Free Radical Biology & Medicine 49 (2010) 227–236

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

NADPH oxidase 4 mediates reactive oxygen species induction of CD146 dimerization in VEGF signal transduction

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ARTICLE INFO

Article history:
Received 5 December 2009
Revised 17 March 2010
Accepted 9 April 2010
Available online 18 April 2010

Keywords:
CD146 dimerization
NADPH oxidase
Small GTPase
VEGF signal transduction
Free radicals

ABSTRACT

CD146 dimerization plays an important role in tumor-induced angiogenesis. Stimulation of target cells with vascular endothelial growth factor (VEGF), a major angiogenic factor produced by tumor cells, elicits a burst of reactive oxygen species (ROS) that enhances angiogenesis. However, the molecular mechanism coupling CD146 dimerization with the VEGF-related oxidant-generating apparatus has not been elucidated. Here, we show that CD146 dimerization is induced by VEGF and is significantly diminished by pretreatment with diphenylene iodonium, an inhibitor of NADPH oxidase, suggesting a potential role for NADPH oxidase (NOX) in VEGF-induced CD146 dimerization. Importantly, we found that overexpression of NADPH oxidase 4 (NOX4), which is the predominant NOX expressed in endothelial cells, significantly enhances VEGF-induced ROS generation and CD146 dimerization. By contrast, these VEGF effects were dramatically attenuated after transfection with siRNA to reduce NOX4 expression. Furthermore, expression of Rac1 N17, a dominant negative mutant of Rac1, a member of the Rho family of small GTPases, suppressed VEGF-induced ROS generation and CD146 dimerization. These studies show for the first time that VEGF alteration of CD146 dimerization is mediated via a NOX4-dependent pathway and provide novel insight into the significant role of NOX in redox regulation of the dimerization of cell adhesion molecules.

Vascular endothelial growth factor (VEGF) has drawn significant attention in the past few years as an endothelial-cell-specific growth and survival factor and as a major inducer of angiogenesis under both physiological and pathological conditions [1]. VEGF is the major angiogenic factor produced by tumor cells and experimental approaches aimed at interfering with VEGF signaling have proven successful in counteracting tumor growth in vivo [2]. Studies on the VEGF signaling pathway have indicated that VEGF binding initiates the tyrosine kinase activity of the vascular endothelial growth factor receptor (VEGFR), which is then autophosphorylated, associating with itself to form a dimer. The activated receptor tyrosine kinases phosphorylate several cellular signaling proteins and form receptor complexes composed of Grb2, Shb, and TSAd, which result in activation of key angiogenic signaling enzymes including MAP kinases and Akt [1,3]. Despite many reports about the VEGF signaling pathway, the mechanisms regulating these pathways are not fully understood.

Recently, several lines of evidence have suggested that VEGF enhances the generation of reactive oxygen species (ROS) as second messengers in cell signaling and that ROS are involved in VEGFR2-mediated signaling, which is linked to endothelial cell migration and proliferation [4–6]. These reports indicate that cells possess cross-talk systems linking VEGF signaling pathways and the cellular redox state via the production of ROS. ROS are produced in mammalian cells in response to the activation of various cell surface receptors and contribute to intracellular signaling processes, which in turn regulate various biological activities including host defense and angiogenesis [7]. Superoxide anion ($O_2^{\bullet-}$), as one kind of the most active ROS, is mainly generated by NADPH oxidase (NOX) [8]. The radical anion is spontaneously or enzymatically converted to hydrogen peroxide (H_2O_2) in cells. To date, seven homologs (NOX1, NOX3, NOX4, NOX5, Duox1, and Duox2) of gp91phox (NOX2) have been identified in various nonphagocytic cells [9], of which NOX4 is most highly expressed in endothelial cells [5,6,10]. Rac1, a member of the Rho family of small GTPases, has been known to mediate multiple cellular responses such as cell adhesion, cell migration, actin reorganization, and cell cycle progression. Recent studies suggest that Rac1 plays an important role in the NOX regulation pathway [11] and that VEGF-induced ROS generation is mediated via Rac1 in endothelial cells [12]. These findings strongly suggest that both NOX4 and Rac1 are critical for VEGF-induced endothelial $O_2^{\bullet-}$ generation. Many adhesion molecules have been reported to be effector molecules in the VEGF signaling pathway, for example, VEGF up-regulates expression of intercellular adhesion molecule 1 (ICAM-1) via the PI3K/Akt/NO

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doi:10.1016/j.freeradbiomed.2010.04.007

pathway [13]. However, the molecular mechanisms coupling the signaling pathway involved in regulating the expression of some adhesion molecules with the VEGF-related oxidant-generating apparatus have not yet been elucidated.

CD146 was originally cloned from human melanoma cells as a melanoma-specific cell adhesion molecule [14] and was then shown to be expressed on circulating endothelial cells and to play an important role in angiogenesis [15]. We previously observed the dimerization of CD146 in living cells using the FRET method [16] and found that its dimerization could be induced and regulated in tumor-induced angiogenesis by the NF-κB pathway [17]. Many previous studies have reported that VEGF mediated tumor angiogenesis via the generation of ROS [4]. Based on these investigations, we hypothesize that CD146 dimerization has a possible link to the production of ROS in response to VEGF. Therefore, the goal of our study was to determine the potential role of ROS in VEGF-induced CD146 dimerization and the involvement of CD146 dimerization in the VEGF signaling pathway underlying VEGF-induced generation of ROS via NOX4 and Rac1.

Materials and methods

Materials

Human recombinant VEGF-A165 was obtained from Upstate Biotechnology. Mouse anti-human CD146 mAbs AA98 and AA1 were either used as hybridoma culture supernatants for biochemical analysis or purified from ascites using protein G–Sepharose (Santa Cruz Biotechnology) and used for functional assays. Rabbit anti-human CD146 polyclonal antibody RαCD146 was purified from rabbit polyclonal antiserum. Anti-human NF-κB p50 and IκBα were purchased from Santa Cruz. Anti-human phospho-NF-κB p65 was from Cell Signaling. HRP-conjugated goat anti-mouse or rabbit IgG antibodies and enhanced chemiluminescence assay kits were purchased from Pierce. 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), and FITC–phalloidin were from Molecular Probes. Diphenylene iodonium (DPI), vitamin C (Vc), *N*-acetyl-l-cysteine (NAC), catalase, anti-human β-actin, and normal mouse IgG were purchased from Sigma. Trizol reagent was from Invitrogen.

Cell culture and transfection

Primary human umbilical vein endothelial cells (HUVECs) were prepared from human umbilical cords as previously described [18]. The human embryonic kidney cell line HEK293T (ATCC CRL-1573) was cultured in Dulbecco's modified Eagle's medium (Gibco) with 1 g/L glucose, 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were grown at 37 °C and 5% CO_2. Fugene HD (Roche)-mediated transfection was performed according to the manufacturer's instructions.

Construction of NOX4 and Rac1

A cDNA encoding full-length human CD146 with its signal peptide was amplified by PCR using the pUC18-CD146 plasmid as a template (kindly provided by Dr. Judith P. Johnson, University of Munich, Germany). The PCR products of the CD146 gene were then inserted into the pCDNA3.1(−)b vector. cDNAs encoding human Rac1 and NOX4 were prepared as previously described [19]. The amplified PCR products encoding human Rac1 and NOX4 were inserted into a pCDNA3.1(−)b vector. Rac1 N17 was generated using site-directed mutagenesis in the pcDNA3.1(−)b vector.

Determination of ROS

ROS production was measured using the DCFH-DA assay as described previously [20,21]. In brief, HUVECs treated with or without VEGF for 15 min were washed with RPMI 1640 medium and were incubated in the dark with DCFH-DA (5 μM) for 15 min at 37 °C. The cells were harvested, washed once, and resuspended in PBS. Fluorescence was monitored using a flow cytometer (Becton–Dickinson FACSort). Mean DCF fluorescence intensity was obtained from 10,000 cells using excitation and emission wavelengths of 480 and 540 nm, respectively. Fluorescence levels were expressed as percentage increases over the control.

Western blotting

Total cellular protein extracts were prepared in radioimmunoprecipitation (RIPA) lysis buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 8.0, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 25 μg/ml aprotinin) and were transferred to Hybond membranes after separation by 8% SDS–PAGE. The membranes were blocked with 5% milk in PBS for 1 h, incubated for 2 h with primary antibodies, and then probed for 1 h with HRP-conjugated anti-mouse or anti-rabbit IgG. After extensive washes with PBS containing 0.05% (v/v) Tween-20 (PBST), the target proteins were detected on the membranes by enhanced chemiluminescence.

Immunoprecipitation

HUVECs were lysed in a culture dish by adding 0.6 ml ice-cold RIPA lysis buffer. The supernatants were collected by centrifugation at 12,000 *g* at 4 °C for 10 min and then precleared with protein G–Sepharose to remove the protein G-bound proteins. The total amount of protein in the precleared supernatants was measured using a Bradford assay kit (Bio-Rad). Each sample was immunoprecipitated with either mAb AA98 or control mIgG at 4 °C for 2 h, followed by incubation with protein G–Sepharose for 1 h. Immunoprecipitates were washed twice with lysis buffer and then boiled for 5 min in loading buffer.

Confocal immunofluorescence microscopy

HUVECs were plated on coverslips and cultured in six-well plates. After stimulation for 24 h with VEGF (50 ng/ml), the cells were washed with PBS, fixed in 4% cold paraformaldehyde (PFA) in PBS for 10 min, and then permeabilized with 0.1% Triton X-100. After being washed with PBS, the cells were blocked in 5% normal goat serum for 30 min and then incubated with FITC-conjugated phalloidin at 37 °C for 30 min. Finally, the coverslips were examined with a confocal laser scanning microscope (Olympus, Tokyo, Japan). For the analysis of F-actin stress fiber rearrangement, the scoring method described by Peiffer et al [22] was used. In brief, cells that showed a well-organized F-actin network were scored as 1. Cells that showed atypical or equivocal F-actin disorganization were scored as 0.5. Cells that showed no well-organized F-actin network were scored as 0. More than 200 individual cells were examined for each assay. Apical F-actin alteration indices were calculated by dividing total points by the total number of cells examined and multiplying the result by 100.

NOX4 siRNA and reverse transcription PCR

RNAi was performed according to the method of Elbashir et al. [23]. The double-stranded siRNA targeting NOX4 was prepared by Invitrogen. siRNA sequences were as follows: 5′-GUCAACAUCCAGCUGUACCdtdt-3′ (sense), 5′-GGUACAGCUGGAUGUUGACdtdt-3′ (antisense). RNA was subjected to reverse transcription PCR (RT-PCR) using a first-strand cDNA synthesis kit (Invitrogen) with a pair of specific primers: NOX1, forward primer, 5′-GCAAGATCTGTTGTTATGCACCCATCCAA-3′, and reverse primer, 5′-GCTGGTACCTCAAAAATTTTCTTTGTTGAAGT-3′; NOX2, forward primer, 5′-GGAGGATCCGTGGTCACTCACCCTTTCAA-3′, and reverse primer, 5′-CCACTCGAGCTCATGGAAGAGACAAGTTAG-3′; NOX3,