Recent evidence shows the involvement of reactive oxygen species (ROS) in the mitogenic cascade initiated by the tyrosine kinase receptors of several growth factor peptides. We have asked whether also the vascular endothelial growth factor (VEGF) utilizes ROS as messenger intermediates downstream of the VEGF receptor-2 (VEGFR-2)/KDR receptor given that the proliferation of endothelial cells during neangiogenesis is physiologically regulated by oxygen and likely by its derivative species. In porcine aortic endothelial cells stably expressing human KDR, receptor activation by VEGF is followed by a rapid increase in the intracellular generation of hydrogen peroxide as revealed by the peroxidesensitive probe dichlorofluorescein diacetate. Genetic and pharmacological studies suggest that such oxidant burst requires as upstream events the activation of phosphatidylinositol 3-kinase and the small GTPase Rac-1 and is likely initiated by lipoxygenases. Interestingly, ROS generation in response to VEGF is not blocked but rather potentiated by endothelial nitric oxide synthase inhibitors diphenyleneiodonium and NG-methyl-L-arginine, ruling out the possibility of nitric oxide being the oxidant species here detected in VEGF-stimulated cells. Inhibition of KDR-dependent generation of ROS attenuates early signaling events including receptor autophosphorylation and binding to a phospholipase C-γ-glutathione S-transferase fusion protein. Moreover, catalase, the lipoxygenase inhibitor nordihydroguaiaretic acid, the synthetic ROS scavenger EUK-134, and phosphatidylinositol 3-kinase inhibitor wortmannin all reduce ERK phosphorylation in response to VEGF, and antioxidants prevent VEGF-dependent mitogenesis. Finally, cell culture and stimulation in a nearly anoxic environment mimic the effect of ROS scavenger on receptor and ERK phosphorylation, reinforcing the idea that ROS are necessary components of the mitogenic signaling cascade initiated by KDR. These data identify ROS as a new class of intracellular angiogenic mediators and may represent a potential premise for new antioxidant-based antiangiogenic therapies.

An emerging concept in signal transduction is that reactive oxygen species (ROS) such as superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂) may serve as intracellular messenger responding to receptor activation by a variety of bioactive peptides including growth factors, cytokines, and hormones (1, 2). While the modality of signal transduction by oxygen species is far from being completely elucidated, it has been clearly shown that these compounds, generated in a ligand-dependent fashion by nearly all the cell types, are necessary components in mitogenic and inflammatory signaling cascades and impinge mostly on key signaling events represented by protein tyrosine phosphorylation (3) (likely by inhibition of tyrosine phosphatase activities) and activation of a number of transcription factors (4). Oxygen species generated upon receptor activation by either NADPH oxidase-like membrane complexes (5) or through the oxidative metabolism of free arachidonic acid released by ligand-dependent phospholipases (6) have a major role in transducing intracellular signals by activated growth factor receptors. Cellular receptors for epidermal growth factor, platelet-derived growth factor, insulin, and immunological stimuli are in fact all linked to ROS-generating systems, the block of which, as well as the removal of oxygen radicals by chemical or enzyme antioxidants, severely compromises cell response to mitogenic stimulation (7–10). It is therefore conceivable that signal transduction by oxygen species (“redox signaling”) represents a general and conserved component of the biochemical machinery triggered by tyrosine kinase receptors and leading, eventually, to cell proliferation.

Among many known growth factors, the vascular endothelial growth factor (VEGF)/permeability factor has in the last few years drawn significant attention as an endothelial cell-specific growth and survival factor and as a major inducer of vasoangiogenesis and neangiogenesis in both physiological and pathological settings (11). In particular, for its capability to promote the formation of new blood vessels in the adult body, VEGF has gained central importance as a putative tool for the treatment of cardiovascular diseases and, on the other side, as a potential target for tumor-suppressive therapies based on the inhibition of tumor vascularization (12). As for the latter point, it is relevant to note that VEGF is the major angiogenic factor produced by tumor cells and that experimental approaches aimed to interfere with VEGF signaling have proven successful in counteracting tumor growth in vivo (13, 14).
VEGF signals to endothelial cells mainly through two tyrosine kinase receptors, VEGFR-1 or Flt-1 and VEGFR-2 or Flk-1/KDR (10). Both receptors are necessary for normal mouse development, but relevant differences have been identified in their signaling properties, the stimulation of Flt-1 being predominately linked to cell migration, while Flk-1/KDR receptor activation is associated to both endothelial cell migration and proliferation and is followed, unlike for Flt-1, by the activation of the mitogen-activated protein kinase cascade (15).

Much evidence suggests that ROS may play a role in angiogenesis. The up-regulation of both VEGF production and KDR expression are part of cell hypoxic response, which may involve as trigger the generation of hydrogen peroxide by mitochondria in low (1.5–2%) oxygen (16); moreover, ROS increase the DNA binding activity of the hypoxia-inducible factor 1α, a transcription factor responsible for cell adaptation to hypoxia and in charge of the oxygen-dependent expression of the VEGF gene (16). Finally, generation of ROS follows cell exposure to alternate hypoxia-reoxygenation, a condition representing a powerful proangiogenic stimulus (17). Despite this evidence, however, the possibility that oxygen species may also contribute, as for other growth factors, to intracellular signaling by VEGF has not been investigated so far. It is known, however, that nitric oxide, a nitrogen-derived reactive species closely related to ROS, is produced by endothelial cells in response to VEGF and is part of the signaling cascade triggered by this factor (18). Moreover another angiogenic factor, basic fibroblast growth factor, has been shown to promote the generation of ROS in nonendothelial cells (19).

On the basis of the above considerations, we evaluated whether ROS are generated in response to VEGF in endothelial cells expressing human KDR and whether a physiological role could be identified for these reactive species in VEGF-mediated angiogenic signaling.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents**—The generation of porcine aortic endothelial (PAE) cells stably expressing human KDR has been described elsewhere (15). Cells were routinely maintained in Ham's medium supplemented with 10% FCS (Eurobio, Les Ulis, France), glutamine, penicillin-streptomycin, and 400 μM sodium orthovanadate (Sigma). The construct encoding the Myc-tagged N17Rac-1 cDNA and the GST-human SHP-1 fusion protein (GST-SHP1) were kind gifts of Dr. A. Hall (Medical Research Council, London, United Kingdom). The Escherichia coli strains expressing the two SH2 domains of human PLC-γ fused with glutathione S-transferase (GST-PLC(2SH2)) and the GST-human SHP-1 fusion protein (GST-SHP1) were kind gifts of Dr. A. Pawson and Dr. K. A. Siminovic (Samuel Lunenfeld Research Institute, Toronto, Canada), respectively. The fusion proteins were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (Advanced Biotechnology) for 3 h and purified from bacterial lysate with Sepharose 4B (Amersham Biosciences, Inc.) in accordance with the recommendations of the manufacturer. Antibodies used in the present study were anti-KDR (Sc-504; Santa Cruz Biotechnology), anti-phosphotyrosine (clone 4G10; Upstate Biotechnology), anti-actin (Sc-1615; Santa Cruz Biotechnology), anti-c-Myc (clone 9E10, Sc-40; Santa Cruz Biotechnology), anti-phospho-ERK and anti-phospho-Akt (Ser-473 and Thr-308) (New England Biolabs), and horseradish peroxidase-conjugated secondary reagents (anti-mouse IgG from Amersham Biosciences, Inc. and anti-rabbit IgG from Bio-Rad).

Human recombinant VEGF was purchased from Upstate Biotechnology. Bovine liver catalase was purchased from Sigma. Chemicals and enzyme inhibitors were all from Sigma unless stated differently. The peroxide-sensitive fluorescent dye 2′,7′-dichlorofluorescein-diacetate (DCF-DA) was from Molecube, Inc. (Eugene, OR). The synthetic superoxide/peroxide scavenger EUK-134 was generously provided by Eukarion Inc. (Bedford, MA). Autoradiography films were from Eastman Kodak Co.

**Cell Transfection**—2 × 105 PAE-KDR cells/well (six-well plate) were seeded in complete medium 16 h before transfection. Transfection was performed with the Effectene reagent (Qiagen) according to the recommendations of the manufacturer with 1 μg of total DNA (RacN17 or vector) per sample. 24 h after transfection cells were trypsinized and replated in Ham's medium containing 1% FCS for further processing. The efficiency of transfection (about 40%) was determined using an equal amount of a plasmid encoding the green fluorescent protein under the cytomegalovirus promoter. The percentage of fluorescent cells was determined 48 h after transfection as the percentage of fluorescent cells (determined as decrease in fluorescence) by flow cytometry (Coulter EPICS equipped with an argon laser lamp; emission, 480 nm; band pass filter, 530 nm). Mean fluorescence data were expressed as percent increase over unstimulated samples (Stimulated/Unstimulated × 100) or as net fluorescence increase (Stimulated – Unstimulated).

In some experiments cells were pretreated with specific enzyme inhibitors (catalase, heat-inactivated catalase, CuZn-superoxide dismutase, rotenone, nrdihydroguaiaretic acid (NDGA), diphenylenedio- nium (DPI), and Nω-methyl-l-arginine (l-NMMA)) or relative vehicles in Ham's medium containing 1% FCS for 1 h before the wash and stimulation in HBSS. Wortmannin (1 μM) was added to cells 10 min before VEGF directly in the stimulation buffer (serum-free HBSS).

In transfection experiments, changes in mean cell fluorescence were normalized for the percentage of transfected cells (determined as described above), according to the following formula: % fluorescence decrease (normalized) = % fluorescence decrease (raw)% × 100% of transfected cells.

**Cell Stimulation and Lysis**—For biochemical studies, PAE-KDR cells were seeded at 5 × 105 cells/well in a six-well plate and incubated for 16 h in Ham’s medium containing 1% FCS. Stimulation with VEGF (50 ng/ml) or vehicle (4 mM HCl, 125 μM bovine serum albumin; Sham) was performed in HBSS for 10–15 min. Stimulation with pervanadate (1 mM vanadate + 1 mM hydrogen peroxide for 5 min on ice) was for 5 min. Pretreatment with catalase and enzyme inhibitors was performed as described in the previous section. After stimulation cells were lysed in 500 μl of 1% Triton lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 8, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, leupeptin, and pepstatin, 1 mM sodium ortho- vanadate) with the help of a rubber scraper. After 30 min on ice, cell debris was centrifuged (14,000 rpm at 4°C for 20 min), and the supernatant was immediately used for SDS-PAGE and immunoprecipitation studies or was stored at −80°C.

**Analysis of Protein Tyrosine Phosphorylation**—For determination of total protein tyrosine phosphorylation and of VEGF-dependent phosphorylation of Akt and ERK, equal amounts of protein samples were subjected to SDS-PAGE, transferred to nitrocellulose membrane (Sartorius), and immunoblotted with specific antibodies against phosphorylase (4G10; 1:5000), phosphoserine Akt + phosphothreonine Akt (1:2000 + 1:2000) or phospho-ERK (1:1000) followed by horseradish peroxidase-conjugated secondary reagents. Immunocomplexes were detected by enhanced chemiluminescence (ECL; Amersham Biosciences, Inc.) and autoradiography. Equal protein loading in all lanes was confirmed by reversible Ponceau S staining of nitrocellulose filters or, occasionally, by immunoblotting with a protein-specific primary antibody and G-Sepharose beads or GST-PLC(2SH2) binding studies. 1 mg of total protein lysate was precleared with empty Sepharose beads and incubated with either 1 μg of anti-KDR antiserum and 10 μl of protein G-Sepharose beads or 5 μg of Sepharose-bound fusion protein for 3 h at 4°C. Immunocomplexes were washed three times in lysin buffer, resuspended in SDS-PAGE loading buffer, and further processed for analysis of protein tyrosine phosphorylation.
stopped after 5 min of incubation with 4 volumes (800 μM concentration of 10 mM. In some samples 10 mM dithiothreitol was included in control cells. Dependent protein phosphorylation is evicted form of the VEGF receptor. No VEGF-dependent protein phosphorylation is evident in control cells. B, PAE-KDR cells were stimulated for 15 min with 50 ng/ml human recombinant VEGF in serum-free HBSS. Cells were labeled with 20 μg/ml DCF-DA for 5 min, detached from the substrate, and immediately analyzed by flow cytometry. No oxidative response was observed in PAE-Neo cells. Columns indicate percent increase of mean cell fluorescence with respect to the untreated (no VEGF) samples (average ± S.D. of duplicate assays). C, cells were exposed to 50 ng/ml VEGF for the indicated times, and cell fluorescence was analyzed after a short (5-min) labeling with DCF-DA. The rise in intracellular oxidants, which peaks at about 15 min and starts to decline at 30 min, is completely blocked in cells preloaded with catalase (see "Experimental Procedures"). Values are percent increases of mean cell fluorescence (average ± S.D. of duplicate samples). Ctrl, control; H.I., heat-inactivated.

Cell Proliferation Assay—For proliferation studies, 24-well plates were precoated with FCS for 2 h at 37 °C to ensure cell adhesion in serum-free medium; after careful removal of residual FCS, cells were seeded at 10^4 cells/well in serum-free Ham's medium containing 0.01% bovine serum albumin in the presence of MeSO_4, 5 μg/ml NDGA, or 100 μM EUK-134. VEGF (50 ng/ml) or empty vehicle was added immediately after cell plating. After 4 days in culture adherent cells were trypsinized, mixed with the supernatant cells, quickly stained with propidium iodide, and subjected to flow cytometry. The live cell population was gated on the forward scatter plot, and the absolute number of cells was determined by using an internal fluorescent standard (Flow-count fluorospheres; Coulter). The percentage of dead (propidium iodide-positive) cells was determined on the number of total events (live cells + dead cells) after gating out cell debris.

Cell Stimulation in Very Low Oxygen—For experiments in an anoxic environment, a controlled atmosphere incubator (Forma Scientific model 3103) was supplied with a blend of 85% N_2, 10% H_2, and 5% CO_2. Such equipment allows cell manipulation from outside the anoxic chamber. Cells were incubated in anoxia for 16 h in the normal incubator (Normoxia) or in very low oxygen (Anoxia) and stimulated with VEGF (50 ng/ml, 10 min) or pervanadate (1 μM, 5 min) or were left untreated. After lysis (anoxic cells were exposed to oxygen only after addition of cold lysis buffer) samples were processed as described above. Cell viability was higher than 90% after overnight incubation in very low oxygen.

SHP-1 Phosphatase Assay—Equal amounts (1 μg) of GST-SHP-1 fusion protein bound to glutathione-Sepharose beads were suspended in 200 μl of standard or nitrogen-purged (anoxic) phosphatase buffer (62 mM Heps, pH 7.4, 6.25 mM EDTA) in the absence of substrate. After a 3-h incubation at 37 °C p-nitrophenyl phosphate was added at the concentration of 10 mM. In some samples 10 mM dithiothreitol was added 30 min before p-nitrophenyl phosphate. Color development was stopped after 5 min of incubation with 4 volumes (800 μl) of 200 mM NaOH, and p-nitrophenyl phosphate dephosphorylation was quantified spectrophotometrically at 410 nm.

RESULTS

VEGF-induced Generation of ROS in PAE Cells Expressing Human KDR—Immortalized PAE cells, despite their endothelial origin, express negligible amounts of endogenous VEGF receptors. Stable expression of either human VEGFR-1/Flt-1 or VEGFR-2/KDR in this cell line has therefore made it possible to dissect individually the signaling pathways initiated by the two VEGF receptors (13). PAE-KDR cells in particular, like human primary endothelial cells, proliferate in response to VEGF in a fashion that correlates with an increase in the activity of the kinases ERK 1 and 2. Moreover, PAE-KDR cells, like their normal human counterparts, increase their proliferative response to VEGF in hypoxia and up-regulate, at a post-transcriptional level, the expression of KDR in low oxygen (20). This transfectant cell line retains therefore the features of human endothelial cells with respect to physiological modulation of KDR signaling and represents a suitable model for signal transduction studies on this receptor.

Unlike parental PAE cells, KDR transfectants respond to VEGF with an increase in protein tyrosine phosphorylation involving mainly two protein species at 150 and 200 kDa, the latter likely representing the phosphorylated form of the KDR receptor (Fig. 1A). Exposure of PAE-KDR cells to VEGF is also accompanied, within minutes, by a significant rise in intracellular ROS as revealed by a net increase in cell fluorescence after cell loading with the peroxide-sensitive fluorescent dye DCF-DA (Fig. 1B). Oxidative response clearly occurs via KDR stimulation since it is completely absent in PAE parental cells (Fig. 1B); a time course analysis of ROS generation following addition of VEGF revealed that the generation of intracellular peroxide is very rapid and sustained over 30 min of observation (Fig. 1C), a kinetic that parallels that of receptor autophosphorylation (not shown and Fig. 4B). The oxygen species responsible for DCF-DA oxidation is most likely hydrogen peroxide since ligand-induced oxidation is completely abolished in cells loaded with the specific scavenger enzyme catalase (Fig. 1C) but not with heat-inactivated enzyme (Fig. 1C) or with native superoxide dismutase (not shown). Note that DCF-DA detects mostly hydrogen peroxide or other organic peroxides and is relatively insensitive to superoxide or nitric oxide (21).

Recent studies have shown that NADPH oxidase (sensitive to DPI) and lipoxygenases (inhibited by NDGA and 5,8,11-eicosatriynoic acid) are two enzyme sources frequently involved in ligand-dependent generation of oxygen species (5, 6). In PAE-KDR cells ROS production following stimulation by VEGF was resistant to inhibition of the mitochondrial respiratory chain (Fig. 2A, rotenone) but was significantly reduced by both the peroxide scavenger and lipoxygenase inhibitor NDGA and...
the highly specific lipoygenase inhibitor 5,8,11-eicosaetoynoic acid, suggesting that oxidative metabolism of arachidonic acid into leukotrienes may have a role in the observed oxidative phenomena (Fig. 2A). Interestingly, both the NADPH oxidase inhibitor DPI and the nitric-oxide synthase inhibitor l-NMMA were consistently found to potentiate the basal (not shown) and VEGF-induced generation of peroxides in KDR cells (Fig. 2B). While this finding argues against a NADPH oxidase-like complex playing a major role in peroxide generation by VEGF, the possibility that oxidations detected by DCF-DA in response to VEGF are due to generation of nitric oxide (a phenomenon already reported in this cell line (22)) is also ruled out in view of the paradoxical effect of l-NMMA. It should be noted that DPI, which blocks flavin-containing enzymes, is also a potent inhibitor of endothelial nitric-oxide synthase (23) (see "Discussion").

The Generation of ROS in Response to VEGF Requires the Activity of PI 3-Kinase and Rac-1—PI 3-kinase associates with KDR in endothelial cells and is necessary for VEGF-mediated cell cycle progression (24). Since PI 3-kinase is necessary for ROS production in fibroblasts stimulated by platelet-derived growth factor (25), likely through the activation of the small GTPase Rac-1, we thought to evaluate whether this enzyme is also involved in redox events elicited by VEGF in endothelial cells. A brief (15-min) exposure to the PI 3-kinase inhibitor wortmannin (1 \text{ \mu M}) nearly completely abolished the generation of peroxide in stimulated PAE-KDR cells, confirming that, as for platelet-derived growth factor, PI 3-kinase is located upstream of a ROS source activated by VEGF (Fig. 3A, a). Note that 1 \text{ \mu M} wortmannin was not toxic for PAE-KDR cells in the indicated time frame. Another unrelated PI 3-kinase inhibitor, LY294002, had an effect similar to that of wortmannin (not shown). As a positive control for the effective inhibition of PI 3-kinase, the VEGF-dependent phosphorylation of Akt, a downstream target of PI 3-kinase, was also abolished by wortmannin in the same experimental conditions in which the elevation of intracellular ROS was inhibited (Fig. 3A, b).

A dominant-negative mutant of the small GTPase Rac-1, a molecule crucial for both lipid- and lipoygenase activation in several cellular models, also inhibits significantly VEGF-induced oxidized burst when overexpressed by transient transfection in PAE cells (Fig. 3B, a and b), suggesting a role for Rac-1 in transducing redox signals by KDR. The inhibitory effect of RacN17 is particularly evident when the reduction in mean cell fluorescence (about 35%) is normalized for the effective percentage of transfected cells (40%) (Fig. 3B, a).

**KDR Autophosphorylation and Signaling after Removal of Hydrogen Peroxide—Like other growth factor receptors, KDR rapidly autophosphorylates upon ligand binding, thereby creating the docking sites for downstream signaling effectors (10). Previous studies have shown that, in cells stimulated by epidermal growth factor, removal of oxygen species significantly reduces the level of receptor phosphorylation likely by favoring its active dephosphorylation by receptor-directed tyrosine phosphatases (8, 26). To evaluate the importance of ligand-induced ROS for KDR autophosphorylation/activation, PAE-KDR cells were pretreated with catalese (and with heat-inactivated catalese as control) or with NDGA, which efficiently prevents oxidative response to VEGF, and subsequently exposed to VEGF. As indicated in Fig. 4, VEGF-induced protein tyrosine phosphorylation (Fig. 4A and B), the phosphorylation of the KDR receptor itself (Fig. 4C) and its capability to bind and recruit PLC-\gamma (Fig. 4D) were all significantly reduced following elimination of oxygen species. This effect conceivably involves tyrosine phosphatase activities; in fact, protein tyrosine phosphatase inhibition by pervanadate is able per se to elicit massive protein tyrosine phosphorylation (Fig. 4A, A and B) and significant KDR phosphorylation (Fig. 4C) in PAE cells, consistent with a major role for tyrosine phosphatases in modulating the activation cascade initiated by KDR in this cell model. Moreover, phosphatase inhibition restores protein phosphorylation in the presence of catalese (Fig. 4A), reinforcing the idea that removal of peroxide deregulates phosphatase activities.

The superoxide/peroxide synthetic scavenger EUK-134 (27) also inhibited, although to a lesser extent, receptor phosphorylation in response to VEGF, and a similar effect was observed with the PI 3-kinase inhibitor wortmannin (data not shown). None of the above treatments changed significantly the level of expression of the VEGF receptor in PAE cells (not shown), indicating that modifications in anti-phophotyrosine reactivity are due to differences in protein phosphorylation/dephosphorylation.

**Activation of ERKs by VEGF Is Attenuated by Antioxidants—**VEGF strongly activates ERKs in endothelial cells; such activation is mediated by the KDR receptor and is required for VEGF-dependent mitogenesis (22). Since removal of ROS appears to attenuate early signaling events following KDR stimulation, we examined the phosphorylation of ERKs as a downstream parameter of VEGF-dependent cell activation directly linked to the entry into the cell cycle. Dual serine-tyrosine phosphorylation of ERKs, a biochemical hallmark of kinase activation, was consistently reduced, although not abolished, by antioxidant treatments represented by catalese (Fig. 5A, a), NDGA (Fig. 5A, b), and EUK-134 (Fig. 5A, c) or by upstream inhibition of redox signaling through the PI 3-kinase inhibitor wortmannin (Fig. 5B). The effect of all these treatments was...
PAE-KDR cells were pretreated for 15 min with wortmannin (Wort) in serum-free HBSS. At the end of the preincubation VEGF was added at the final concentration of 50 ng/ml for 15 min. Cells were loaded with DCF-DA, and fluorescence was analyzed as described in Fig. 1A. Values indicate the VEGF-dependent percent increase in mean cell fluorescence. b, inhibition of Akt phosphorylation by wortmannin. PAE-KDR cells were pretreated as in A and stimulated with VEGF. After 15 min cells were lysed, and an equal amount of proteins was subjected to SDS-PAGE and immunoblotting with a mix of two anti-phospho-Akt-specific antisera directed to the two different sites of phosphorylation (Ser-473 and Thr-308). Equal protein loading throughout the gel was verified by reversible Ponceau S staining. B, a, effect of a dominant-negative mutant of Rac-1 on KDR-dependent generation of peroxide. The N17V12 mutant of Rac-1 was overexpressed by transient transfection, and the generation of ROS was evaluated 48 h later as described above. VEGF-induced fluorescence increases (mean fluorescence VEGF − mean fluorescence unstimulated) were evaluated in RacN17 and mock-transfected cells, and values are expressed as percentage of control (mock-transfected) cells. Fluorescence decrease induced by RacN17 was expressed as raw data (RacN17) or as data normalized for the efficiency of cell transfection (assumed as 40%; RacN17 normalized) as described under “Experimental Procedures.” Numbers are mean ± S.D. of four independent experiments. b, expression of the Myc-tagged Rac-1 mutant was verified by anti-c-Myc immunoblotting 48 h after cell transfection.

DISCUSSION

The mechanisms of signal transduction by the vascular endothelial growth factor receptors have recently received growing attention from the scientific community, especially in relation with the possibility to inhibit cancer cell growth by...
blocking tumor angiogenesis (12). We have here identified reactive oxygen species and hydrogen peroxide in particular as novel putative mediators involved in signal transduction by human VEGFR-2/KDR. This finding extends to angiogenic signaling some recent observations on the role of oxidants in signal transduction by tyrosine kinase receptors (Refs. 2 and references therein) and may have important applicable ramifications since many compounds as well as dietary constituents are well known to reduce the content of oxygen species within cells. Moreover, the observation that oxygen-derived reactive species can modulate VEGF signaling appears physiologically relevant in view of the fact that angiogenesis is a process normally regulated by oxygen tension.

In favor of the idea that oxidants act as messengers downstream of KDR activation is the observation that a rapid rise in intracellular peroxides can be detected following cell exposure to VEGF and that inhibition of such oxidative burst impairs downstream VEGF signaling in terms of receptor phosphorylation, activation of the ERK cascade, and mitogenesis/survival. The inhibitory effect of exogenously added catalase on both oxidant increase and protein phosphorylation induced by KDR has allowed us to identify hydrogen peroxide as the reactive species involved in redox signaling by VEGF. This finding extends to angiogenic signaling some recent observations on the role of oxidants in signal transduction by tyrosine kinase receptors (Refs. 2 and references therein) and may have important applicable ramifications since many compounds as well as dietary constituents are well known to reduce the content of oxygen species within cells. Moreover, the observation that oxygen-derived reactive species can modulate VEGF signaling appears physiologically relevant in view of the fact that angiogenesis is a process normally regulated by oxygen tension.

In favor of the idea that oxidants act as messengers downstream of KDR activation is the observation that a rapid rise in intracellular peroxides can be detected following cell exposure to VEGF and that inhibition of such oxidative burst impairs downstream VEGF signaling in terms of receptor phosphorylation, activation of the ERK cascade, and mitogenesis/survival.

The inhibitory effect of exogenously added catalase on both oxidant increase and protein phosphorylation induced by KDR has allowed us to identify hydrogen peroxide as the reactive species involved in redox signaling by VEGF. Interestingly, neither the heat-inactivated enzyme nor the superoxide scavenger superoxide diamutase (not shown) have significant effects on cell signaling, demonstrating the functional specificity of H$_2$O$_2$. Since cells, after overnight incubation with the enzyme, were washed before being exposed to VEGF, it is conceivable that a fraction of the enzyme has been internalized and acts on intracellular peroxide as is consistent with previous observations (7). The possibility also exists that extracellular, membrane-absorbed catalase contributes to the observed phenomena by removing peroxide from the extracellular com-

![Fig. 4. Effect of catalase and NDGA on KDR phosphorylation and signaling.](http://www.jbc.org/)

![Fig. 5. ERK phosphorylation in response to VEGF is reduced by antioxidants.](http://www.jbc.org/)
partment; this could still result in decrease of intracellular H$_2$O$_2$ since this oxidant species diffuses freely across the plasma membrane.

While transfection experiments with the dominant-negative Rac-1 mutant have clearly shown that this transducer is part of the molecular machinery that produces hydrogen peroxide upon KDR stimulation, the role of different potential peroxide sources controlled by Rac-1 (mainly NADPH oxidases and lipoxygenases (30)) has been addressed using selective enzyme inhibitors.

It is a general concern that definitive conclusions from drug-based experiments should be drawn very cautiously since many compounds often display additional biological activities other than those for which they have been selected and utilized. This is particularly true for experiments with the lipoxygenase inhibitor and peroxide scavenger NDGA since both activities may be contributing to the observed effects on VEGF signaling. Although another lipoxygenase inhibitor, 5,8,11-eicosatriynoic acid, also displays similar activities as NDGA (Fig. 2A) and lipoxygenases are a physiological target for Rac-1 signaling (30), the importance of lipoxygenases in angiogenic signaling may require further investigation, especially in view of potential pharmacological applications. On the other hand, it should be noted that the fact that oxygen species are required for KDR activity has been demonstrated here not only using chemical or enzymatic antioxidants but also with identical results in experiments performed on cells stimulated in very low oxygen (Fig. 7).
While data on redox signaling by KDR parallel previous observations made with epidermal growth factor, platelet-derived growth factor, and other growth factors, the picture is further complicated by the fact that VEGF also induces the formation of nitric oxide (31) and that this species can potentially react with ROS (mainly superoxide, the precursor of hydrogen peroxide) (32) and has been shown to participate, like ROS, in ERK activation and mitogenesis (18). Therefore it is noteworthy that inhibition of nitric-oxide synthase by either DPI or L-NMMA increases DCF-DA oxidation following cell exposure to VEGF (Fig. 2B). This observation may suggest that in VEGF-stimulated cells ROS are normally scavenged to some extent by NO. Since block of either NO (18) or ROS (present data) interferes with activation of ERK, it is possible that both classes of radical species, or a product of their combination (such as, for instance, peroxynitrite), are required for mitogenic signaling by KDR. Although largely speculative, this hypothesis has some experimental support (33, 34) and certainly deserves further investigation.

The effect of VEGF-induced ROS on KDR signaling seems to impinge mostly on protein tyrosine phosphorylation events (Figs. 4, 5, and 7). This is again in line with previous observations on the role of oxygen species as endogenous modulators of tyrosine phosphatase activities during growth factor signaling (9, 26). However, a new element in this picture is introduced by data (shown in Fig. 7) suggesting that an increase in protein tyrosine phosphatase activity may inhibit VEGF signaling in very low oxygen. KDR associates with at least two tyrosine phosphatases, SHP-1 and SHP-2, which likely dephosphorylate the receptor itself or its key substrates (15). Transient inactivation of these enzymes may therefore be necessary for KDR activation. The complete absence of oxygen could interfere with this redox circuitry leading to excess protein dephosphorylation and impaired mitogenic signaling in analogy with what has been suggested for contact inhibition of fibroblast growth (9). Complete anoxia is in fact often associated with cell cycle arrest even in the absence of cell death (35). Protein tyrosine phosphatase may therefore have a role in cell response to extremely low oxygen.

On a similar line of thinking, protein tyrosine phosphatase inhibitor vanadate was here shown to restore ERK response to VEGF in cells in which PI 3-kinase has been inhibited by wortmannin blocks the generation of ROS in response to VEGF in cells in which PI 3-kinase has been inhibited by wortmannin, suggesting that PI 3-kinase may therefore have a role in cell response to extremely low oxygen.

Role of ROS in VEGF Signaling

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