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,

forward primer, 5′-GCAGGATCCGTGGTAAGCCACCCCTCTG-3′, and reverse primer, 5′-GCTGAATTCAGAAGCTCTCCTTGTTGTAAT-3′; NOX4, forward primer, 5′-GCAGGATCCGTCATAAGTCATCCCTCAGA-3′, and reverse primer, 5′-GCTGTTAACGTCGACTCAGCTGAAAGACTCTTTAT-3′; NOX5, forward primer, 5′-GCAGGATCCACTATCTGGCTGCACATTCG-3′, and reverse primer, 5′-GCTGAATTCCTAGAAATTCTCTTGGAAAAATC-3′; p22phox, forward primer, 5′-ATGGAGCGCTGGGGACAGAAGCACATG-3′, and reverse primer, 5′-GATGGTGCCTCCGATCTGCGGCCG-3′.

Tube formation

The tube formation assay was performed as described by Nagata et al. [24]. Briefly, 24-well culture plates (Costar, Corning, Inc.) were coated with growth factor-reduced Matrigel (BD Biosciences) in a total volume of 200 μl/well and allowed to solidify for 30 min at 37 °C. Cells transiently transfected with the appropriate siRNA or plasmids were trypsinized, harvested, and resuspended at $5\times s10^5$/ml in complete RPMI 1640 medium. Then 200 μl of this cell suspension was added to each well and the cells were incubated at 37 °C overnight. In the case of testing the effect of CD146 dimerization on VEGF-induced tube formation, appropriate concentrations of VEGF were added directly to the cell suspensions, which were then seeded into the corresponding wells. Tube formation was observed under an inverted microscope (Eclipse Model TS100; Nikon, Japan). Images were captured with a CCD color camera (Model KP-D20AU; Hitachi, Japan) attached to the microscope and tube length was measured using NIH ImageJ software.

Cell migration assay

Cell migration was assayed using a modified Boyden chamber assay as described (8-μm pore size; Costar, Corning, NY) [25]. After the appropriate transfections, cells were trypsinized, washed, and resuspended in fresh serum-free medium (10,000 cells per well). Lower chambers contained fresh medium containing 20% FCS. VEGF (50 ng/ml) was simply added to the upper chambers when cells were seeded. After incubation at 37 °C overnight, cells remaining at the upper surface of the membrane were removed using a swab, whereas the cells that migrated to the lower membrane surface were fixed with 4% PFA and stained with Giemsa solution. The number of cells migrating through the filter was counted and plotted as the number of migrating cells per optic field (×20).

Statistical analysis

All experiments were done in triplicate. Representative experiments or mean values ± SD are shown. Statistical differences were determined with the Student *t* test. A *p* value of 0.05 was considered significant.

Results

CD146 dimerization is required for VEGF-induced tube formation and signal transduction

Our group has previously found that CD146 dimerization in endothelial cells is inducible and regulatable by the NF-κB pathway in tumor-induced angiogenesis [16]. To study tumor-induced angiogenesis, VEGF, an important tumor-secreted cytokine, was used to stimulate human endothelial cells. We utilized a CD146 mutant, CD146/C452A, which is not able to form dimers during tumor-induced angiogenesis, to test the role of CD146 dimerization in VEGF-induced angiogenesis [17]. The tube formation assay, an in vitro test for angiogenesis, was employed to examine changes in the morphology and function of endothelial cells transfected with CD146/C452A under treatment with VEGF-A165, a prominent growth factor involved in angiogenesis. The CD146/C452A overexpression in transfected HUVECs was analyzed by Western blotting (Supplemental Fig. 1). HUVECs transfected with various plasmids were placed on Matrigel overnight and the results showed that VEGF promoted tube formation. However, cells transfected with CD146/C452A did not form capillary-like tubes in response to VEGF (Figs. 1A and D). Moreover, transwell migration assays also confirmed that the increased motility of endothelial cells in response to VEGF required CD146 dimerization (Figs. 1B and E). Because recently reported evidence suggests that VEGF induces actin stress fiber formation [26], we analyzed the roles of CD146 dimerization in VEGF-induced actin cytoskeletal reorganization using confocal microscopy. We found that CD146/C452A significantly reduced VEGF-stimulated actin stress fiber formation (Figs. 1C and F). Therefore, our data indicate that CD146 dimerization plays a crucial role in the pathological angiogenesis stimulated by VEGF.

Previous studies indicated that inhibition of NF-κB activation blocks angiogenesis [27] and knockdown of CD146 expression could suppress NF-κB activation [17]. To investigate whether CD146 dimerization contributed to the activation of the NF-κB signal pathway, HUVECs transfected with CD146/C452A were treated with VEGF, and then the whole-cell lysates were subjected to Western blotting analysis. As shown in Figs. 2A–C, the phosphorylation of NF-κB p65 and the decreasing expression of IκBα from treatment with VEGF were inhibited by CD146/C452A. Immunofluorescent staining of NF-κB p50 also confirmed that the nuclear translocation of NF-κB p50 induced with VEGF was suppressed by CD146/C452A (Fig. 2D). In conclusion, CD146 dimerization was essential for VEGF-induced NF-κB activation.

VEGF stimulates CD146 dimerization but not overexpression in HUVECs

Because the dimer has been implicated as the functional form of CD146 in tumor-induced angiogenesis and signal transduction [17], we investigated whether VEGF affected CD146 dimerization in HUVECs. CD146 dimerization was detected by coimmunoprecipitation (Co-IP) after HUVECs were treated with VEGF for various times. Relative dimerization efficiency was calculated from band densities of Western blot results as described under Materials and methods. Co-IP and Western blot assay results show that VEGF markedly enhanced the dimerization of CD146 after treatment for 1 h (Figs. 3A and B). Because VEGF can induce the overexpression of many adhesion molecules, such as ICAM-1 [13,28], we investigated whether CD146 was up-regulated after the treatment with VEGF. HUVECs were incubated with VEGF for various lengths of time and its effects on CD146 expression at the protein (Fig. 3C) and mRNA levels (Fig. 3D) were analyzed by Western blotting or RT-PCR. The results showed that VEGF could not induce CD146 overexpression in HUVECs at the protein or mRNA level.

VEGF-induced CD146 dimerization is accompanied by ROS production

As VEGF has previously been reported to trigger ROS production in HUVECs [4], we examined whether that was also the case in our experiments. ROS production in HUVECs treated with VEGF was measured using a fluorescence-based DCFH-DA assay and analyzed by FACS. HUVECs treated with VEGF showed a time-dependent increase in the intensity of DCF fluorescence. The fluorescence increased 1.5-fold after 15 min of stimulation and declined after 30 min (data not shown). VEGF could induce a burst of ROS as early as 5 min after treatment; the explanation of this phenomenon is that VEGF rapidly promotes translocation of Rac1 from the cytosol to the membrane, thereby activating NADPH oxidase in endothelial cells [29]. Furthermore, vitamin C, NAC, or catalase (CAT) could abolish VEGF-induced ROS production (Figs. 4A, B, E, and F). Because ROS play an important role in VEGF signal transduction [4], the role of ROS in VEGF-stimulated CD146 dimerization was then investigated. HUVECs were

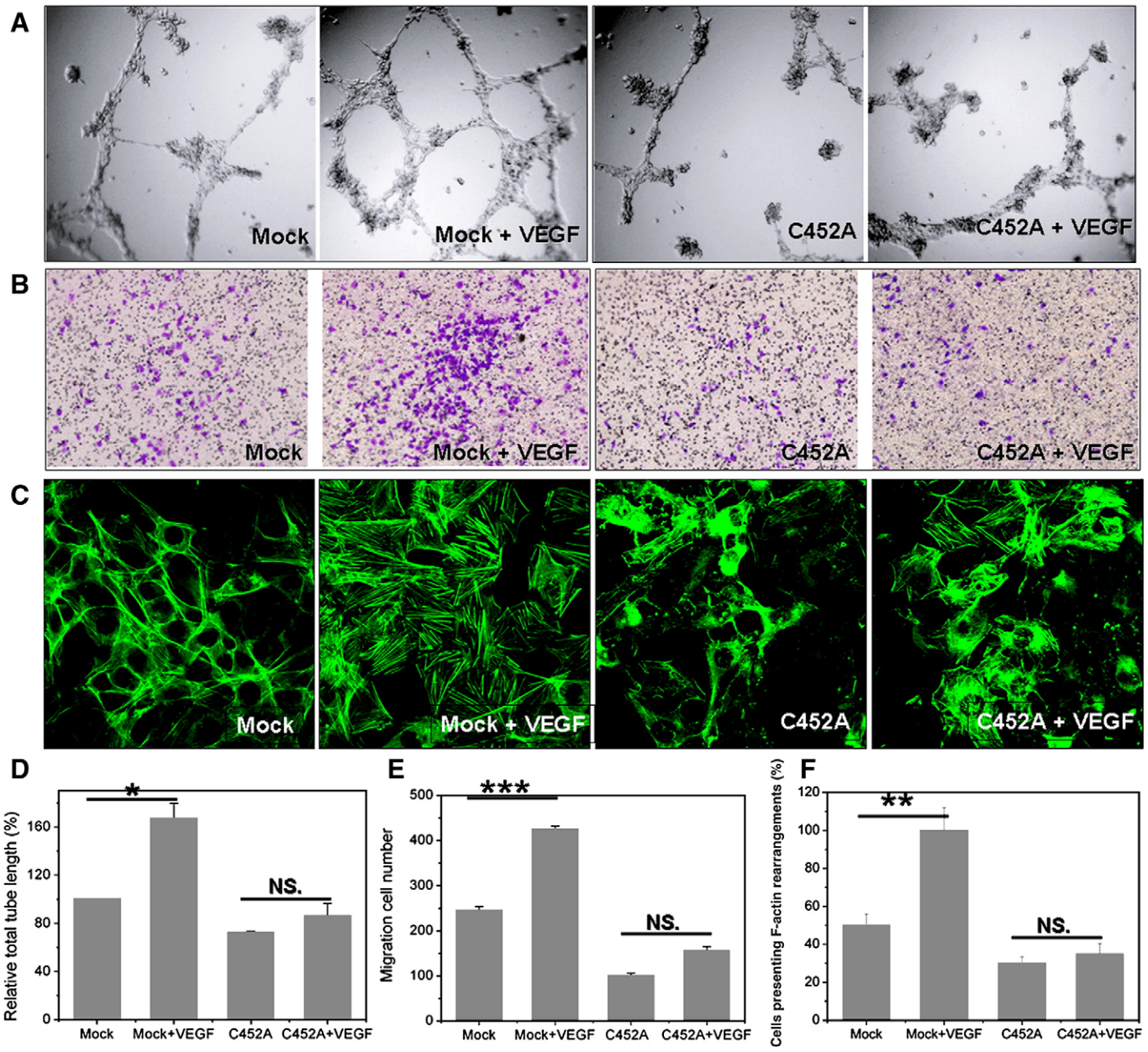

Fig. 1. CD146 dimerization is required for VEGF-induced tube formation. VEGF was used to stimulate HUVECs transfected with empty vector (mock) or CD146/C452A. (A) Cells given the treatments indicated were subjected to tube formation assays, (D) the results of which were analyzed by calculating total tube lengths. The bar graph presents the relative total tube length from at least three independent tests. (B) Cells were also subjected to migration assays using a transwell system, and (E) the number of cells migrating through the filters was counted. Results from at least three independent assays are presented in the bar graph as means ± SD of the number of cells per optic field. (C) Cells were stained with FITC–phalloidin, and actin cytoskeletal reorganization was observed by confocal microscopy, and (F) the F-actin rearrangements were quantified as described by Peiffer et al. [22].

preincubated with Vc, NAC, or CAT for 15 min and then treated with VEGF for 24 h. Co-IP and Western blotting were performed and results showed that Vc, NAC, or CAT inhibited VEGF-induced CD146 dimerization by reducing the production of ROS (Figs. 4C, D, G, and H).

To study whether H_2O_2 could directly induce CD146 dimerization, HUVECs were treated with various concentrations of H_2O_2 for 15 min or with 100 μM H_2O_2 for various times. The results indicated that CD146 dimerization was induced at higher H_2O_2 concentrations and longer treatment times (Figs. 5A–D).

NOX4 is indispensable in VEGF-induced CD146 dimerization

Recent studies have shown that NOX is frequently involved in ligand-dependent generation of ROS [4]. We therefore investigated the role of NOX in VEGF signal transduction, using DPI as a specific pharmacological inhibitor of NOX. HUVECs were preincubated with DPI for 15 min, and intracellular ROS levels were investigated after treatment with VEGF for 15 min. Upon preincubation with NOX inhibitors, the VEGF-induced ROS production was totally abolished, clearly demonstrating that NOX contributed to ROS production in VEGF signal transduction (Figs. 6A and B). We also found that DPI could reduce VEGF-enhanced CD146 dimerization (Figs. 6C and D). These results suggest that VEGF stimulates ROS production and CD146 dimerization in HUVECs via NOX.

Several isoforms of NOX, such as NOX2 and NOX4, have been identified in endothelial cells. NOX4 has been shown to be the predominant isoform in HUVECs [6] and a distinct NOX4 band was also identified by RT-PCR in our studies (Supplemental Fig. 2A). However, NOX2, which has been reported to contribute to endothelial ROS production and proliferation, was not detected in HUVECs but

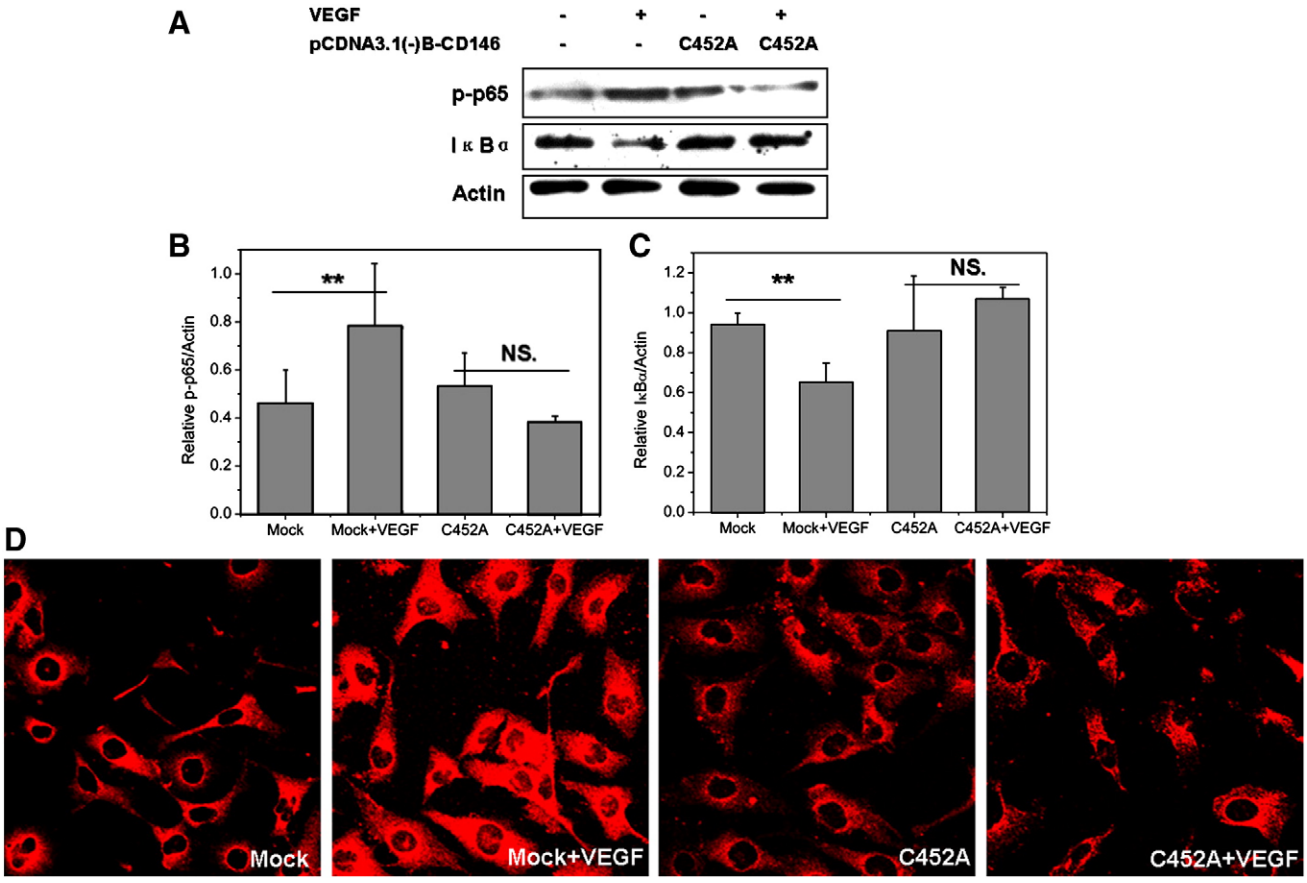

Fig. 2. CD146 dimerization is essential for VEGF signal transduction. VEGF was used to stimulate HUVECs transfected with empty vector (mock) or CD146/C452A. Cells were employed in (A) Western blotting using whole-cell extracts or (D) immunostaining with specific anti-human NF-κB p-50 antibody. (B and C) The results of Western blotting were quantified by measuring the band density and then normalized to actin. The bar graphs present the relative values from at least three independent tests.

was observed at high levels in HEK293T cells as a positive control (Supplemental Fig. 2A). Our conclusion is that NOX4 is the predominant oxidase: transcripts of NOX homologs other than NOX4 were barely detectable in endothelial cells. To demonstrate the role of NOX4 in mediating VEGF-induced ROS, we employed a vector encoding NOX4. NOX4 overexpression in transfected HUVECs was confirmed by RT-PCR analysis (Supplemental Fig. 2C). The level of NOX4 mRNA was reduced by over 80% in HUVECs transfected with NOX4 siRNA compared to control cells transfected with scrambled RNA (Supplemental Fig. 2B). We found that NOX4 overexpression

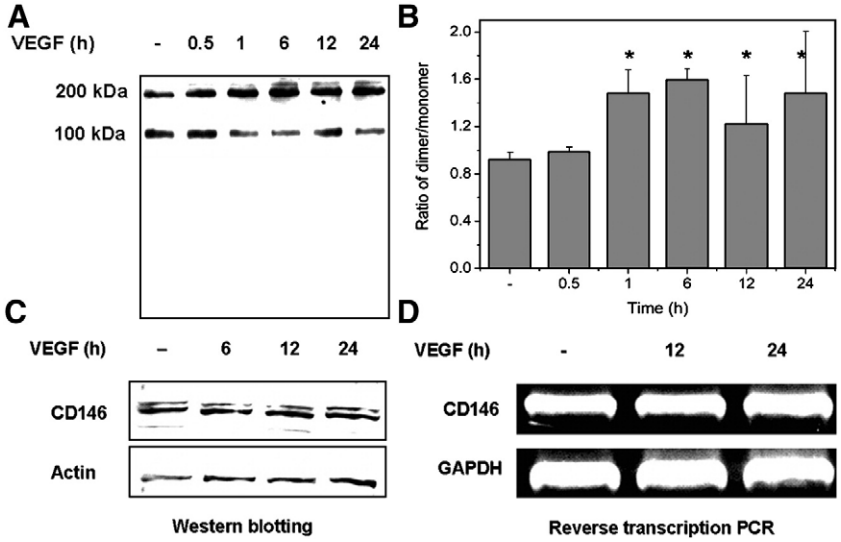

Fig. 3. CD146 dimerization is enhanced by VEGF. VEGF was used to stimulate HUVECs for the times indicated. Whole-cell lysates were immunoprecipitated with anti-CD146 mAb AA98, followed by Western blotting with rabbit anti-CD146 polyclonal antibody. (A) The CD146 dimer (200 kDa) and monomer (100 kDa) were specifically recognized by the two antibodies. (B) The ratio of CD146 dimers to monomers was calculated from band densities. The bar graph presents the means ± SD of relative dimerization efficiencies from three independent tests. Cells were treated with VEGF for the times indicated, and expression of CD146 at the (C) protein and (D) mRNA levels was analyzed.

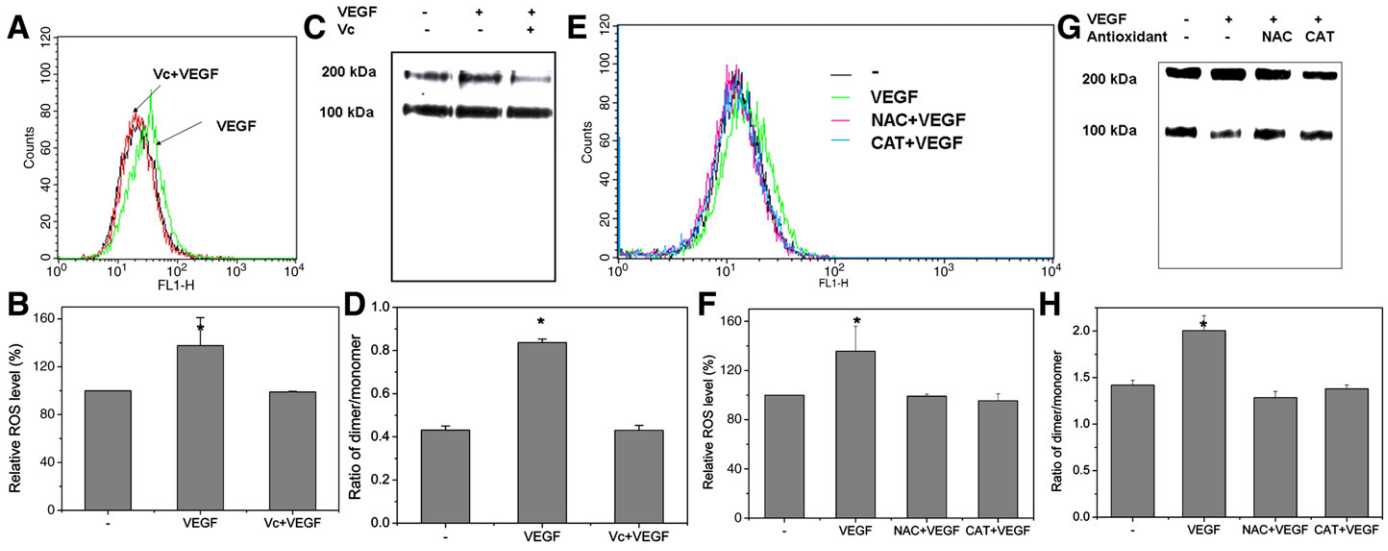

Fig. 4. VEGF-induced ROS generation is required for CD146 dimerization. HUVECs were preincubated with (A) Vc or (E) NAC or catalase and then treated with VEGF for indicated times. ROS generation in cells given the treatments indicated was monitored by FACS analysis of DCF fluorescence. (B, F) The fluorescence intensity relative to unstimulated cells is shown. (C, G) CD146 dimerization efficiency was analyzed by immunoprecipitation with the anti-CD146 mAb AA98, followed by Western blotting with rabbit anti-CD146 polyclonal antibody. (D, H) The ratio of CD146 dimers to monomers was calculated from the band density. The bar graphs present the means ± SD of relative dimerization efficiencies from three independent tests.

enhanced VEGF-induced ROS generation, whereas NOX4 siRNA completely blocked it, suggesting that NOX4 is essential for VEGF-induced ROS production in HUVECs (Figs. 7A–D). To determine the role of NOX4 in CD146 dimerization, NOX4 and NOX4 siRNA were transfected. Induction of CD146 dimerization by VEGF was significantly enhanced by NOX4 transfection; however, VEGF-enhanced CD146 dimerization was completely blocked by NOX4 siRNA (Figs. 7E–H). In conclusion, NOX4 played an important role in VEGF-induced CD146 dimerization.

Data in this study clearly show that the rapid induction of ROS generation requires activation of NOX. The activation of NOX is apparently followed by feedforward up-regulation of the NOX complex. We have previously demonstrated that VEGF-induced ROS generation is enhanced by NOX4 overexpression and inhibited by NOX4-specific siRNA, suggesting that NOX4 plays a major role in VEGF-induced ROS generation and CD146 dimerization. To assess whether VEGF induction of ROS could feedforward the up-regulation of NOX4, RT-PCR of NOX4 was performed after VEGF treatment for the

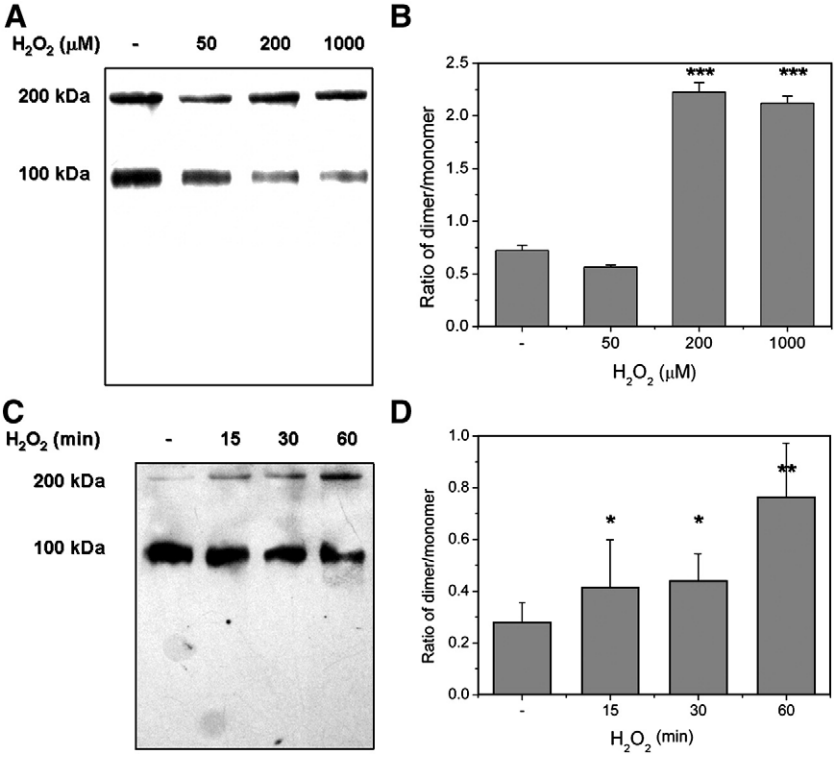

Fig. 5. Dose and time response of H_2O_2-induced CD146 dimerization. HUVECs were incubated (A) with various concentrations of H_2O_2 for 15 min or (C) with 100 μM H_2O_2 for various times, and immunoprecipitation and CD146 dimerization were analyzed by Western blotting. (B, D) The ratio of CD146 dimers to monomers was calculated from the band density. The bar graphs present the means ± SD of relative dimerization efficiencies from three independent tests.

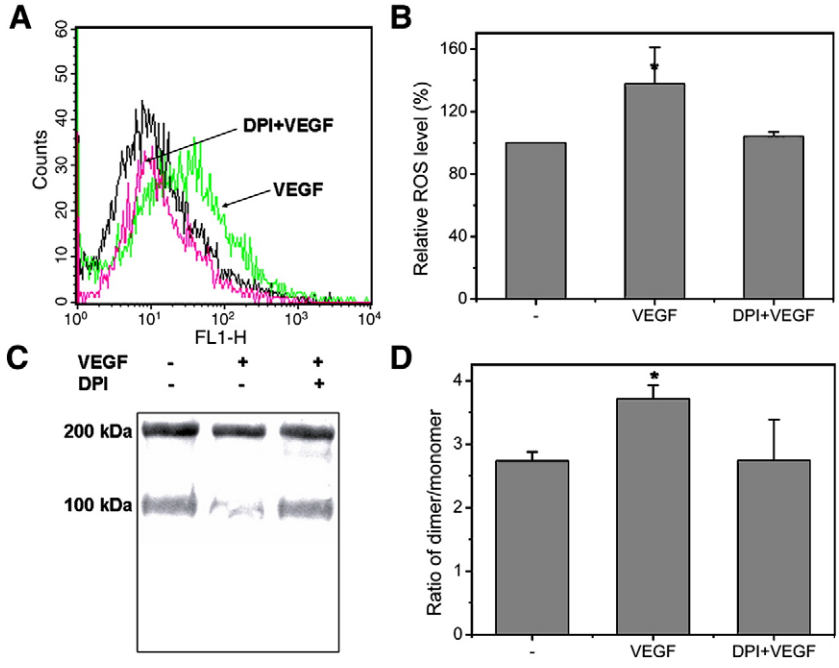

Fig. 6. NOX homologs are indispensable for VEGF-induced CD146 dimerization. HUVECs were preincubated with DPI (5 μM), a NOX inhibitor, and then treated with VEGF. (A) ROS generation in cells given the treatments indicated was monitored by FACS analysis of DCF fluorescence. (B) The fluorescence intensity relative to unstimulated cells is shown. (C) CD146 dimerization efficiency was analyzed by immunoprecipitation with anti-CD146 mAb AA98, followed by Western blotting with rabbit anti-CD146 polyclonal antibody. (D) The ratio of CD146 dimers to monomers was calculated from band densities. The bar graphs present the means ± SD of relative dimerization efficiency from three independent tests.

lengths of time indicated. VEGF significantly increased NOX4 expression, supporting the notion that VEGF treatment enhanced intracellular ROS by increasing the activity and/or expression of NOX4 (Figs. 8A and B). The expression of an important component of NOX4, p22phox, was analyzed after VEGF treatment. VEGF obviously increased p22phox expression at the transcript level after 24 h treatment (Figs. 8A and C).

Rac1 plays crucial roles in VEGF-induced CD146 dimerization

Recently, there has been intense interest in the role of Rac1, a member of the Rho family of small GTPases in signal transduction. It has emerged that the signaling pathways affected through Rho GTPase activation are not limited to those related to the actin cytoskeleton as originally described, but also include, among others, regulation of ion

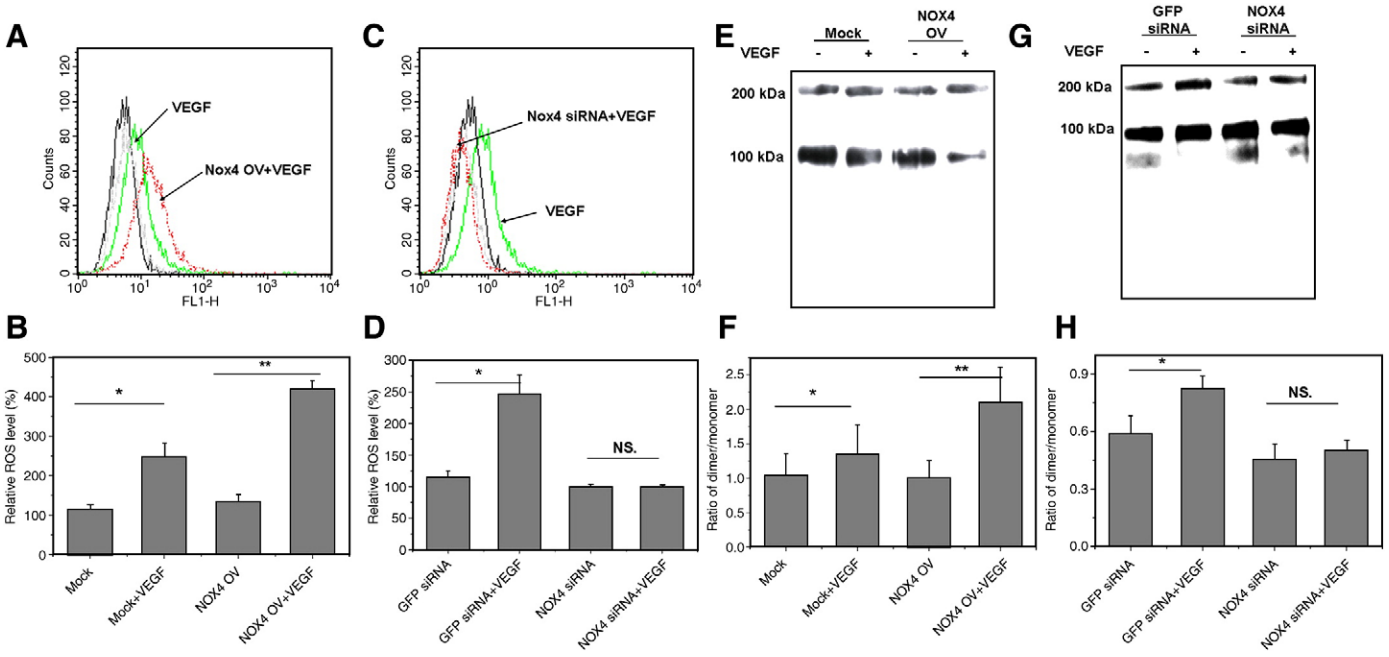

Fig. 7. Effects of NOX4 overexpression and knockdown on VEGF-mediated CD146 dimerization. HUVECs transfected with (A) NOX4 or (C) NOX4 siRNA for 24 h were incubated with or without VEGF. ROS generation was monitored by FACS analysis of DCF fluorescence in the absence or presence of VEGF. (B, D) The fluorescence intensity relative to unstimulated cells is shown and (E, G) CD146 dimerization efficiency was analyzed by immunoprecipitation with anti-CD146 mAb AA98, followed by Western blotting with rabbit anti-CD146 polyclonal antibody. (F, H) The ratio of CD146 dimers to monomers was calculated from the band density. The bar graphs present the means ± SD of relative dimerization efficiencies from three independent tests.

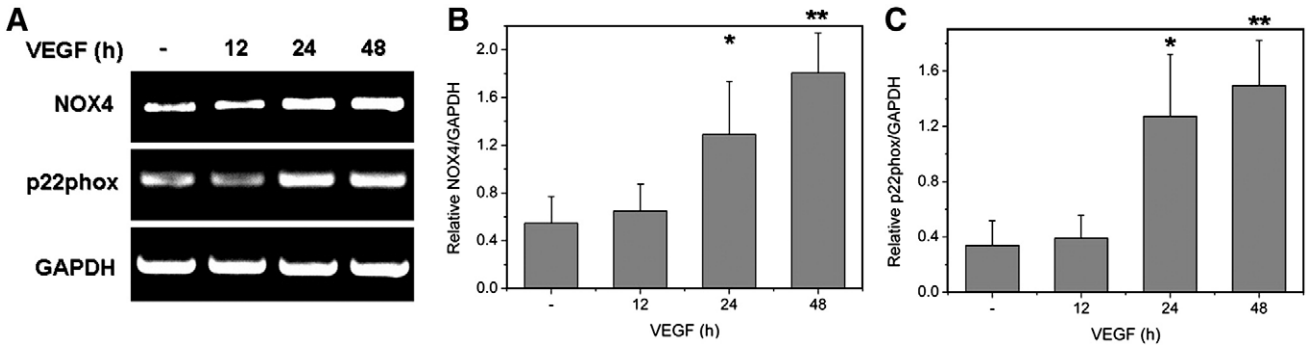

Fig. 8. Effects of VEGF on NOX4 and p22phox expression. (A) HUVECs were stimulated with VEGF for the times indicated, and NOX4 and p22phox expression was quantified by reverse transcription PCR. (B, C) The results of reverse transcription PCR were quantified by measuring the band density and then normalized to GAPDH. The bar graphs present the relative values from at least three independent tests.

channel activity and endothelial permeability [30]. Moreover, Rho GTPases have been implicated in ROS production. Rac1 is a component of the NOX complex and has been shown to contribute to oxygen sensing [11]. To investigate the role of Rac1 in VEGF-induced ROS generation, we infected HUVECs with Rac1 N17, a dominant negative mutant of Rac1 [20]. Expression of Rac1 N17 in the infected HUVECs was confirmed by RT-PCR analysis (Supplemental Fig. 2D). HUVECs expressing Rac1 N17 showed no increase in ROS generation in response to VEGF treatment (Figs. 9A and B). We then analyzed the effects of Rac1 N17 on VEGF-induced CD146 dimerization and found that VEGF-induced CD146 dimerization was completely blocked by Rac1 N17 (Figs. 9C and D). These results indicate that Rac1 plays an important role in VEGF-induced CD146 dimerization.

Discussion

Many previous studies have found that the dimerization of cell adhesion molecules is crucial for cell function and signal transduction. For example, ICAM-1 homodimers support greater levels of lymphocyte functional antigen-1-dependent cell adhesion than the monomer form [31] and clustered PECAM-1 activates the integrin-requiring adhesive properties and signal transduction of vascular cells [32]. Our group has also found that CD146 dimerization plays an important role in tumor-induced angiogenesis [17]. In this study, we used CD146/C452A to disrupt CD146 dimerization in the process of VEGF-induced angiogenesis and demonstrated that CD146 dimerization was required for VEGF-induced tube formation, migration, and actin rearrangement (Fig. 1). Given these observations, we concluded that CD146 dimerization or oligomerization is essential for VEGF-induced angiogenesis.

It has been found that inflammatory cytokines and growth factors regulate the expression of some adhesion molecules through signal transduction. Expression of leukocyte–endothelial adhesion molecules such as ICAM-1 is up-regulated by the VEGF-mediated PI3K/Akt/NO pathway [13], and lipopolysaccharides (LPS) can induce CD44 overexpression mediated by the JNK signaling pathway [33]. But the

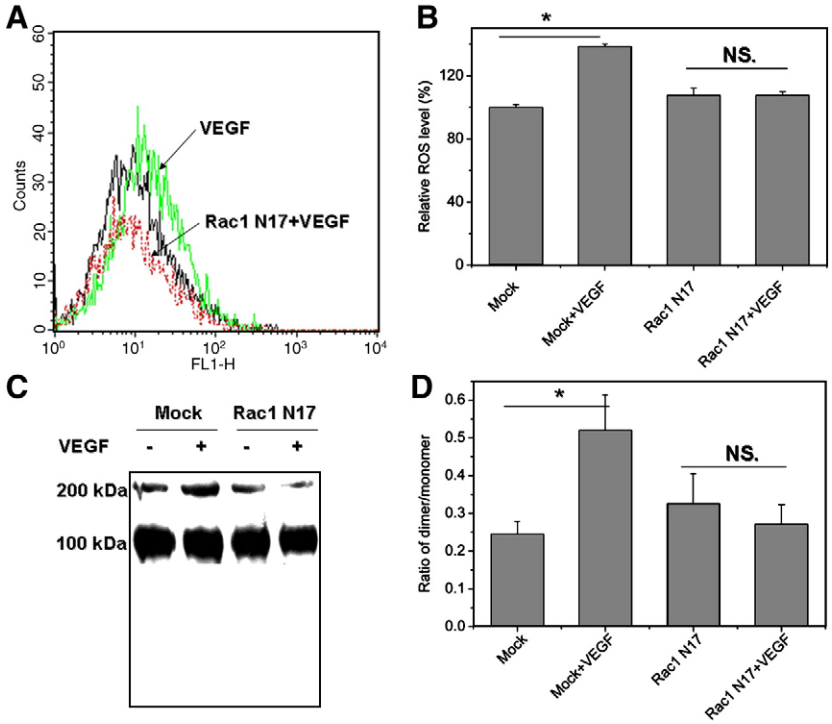

Fig. 9. Effect of Rac1 on VEGF-mediated CD146 dimerization. HUVECs transfected with a dominant negative mutant of Rac1, Rac1 N17, for 24 h were treated with or without VEGF. (A) The generation of ROS was monitored by FACS analysis of DCF fluorescence in the absence or presence of VEGF. (B) The fluorescence intensity relative to unstimulated cells is shown and (C) CD146 dimerization efficiency was analyzed by immunoprecipitation with anti-CD146 mAb AA98, followed by Western blotting with rabbit anti-CD146 polyclonal antibody. (D) The ratio of CD146 dimers and monomers was calculated from the band density. The bar graphs present the means ± SD of relative dimerization efficiencies from three independent tests.

expression of CD146 is not regulated by cytokines such as LPS, endothelial cell growth factor, or interleukin-6 [15]. Recently, Mangahas et al. reported that endothelin-1 up-regulates CD146 expression in primary human melanocytes, probably through the phosphorylation of cAMP-responsive element binding protein [34]. The need for up-regulation of CD146 expression may simply reflect a quantitative requirement for VEGF signal transduction; however, VEGF treatments applied here did not enhance the expression of CD146 (Figs. 3C and D), indicating that CD146 overexpression is not essential for VEGF signal transduction. In addition to changes in expression levels, the extent of dimerization varied in different cell lines or under different cell conditions, suggesting that this process is under cellular control and plays an important role in signal transduction. In fact, VEGF triggered CD146 dimerization, indicating that VEGF induction of tube formation is due to its interference with the equilibrium between monomeric and dimeric forms, it favoring CD146 dimerization (Figs. 3A and B). These results also suggest that CD146 dimerization is regulated by VEGF. However, the detailed mechanisms of the regulation of CD146 dimerization in VEGF signal transduction require further investigation.

It is known that the redox state plays a role in the regulation of the dimerization of adhesion molecules/receptors and that antioxidants are potential agents for inhibiting their dimerization. Kato et al. [35] have reported that ultraviolet light (UV) induces Ret kinase dimerization through the production of UV-induced free radicals. Kamata et al. [36] have demonstrated that the antioxidant NAC markedly suppresses epidermal growth factor-induced epidermal growth factor receptor (EGFR) dimerization. Here, we found that antioxidants such as Vc, NAC, and catalase could abrogate VEGF-induced CD146 dimerization (Figs. 4C, D, G, and H). In contrast, H_2O_2, a major ROS, dramatically enhanced CD146 dimerization, indicating that CD146 dimerization is regulated by redox state (Figs. 5A–D). Furthermore, NOX is a critical component for ROS generation [4] and is required for up-regulation of cell adhesion molecules in endothelial cells [37,38]. For example, the lipid raft-dependent NOX/JAK/EGFR signaling pathway regulates the expression of cell adhesion molecules in brain endothelial cells and adhesion of leukocytes to endothelial monolayers [37]. Up-regulation of E-selectin expression in human coronary artery endothelial cells is dependent on the activation of endothelial NOX via an enhanced membrane translocation of p47phox [38]. Because NOX4 is the major NOX component present in HUVECs, we transfected siRNA to reduce NOX4 expression and research NOX4 function in CD146 dimerization. Down-regulation of NOX4 attenuated VEGF-induced ROS generation and CD146 dimerization (Figs. 7C, D, G, and H). These results suggest that NOX4 is the major source of VEGF-induced ROS generation, which is important for CD146 dimerization. Moreover, Rac1 has been reported to be involved in the activation of NOX in endothelial cells and is indispensable for ROS generation in response to VEGF [11]. In the present work, transfection of Rac1 N17, a dominant negative mutant of the Rho family of small GTPases (Rac1), abrogated VEGF-induced ROS generation and CD146 dimerization (Fig. 9), indicating that Rac1 is required for VEGF-induced ROS generation and CD146 dimerization. These results demonstrate that NOX4 and Rac1 are essential for VEGF-induced CD146 dimerization.

To summarize our results, we propose a model for the regulation of CD146 dimerization in the VEGF signaling pathway. The binding of VEGF to VEGFR2 induces VEGFR2 dimerization, which, in turn, activates Rac1 to form a complex with NOX4. The formation of NOX4–Rac1 complexes results in a burst of ROS that enhances CD146 dimerization and further promotes angiogenesis (Fig. 10). Our findings indicate that VEGF alteration of CD146 dimerization is mediated via a NOX4-dependent pathway. Importantly, it provides novel insights into the role of NOX as a major player in redox-regulation of the dimerization of cell adhesion molecules.

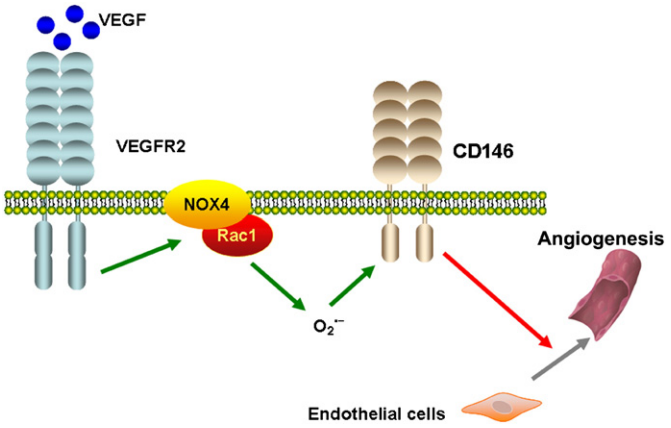

Fig. 10. A proposed model for the regulation of CD146 dimerization in the VEGF signaling pathway. The binding of VEGF to VEGFR2 induces VEGFR2 dimerization, which, in turn, activates Rac1 to form a complex with NOX4. The formation of NOX4–Rac1 complexes results in a burst of ROS that enhances CD146 dimerization and further promotes angiogenesis.

Acknowledgments

This work was partly supported by the National High-Tech R&D Program of China (863 Program) (2006AA02A245), the National Basic Research Program of China (973 Program) (2006CB910901, 2006CB933204, 2009CB521704), National S&T Major Project (2009ZX09102-247, 2008ZX10002-017), the National Natural Science Foundation of China (30672436), and the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-YW-R-121, KSCX2-YW-G-044, KSCX2-YW-R-173). This work is also supported by a contract research fund from the Ministry of Education, Culture, Sports, Science and Technology for Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases (2006DFB32010).

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2010.04.007.

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