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Regulation of Caveolin-1 Expression and Phosphorylation by VEGF in Ovine Amnion Cells

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Abstract

Vascular endothelial growth factor (VEGF) has been implicated in the regulation of vesicular transport of amniotic fluid via caveolae across the amnion. This study tested the hypothesis that VEGF regulates caveolar function by stimulating caveolin-1 expression and phosphorylation in ovine amniotic epithelial cells (oAECs). Using primary cultures of oAECs, caveolin-1 was identified by immunofluorescent staining. Caveolin-1 messenger RNA (mRNA) abundance was determined by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and protein by Western blotting. The effects of $VEGF_{165}$ on caveolin-1 expression and phosphorylation were determined. Caveolin-1 immunoreactivity was detected in oAECs. In response to 10 ng/mL $VEGF_{165}$, caveolin-1 mRNA levels increased whereas the protein levels were unaffected. Furthermore, VEGF stimulated caveolin-1 phosphorylation, an effect abrogated by the inhibition of c-*Src* protein kinase. These data suggest that VEGF upregulates caveolin-1 activity through c-*Src* signaling pathways. Our observations support the hypothesis that VEGF regulates amniotic fluid transport across the amnion by stimulating caveolin-1 activity to mediate caveolar function in amnion cells.

Keywords

amnion fluid volume; growth factor; caveolin-1; gene expression; phosphorylation

Introduction

During pregnancy, the volume of amniotic fluid is typically maintained at an optimal level to sustain normal development of the fetus. Amniotic fluid volume is regulated primarily by modulating the rate of amniotic fluid transport across the intramembranous pathway.[1-3] This transport pathway consists of the amnion, chorion, and a network of intramembranous fetal blood vessels that perfuse the fetal surface of the placenta and, in ungulates, the fetal membranes as well. Amniotic fluid is absorbed across the amnion and the vascular endothelium into the fetal blood vessels, with the amnion as the rate limiting layer for this

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transport. The absorption process is consistent with the concept of transcytotic transport of fluid involving vesicle formation and mobilization within the amnion cells.[3-6] Vascular endothelial growth factor (VEGF) has been suggested to play a pivotal role in the regulation of intramembranous absorption presumably through the activation of vesicular transcytosis in the amnion.[7] We have previously demonstrated the expression of VEGF in ovine amnion and chorion with $VEGF_{165}$ as the major isoform expressed.[8-10] We have also documented the expression of VEGF receptor R-1 (Flt-1) and R-2 (KDR) in the ovine amniotic epithelium.[11,12] Furthermore, VEGF expression in the amnion was upregulated under conditions of experimentally induced increases in intramembranous absorption.[7] These findings support the concept that VEGF regulates amniotic fluid absorption by directly targeting the amnion for the transport process.

In vascular endothelial cells, specialized membrane micro-domains termed caveolae are plasma membrane invaginations that, on internalization and release from the cell surface, form vesicles of approximately 50 nm in diameter that exist as free structures in the cytoplasm.[13,14] Cavoelae lack a membrane coat but contain the 21-kd protein caveolin-1, a member of the family of structural proteins referred to as caveolins.[15] In addition to their role in the regulation of cholesterol metabolism and signal transduction events that initiate cell differentiation and proliferation, caveolae are involved in transcytosis of fluid and macromolecules[16] as well as in the regulation of vascular permeability.[17] This process likely involves the formation of caveolae at the luminal cell surface, their internalization and mobilization across the cell, and eventual fusion to the abluminal surface to expel their contents resulting in transport.[17] In microvascular endothelial cells, VEGF increases cell permeability by enhancing caveolae-mediated transcytotic transport of fluids.[17,18] The VEGF stimulation of transcytosis by caveolae appears to be regulated primarily by a c-*Src* signal transduction pathway[19] that activates downstream phosphorylation of caveolin-1 to initiate caveolar transport. In addition, other signaling intermediates such as protein kinase C (PKC) may participate in the transcellular transport process.[20]

Although caveolae and caveolin-1 normally are found in abundance in vascular endothelial cells, their existence in other cell types including epithelial cells has also been described.[21] Thus, it is likely that caveolae are present in amniotic epithelial cells. In humans, caveolae and caveolin-1 have been localized in term villous cytotrophoblasts.[22,23] In addition, the caveolin-1 protein is expressed in vascular endothelial cells of chorionic villi and in the amniotic epithelium.[24] However, even though these findings support a functional role for caveolae in the amnion, whether caveolae similarly serve as signal transduction platforms in epithelial cells is not clear and their role in mediating fluid transport in the amnion has not been explored. Further the potential role of VEGF in regulating caveolar transcytosis of amniotic fluid has not been elucidated. Therefore, we undertook the current study to determine the expression of caveolin-1 in ovine amniotic epithelial cells (oAECs). In addition, we investigated the effects of VEGF on caveolin-1 expression and phosphorylation in these cells. We tested the hypothesis that VEGF induces caveolin-1 activity in amnion cells, thereby supporting a role for VEGF in regulating intramembranous transport of amniotic fluid across the amniotic membrane.

Methods

Animals and Amnion Cell Preparation

Pregnant sheep with singleton or twin fetuses at 120 to 132 days gestation were used for the study. The ewe was euthanized and the uterus including the fetus was delivered through an abdominal incision. The fetal membranes were removed and the amnion carefully separated from the chorion. The amniotic membrane was placed in sterile Dulbecco's Modified Eagle Medium (DMEM)/F12 cell culture medium supplemented with antibiotics and used for

amniotic epithelial cell preparation as described in our recent report.[25] Briefly, ovine amniotic membranes were minced and subjected to 3 successive digestions in DMEM/F12 containing trypsin (0.625%). The final cell suspension was passed through a 70-μm cell strainer to remove undigested tissues and cellular debris. The cell pellet was resuspended and plated onto 75 cm^2 tissue culture flasks in DMEM/F12 supplemented with 10% fetal bovine serum and antibiotics containing 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. The cells were maintained in an incubator at 37°C in a humidified atmosphere of 5% carbon dioxide in air. Typically the experiments were conducted in oAECs from the second passage.

Immunostaining of Caveolin-1 and VEGF Receptors in Ovine Amnion Cells

Ovine AECs were plated onto coverslips and maintained in culture. At 50% confluence, the cells were fixed in 3.7% paraformaldehyde. After washing with cold phosphate-buffered saline (PBS) containing 0.1% Triton X-100, the cells were incubated with 2% goat serum in PBS to block nonspecific binding. For caveolin-1 immunostaining, the fixed cells were treated with a rabbit polyclonal anti-human caveolin-1 antibody specific for the α-caveolin-1 isoform (*sc-894*, Santa Cruz Biotechnology, Inc, Santa Cruz, California) at 1:200 dilution overnight at 4°C. The cells were then incubated with a Cy3 conjugated goat anti-rabbit immunoglobulin G ([IgG] Jackson Immunoresearch Laboratories, West Grove, Pennsylvania) at 1:200 dilution for 1 hour in the dark. Ovine AECs were similarly stained for VEGFR-1 and VEGFR-2 using a rabbit polyclonal anti-human Flt-1 antibody (*sc-316*, Santa Cruz Biotechnology, Inc) or a mouse monoclonal anti-human Flk-1/KDR antibody (*sc-6251*, Santa Cruz Biotechnology, Inc) at 1:200 dilution for 4 to 24 hours at 4°C. As a secondary antibody for flt-1 and KDR, an anti-rabbit or anti-mouse IgG, respectively, conjugated to Alexa $Fluor^{488}$ (Molecular Probe, Invitrogen, San Diego, California) was used at 1:200 dilution for an 1-hour treatment in the dark. The coverslips were mounted on glass microscope slides and viewed under a fluorescence microscope (Nikon Labophot-2, Melville, New York). Images of the labeled cells were captured and digitized using a SPOT CCD digital camera and associated software.

Determination of Caveolin-1 Messenger RNA Expression

Ovine AEC at 85% to 90% confluence were plated onto 100 mm culture dishes (BD Falcon, Franklin Lakes, New Jersey) for gene expression studies. To determine the effects of VEGF treatment, the culture medium was replaced with serum-free DMEM/F12 supplemented with antibiotics, glutamine, and albumin (Cellgro, Mediatech, Voigt Global Distribution, Inc, Lawrence, Kansas). Recombinant human $VEGF_{165}$ (VEGF, R & D Systems, Minneapolis, Minnesota) at 0, 1, 5, 10, or 25 ng/mL were added to the culture medium and the cells were incubated in the presence of VEGF for 6, 12, or 24 hours. At the end of each time period, cells were collected for RNA extraction to analyze caveolin-1 messenger RNA (mRNA) expression, or lysed with a protein lysis buffer to determine caveolin-1 protein levels.

Cellular total RNA from oAEC was extracted using an RNeasy Kit (Qiagen, Inc Valencia, California). The RNA was quantified spectrophotometrically at OD 260/280. Total RNA (2 μg per sample) was reverse transcribed using 10 units of MuLV reverse transcriptase in the presence of 250 ng of random hexamers. The expression level of caveolin-1 mRNA was determined by semi-quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) using L-19 as the internal reference in the same reaction as previously described.[26] The sense and antisense primers used for amplification of ovine caveolin-1 were as follows:

5′-AACATCTACAAGCCCAACAACAAG-3′, and 5′-CGCATCAACACGCAGAAAGAAATA-3′, respectively.

For L-19, the sense and antisense primers were, 5′-ATCGC-CAATGCCAACTCCC-3′ and 5′-GTCTGCCTTCAGCTTGT GG-3′, respectively. The amplification was carried out in the presence of 1.5 mmol/L Mg Cl_2 and 0.2 mmol/L deoxynucleotide triphosphate (dNTP) in PCR buffer for 28 cycles of 55°C for 30 seconds and 72°C for 30 seconds with a final extension at 72°C for 9 minutes. The amplified products were separated by gel electrophoresis in 2% to 3% agarose and visualized with ethidium bromide staining. The intensity of the signal was quantified under ultraviolet light. Digital images were captured with the ChemiImager Imaging System (Alpha Innotech Corp, San Leandro, California) using a high-resolution charge-coupled device camera and analyzed by the ChemiImager 4400 software (Alpha Innotech Corp). The caveolin-1 mRNA abundance was quantified and normalized to the respective L-19 signal.

Analysis of Caveolin-1 Protein Levels

The level of caveolin-1 protein in oAEC was determined by Western blot analysis using procedures as previously described.[27] Whole cell extracts were prepared with a nondenaturing lysis buffer and protein concentrations measured using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, Illinois) and bovine serum albumin (BSA) as the standard. The samples were boiled in sodium dodecyl sulfate (SDS) sample buffer and the proteins were separated by SDS polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes and exposed to a rabbit polyclonal anti-human caveolin-1 antibody (*sc-894*, Santa Cruz Biotechnology, Inc) at 1:40,000 dilution. After treatment with an anti-rabbit IgG peroxidase conjugated secondary antibody, the caveolin-1 protein was visualized using the ChemiGlow Chemiluminescent substrate with the ChemiImager Imaging System and ChemiImmager 4400 software (Alpha Innotech Corp.). For the internal reference, the membrane was stripped and re-probed with a mouse anti-human β-actin monoclonal antibody (Ambion Inc, Applied Biosystems, Foster City, California) at 1:200 dilution. The caveolin-1 levels were normalized to the respective β-actin signals.

Caveolin-1 Tyrosine Phosphorylation

For caveolin-1 phosphorylation experiments, oAEC were plated onto 100 mm culture dishes (Sarstadt, Inc, Newton, North Carolina). At the second passage with 80% to 90% confluence, the cells were serum withdrawn overnight and the medium replaced with phenol red free M-199 containing 0.1% BSA and 25 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Prior to experimentation, the medium was again replaced with fresh medium and equilibrated for 1 hour at 37°C. The cells on culture dishes were treated with agonists for 20 minutes, and the reaction was terminated by placing the dishes on ice. The cells were washed twice with ice-cold PBS and extracted with a nondenaturing lysis buffer on ice with continuous shaking for 30 minutes. The whole cell lysates were collected and protein content was measured by the BCA Protein Assay Kit. Total tyrosine phosphorylated proteins were analyzed by Western blot.[28] Briefly, equal amounts of proteins (200-400 μg/sample) were immunoprecipitated with a specific anti-phosphotyrosine monoclonal IgG (2 μg, PY-99, *sc-7020*, Santa Cruz Biotechnology, Inc) overnight at 4°C with end-over-end rotation. Protein A agarose beads (50/50 slurry, 50 μL) were then added for 2 hours at 4°C. The immunoprecipitates containing total tyrosine phosphorylated proteins were recovered by centrifugation, washed, resuspended in SDS sample buffer, and heat denatured. The proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon-P membranes electrically at 0.3 A for 1.5 hours using a semi-dry blotter. To identify phosphorylated caveolin-1, the membranes were probed with a rabbit polyclonal anti-human caveolin-1 antibody at 1:40,000 dilution (*sc-894*, Santa Cruz Biotechnology, Inc) overnight at 4°C with continuous rotation. The membranes were washed and re-probed with an anti-rabbit peroxidase-conjugated IgG at 1:3,000 at room temperature for 1 hour. After washing, the tyrosine

phosphorylated caveolin-1 was visualized with ChemiGlow Chemiluminescent substrate and quantified as mentioned above. For total caveolin-1 protein, cell lysates (20 ug/sample) were boiled in Laemmli buffer and similarly subjected to SDS-PAGE and immunoblotting. The levels of total caveolin-1 were similarly quantified.

In preliminary studies, we tested the kinetic characteristics of caveolin-1 phosphorylation in amnion cells by determining the dose–response effect of VEGF on caveolin-1 phosphorylation. Regression analysis showed that at concentrations ranging from 1 to 25 ng/mL, $VEGF_{165}$ induced caveolin-1 tyrosine phosphorylation in a dose-dependent manner (unpublished observation). The effect was consistent with the first-order Michalis-Menton kinetics, which predicted a half-maximal effective concentration of 3.8 ng/mL ($R = .98$, $P < .02$). Based on this data, we selected a dose of 10 ng/mL for the studies of VEGF effects on caveolin-1 phosphorylation. In addition, the effects of hydrogen peroxide and phorbol 12-myristate 13-acetate (PMA) on caveolin-1 phosphorylation were tested. Cell lysates were analyzed for total and phosphorylated caveolin-1 levels.

Statistical Analysis

The data are presented as the mean ± SE. The phosphorylation of caveolin-1 was expressed as the ratio of phosphorylated caveolin-1 (IP) to total caveolin-1 (total) level. Results from the experiments on oAEC were analyzed by a 2-factor analysis of variance with treatment and individual experiments as the 2 factors. Individual differences were determined by post hoc testing.

Results

Immunolocalization of Caveolin-1 and VEGF Receptors in Amnion Cells

Visualization by fluorescence microscopy of oAEC immunostained with anti-caveolin-1 antibody revealed the presence of caveolin-1 protein distributed throughout the cell surface (Figure 1A). Under high magnification, small punctuated loci (cell membrane structures) of caveolin-1-positive immunostaining were observed along the periphery of the cell resembling aggregates of microdomains, suggestive of caveolin-1-enriched vesicles (Figure 1B). Immunostaining of oAEC with anti-VEGFR-1 and VEGFR-2 antibodies demonstrated the expression of both VEGF receptors R-1 (Figure 1C) and R-2 (Figure 1D) proteins localized to the entire cell surface.

Effects of VEGF on Caveolin-1 Gene Expression

Using a semiquantitative RT-PCR method, the relative levels of caveolin-1 mRNA in oAEC were determined. The analysis showed that treatment of oAEC with $VEGF_{165}$ induced a time-dependent increase in caveolin-1 mRNA levels. At 6 hours, caveolin-1 mRNA levels were elevated in response to VEGF treatment, but the increase was not statistically significant. By 12 hours, VEGF at concentrations of 1 to 25 ng/mL stimulated increases in caveolin-1 mRNA levels with a maximum rise to 1.4 times control levels ($F = 3.50$, $P < .02$; Figure 2A). The concentration of VEGF that induced the maximal stimulatory effect on caveolin-1 mRNA was found to be 10 ng/mL, because a higher concentration of 25 ng/mL did not produce a greater effect on caveolin-1 levels. At 24 hours, the VEGF-induced increase in caveolin-1 mRNA levels diminished and was no longer significantly different from the control. Although a more quantitative method such as real-time RT-PCR might have revealed a greater change in caveolin-1 mRNA levels, the observed modest effect of VEGF on caveolin-1 expression was significant. In contrast to the mild stimulatory effect of VEGF on mRNA abundance, caveolin-1 protein levels in oAEC were not elevated significantly in response to VEGF at 1 to 25 ng/mL during the 24-hour treatment period (Figure 2B).

Effects of VEGF on Caveolin-1 Phosphorylation

In oAEC, treatment with 10 ng/mL $VEGF_{165}$ significantly stimulated caveolin-1 phosphorylation to 2.2 times the control levels ($P < .05$; Figure 3). The effective concentration of VEGF in inducing caveolin-1 phosphorylation is consistent with the dose of VEGF required for the stimulation of ERK2/1activation in oFPAE cells.[29] The activation of caveolin-1 in response to 500 mmol/L hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS), was highly significant with an increase in caveolin-1 phosphorylation to 7 times control levels (Figure 3). Treatment with the PKC activator PMA at 100 nmol/L induced an increase in caveolin-1 phosphorylation to 2.5 times control ($P < .05$; Figure 3). This level of activation was similar to the stimulation (2.2 times) observed in response to 10 ng/mL VEGF treatment. To determine whether the VEGF induction of caveolin-1 phosphorylation was mediated through the c-*Src* protein kinase pathway, oAEC were treated with the *Src* protein kinase inhibitor pyrazolopyrimidine 2 (PP2). The stimulatory effects of 10 ng/mL VEGF on caveolin-1 phosphorylation were significantly reversed by PP2 treatment in a dose-dependent manner (Figure 4).

Discussion

To explore the possible involvement of caveolae in the transport of amniotic fluid across the amnion, we determined the expression and activation of caveolin-1, the structural protein of caveolae, in cultured oAEC. Our observations showed that caveolin-1 immunoreactivity was present across the amnion cell surface as small dense and discrete vesicle-like structures on the periphery of the cell. Although we have not distinguished the subcellular localization of caveolin-1 based on light microscopy used in this study, the results are consistent with the presence of caveolin-1-enriched vesicles in oAEC. We have further demonstrated the expression of caveolin-1 mRNA and protein in the oAEC. These findings suggest that caveolae are present in oAEC similar to those in vascular endothelial cells and may be involved in fluid and solute transport across the amnion. This assumption is consistent with the studies in vascular endothelial cells where caveolar transcytosis is the primary mechanism for transcellular albumin and fluid transport.[16,30-32] Although transport across epithelial cells has been less studied, the involvement of vesicular transcytosis in these cells is suggested by a report in human alveolar epithelial cells where transport of serum proteins including albumin is a nonpassive vesicular mechanism.[33] This report together with our recent demonstration of active transport of solutes across oAEC monolayers in culture[6] indicates that caveolae are very likely the mediator for the transport of fluid and solutes across the amniotic membrane.

In vascular endothelial cells, the regulation of caveolar transport has been extensively studied and the observations documented that VEGF is a major regulatory factor that mediates transcytosis by caveolae.[17] Binding of VEGF to VEGFR-2 leads to activation of downstream signaling events to elicit transcellular transport via caveolae.[18] Based on our previous experimental evidence in the ovine fetus that VEGF expression in the amnion was upregulated under conditions of increased intramembranous absorption,[4,34] it is reasonable to speculate that VEGF plays a similar role in inducing transcytosis of amniotic fluid by regulating the activity of caveolae in amnion cells. Our previous reports on VEGF expression[10,11] and VEGF receptor binding activities[12] in the ovine amniotic membrane as well as the present demonstration of VEGFR-1 and VEGFR-2 immunoreactivity in oAEC support this concept. We further evaluated the effect of VEGF on caveolin-1 gene expression in oAEC. Our results indicated that VEGF induced a time-dependent upregulation of caveolin-1 mRNA expression that was not associated with significant increases in caveolin-1 protein levels. It is possible that the 24-hour experimental period used in our study was insufficient for an effect of VEGF on caveolin-1 protein induction to

be detectable. Alternatively, because VEGF mediates its effects presumably at the cell surface where the caveolae are localized, it is possible that the stimulatory effect of VEGF on caveolin-1 expression is masked by the use of whole cell extracts for RT-PCR and whole cell lysates for Western blot, instead of using enriched and purified caveolae preparations. Nevertheless, because VEGF induced a modest increase in caveolin-1 mRNA expression without an accompanying effect on protein levels, it is likely that the major effect of VEGF on caveolin-1 is not through upregulation of gene expression. In addition, our present result demonstrating VEGF stimulation of caveolin-1 mRNA levels in oAEC differs from the reports in vascular endothelial cells in that VEGF was found to either have no effect on caveolin-1 mRNA or protein levels in ovine placental artery endothelial cells[29] or downregulated caveolin-1 protein expression in human umbilical vein endothelial cells (HUVECs).[35] Because the VEGF dose range and time course were similar in these studies, it is difficult to reconcile the differences in these findings with the exception that our studies were carried out in epithelial cells derived from the ovine amniotic membrane rather than vascular endothelial cells.

In the current study, we have explored the effects of VEGF on the activation of caveolin-1 by determining the phosphorylation of caveolin-1 protein in oAEC. We observed a significant upregulation of caveolin-1 tyrosine phosphorylation in response to VEGF stimulation. The effective concentrations of $VEGF_{165}$ were within the physiological range of VEGF in amnion tissues and are similar to the concentrations used in other studies on VEGF biological actions.[19,29] In comparison to the effect on caveolin-1 mRNA levels, the stimulatory effect of VEGF on caveolin-1 phosphorylation was much greater. These observations suggest that in oAEC, the primary effect of VEGF on caveolin-1 is exerted on protein activation, whereas the stimulatory effect on gene expression is secondary to the activation of protein. More importantly, the VEGF-induced activation of caveolin-1 phosphorylation could be reversed by the *Src* protein kinase inhibitor PP2. Thus, it appears that the VEGF activation of caveolin-1 phosphorylation was mediated through c-*Src* protein kinase signaling. Of note, our preliminary studies suggested that the tyrosine phosphorylation of c-*Src* kinase is inducible by VEGF treatment (unpublished observation). Because the c-*Src* signaling cascade was shown to be a crucial pathway required for caveolar endocytosis and transcytosis in vascular endothelial cells,[36-38] and that VEGF acting through VEGFR-2 was capable of increasing c-*Src* activity to presumably mediate caveolar transcytosis,[19] it is possible to propose that, in amniotic epithelial cells, VEGF similarly initiates caveolae transport by activation of VEGFR-2 to phosphorylate caveolin-1 through c-*Src* signal transduction pathways.

Reactive oxygen species such as H_2O_2 are known to activate the phosphorylation of caveolin-1 through the c-*Src* protein kinase pathway[28] to initiate many of the endothelial cell functions.[39] Our demonstration that H_2O_2 induced caveolin-1 phosphorylation in oAEC confirms a similar role for H_2O_2 in epithelial cells. In addition to ROS, PKC has been shown to interact with caveolin-1 in lipid rafts of lens epithelial cells.[40] In the current study, we observed that PMA stimulated caveolin-1 phosphorylation, suggesting that PKC may be an additional signaling intermediate for caveolin-1 activation in oAEC. Because inhibition of PKC activation in fibroblasts prevented endocytosis[41] and loss of PKC from caveolae reduced caveolae internalization,[42] PKC may participate in caveolae-mediated transcytosis through activation of caveolin-1. Furthermore, because the VEGF-induced vascular permeability in HUVECs[43] involves PKC signaling, it is possible that a similar mechanism for VEGF stimulation of transcellular amniotic fluid transport is facilitated by PKC activation of caveolin-1 in oAEC.

In summary, we have demonstrated the presence of caveolin-1 in oAEC and that its activation was upregulated by VEGF primarily through the induction of caveolin-1 protein

phosphorylation. Vascular endothelial growth factor secondarily enhanced caveolin-1 mRNA expression, thus consistent with the effects on caveolin-1 phosphorylation. More importantly, these observations corroborate the concept that VEGF regulates amniotic fluid absorption across the amnion by initiating a caveolae-dependent transcytotic transport mechanism involving c-*Src* and PKC signaling events.

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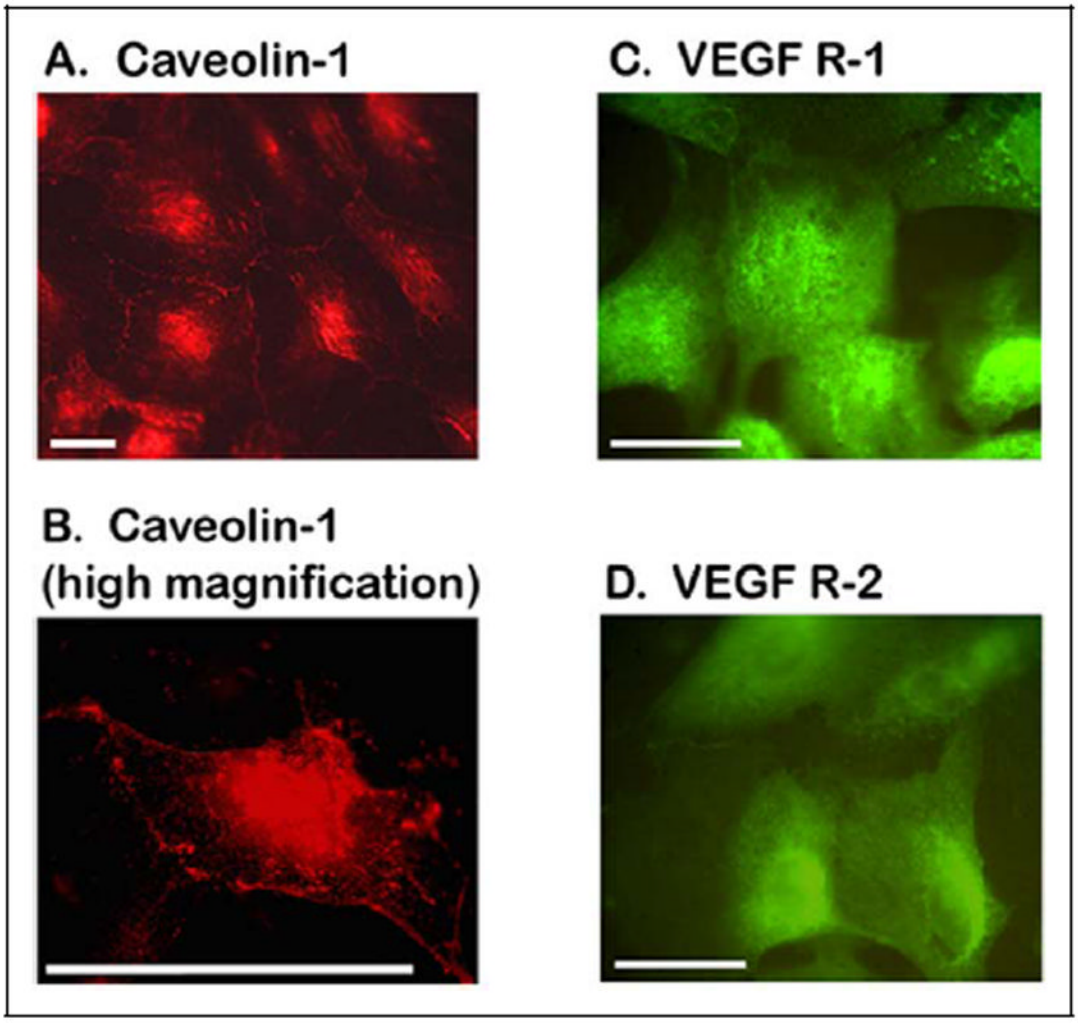

Figure 1.

Immunofluorescent labeling of ovine amniotic epithelial cells for caveolin-1, VEGFR-1, and VEGFR-2 proteins. Ovine amnion cells fixed on coverslips were reacted with specific antibodies and visualized under the florescence microscope. The images were captured and digitized. A. oAECs treated with anti-caveolin-1 polyclonal antibody at 1:200 followed by secondary antibody conjugated to Cy3. B. High magnification of oAEC immunostained for caveolin-1. C. oAECs immunostained for VEGFR-1 with anti-Flt-1 polyclonal antibody followed by secondary antibody conjugated to Alexa Flour 488. D. oAECs immunostained for VEGFR-2 with anti-Flk-1/KDR monoclonal antibody followed by secondary antibody conjugated to Alexa Flour 488. VEGFR indicates vascular endothelial growth factor receptor; oAECs, ovine amniotic epithelial cells.

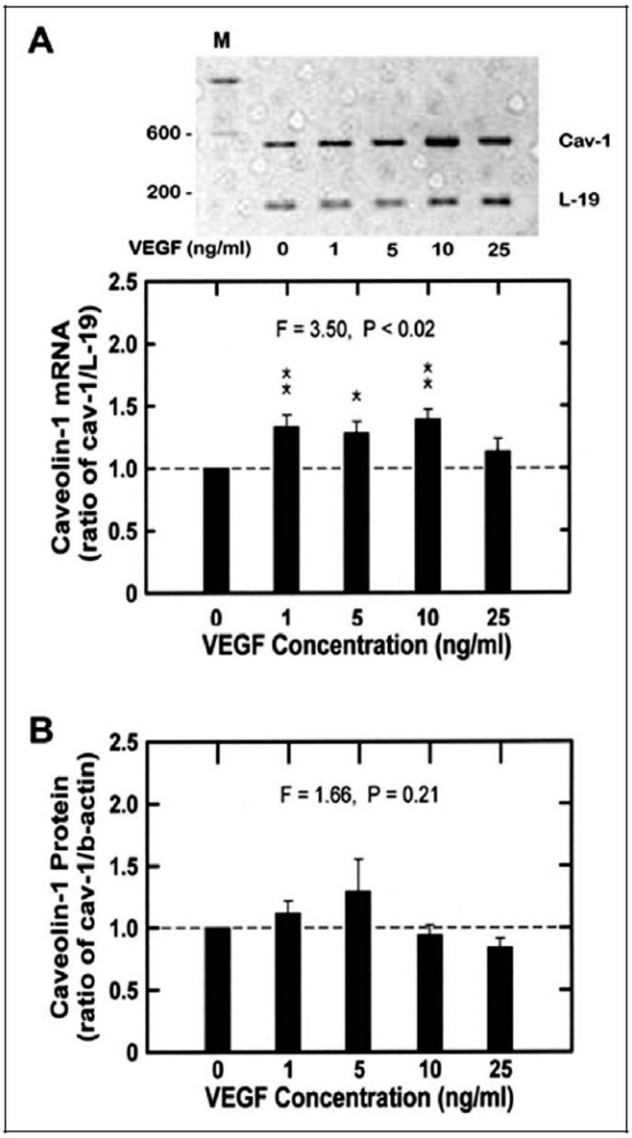

Figure 2.

Vascular endothelial growth factor (VEGF) regulation of caveolin-1 expression in oAEC. The cells were treated with $VEGF_{165}$ at 1 to 25 ng/mL for 6 to 24 hours, and caveolin-1 mRNA and proteins levels were analyzed. A. Caveolin-1 mRNA levels as determined by semi-quantitative RT-PCR and expressed as the ratio of caveolin-1 abundance to the internal reference L-19 at 12 hours of VEGF treatment ($F = 3.5$, $P < .02$, 2-factor analysis of variance); *$P < .05$; **$P < .01$. Top panel, representative agarose gel stained with ethidium bromide for the caveolin-1 and L-19 PCR products. B. Western immunoblot determination of caveolin-1 protein levels referenced to the respective α-actin levels at 12 hours of VEGF treatment. ($F = 1.66$, P = nonsignificant, 2-factor analysis of variance). Results are mean ± SE, n = 4 experiments. M indicates, 100 bp ladder; mRNA, messenger RNA; SE, standard error; cav-1, caveolin-1.

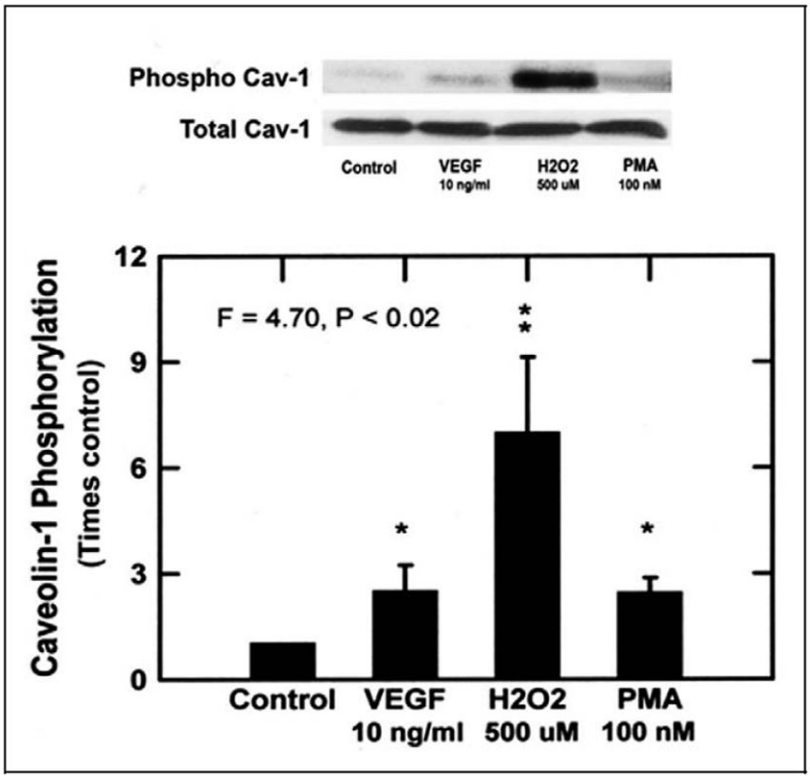

Figure 3.

Agonist activation of caveolin-1 phosphorylation in oAECs. Results are expressed as caveolin-1 phoshorylation (IP/total) and presented as the increase from the control level (F = 4.70, P < .02, 2-factor analysis of variance). Top panel, SDS-PAGE analysis of phosphorylated caveolin-1 protein and total caveolin-1 protein. Data are mean ± SE, n = 6 experiments. *P < .05; **P < .01. H_2O_2 indicates hydrogen peroxide; VEGF, $VEGF_{165}$; PMA, phorbol 12-myristate 13-acetate; oAECs, ovine amniotic epithelial cells; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Cav-1, caveolin-1.

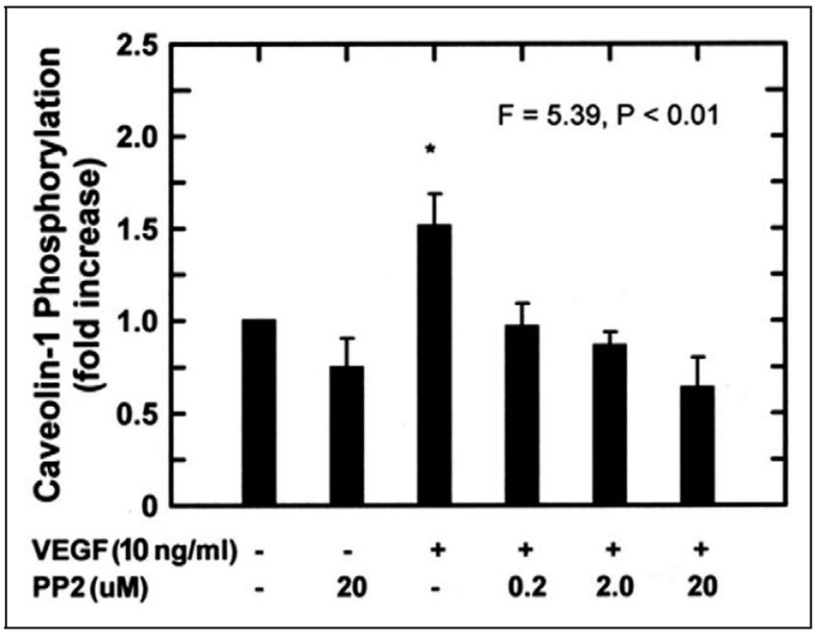

Figure 4.

Effect of c-*Src* inhibition on VEGF165-induced caveolin-1 phosphorylation. Levels of caveolin-1 phosphorylation (IP/total) are presented as the increase from control level (F = 5.39, P < .01, 2-factor analysis of variance). Data are mean ± SE, n = 3 experiments. *P < .05. PP2 indicates pyrazolopyrimidine 2; VEGF, vascular endothelial growth factor.