Early Signaling by Vascular Endothelial Growth Factor and Placental Growth Factor in Human Bone Marrow-Derived Endothelial Cells Is Mediated by Superoxide

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Key Words

Endothelium • Reactive oxygen species • Placental growth factor • Vascular endothelial growth factor • Superoxide dismutase • Receptor phosphorylation • Angiogenesis

Abstract

Aims: We investigated the role of superoxide O_2^- during the initiation of vascular endothelial growth factor (VEGF)- and placental growth factor (PlGF)-mediated signal transduction in bone marrow-derived endothelial cells. **Methods:** BMhTERT cells were treated with VEGF or PlGF in the presence or absence of antioxidants. The signaling pathways downstream were analyzed by immunoprecipitation and Western blotting. Superoxide and reactive oxygen species (ROS) were measured using Superluminol or 2',7'-dichlorofluorescein fluorescence measurements. **Results:** We show here that VEGF and PlGF generate extracellular and intracellular O_2^- that regulates their downstream signaling transduction pathways. Indeed, the extracellular O_2^- generated treatment of endothelial cells (using hypoxanthine/xanthine oxidase) was sufficient to initiate receptor phosphorylation of VEGF receptor 2. The PlGF treatment of endothelial cells increased the generation of intracellular ROS in an extracellular O_2^- dependent manner. Quenching of intracellular ROS by resveratrol inhibits PlGF- and VEGF-dependent induction of MAP kinase phosphorylation. Additionally, we found that

the interaction of VEGF and PlGF with their specific receptors generates O_2^- in a cell-free system. Endothelial cells treated with VEGF stop proliferation in the presence of extracellular catalase, superoxide dismutase or peroxiredoxin IV. **Conclusion:** Our studies underscore the role of O_2^- as a critical regulator of VEGF and PlGF signal transduction initiation in endothelial cells.

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Introduction

The vascular endothelial growth factor (VEGF) gene family currently includes 6 members: VEGF-A, placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E [1]. Three receptors with tyrosine kinase activities have been identified as VEGF receptors: VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) [2] with apparent molecular weights of 180 and 230 kDa, respectively, and VEGFR-3 [3]. In addition to VEGF, PlGF has also emerged as an important proangiogenic factor. Unlike VEGF-A which binds both VEGFR-1 and VEGFR-2, PlGF specifically binds VEGFR-1. Studies of PlGF-deficient mice have shown that PlGF is required for pathologic angiogenesis, including retinal and choroidal neovascularization [4].

Reactive oxygen species (ROS), like H_2O_2 , superoxide (O_2^-) and the hydroxyl radical, have been identified as es-

sential mediators that regulate the transduction of signals from the membrane to the nucleus via oxidation and reduction in proteins [5, 6]. ROS are produced by vascular cells following stimulation with growth factors including epidermal growth factor, granulocyte macrophage colony-stimulating factor (GM-CSF) and platelet-derived growth factor [7, 8]. While NADPH oxidase is the primary source of O_2^- in endothelial cells [9, 10], we investigate whether other sources contribute to the formation of O_2^- .

Our previous work showed that GM-CSF receptor α -subunit, lacking the cytoplasmic domain, was able to signal in an extracellular H_2O_2 -dependent manner [11] and that GM-CSF interaction with its ligand leads to the generation of H_2O_2 extracellularly [12]. In this study, we investigated the role of extracellular ROS in the initiation of signal transduction mediated by VEGF and PlGF. We identified that VEGF induced VEGFR phosphorylation and its downstream signaling pathways are dependent on extracellular superoxide, and that VEGF-VEGFR binding generates O_2^- . We propose that extracellular O_2^- formed by the VEGF-VEGFR-1 and PlGF-VEGFR-1 interaction is critical for the maintenance of the signaling transduction pathways and transcriptional events observed during angiogenesis.

Materials and Methods

Materials used include: anti-VEGFR-2 and VEGFR-1 polyclonal antibody (Santa Cruz); phosphotyrosine antibody 4G10, A/G Plus-Agarose (R&D Systems, Minneapolis, Minn., USA); anti-phospho p44/42-MAP kinase and anti-p44/42 MAP kinase (Upstate); Western blotting detection system (Amersham Biosciences, Piscataway, N.J., USA); phospho-AKT (Ser473), phospho-AKT (Thr308) and AKT (Cell Signaling Technologies, Inc.); Superluminol Assay Kit (World Precision Instruments); and Cell-Titer-Glo[®] reagent (Promega). Human VEGFR and VEGFR-1 cDNA were a gift from Dr. L.C. Walsh (University of Upsala, Upsala, Sweden).

Cell Culture

The BMhTERT cell line was cultured in EBM2 medium with growth factor supplement (Clonetics). 293T (human kidney epithelial) cells were grown in DMEM-high glucose supplemented with 10% heat-inactivated FBS, 1% L-glutamine and 1% penicillin/streptomycin. cAMP responsive element (CRE)-dependent signaling was performed using 293T cells, which were transiently transfected with VEGFR-2 cDNA, CRE-luciferase reporter plasmid (Clontech), by Superfect (Stratagene). SV40-Renilla luciferase cotransfection was included as a control. Cells were washed twice with PBS, lysed in passive lysis buffer, and luciferase activity was analyzed using the dual luciferase reporter system (Promega). Luciferase activity was normalized relative to the SV40-Renilla control.

Immunoprecipitations

Cultured cells were starved in serum-free medium for 16 h. Cells were washed with PBS and suspended and lysed in cell lysis buffer containing 150 mM NaCl, 2.5 mM $MgCl_2$, 0.05 mM EDTA, 50 mM HEPES (pH 7.4), 0.5% Triton, 10% glycerol and protease inhibitors (Boehringer Mannheim). Total cell lysates were clarified by centrifugation at 10,000 g for 10 min. Total cell lysate was incubated overnight with anti-receptor antibody for VEGFR-1 and VEGFR-2 and recovered by incubation with protein A/G Plus-Agarose for 2 h at 4°C. The proteins were eluted by boiling and analyzed by SDS polyacrylamide gel electrophoresis followed by immunoblotting. Hypoxanthine (125 μ M/xanthine oxidase 0.75 mU/ml) for 1 min was used as source of superoxide.

Superoxide Measurements

Superoxide was detected using Superluminol (WPI). Superluminol (40 μ l) and 20 μ l of WPI enhancer were mixed followed by the addition of 200 nM receptor or 1 μ M cognate ligand or a combination of the 2 in the presence of 50 U/ml superoxide dismutase (SOD). Superoxide production was measured by luminescence counts for 5–10 measurements with an integration time of 5–15 s using the Lumat LB9501 luminometer. The background luminescence was determined by mixing Superluminol and enhancer in buffer without proteins.

Measurement of Intracellular ROS

BMhTERT cells were plated at subconfluence on 96-well dishes. Twenty-four hours later, the media were replaced with serum-free media and the cells cultured for 16 h. The cells were washed with PBS, 20 μ g/ml 2',7'-dichlorofluorescein (DCF) diacetate was added for 20 min and washed and then stimulated with 50 ng/ml PlGF in the presence or absence of SOD 100 U/ml. DCF fluorescence was measured every minute.

Cell Proliferation Assay

Cells were seeded in a 96-well plate with 5,000 cells per well. Cells were then made quiescent for 16 h and treated with VEGF in the presence or absence of catalase, SOD or peroxiredoxin IV (PRDXIV) for 24 h, after which an equal volume of CellTiter-Glo reagent was added to each well of cell culture medium, and the luminescence is read with an integration time set for 0.25 to 1 s.

Statistical Analysis

Statistical significance was set at $p < 0.005$. One-way ANOVA was performed for statistical comparisons followed by Bonferroni posttests.

Results

Superoxide Induces the Tyrosine Phosphorylation of VEGFR-1 and VEGFR-2 in BMhTERT Cells

It has been described that extracellular treatment of endothelial cells by catalase inhibits VEGF-induced cell signaling [13]. Catalase is a tetrameric protein of 240 kDa, and hence, an extracellular antioxidant and cell impermeable [14]. The results showed that in the endothelial cell line BMhTERT [15], VEGF triggered the phosphory-

Fig. 1. Ligand-dependent receptor phosphorylation is attenuated by extracellular SOD and catalase (CAT). * $p < 0.05$ indicates statistical significance, using one-way ANOVA followed by Bonferroni's multiple comparison test. **a** Quiescent BMhTERT cells were stimulated with 20 ng/ml of VEGF for 2 min. Cell lysates were subjected to immunoprecipitation with anti-VEGFR-1 antibody followed by Western blotting with phosphotyrosine antibody (4G10). Loading of the receptor was detected by Western blotting with VEGFR-1 antibody. Catalase (100 and 1,000 U/ml) and SOD (100 and 500 U/ml) were added to the cells immediately before VEGF treatment. **b** Tyrosine phosphorylation of VEGFR-2 in BMhTERT cells stimulated with 20 ng/ml of VEGF was detected by immunoprecipitation with anti-VEGFR-2 antibody followed by phosphotyrosine antibody (4G10). Catalase (100 and 1,000 U/ml) and SOD (100 and 500 U/ml) were added to the cells immediately before VEGF treatment. Each experiment was performed in triplicate, and a representative figure is shown. The histograms represent the values of the ratio between the phosphorylated protein and the total protein.

lation of VEGFR-1 and VEGFR-2 that was attenuated by extracellular catalase and SOD (fig. 1).

Although it is known that H_2O_2 enhances phosphorylation of VEGFRs [13], we hypothesize that the O_2^- species itself could induce the phosphorylation of VEGFR-2. As shown in figure 2a, treatment with extracellular superoxide induced tyrosine phosphorylation of VEGFR-2, and it was inhibited by SOD. In contrast, PlGF did not induce phosphorylation of VEGFR-2.

Having demonstrated that VEGFR phosphorylation can be initiated by extracellular O_2^- alone, we investigated the effect of extracellular SOD during VEGF-dependent transcription. Transcription gene targets of VEGF-mediated signaling include genes containing CRE in their promoters [16]. Transiently transfected 293T cells with a CRE-luciferase reporter vector were treated with VEGF and the reporter activity determined in the presence or absence of SOD. VEGF treatment triggered a 50% increase in CRE-luciferase activity; however, extracellular SOD blocked the reporter activity (fig. 2b).

We further evaluated the regulation of cell proliferation in response to the ROS present outside the cell.

BMhTERT cells were grown in 96-well plates and treated with VEGF in the presence or absence of catalase or SOD and the cell proliferation was assessed. To ensure continuous expression of the antioxidant in the extracellular medium, PRDXIV cDNA was transfected in BMhTERT cells, and the proliferation of transfected cells was compared with that of untransfected cells. The presence of PRDXIV in the conditioned medium was ascertained by Western blotting (data not shown). As shown in figure 2c, VEGF-dependent cell proliferation was completely stopped in the presence of extracellular antioxidants. These results complement studies showing inhibition of endothelial proliferation by antioxidants, although in most studies, intracellular scavengers of ROS have been used [17, 18].

Extracellular O_2^- Regulates Signaling Events Mediated by PlGF

We investigated the nature of ROS involved in perpetuating the signaling axis inside the cell using specific cell-permeable antioxidant-like resveratrol, a quencher of hydroxyl and O_2^- species. The results show that phosphor-

Fig. 2. Superoxide-induced VEGFR-2 phosphorylation. * $p < 0.05$ indicates statistical significance, using one-way ANOVA followed by Bonferroni's multiple comparison test. **a** BMhTERT cells were exposed to HXXO mixture or 20 ng/ml PIGF for 1 min. Cell lysates were immunoprecipitated with anti-VEGFR-2 antibody and then analyzed by Western blot using phosphotyrosine antibody. SOD was added just prior to the addition of HXXO. The histogram represents the values of the ratio between the phosphorylated protein and the total protein. **b** Luciferase activity of 293T

cells transfected with VEGFR-2 and CRE-luciferase construct, incubated for 5 h with 20 ng/ml VEGF, with or without SOD (100 and 500 U/ml). These experiments were performed in triplicate and representative figures are shown. **c** Quiescent BMhTERT cells plated in 96-well plates were stimulated to grow with VEGF in the presence or absence of catalase (Cat) or SOD for 48 h. Fold increase in cell number over cells at day zero is shown. BMhTERT cells were transfected with PRDXIV cDNA and cellular proliferation was measured after 24 or 48 h of transfection.

ylation of the VEGFR-1 was inhibited by resveratrol indicating that intracellular O_2^- species also play a critical role (fig. 3a).

Then, we asked whether PIGF stimulates intracellular ROS levels. Endothelial cells were treated with PIGF in the presence or absence of extracellular SOD. Figure 3b shows a significant increase in intracellular ROS in

BMhTERT that was inhibited by extracellular SOD. Since PIGF treatment led to increased levels of intracellular ROS, we hypothesize that an intracellular scavenger of ROS should downregulate the phosphorylation of MAP kinase activation. Indeed, both PIGF- and VEGF-induced phosphorylation of p44/42 MAP kinase in these cells was inhibited by resveratrol (fig. 3c, d).

Fig. 3. VEGFR-1 phosphorylation is inhibited by intracellular superoxide scavengers. * $p < 0.05$ indicates statistical significance, using one-way ANOVA followed by Bonferroni's multiple comparison test. **a** BMhTERT cells were treated with indicated amounts of resveratrol followed by stimulation with VEGF for 3 min. The histogram represents the values of the ratio between the phosphorylated protein and the total protein. **b** PI3G-induced ROS generation in BMhTERT cells. DCF fluorescence was measured 1 min after PI3G addition. The percent increase in cell fluorescence compared with cells not treated with the growth factor is shown. Data represent the mean from 3 experiments; each

condition was in quadruplicate. **c** BMhTERT cells were treated with cell-permeable resveratrol, 1 or 5 μ M, before the addition of PI3G. Western blot analysis was done for phosphorylated p42/p44. Loading of the proteins as determined, using anti-p44/42 antibody on a duplicate blot. **d** BMhTERT cells were treated with cell permeable 1 or 5 μ M resveratrol, before the addition of VEGF. Western blot analysis was done for phosphorylated AKT. Loading of the proteins as determined, using anti-p44/42 antibody on a duplicate blot. The histogram represents the values of the ratio between the phosphorylated protein and the total protein.

Fig. 4. Generation of superoxide upon receptor-ligand interactions. * $p < 0.05$ indicates statistical significance, using one-way ANOVA followed by Bonferroni's multiple comparison test. **a** Equal number of A431 cells were treated with 20 ng/ml VEGF or albumin 50 ng/ml. DPI (100 μ M) was added 5 min before addition of the ligand. The histogram indicates O_2^- production over 1 min measured by luminescence emission upon incubation with

Superluminol. **b** VEGF, PlGF, VEGFR-1 and VEGFR-1 with VEGF or PlGF, VEGFR-1 and VEGF with SOD in the presence of superluminol luciferase activity measured for 5 s is shown. **c** VEGF, PlGF, VEGFR-2 alone or VEGFR-2 with VEGF or PlGF luciferase activity measured for 5 s is shown. These experiments were performed twice in triplicate. The histograms represent the measurement of luminescence.

Detection of Extracellular O_2^-

Having demonstrated that extracellular O_2^- was essential for VEGFR-1 and VEGFR-2 signaling, we investigated whether extracellular O_2^- was a consequence of ligand addition to the cells. While VEGFR is expressed in endothelial cells, expression of VEGFR-2 is also upregulated in A431 cells [19]. To detect the generation of extracellular O_2^- upon ligand-receptor interaction, A431 cells were incubated with VEGF or albumin as control (see Material and Methods). We observed O_2^- generation seconds after addition of the ligand (fig. 4a). To rule out the contribution of NADPH membrane oxidase in the generation of O_2^- , A431 cells were pretreated with diphenyleneiodonium (DPI; 100 μ M), an inhibitor of NADPH oxidase. Treatment with DPI had no significant influence on the ligand-dependent generation of O_2^- (fig. 4a). Our results rule out the contribution of membrane NADPH oxidase in the generation of O_2^- measured and raise the possibility that receptor-ligand interaction alone is sufficient to initiate the formation of O_2^- species. In order to further establish that VEGF and PlGF generate O_2^- , we used a cell-free system where recombinant extracellular VEGFR1-Fc chimera was incubated with recombinant VEGF or PlGF and the superoxide generation measured with Superlu-

minol. PlGF and VEGF binding to VEGFR-1 triggered the production of O_2^- (fig. 4b), and the species measured is indeed superoxide since treatment with SOD abrogates the increase. However, VEGFR-2 generated O_2^- only when combined with VEGF but not with PlGF (fig. 4c). These results confirmed that specific ligand-receptor interaction is critical for the generation of O_2^- .

Discussion

In this study, we addressed the role of extracellular O_2^- during VEGF- or PlGF-mediated receptor activation. The cellular response(s) to each species of ROS appear to be tightly regulated. For instance, O_2^- production by NADPH oxidase is critical for the feed forward mechanism of signaling involving c-Src activation leading to sustained oxidase activation [20]. ROS as O_2^- has been implicated in endothelin-1-induced venous contraction, whereas ROS as H_2O_2 does not contribute to the same [21]. Induction of hypoxia-inducible factor 1 α gene by VEGF is mediated primarily by the superoxide species [22]. Additionally, angiopoietin-induced angiogenesis is known to be dependent on superoxide from NADPH

[23]. Similarly, low-density lipoprotein-induced VEGF and VEGFR overexpression was prevented by vitamin C, suggesting a role of intracellular superoxide in the process [24]. Therefore, the role of the O_2^- species is emerging as necessary for maintaining several cellular processes.

Our work demonstrates that extracellular O_2^- generated by VEGF and PlGF is sufficient to initiate receptor phosphorylation independent of the NADPH oxidase activity. We showed that extracellular application of SOD can inhibit phosphorylation of VEGFR-1. Earlier work has shown that catalase, a scavenger of H_2O_2 , inhibits VEGFR-1 phosphorylation, and the source of the peroxide can be intracellular as peroxide is cell permeable, or it can be a dismutation product of O_2^- species generated upon receptor-ligand interaction [13]. It has also been shown that NADPH oxidase activity is not required for early phosphorylation of VEGFR-2/KDR by VEGF but may play a role in maintaining the receptor in the phosphorylated state [20]. DPI, an inhibitor of flavin-containing oxidases [25], has been frequently used to block NADPH oxidase activity. In this work, we show that superoxide burst is produced in A431 cells upon VEGF treatment even in the presence of DPI, further supporting earlier observations that superoxide formation is not dependent on NADPH activity [26]. O_2^- species which are present extracellularly have the potential to contribute to the cell-permeable H_2O_2 species which can modulate the activity of intracellular protein tyrosine phosphatases [27, 28], thus modulating the phosphorylation of the receptor.

The role of PlGF in angiogenesis and endothelial cell biology is less known. We show here that PlGF treatment

induces intracellular ROS and that the increased ROS was dependent on extracellular O_2^- . Squelching of intracellular hydroxyl and/or O_2^- species by resveratrol inhibited the phosphorylation of p44 MAP kinase induced by PlGF in endothelial cells.

It can be speculated that superoxide may modify the lipid microdomain of the receptor that makes intracellular kinase activation permissive and initiate the assembly of a signaling complex by modification of the adaptor molecules. Recent reports show that VEGFR-2 is localized within caveolae/lipid rafts in the basal state [29]. Caveolin-1 binds to VEGFR-2 and negatively regulates receptor activity, and VEGF stimulates tyrosine phosphorylation of caveolin.

Taken together, our study provides evidence suggesting a significant role of extracellular O_2^- in mediating and initiating intracellular signaling due to PlGF/VEGFR-1 interaction, perhaps enhancing the established role of intracellular superoxide during ligand-receptor signaling. More important, this study revealed a new therapeutic target where an antioxidant might be sufficient to inhibit tumor cellular proliferation especially by inhibiting tumor vasculature.

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