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VEGF Induces Nuclear Translocation of Flk-1/KDR, Endothelial Nitric Oxide Synthase, and Caveolin-1 in Vascular Endothelial Cells

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VEGF increases endothelial cell permeability and growth by a process requiring NOS activity. Because eNOS activity is regulated by its interaction with the caveolar structural protein caveolin-1, we analyzed VEGF effects on structural interactions between eNOS, caveolin-1 and the VEGF receptor Flk-1/KDR. Confocal immunolocalization analysis of the subcellular distribution of Flk-1/KDR, caveolin-1 and eNOS showed that VEGF stimulated the translocation of all three proteins into the nucleus. This result was confirmed by cell fractionation and immunoblotting studies showing that levels of all three proteins within the caveolar compartment declined progressively after 30 and 60 min of VEGF treatment. The pattern was reversed for nuclear fractions. Protein levels were lowest in the control cultures, but increased progressively after 30 and 60 min of treatment. Nuclear translocation of eNOS and Flk-1/KDR within caveolae may represent a mechanism for targeting NO production to the nuclear compartment where it could influence transcription factor activation.

Vascular endothelial cell growth factor (VEGF) was first discovered as a tumor-secreted protein that induced a transient and reversible hyperpermeability (1). VEGF was subsequently recognized as a potent mitogen that stimulates both growth and migration of vascular endothelial cells (2,3). VEGF acts on endothelial cells through two receptor tyrosine kinases known as Flk-1/KDR and Flt-1. Flk-1/KDR is involved primarily in signaling the vascular permeability and mitogenic responses, whereas Flt-1 is required for endothelial cell morphogenesis (4-10) for review see (11). VEGF signaling is initiated by ligand-induced receptor autophosphorylation and tyrosine phosphorylation of various cellular substrates, followed by a transient accumulation of intracellular Ca^{2+} and inositol 1,4,5-trisphosphate and tyrosine kinase-dependent activation of endothelial nitric oxide synthase (eNOS, 12).

NOS activity has been shown to be required for VEGF's vascular growth promoting actions under experimental conditions in vivo and in vitro (13,14). VEGF's effects in increasing permeability and producing vasorelaxation of isolated coronary arteries and in enhancing permeability of subcutaneous vessels also require NOS activity (10,15). Recently, we have demonstrated that VEGF increases the permeability of cultured microvascular endothelial cells by stimulating caveolae-mediated transyctotic transport in a NOS-dependent process (16).

Caveolae are specialized membrane microdomains which appear as flask-shaped invaginations on the cell surface of many cell types. They contain one of a family of structural proteins called caveolins, as well as numerous signaling proteins and a high cholesterol content. Caveolae were originally described as an intracellular compartment involved in endocytotic and transcytotic transport, but now are recognized as having the additional functions of 1) concentrating and internalizing small molecules by a process called potocytosis and 2) serving as signal transduction organizing centers for segregating and concentrating membrane receptors with downstream effectors (17-22). Because a significant portion of eNOS is localized to caveolae, it is thought that NO signaling in endothelial cells also occurs within the caveolar compartment (23-26). Moreover, agonist-induced activation of eNOS in aorta endothelial cells has been found to involve changes in eNOS protein-protein interactions with caveolin-1 (26,27).

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Abbreviations: eNOS, endothelial nitric oxide synthase; NO, nitric oxide; VEGF, vascular endothelial growth factor, BRE, bovine retinal endothelial cells; BAE, bovine aorta endothelial cells; PDGF, platelet derived growth factor; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor.

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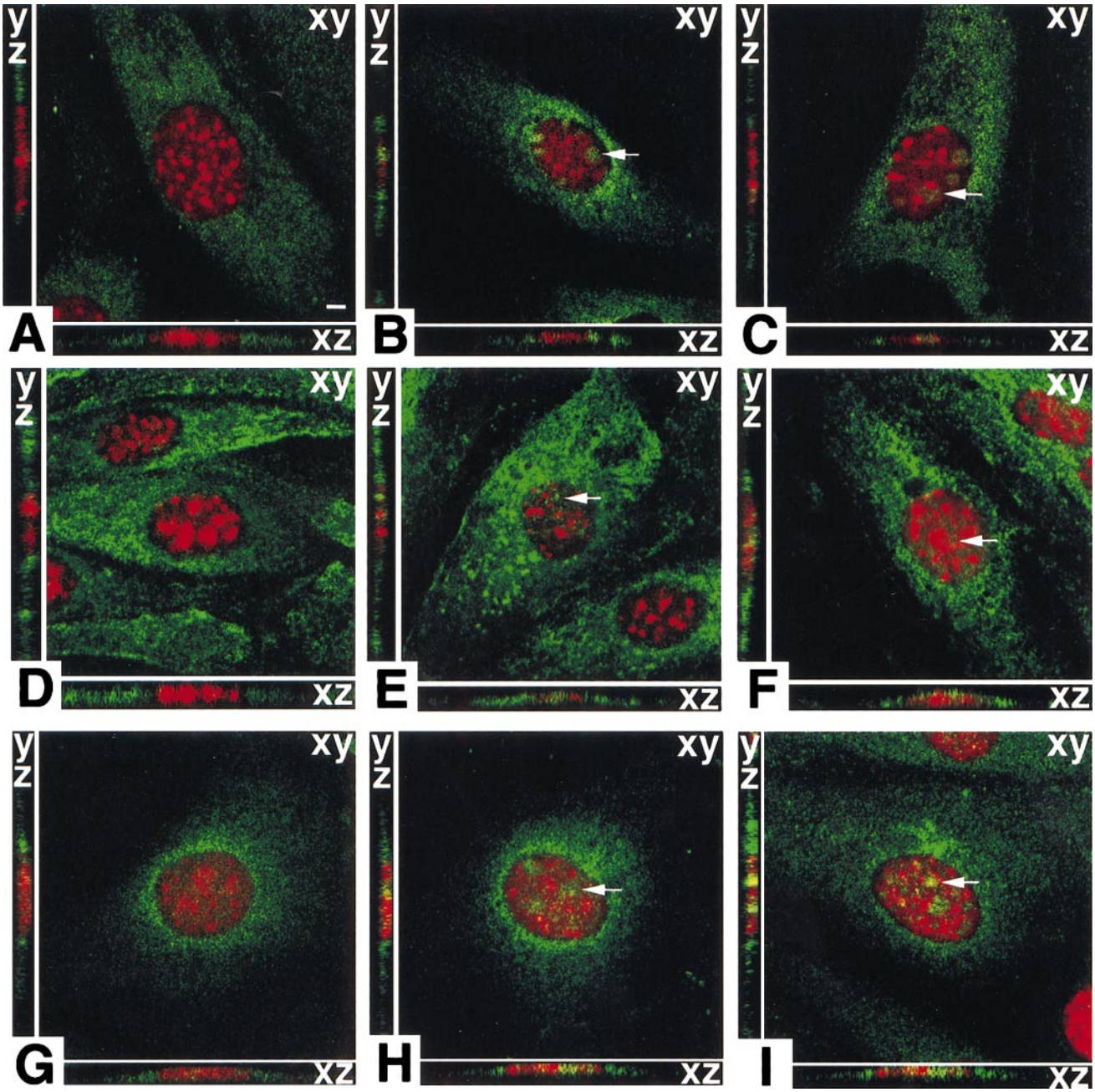

FIG. 1. VEGF induces nuclear translocation of Flk-1/KDR, caveolin-1 and eNOS. Cells prepared as described in the methods section were treated with VEGF (10ng/ml) for 0, 10, 30 min and double labeled with antibodies against Flk-1/KDR (green in A,B,C), caveolin-1 (green in D,E,F) or eNOS (green in G,H,I) in combination with propidium iodide (red) to stain the nuclei. Confocal microscope techniques were used to generate optical sections through the center of the nucleus in three planes (x-y, x-z, y-z). This analysis showed that the nuclei of untreated control cells contain little labeling for Flk-1/KDR (A) or caveolin-1 (D) and only weak, diffuse labeling for eNOS (G). However, after 10 and 30 minutes of VEGF treatment, the nuclei contain large and small clusters of immunoreactivity (arrows) for Flk-1/KDR (B,C), caveolin-1 (E,F) and eNOS (H,I). (Bar = 2 μm)

The caveolar compartment appears to serve in very different targeting and delivery capacities—including both transcellular and intracellular transport as well as delivery of signaling molecules to their targets. The relationship between these functions is not yet understood, and considerable controversy exists concerning whether caveolae represent a static or mobile intracellular compartment. While it is conceivable that different subpopulations of caveolae may exist to subserve the different transport and signaling functions, recent studies of mitogenic signaling molecules suggest that some signaling functions may also involve processes of

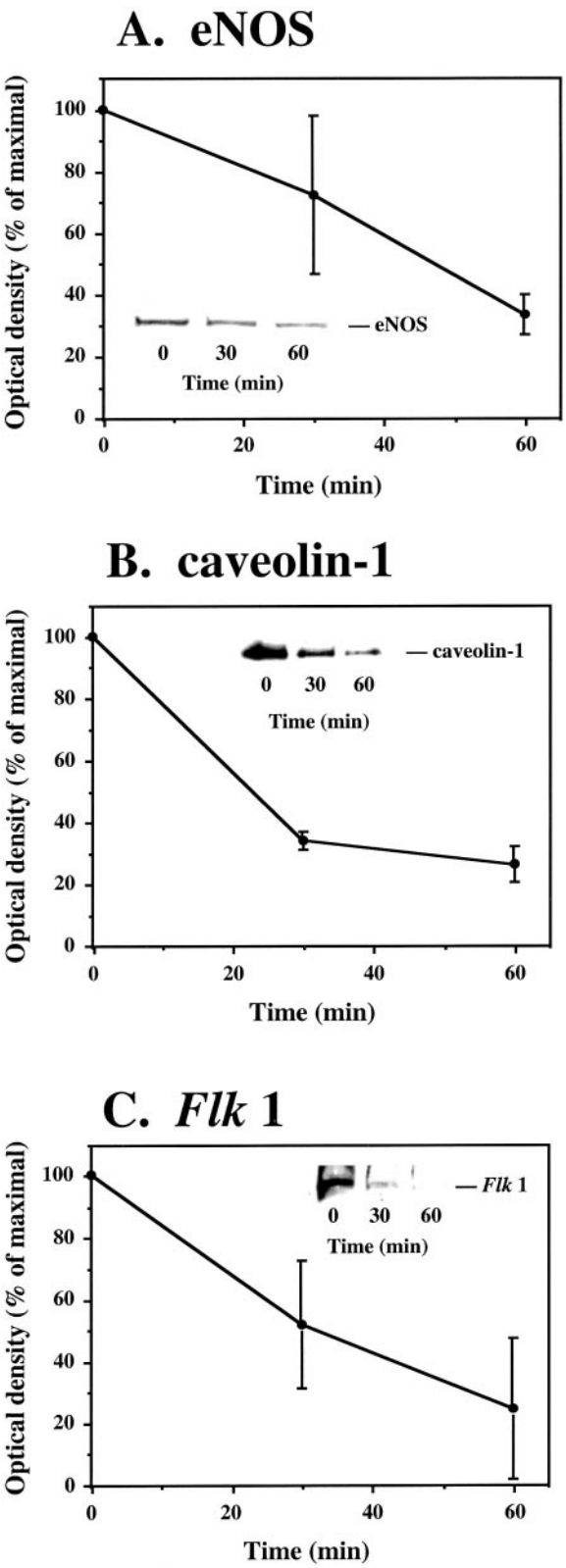

FIG. 2. Effect of VEGF treatment on the amount of eNOS, caveolin-1, and Flk-1/KDR in caveolar fractions. Cells were treated with VEGF (10 ng/ml) for 0, 30 or 60 min, homogenized and fractionated as described in the legend to Figure 9. Caveolar fractions were pooled, precipitated using TCA and analyzed by immunoblotting for the content of eNOS (A), caveolin-1 (B) and Flk-1/KDR (C). Shown are representative immunoblots (inset) and densitometric analysis of blots from three separate experiments (mean ± S.E.).

endocytosis and intracellular transport. For example, receptors for both PDGF and EGF are localized in caveolae and both growth factors and receptors have been shown to undergo translocation to the nucleus via a receptor-mediated endocytotic pathway (28,29). Nuclear translocation has also been observed for basic fibroblast growth factor receptors which have not yet shown to be localized in caveolae (30).

In our previous study of VEGF-induced permeability increases in cultured endothelial cells, we found that the VEGF receptor Flk-1/KDR colocalizes with endothelial NOS and the caveolar structural protein caveolin-1 (16). Taken with the previous work indicating a role for the caveolar compartment in transducing growth factor receptor tyrosine kinase signals to the nucleus, this suggests that the caveolar compartment is also involved in transducing VEGF signals. In order to test this concept, we have now determined VEGF effects on the subcellular distribution of Flk-1/KDR, eNOS and caveolin-1 by using immunolocalization and cell fractionation methodologies. Here we demonstrate that VEGF induces the translocation of all three proteins to the nucleus.

MATERIALS AND METHODS

Cell culture. These experiments were done using freshly isolated cultures (passages 2-5) of bovine retinal endothelial (BRE) and bovine aorta endothelial (BAE) cells. Retinal and aortic endothelial cells were prepared from bovine vessels obtained as described previously (16,31,32).

Laser confocal microscopic analysis. Cells were plated sparsely ($1 \times 10^3/cm^2$) on glass chamber slides coated with collagen and fibronectin. The cultures were serum-deprived overnight and then treated with VEGF (10 ng/ml, recombinant human VEGF165, R & D Systems, Minneapolis, MN) for 0, 10, 30 or 60 min. After treatment the cultures were processed for immunocytochemistry as described (16). Subcellular distribution of Flk-1/KDR, caveolin-1 and eNOS were analyzed by confocal immunofluorescence microscopy after labeling with monoclonal anti-eNOS, polyclonal anti-caveolin-1 (both from Transduction Laboratories, Lexington, KY) or monoclonal anti-Flk-1/KDR antibodies (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Primary antibodies were detected using Oregon Green- and Texas Red-labeled secondary antibodies (Molecular Probes, Eugene, OR). Control experiments in which the primary antibodies were omitted were negative. For visualization of nuclei, cells were stained with propidium iodide (20 μg/ml, Molecular Probes).

A MultiProbe 2001 confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, CA) fitted with a argon/krypton laser was used for the analysis. The microscope was configured for dual channel fluorescent imaging with pinhole 200 μm, 488/568 nm excitation, 488/568 nm primary beamsplitter, 10% laser attenuation, 12-15 milliwatt laser power. A 565 nm secondary beamsplitter passed fluorescent light emitted from organelles labeled with Texas Red or Propidium Iodide to a photomultiplier tube fitted with 600 DF40 nm

band pass filter. Short wave length light (<595 nm) emitted from Oregon Green-labeled organelles was directed to a second photomultiplier tube fitted with a 530 DF30 nm band pass filter and simultaneously recorded. Fields of BRE cells were brought into focus using a 40X/1.4 Plan Apo objective and 0.48 μm z-series sections were collected and analyzed with Image Space software v3.21 (Molecular Dynamics).

Cell fractionation. Cell fractionation studies were done using confluent BRE and BAE cell cultures which had been serum starved overnight and then treated with VEGF (10ng/ml) for 0, 30 or 60 min. Caveolae membranes were isolated and characterized as described previously with minor modifications (33). All solutions used in the isolation were buffered at pH 7.5 (26). In addition, caveolar fractions were TCA precipitated prior to immunoblotting. For preparation of nuclear fractions, cultures were then washed 2 times with phosphate-buffered saline containing 1 mM sodium orthovanadate (PBSV). Cells were harvested by scraping in PBSV and centrifugation at 500 × g for 5 min. Cell pellets were then resuspended in buffer containing 10 mM Tris HCl, pH 7.4, 3 mM CaCl2, 2 mM MgC12, 1mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and allowed to swell on ice for 20 min. Cells were then centrifuged at 500 × g for 5 min and cell pellets were resuspended in the above buffer containing 0.5% Triton X-100 and again allowed to swell on ice for another 20 min. Cells were broken by homogenization using 30 strokes with a tight fitting Dounce homogenizer and very lightly sonicated. Nuclei were separated from other cellular components by centrifugation through 350 mM sucrose in lysis buffer cushion (500 × g for 5 min). Nuclei were resuspended in SDS sample buffer and subjected to SDS-PAGE gel electrophoresis and Western blotting. Integrity and quality of the nuclear fractions were evaluated at each stage of the separation by microscopic analysis of fraction samples. Fraction purity was assessed by immunoblotting with an anti-β 1,4 galactosyltransferase antibody and by assaying for acid phosphatase as markers for Golgi and lysosomal contamination, respectively.

RESULTS AND DISCUSSION

The 21-24 kDa integral membrane protein caveolin-1 is the principal protein component of endothelial cell caveolae (34). Various mammalian cell types have been shown to have 90% of caveolin-1 localized in caveolae with the remaining 10% being found in the Golgi apparatus. Therefore redistribution of caveolin from the plasma membrane to the cellular interior has been used by many researchers to study the caveolae-mediated transport processes of endocytosis and transcytosis (35-37). Our previous work has shown that caveolae are involved in transcytotic transport in cultured BRE cells. We also found that caveolin-1 colocalizes with both Flk-1/KDR and eNOS, suggesting that VEGF signaling occurs within the caveolar compartment (16). In order to test this concept and examine the potential role of the caveolar compartment in the VEGF signal transduction process, we have now determined VEGF effects on the subcellular distribution of Flk-1/KDR, caveolin-1 and eNOS by using two independent techniques: confocal laser scanning microscopy and subcellular fractionation analysis.

We used confocal laser scanning microscopy and optical sectioning techniques to ascertain whether or not VEGF causes the translocation of caveolin-1, Flk-1/

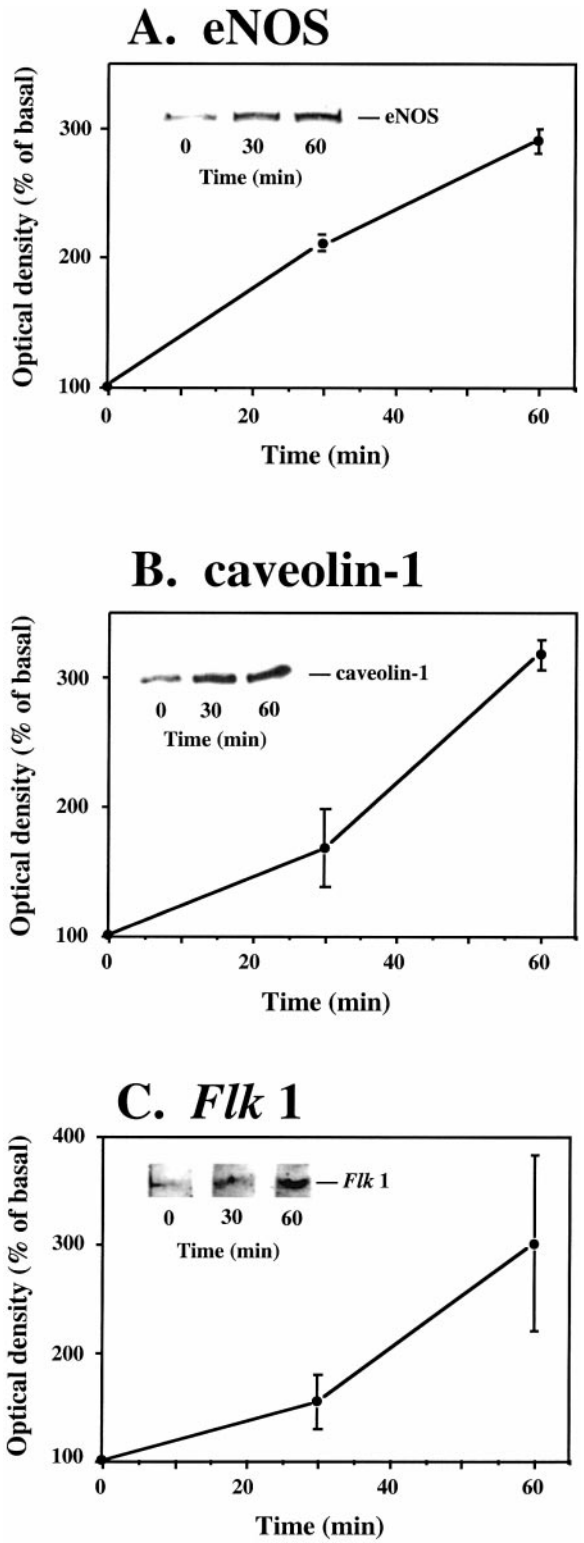

FIG. 3. Effect of VEGF treatment on the amount of eNOS, caveolin-1 and Flk-1/KDR in nuclear fractions. Cells were treated with VEGF (10 ng/ml) for 0, 30 or 60 min, homogenized and fractionated as described in the methods section. The nuclear fraction was analyzed by immunoblotting for the content of eNOS (A), caveolin-1 (B) and Flk-1/KDR (C). Shown are representative immunoblots (inset) and densitometric analysis of blots from three separate experiments (mean ± S.E.).

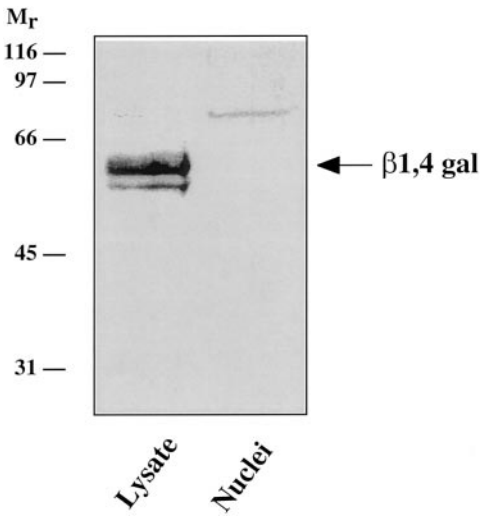

FIG. 4. Nuclear fractions are free of Golgi contamination. Immunoblots were prepared as described in the methods section using equal amounts of protein from whole cell lysates and nuclear fractions and probed with an antibody against the Golgi apparatus protein β 1,4 galactosyltransferase. The whole cell lysate is strongly positive for β 1,4 galactosyltransferase, whereas the nuclear fraction is negative.

KDR and eNOS into the nucleus. Cultured BRE cells were double labeled for either Flk-1/KDR, eNOS or caveolin-1 in combination with propidium iodide to localize the exact position of the cell nuclei. Then optical slices were taken through the center of the nucleus. Images taken through the x-y, x-z and y-z planes showed that VEGF treatment caused translocation of all three proteins into the nucleus (Fig. 1). The nuclei of the untreated control cells contained little labeling for Flk-1/KDR or caveolin-1 and weak, diffuse labeling for eNOS. However, after 10 and 30 minutes of VEGF treatment, BRE cell nuclei contained large and small clusters of immunoreactivity for Flk-1/KDR, caveolin-1 and eNOS.

The imaging data strongly suggested that VEGF signaling involves the nuclear transport of Flk-1 and eNOS by caveolae. In order to confirm these results, cell fractionation techniques were used to determine the effects of VEGF treatment on the subcellular distribution of caveolin-1, eNOS and Flk-1/KDR. These experiments showed that following 30 and 60 min of VEGF treatment, amounts of all three proteins within the caveolar fraction were reduced (Fig. 2). When a similar experiment was performed using nuclear fractions isolated from VEGF-treated and untreated cells this pattern was reversed. Immunoreactivity for all three proteins in the nuclear fractions was lowest in the untreated control cultures, whereas immunoreactivity within the nucleus increased progressively after 30 and 60 min of VEGF treatment (Fig. 3). In order to rule out the possibility that these results could be due to contamination of the nuclear fractions by material from the Golgi apparatus, control cell fractionation and immunoblotting experiments were done using antibodies against β 1,4 galactosyltransferase as a marker for Golgi proteins (Fig. 4). The nuclear fractions were negative for this protein. Parallel experiments performed using BAE cells showed results comparable with the above data for BRE cells. Thus, the nuclear translocation of Flk-1/KDR, caveolin-1 and eNOS appears to be a general feature of VEGF signaling in endothelial cells.

Taken with our previous analyses showing the colocalization of Flk-1/KDR and eNOS with caveolin-1 this demonstration that VEGF stimulation causes the translocation of all three proteins into the nucleus suggests that interactions among the three proteins have an important role in the VEGF signal transduction processes. A requirement for eNOS activity in signaling VEGF's effects on both angiogenic and permeability enhancing responses has been well established in a variety of different experimental model systems (10,13-15). Our previous analyses have shown that eNOS activity is also required for VEGF's effect in increasing permeability of cultured BRE cells. Our experiments also have shown that VEGF causes increased NO release by BRE cells in a process that is totally blocked by inhibitors of either NOS or tyrosine kinase activity (38).

The involvement of the caveolar compartment in regulating eNOS activity is well established. Subcellular targeting of eNOS to caveolae is thought to influence signal transduction mechanisms by restricting NO signaling to specific sites within the cell (23,24). Activity of eNOS has been shown to depend on its transport in caveole to a Golgi-associated, perinuclear subcellular compartment as well as on Ca^{2+}-calmodulin binding (23-25,39). Furthermore, recent work done by others and by us has shown that the activity of purified eNOS is markedly and reversibly attenuated by its interaction with caveolin (40-44), suggesting that caveolin serves as a negative regulator of eNOS activity.

The caveolar compartment has also been implicated in growth factor signal transduction processes. A variety of tyrosine kinase receptors and effector molecules have been shown to be localized in caveolae, including the PDGF receptor, the EGF receptor, PI-3 kinase, PLCγ and protein kinase C (45,46). Moreover, the translocation to the nucleus via a receptor-mediated endocytotic pathway is a common feature in the growth-promoting effects of several angiogenic growth factors, including angiogenin, PDGF, EGF abd basuc abd acuduc FGF (47). Thus, we hypothesize that ligand binding of VEGF receptors promotes VEGF receptor internalization within caveolae together with eNOS and translocation of both molecules to the nucleus. Translocation of eNOS to the nucleus within caveolae may represent a mechanism for targeting NO produc-

tion to the nuclear compartment where it can activate transcription factors and promote cell growth. NO has been shown previously to induce the early growth response gene, c-fos (48,49) and may affect other transcription factors as well.

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