRNA and HIF-1α protein and nuclear translocation. Autologous endothelial cell VEGF mRNA and protein were also increased upon exposure to exogenous VEGF.

The signaling implication of reactive oxygen species was examined by comparison with H2O2 and hypoxanthine/xanthine oxidase and by the superoxide dismutase mimetic, MnTMPyP, the Rac1-NAD(P)H oxidase complex inhibitor, apocynin, transfection of a dominant negative Rac1 mutant, and transfection of a p67phox antisense oligonucleotide. Superoxide anion, largely composed by two subunits (HIF-1α and HIF-1β) with apparent molecular masses of 120–130 and 91–94 kDa, respectively (6). The mRNA of both subunits is expressed constitutively, albeit in variable amounts. However, HIF-1α protein and HIF-1 activity are only detectable in cells exposed to hypoxia (7).

Even though VEGF expression is regulated by a number of stimuli, hypoxia is the principal trigger to increase VEGF gene transcription. This increment is mainly mediated by a specialized transcription factor, HIF-1 (5). HIF-1 is a heterodimer composed by two subunits (HIF-1α and HIF-1β) with apparent molecular masses of 120–130 and 91–94 kDa, respectively (6). The mRNA of both subunits is expressed constitutively, albeit in variable amounts. However, HIF-1α protein and HIF-1 activity are only detectable in cells exposed to hypoxia (7).

Recently, several growth factors and cytokines have also been reported to induce HIF-1α protein synthesis and HIF-1 activity in normoxia (8–14). The major signaling pathways identified as contributing to the HIF-1α induction by these growth factors include phosphatidylinositol 3-kinase/Akt (protein kinase B) and Ras/MAPK/phosphatidylinositol 3-kinase (MEK) (10, 11, 14–16). In these circumstances the transactivated HIF-1 leads to increased VEGF synthesis and/or protein stabilization, therefore amplifying the angiogenic signal. The limits and potential importance of this type of HIF-1 activation have not yet been accurately appraised.

Among their numerous effects, ROS participate in activation pathways for redox-sensitive transcription factors mainly through protein tyrosine phosphorylation (17–19). Among these activations, that of HIF-1 is singularly relevant. In addition, the interaction of certain growth factors with their tyrosine kinase receptors leads to ROS production. ROS act as ubiqui-

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2 The abbreviations used are: VEGF, vascular endothelial growth factor; VEGF/VEGF receptor; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; JNK, c-Jun NH2-terminal kinase; CHX, cycloheximide; HIF-1, hypoxia-inducible factor 1; EC, endothelial cell(s); PKG, placental growth factor; ESA, erythropoietic-stimulating agents; HX, hypoxanthine; XO, xanthine oxidase; PBS, phosphate-buffered saline; mAb, monoclonal antibody; L-NAME, Nω-nitro-L-arginine methyl ester.
uitous intracellular messengers, e.g. O$_2^-$ and H$_2$O$_2$ in mitogenic and cytoprotective responses (20–23). In terms of mechanism, the main pathway of receptor tyrosine kinase-mediated ROS production involves the Rac1-NAD(P)H oxidase-like membrane complex (24–27). Colavitti et al. (28) have demonstrated the effect of VEGF-induced ROS production as a downstream angiogenic signal, which is conveyed through the VEGFR2 (29).

Taking into account the aforementioned role of Rac1-NAD(P)H oxidase complex as a main source of ROS in vascular tissue (30) as well as the capability of VEGF for signaling through ROS and the described effect of ROS in HIF-1 activation, the hypothesis was raised that the products of HIF-1-activated genes, e.g. VEGF or erythropoietin, may act on the expression of HIF-1 itself. A series of experiments has been performed to examine this hypothesis and to clarify the signaling mechanisms involved. The potential importance of the pathway we examined herein can be traced to an ample setting of non-hypoxic inflammatory conditions and to neoplastic cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Bovine aorta EC were obtained, cultured up to confluence, and characterized as previously described (31). These cells, named simply as EC throughout the manuscript, were used between the second and fifth passages. Under these conditions, EC response has been tested by growth rate assays in the presence of recombinant human VEGF-A$_{165}$ (hereafter designed as VEGF) (31, 32).

**Experimental Maneuvers**—After 24 h of depletion in minimum essential medium/d-Val without calf serum, cells were incubated for different times with VEGF (R&D Systems, Minneapolis, MN), darbepoetin α (Aranesp$^{	ext{NB}}$), a gift of Amgen SA (Barcelona, Spain), or erythropoietin α (Eprex$^{	ext{NB}}$), a gift of Janssen-Cilag (Madrid, Spain). When indicated, EC were pretreated with specific drugs or their vehicles for 30 min. Drugs used were the NAD(P)H oxidase inhibitor, apocynin (1 mM), the cell-permeable superoxide dismutase mimetic, MnTMPyP (25 μM), the p38 MAPK inhibitor, SB203580 (20 μM) (Calbiochem), the JNK inhibitor, dicumarol (20 μM), the p42/p44 MAPK inhibitor, PD98059 (100 μM), and the phosphatidylinositol 3-kinase inhibitor, wortmannin (400 nM) (Sigma-Aldrich). The nitric-oxide (NO) synthase inhibitor L-NAME (400 nM, Sigma-Aldrich) was used to study NO and peroxynitrite (ONOO$^-$) implication. Protein synthesis was inhibited by cycloheximide (CHX, 10 μg/ml), CoCl$_2$ was used as an hypoxic equivalent, and HX (2 mM)/XO were used as donors of O$_2^-$ (Sigma-Aldrich). The efficacy of H$_2$O$_2$ exposure in this system has been previously checked by electrophoretic mobility shift assay of NFκB.

**Protein Extraction**—After the different treatments, the EC were washed and scrapped in 1 ml of cold PBS. Cytoplasmic and nuclear proteins for Western blots were extracted using the method described by Schreiber et al. (33) and quantified by Coomasie Plus Protein Assay Reagent method (Pierce). Extraction buffers contained 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 2.5 μg/ml aprotonin, peptatin A, and leupeptin.

**Western Blot Analysis**—Proteins (30 μg) were electrophoretically separated by SDS-PAGE and transferred to nitrocellulose (Trans-Blot Transfer Medium, Bio-Rad). Loading and transfers were verified by staining with Ponceau S Solution (Sigma-Aldrich). Blocked membranes were incubated with blocking buffer containing mouse mAb anti-HIF-1α (1:250, 4 °C, overnight, BD Transduction Laboratories) or mouse mAb anti-α-tubulin (1:2000, 4 °C, overnight, Sigma-Aldrich) then with blocking buffer containing goat mAb anti-mouse HRP-conjugated (1:6000, Bio-Rad) and revealed using the ECL system and exposed to Hyperfilm ECL (Amersham Biosciences). Quantifying of ECL images was done using ImageJ software (National Institutes of Health, Bethesda, MD).

**Plasmids and Transfections**—For expression vectors a dominant negative mutant of Rac1 (pcDNA3-Rac1N17) was transferred and overexpressed to inhibit superoxide production dependent from Rac1-NAD(P)H oxidase. This vector was kindly provided by Dr. Alberto Muñoz (Instituto de Investigaciones Biomédicas de Madrid). A pcDNA3-empty vector was used as the control.

**Antisense Oligonucleotide for p67phox**—Antisense or sense oligonucleotides for the Rac1-NAD(P)H oxidase complex protein, p67phox (antisense oligonucleotide, 5'-CGGCGAGGA-CATTGTCAGG-3'; sense oligonucleotide, 5'-CCTGACCAT-GTCCCCTGGCCG-3' (Metabion, Martinsried, Germany)) were added to primary bovine aorta EC at a 2 μM concentration and incubated for 72 h before treatment with 40 ng/ml VEGF for 6 h. Sense oligonucleotide was used as control.

**Detection of O$_2^-$ Production by Dihydroethidium**—After 24 h of serum deprivation, confluent EC cultured in poly-L-lysine-coated coverslides (BD Biosciences) were treated with 40 ng/ml VEGF or vehicle for 1 h and thereafter incubated with 10 μM dihydroethidium (Calbiochem). After that, EC were washed twice with cold PBS and fixed with Merckofix (Merck). To estimate the amount of O$_2^-$ production, confluent EC with the same treatment as just described were incubated in the presence of the O$_2^-$-generating system, HX/XO. Fluorescence of the oxidized form of dihydroethidium, i.e. ethidium, was measured using an Infinite F200 fluorometry system (Tecan Ibérica, Barcelona, Spain). Images were obtained using a fluorescence microscope (Nikon, Madrid, Spain). Quantifying of images was done using ImageJ software (NIH, Bethesda, MD).

**Reporter Plasmids**—The reporter construct VEGF-luc was kindly provided by Dr. Gregg Semenza (John Hopkins Hospital). This construct contains the human VEGF sequence from −2274 to 379 relative to the transcription initiation site (5) ligated into the KpnI-MluI sites of pGL2-Basic (Promega Biotech Ibérica, Madrid, Spain), a firefly luciferase reporter vector. Cells were co-transfected with each reporter construct (VEGF-luc and pGL2-Basic) and the renilla luciferase vector pRL-CMV (Promega Biotech Ibérica) stimulated with or without VEGF (40 ng/ml) or XO (2.5 millinits/ml) for 12 h and then treated with active lysis buffer according to the Dual-Luciferase assay. The results were expressed as relative luciferase activity.

**Transient Expression Assays**—Transient expression of EC was carried out using CaCl$_2$ method. Briefly, the EC were grown to near confluence (80–90%). The optimal transfection was achieved at a CaCl$_2$ to DNA ratio of 6.5 mM to 10 μg/ml, respectively. For the reporter assays the DNA quantities were 0.1 and 10 μg/ml for pRL cyanomagalovirus and VEGF-luc/pGL2-Basic, respectively. The CaCl$_2$-DNA complex was performed in HBS
buffer (21 mM HEPES, 0.14 mM NaCl, 5.5 mM D(+)-glucose, 50 mM KCl, 1.32 mM Na₂HPO₄ at pH 7.1). The transfection mix remained during an additional time of 6 h; hence, cells were washed with PBS × 2, and growth medium was added until confluence (24–48 h). Cell stimulations were performed as described. To evaluate transfection efficiency, all assays were also transfected using pEGFP-N1 vector (BD Biosciences). Transfection efficiency was more than 50% in all cases.

**RNA Isolation and Quantitative Reverse Transcription-PCR**—RNA was extracted using the Tripure Isolation Reagent (Roche Diagnostics) according to the manufacturer’s instructions. Total RNA was extracted at 6, 12, and 24 h after treatments based on previous studies in our laboratory. One µg of total RNA from each sample was retrotranscribed to cDNA (Improm-II reverse transcriptase; Promega). 1–3 µl of cDNA samples were used as the template for amplification reactions carried out with the LC Fast Start DNA master SYBR Green I kit (Roche) following the manufacturer’s instructions. PCR amplifications were carried out in a Light Cycler System (Roche Diagnostics), and data were analyzed with LightCycler software 3 Version 3.5.28 (Idaho Technology Inc., Salt Lake City, UT). For analysis purposes, the ampiclon for each of the analyzed genes was cloned, and known amounts of the cloned product were used to generate a standard curve. The number of copies of the gene of interest in each sample was extrapolated from the corresponding standard curve by the indicated software. For each sample, duplicate determinations were made, and the gene copy number was normalized by the amount of β-actin. The primer pairs used in this study are reported elsewhere (35).

**Immunofluorescence Assay**—EC were grown and treated on Falcon Culture Slides (BD Biosciences) and were washed ×5 with PBS and fixed with Merckofix (Merck). Fixed cells were permeabilized with 0.2% (v/v) Triton X-100 in PBS (15 min), washed, and incubated in blocking buffer for 1 h (PBS with 1% bovine serum albumin). Slides were incubated overnight with mouse mAb anti-VEGF primary antibody (1:500). Slides were then washed and incubated with goat mAb anti-mouse coupled to FITC (1:2000) for 2 h (both, Sigma-Aldrich). Propidium iodide (Sigma-Aldrich) was added (45 min, 50 ng/ml) for visualization of the cell nucleus. Images were obtained using a Confocal System TCS SP20 (Leica, Madrid, Spain). Quantifying of images was done using ImageJ software (National Institutes of Health).

**Statistics**—Results are expressed as the mean ± S.D. Unless stated otherwise, values correspond to a minimum of five experiments done by triplicate. When applicable, Student’s t test or analysis of variance were used to compare variables. Fisher and Scheffe tests for multiple comparisons were used to determine the p value, which was considered significant at < 0.05. The statistic analyses were performed with the Windows SPSS 10.0 package (SPSS Inc. Chicago, IL).

**RESULTS**

**VEGF Increases Nuclear HIF-1α Protein in a Time- and Concentration-dependent Manner**—EC were incubated under different experimental conditions, and cytoplasmic and nuclear extracts were loaded according to protein quantization. As can be seen in Fig. 1A, only trace levels of HIF-1α could be detected in the nuclear extract of untreated EC. VEGF (40 ng/ml) treatment increased nuclear HIF-1α protein in a time-dependent manner (Fig. 1A), reaching the maximum level after 6 h. At all the time points assessed, HIF-1α protein was undetectable in the cytoplasmic extract. Nuclear HIF-1α increased in the presence of VEGF in a concentration–dependent manner (Fig. 1B). An additional experiment was performed to ascertain whether a selective ligand of VEGFRI, i.e. placental growth factor (PIGF), was able of inducing an increase of HIF-1α. As can be observed in Fig. 1C, PIGF increased nuclear HIF-1α protein. Of importance, the concentration-response curve of PIGF was rightward shifted with respect to that of VEGF (p < 0.01). Because VEGF binds to both types of receptors, whereas PIGF binds only to VEGFRI, the difference between the concentration-response curves of both agents suggests that the two types of VEGF receptor are involved in the response.

To test whether the effects observed with VEGF were shared by the product of another HIF-1 transcriptionally activated...
gene, similar experiments were performed using erythropoietic-stimulating agents (ESAs), namely, darbepoetin and erythropoietin (both at final concentrations equivalent to 50, 100, and 200 units/ml of erythropoietin). These experiments revealed no effects of the ESAs on HIF-1α protein levels (Fig. 1D).

Superoxide and Rac1 Are Necessary for VEGF-induced HIF-1α Protein Increase—At a first glance and based on the known regulation of HIF-1α protein, none of the more common VEGF-induced signaling pathways appeared as a probable regulator of HIF-1α increase. However, two facts were already known. First, according to the literature (36) and our own data, VEGF is capable of inducing ROS production and ROS-dependent NFκB activation; second, HIF-1α synthesis can putatively augment by using a ROS-related signaling pathway (12, 37, 38). Accordingly, our analysis was focused on the investigation of whether ROS were involved in the increased VEGF-induced HIF-1α expression. With this purpose we first examined the effect of both exogenously added H₂O₂ and O₂⁻ for 6 h. As shown in Fig. 2A, no effect on HIF-1α protein level was observed within the range of 2–250 μM H₂O₂. On the contrary, a clear-cut effect of O₂⁻ was found with the HX/XO system. In these conditions, HIF-1α protein levels were directly proportional to O₂⁻ production. Of note, because a saturating HX concentration (2 mM) was used, O₂⁻ generation by the HX/XO system was dependent on XO concentration. Based on this background, we further investigated the participation of O₂⁻ in VEGF-induced HIF-1α increase. The main source of O₂⁻ in EC in conditions of normal aerobic metabolism are the oxidases of the Rac1-NAD(P)H complex (36). Therefore, EC were incubated with VEGF or HX/XO in the presence or in the absence of the non-scavenging, specific Rac1-NAD(P)H oxidase complex inhibitor, apocynin (1 mM), and the cell-permeable superoxide dismutase mimetic, MnTMPyP (25 μM). As shown in Fig. 2B, apocynin completely blocked VEGF- but not HX/XO-induced HIF-1α increase, whereas MnTMPyP completely blocked both stimuli. Additionally, we determined whether NO or ONOO⁻ formed from NO + O₂⁻ were implicated in the VEGF- and HX/XO-induced HIF-1α protein increase. With this purpose, EC were also incubated with the nitric-oxide synthase inhibitor, L-NAME (400 nM). No effect of L-NAME was detected in VEGF-treated EC, but a small one could be observed when treated with HX/XO (Fig. 2B).

The effects of apocynin gave us an important clue about the participation of Rac1-NAD(P)H. Therefore, to provide a specific confirmation of its role in VEGF-increased HIF-1α protein level, EC were transfected with a dominant negative mutant of Rac1. As shown in Fig. 2C, expression of a negative form of Rac1 is enough to abolish the VEGF effect on HIF-1α increase. With the purpose of ruling out the remote, albeit real, possibility that the effect of Rac1 inhibition might have been independent of NAD(P)H, EC were transfected with a p67phox antisense oligonucleotide. This antisense oligonucleotide also blocked the VEGF effect (Fig. 2, D and E). Collectively, this set of results supports that the Rac1-NAD(P)H oxidase complex is involved and necessary in VEGF signaling on HIF-1α and that O₂⁻ acts as a second messenger of VEGF in the induction of HIF-1α protein increase. This effect appears to be more probably related to a direct action of O₂⁻ itself rather than by means of ONOO⁻ formation.

VEGFs Increase O₂⁻ Production in EC—To confirm O₂⁻ production by VEGF, we labeled EC with the cell-permeable molecule, dihydrolipoamide. Two different experiments were carried out with this method, both based on the capability of dihydrolipoamide to react specifically with O₂⁻, producing ethidium, which has different fluorescent properties than dihy-

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3C). On the first experiment, cells were fixed after VEGF exposure; on the second experiment, fluorescent detection was carried out in real time, whereas EC were stimulated alive on culture wells. In both cases, VEGF exposure (40 ng/ml) increased ethidium fluorescence, and the cell-permeable superoxide dismutase mimetic MnTMPyP (25 μM) abolished this effect. In setting-up studies, we ruled out the existence of a fluorescence quenching by MnTMPyP.

**VEGF and ROS-induced HIF-1α Protein Increase Requires JNK, p38, and Phosphatidylinositol 3-kinase/Akt Activity**—A number of proteins could be theoretically involved in VEGF signaling on HIF-1α. To test the participation of the main pathways possibly implicated in the process, we tested the role of phosphatidylinositol 3-kinase/Akt, JNK, and MAPKs. For this purpose, EC were pretreated with specific inhibitors of phosphatidylinositol 3-kinase/Akt, JNK, and MAPKs, namely, p38 (SB203580) and p42/44 (PD98059), and then stimulated with VEGF or HX/XO. As shown in Fig. 4A, blockade of all the kinases, except p42/p44, markedly inhibited the effects of both VEGF and HX/XO signaling on HIF-1α increase. Based on these screening findings, we analyzed VEGF capability to activate JNK, p38, and Akt by assessing the status of their phosphorylated forms after VEGF exposure. As shown in Fig. 4B, VEGF increased JNK and p38 phosphorylation after 30 min of stimulation, with an apparent peak at about 60 min. Akt phosphorylation was already detected in the earliest sample (1 min), but no increase was evident under the effect of VEGF (Fig. 4B).

**VEGF Does Not Affect HIF-1α Protein Degradation but Increases HIF-1α mRNA Levels Using O2−**—The findings mentioned above prompted us to study the mechanism of VEGF-related HIF-1α protein increase. HIF-1α half-life was assessed by comparing HIF-1α signal as induced by either CoCl₂ or VEGF in the presence of the protein synthesis inhibitor, an Intracellular Mediator—the findings mentioned above prompted us to study the mechanism of VEGF-related HIF-1α protein increase. HIF-1α half-life was assessed by comparing HIF-1α signal as induced by either CoCl₂ or VEGF in the presence of the protein synthesis inhibitor,

droethidium. Therefore, ethidium formation depends on O₂⁻ levels. On the first experiment, we detected ethidium formation, i.e. O₂⁻ production, by means of fluorescent microscopy (Fig. 3, A and B). On the second experiment, we analyzed ethidium/O₂⁻ production by using a fluorescence detector (Fig.

![FIGURE 3. A, fluorescence microscopy images of dihydroethidium-labeled EC. The red color corresponds to ethidium fluorescence as results from dihydroethidium oxidation by superoxide anion. EC were stimulated with VEGF or XO in the presence or absence of specific inhibitors during 1 h. 1, non-stimulated cells; 2, 2.5 milliunits/ml XO treatment; 3, 40 ng/ml VEGF treatment; 4, 25 μM MnTMPyP treatment; 5, XO treatment in presence of MnTMPyP; 6, VEGF treatment in presence of MnTMPyP. B, relative quantification of fluorescence microscopy images shown in A. 1, non-stimulated cells; 2, 2.5 milliunits/ml XO treatment; 3, 40 ng/ml VEGF treatment; 4, 25 μM MnTMPyP treatment; 5, XO treatment in presence of MnTMPyP; 6, VEGF treatment in presence of MnTMPyP. C, relative fluorescent quantification of dihydroethidium oxidation to ethidium. EC labeled with dihydroethidium were stimulated for 1 h with vehicle (basal) or 40 ng/ml VEGF in the presence or in the absence of the superoxide dismutase mimetic, MnTMPyP (25 μM). Measurements were obtained at different times. **, p < 0.001 of the curve with respect to the vehicle; *, p < 0.05 with respect to the vehicle.
**VEGF Induction of HIF-1α through Superoxide**

**A**

| VEGF | XO |
|------|----|
| Controls | + |
| Dicumarol | + |
| SB203580 | + |
| Wortmannin | - |
| PD98059 | - |

**B**

| VEGF 40 ng/ml |
|--------------|
| Time (Min)   |
| 1 | 5 | 15 | 30 | 60 | 180 |
| p-JNK | 54 | 46 |
| JNK   | 54 | 46 |
| p-p38 |                |
| p38   |                |
| p-Akt |                |
| Akt   |                |

**Figure 4.** A, inhibitory effect on nuclear HIF-1α protein levels by dimercurial (20 μM), SB203580 (20 μM), wortmannin (400 nM), and PD98059 (100 μM). A HIF-1α Western blot from EC nuclear extract is shown. EC were incubated with the inhibitors 30 min before the addition of VEGF (40 ng/ml) or XO (2.5 milliunits/ml) and maintained for the 6 h of the experiment. B, phosphorylated (p)-JNK, p-p38, and p-Akt Western blot from EC total extract. EC were stimulated with 40 ng/ml VEGF for 1, 5, 15, 30, 60, and 180 min. Non-phosphorylated forms of JNK, p38, and Akt were used as Western blot loading control. Images of ECL correspond to the same experiment; therefore, time of exposure is the same. The results shown correspond to one representative set of experiments of three yielding similar results.

CHX. CoCl2 was used as a hypoxia-mimetics, acting as a positive control of inhibited HIF-1α degradation (39). In EC treated with CoCl2 (50 μM) for 6 h, HIF-1α levels remained more stable 60 min after protein synthesis blockade with CHX. In contrast, after VEGF treatment (40 ng/ml, 6 h), HIF-1α protein levels were rapidly decreased to base line in less than 30 min (Fig. 5A).

**Figure 5.** HIF-1α protein half-life and mRNA transcription analysis. A, HIF-1α protein is rapidly degraded after CHX treatment in VEGF-stimulated but not in CoCl2-stimulated EC. A Western blot of HIF-1α from EC nuclear extract is shown. EC were stimulated with VEGF (40 ng/ml) or CoCl2 (50 μM) for 6 h. At 6 h CHX (10 μg/ml) was added for the indicated times without removing the previous stimulus. The experiment shown is one of three yielding similar results. The density values of the protein bands were normalized to the time 0 signal and expressed as percentage. B, HIF-1α mRNA is increased after VEGF or XO treatment but not with CoCl2. EC were stimulated with VEGF (40 ng/ml), XO (2.5 milliunits/ml), or CoCl2 (100 μM) for 6, 12, and 24 h. The HIF-1α mRNA transcription level was analyzed by quantitative reverse transcription-PCR. *, *p < 0.05; **p < 0.01. C, VEGF and XO-related HIF-1α mRNA increase was markedly reduced in presence of 1 mM apocynin or 25 μM MnTMPyP (12 h). *, *p < 0.05, between VEGF and XO treatment alone or in the presence of apocynin or MnTMPyP.

Further experiments were conducted to analyze the mechanism of HIF-1α increase by VEGF. Accordingly, the HIF-1α mRNA production strongly increased after VEGF or HX/XO stimulus (for 6, 12, and 24 h); on the contrary, no changes were observed with CoCl2 (Fig. 5B). The next studies, done by challenging EC with VEGF or HX/XO for 12 h, showed that a VEGF and HX/XO-related HIF-1α mRNA increase was markedy reduced in the presence of apocynin (1 mM) or MnTMPyP (25 μM) (Fig. 5C).

**VEGF Promoter Activity, mRNA, and Protein Levels Are Increased after VEGF Treatment**—With the aim of analyzing the transducational functionality of the VEGF-HIF-1α pathway, we investigated whether exogenous VEGF and its putative
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The main outcome of the present study is the detection of a strong stimulation by VEGF on its leading transcriptional factor, HIF-1α, and also on its own expression. Even though effects of other growth factors on HIF-1 activation have been previously recognized, to the best of our knowledge the present is the first evidence showing this type of effect with VEGF. A cardinal feature of the present investigation is that VEGF exerts its stimulating action on HIF-1α independent of hypoxia; furthermore, our results link the VEGF effect on HIF-1α to O2·-. Both pieces of information reveal the existence of a positive feedback of VEGF on HIF-1, which can putatively operate in inflammatory and high VEGF production conditions, namely, malignant tumors. The effects of VEGF appear to be conveyed through both VEGF1 and VEGF2 receptors, as judged by comparing the effects of PI GF and VEGF.

Specifically aimed experiments provided complementary information on the mechanisms by which VEGF induces HIF-1α activation. The effect of VEGF on HIF-1α did not imply a significant inhibition of HIF-1α degradation but an increase in its synthesis. This increment is timely related to an increased HIF-1α mRNA, therefore tracing this effect to up-regulation of HIF-1α gene expression. As aforementioned, the mechanisms found herein have similarities to those described for the increase of HIF-1α induced by other growth factors; however, our results add new information, disclosing increased HIF-1α gene expression upon VEGF challenge. Collectively, this group of findings provides further support to the existence of substantial differences in HIF-1 activation pathways by either growth factors or hypoxia (11, 12, 14, 16, 40).

In this regard, because Rac1 may be putatively involved in intracellular mediator, O2·-, are able of increasing VEGF levels in EC. As shown in Fig. 6A, exogenous VEGF (40 ng/ml) induced VEGF promoter activity. This induction was eliminated or considerably decreased by using the non-scavenging, specific Rac1-NAD(P)H oxidase complex inhibitor, apocynin (1 mm) and the cell-permeable superoxide dismutase mimetic, MnTMPyP (25 μM). In the same way, both VEGF and XO stimulus increased VEGF mRNA expression in quantitative reverse transcription-PCR assays (Fig. 6B). This expression reached its maximum after 12 h of VEGF treatment and was blocked in the presence of apocynin (1 mm) or MnTMPyP (25 μM) (Fig. 6C). In agreement with this finding, VEGF protein was assessed by confocal microscopy. A clear-cut decrease was detected after 24 h of VEGF treatment (40 ng/ml); this increase was significantly reduced in the presence of apocynin (1 mm) and MnTMPyP (25 μM) (Fig. 7). It should be noted that the marked reduction of VEGF green signal in cells having exogenous VEGF added in the presence of apocynin and MnTMPyP (subsets 7 and 8 of Fig. 7A) practically rules out the possibility that the fluorescent VEGF signal observed in other subsets, e.g. 4, was due to exogenous VEGF.

**DISCUSSION**

The study contains additional new elements. First, the fact that autologous VEGF mRNA and VEGF protein are induced after EC exposure to exogenous VEGF supports the observation that the increase in HIF-1α is functionally meaningful in terms of transactivation of the VEGF gene. However, the possibility exists, albeit with low probability, of HIF-1α-independent effects. This hypothetic point is beyond the scope of the present investigation, and additional studies will be needed.

Specifically aimed experiments provided complementary information on the mechanisms by which VEGF induces HIF-1α activation. The effect of VEGF on HIF-1α did not imply a significant inhibition of HIF-1α degradation but an increase in its synthesis. This increment is timely related to an increased HIF-1α mRNA, therefore tracing this effect to up-regulation of HIF-1α gene expression. As aforementioned, the mechanisms found herein have similarities to those described for the increase of HIF-1α induced by other growth factors; however, our results add new information, disclosing increased HIF-1α gene expression upon VEGF challenge. Collectively, this group of findings provides further support to the existence of substantial differences in HIF-1 activation pathways by either growth factors or hypoxia (11, 12, 14, 16, 40).

The circumstance that O2·- has a critical role as a second messenger, leading to HIF-1α increase by VEGF, opens a new source of interpretation about VEGF signaling process. Stimulation of superoxide production by VEGF has been previously shown by other groups (26, 41–43). However, the consequences of this stimulation in the present experimental setting appear to be rather specific. In fact, the effects of superoxide on HIF-1α are not shared by H2O2. Furthermore, inhibition by the non-scavenging Rac1-NAD(P)H oxidase complex inhibitor, apocynin, supports that Rac1-NAD(P)H oxidase complex is the main source of O2·- in this signaling pathway. Of practical interest, apocynin is increasingly used in experimental therapeutics (44, 45), therefore leaving open the possibility of a relatively simple practical application of the present findings. Further assessment of the meaning of the apocynin results was obtained by means of experiments with transfection of the Rac1 negative form. In this regard, because Rac1 may be putatively involved in

**FIGURE 6. VEGF promoter activity; VEGF mRNA studies.** A, VEGF promoter activity is increased after VEGF exposure, as assessed by luciferase assay using the reporter construct VEGF-luc. After co-transfection with VEGF-luc and the renilla luciferase vector, EC were stimulated with VEGF (40 ng/ml). Promoter activity was markedly reduced in the presence of apocynin (1 mM) or MnTMPyP (25 μM). *, p < 0.05; **, p < 0.01 between VEGF treatment alone or in the presence of apocynin or MnTMPyP. B, VEGF mRNA is increased after VEGF or XO exposure. EC were stimulated with VEGF (40 ng/ml) or XO (2.5 millimicroits/ml) for 6, 12, or 24 h. VEGF mRNA was analyzed by quantitative reverse transcription-PCR; *, p < 0.05. C, VEGF-related and XO-related VEGF mRNA increase was markedly reduced in the presence of 1 mM apocynin or 25 μM MnTMPyP (12 h). *, p < 0.05 between VEGF and XO alone or in the presence of apocynin or MnTMPyP.
other cellular pathways (46), a specifically aimed experiment was performed which demonstrated by similarity of effects by a transfected p67phox antisense oligonucleotide that the effects obtained by Rac1 blockade can be attributed to inhibition of the NAD(P)H oxidase complex. The increase of HIF-1α by superoxide is not a new finding as far hypoxia is concerned; however, the role of superoxide in VEGF-related induction of HIF-1α is hereby communicated for the first time.

The absence of effect of a different HIF-1-dependent growth factor, namely, erythropoietin/ESAs, on HIF-1α suggests that at least in the present conditions, the feedback on HIF-1α described for VEGF is not a generalized mechanism concerning other factors containing hypoxia-responsive elements in their promoter, e.g. erythropoietin. The lack of effect of ESAs on HIF-1α increase is of considerable practical importance due to their widespread therapeutic use. This may be particularly remarkable in terms of the potential actions of ESAs in angiogenic and cytoprotective processes (48). In fact, our results do not support a view of ESAs as amplifying agents acting on the HIF-1 response. The differences between the effects of VEGF and ESAs on HIF-1 can largely be traced to their different intracellular signaling mechanisms (2, 49), but more in depth experiments focused on signaling may be useful to attain additional insight.

In a broader sense, the existence of a communication between Rac1-NAD(P)H oxidase complex and VEGF receptors is implicit in the results. An effort to define the precise characteristics of this interaction as well as the precise microdomain characteristics of the superoxide signal is being done by leading groups in the field (for a state-of-the-art review, see Refs. 26 and 29). Even though our data indicate that the main signaling molecule of the mechanism studied hereby is O$_2^-$, a role of NO or ONOO$^-$, formed by the simultaneous increase of NO (47) and O$_2^-$ in VEGF-targeted cells, could not be ruled out a priori. The experiments using L-NAME were performed with this possibility in mind, and their negative outcome suggests that the interaction between NO and O$_2^-$ on the

### Figure 7

**Detection of autologous VEGF by immunofluorescence.** A, EC were stimulated with VEGF with or without specific inhibitors during 24 h. Then, after extensive washing ($\times$5) slides were subjected to immunofluorescence assay with specific antibodies. The images were obtained by confocal microscopy (400x). Cell nuclei are marked in red with propidium iodide, and VEGF is marked in green by a secondary antibody coupled to FITC. 1, immunofluorescence control, without primary antibody. 2, immunofluorescence control, with nonspecific primary antibody. 3, non-stimulated cells. 4, apocynin treatment (1 mM). 5, MnTMPyP treatment (25 μM). 6, VEGF treatment (40 ng/ml). 7, apocynin (1 mM) + VEGF (40 ng/ml). 8, MnTMPyP (25 μM) + VEGF (40 ng/ml). Results correspond to one representative set of experiments of five yielding similar results. B, relative quantification of green fluorescence signal corresponding to autologous VEGF of microscopy images showed in A. To achieve these values we used ImageJ software (National Institutes of Health). Each image was split in its three colors, and the green channel was quantified after selection of cells perimeter. The green signal, corresponding to VEGF protein immunodetection of each image, has been normalized with respect to non-stimulated cells signal. 1, immunofluorescence control, with nonspecific primary antibody. 2, immunofluorescence control, without primary antibody. 3, non-stimulated cells. 4, apocynin treatment (1 mM). 5, MnTMPyP treatment (25 μM). 6, VEGF treatment (40 ng/ml). 7, apocynin (1 mM) + VEGF (40 ng/ml). 8, MnTMPyP (25 μM) + VEGF (40 ng/ml). Each relative value has been normalized with respect to non-stimulated cells value.

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HIF-1α increase by VEGF is not a relevant one. The studies using dihydroethidium have demonstrated in real time that VEGF induces O₂⁻ production.

A number of signal transduction pathways acting between VEGF receptor stimulation and the increase in HIF-1α protein were examined. The results obtained herein with a set of inhibitors have a screening value and suggest that phosphatidylinositol 3-kinase/Akt, JNK, and p38 MAPK are involved in the VEGF and HX/XO signal, whereas p42/44 is not. This pattern of activation is coincident with others already described in the literature (40). Collectively, the aforementioned results suggest that phosphatidylinositol 3-kinase/Akt, JNK, and p38 MAPK are targeted for activation in the context of VEGF-induced O₂⁻ production and are, therefore, most probably implicated in the increased HIF-1α protein synthesis. The fact that Akt phosphorylation, a previously described effect of VEGF (1), was not detected was rather discordant with the inhibition of VEGF-induced increase of HIF-1α by wortmannin. This result can probably be traced to the existence in the experimental conditions used of Akt phosphorylation at the base line, as can be seen in Fig. 4B (bottom rows). This may diminish the sensitivity of the method to detect an additional increase in phosphorylation by the action of VEGF. Another alternative may be that Akt phosphorylation actually occurred before the first, 1-min sampling. Nonetheless, the actual step order of this signaling sequence cannot be determined from the present results, and further investigation is required to complete this mechanistic framework. In summary, our study reveals the existence of a loop of the main angiogenic growth factor, VEGF, on its principal transcriptional activator, HIF-1. Our results also disclose that VEGF is capable of activating its own expression, an effect presumably involving HIF-1α. Experiments are in course to precisely clarify this latter issue as well as identify which are the transcriptionally operative signals. In particular, no dampening signal has been identified. However, in this regard the results can be directly explained by the possibility that a critical amount of HIF-1 is mandatory for eliciting VEGF induction. Therefore, HIF-1 dampening can simply be enough to act as the off-regulator of the pathway. In other words, no new off signal beyond decrease of high levels of HIF-1 would be necessary for sustaining the logic of the mechanism.

Several putative biological purposes of the present pathway can be proposed, but the amplification of the effects of VEGF in the absence of hypoxia by means of the recruitment of HIF-1-dependent genes is the one that can be more readily envisioned. In this regard the gene repertoire activated by HIF-1 is by far more extensive than that activated by VEGF alone. In fact, bidirectional VEGF-HIF-1 interactions could be particularly meaningful in the case of malignant tumors, in which a simultaneous increase of both VEGF and HIF-1 does occur (34, 50). Experiments aimed at examining the VEGF-on-HIF-1 effect in other conditions, e.g. hypoxia or inflammation, need to be done to further understand the full implications of this type of activation pathway.

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