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Vasostatin 1 Activates eNOS in Endothelial Cells Through a Proteoglycan-Dependent Mechanism

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ABSTRACT

Accumulating evidences point to a significant role for the chromogranin A (CgA)-derived peptide vasostatin 1 (VS-1) in the protective modulation of the cardiovascular activity, because of its ability to counteract the adrenergic signal. We have recently shown that VS-1 induces a PI3K-dependent-nitric oxide (NO) release by endothelial cells, contributing to explain the mechanism of its cardio-suppressive and vasodilator properties. However, the cellular processes upstream the eNOS activation exerted by this peptide are still unknown, as typical high-affinity receptors have not been identified. Here we hypothesize that in endothelial cells VS-1 acts, on the basis of its cationic and amphipathic properties, as a cell penetrating peptide, binding to heparan sulfate proteoglycans (HSPGs) and activating eNOS phosphorylation (Ser1179) through a PI3K-dependent, endocytosis-coupled mechanism. In bovine aortic endothelial cells (BAE-1 cells) endocytotic vesicles trafficking was quantified by confocal microscopy with a water-soluble membrane dye; caveolin 1 (Cav1) shift from plasma membrane was studied by immunofluorescence staining; VS-1-dependent eNOS phosphorylation was assessed by immunofluorescence and immunoblot analysis. Our experiments demonstrate that VS-1 induces a marked increase in the caveolae-dependent endocytosis, ($115 \pm 23\%$ endocytotic spots/cell/field in VS-1-treated cells with respect to control cells), that is significantly reduced by both heparinase III (HEP, $17 \pm 15\%$ above control) and Wortmannin (Wm, $7 \pm 22\%$ above control). Heparinase, Wortmannin, and methyl-β-cyclodextrin (MβCD) abolish the VS-1-dependent eNOS phosphorylation ($P^{Ser1179}$eNOS). These results suggest a novel signal transduction pathway for endogenous cationic and amphipathic peptides in endothelial cells: HSPGs interaction and caveolae endocytosis, coupled with a PI3K-dependent eNOS phosphorylation. J. Cell. Biochem. 110: 70–79, 2010.

KEY WORDS: VASOSTATIN 1; CHROMOGRANIN A; ENDOCYTOSIS; NITRIC OXIDE SYNTHASE; ENDOTHELIAL CELL

Vasostatin 1 (VS-1) is the major and naturally most abundant N-terminal peptide derived from chromogranin A (CgA), a pro-hormone contained in secretory granules of chromaffin and other cells. CgA is cleaved by tissue-specific proteases to numerous peptides, some of them involved in the modulation of different physiological processes, in inflammatory reactions and in the innate immunity. Many experiments strongly suggest that VS-1 acts as a multifunctional regulatory agonist exerting inhibitory influence via autocrine, paracrine, and/or endocrine mechanisms. In particular, VS-1 has been shown to act as a cardio-inhibiting and vasodilating agent, suggesting that this peptide may play a role as a stabilizer of the cardiovascular system, particularly under conditions of sympathetic overstimulation, such as those occurring under stress response [Helle et al., 2007; Tota et al., 2008]. In addition to the effects on cardiac function, it has been previously shown that CgA and CgA N-terminal fragments can modulate in a differential manner fibroblasts and smooth muscle cells adhesion and spreading as well as endothelial cell-to-cell adhesion and permeability [Ferrero et al., 2004].

In previous studies [Gallo et al., 2007; Cerra et al., 2008] we showed that VS-1 induces a PI3K-dependent nitric oxide (NO) release by endothelial cells, providing an unifying working hypothesis for explaining at the cellular level both the mechanism of the cardiac inotropic effect of VS-1 and of the vasodilation observed in some vascular preparations [Aardal and Helle, 1992; Angeletti et al., 1994; Brekke et al., 2002]. However, in the absence of a typical high-affinity membrane receptor, the initiating step required for this intracellular cascade remains unrevealed.

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Regarding this aspect, although binding sites for VSs have been suggested in calf aorta smooth muscle and in bovine parathyroid cells [Angeletti et al., 1994; Russell et al., 1994], a receptor-independent cell penetration or a membrane perturbation-associated mechanism has been postulated [Helle, 2004; Cerra et al., 2006]. This hypothesis was supported by the evidence that the N-terminal domain of VS-1 (CgA_{1-40}) comprises two amphipathic regions, and that the C-terminal domain (CgA_{47-66}) is a cationic and amphipathic peptide, exhibiting potent antifungal activities associated with penetration into the fungi [Lugardon et al., 2001; Maget-Dana et al., 2002]. Therefore, VS-1 amphipathic domains may interfere with subcellular signaling pathways through a plasma membrane interaction mechanism that appear to be involved in toxicity toward microorganisms, while it may inhibit or activate endogenous animal systems in cell- and tissue-specific patterns [Helle et al., 2007].

Grounding on the previous remarks, we propose that in endothelial cells VS-1 interaction with plasma membrane is mediated by proteoglycans because of their negative charge and activates a PI3K-dependent endocytotic machinery. Therefore, as recent reports propose a mechanism of eNOS activation involving caveolae endocytosis [Maniatis et al., 2006; Sanchez et al., 2008; Nishikawa et al., 2009], we investigated on a similar pathway to explain the NO synthase activation exerted by VS-1.

METHODS

CELL CULTURE AND DRUGS

Bovine aortic endothelial (BAE)-1 cells (European Collection of Cell Cultures, Salisbury, Wiltshire, UK) were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS; Biowhittaker, Verviers, Belgium, lot 1SB0019), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine, at 37°C, in a humidified atmosphere of 5% CO_2 in air. Cells were used at passages 2–6 and maintained in 1% FCS 24 h before the experiments.

Methyl-β-cyclodextrin (MβCD) was purchased from Sigma.

We used recombinant human VS-1 [STA-CgA-(1–78)] containing the VS-1 peptide (1–76) previously described [Angeletti et al., 1994].

ANTIBODIES

The expression of total eNOS was detected with a specific monoclonal antibody (BD Biosciences), the $P^{Ser1179}$eNOS and the caveolin-1 with polyclonal antibodies that were purchased, respectively, from Invitrogen and Sigma.

The secondary antibodies employed for immunofluorescence experiments were Alexa Fluor 488 antimouse (Molecular Probes), for total eNOS and Cy3 antirabbit (Sigma) for $P^{Ser1179}$eNOS and caveolin-1. For Western blot experiments we used horseradish peroxidase-conjugated secondary antibodies: antimouse (Amersham) for total eNOS and antirabbit (Amersham) for $P^{Ser1179}$eNOS.

IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY

Cells grown on cover slides were fixed for 30 min in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. After three washes with sodium phosphate buffer (PBS), cells were incubated for 20 min with 0.3% Triton and 1% bovine serum albumin (BSA; Sigma) in PBS and stained with the primary antibody 24 h at 4°C. Cover slides were washed twice with PBS and incubated for 1 h at room temperature with the secondary antibody. After two washes in PBS cover slides were mounted on standard slides with DABCO (Sigma) and observed after 24 h under confocal microscope. Confocal fluorimetric measurements were performed using an Olympus Fluoview 200 laser scanning confocal system (Olympus America, Inc., Melville, NY) mounted on an inverted IX70 Olympus microscope, equipped with a 60× Uplan FI (NA 1.25) and a 100× Uplan FI (NA 1.3) oil-immersion objectives. Image processing and analysis were performed with ImageJ software (W.S. Rasband, U. S. National Institutes of Health, Bethesda, MA, http://rsb.info.nih.gov/ij/, 1997–2008).

WESTERN BLOT ANALYSIS

For Western blot analysis, BAE-1 cells were lysed with lysis buffer (100 mM Tris–HCl, pH 8.0, 1 mM $MgCl_2$, plus inhibitor cocktail) and incubated at −80°C overnight. An equal volume of sucrose buffer containing 20 mM Tris–HEPES, pH 7.4, 315 mM sucrose plus inhibitor cocktail, was added and cell lysate was forced through a 1-ml syringe needle for several times. The inhibitor cocktail contained 2 μg/ml aprotinin, 0.1 mM PMSF, 1 mM sodium orthovanadate, 20 mM sodium fluoride. The protein lysates (15 μg of protein per lane) were run on 8% gradient SDS–PAGE gel, transferred to a polyvinylidene fluoride membrane (PVDF; Millipore), and blocked overnight in TBST (10 mM Tris–HCl, pH 7.5, 0.1 M NaCl, 0.1% Tween 20) plus 5% non-fat dry milk (Bio-Rad). PVDF was incubated, with gentle agitation, for 1 h at 30°C with a monoclonal anti-eNOS antibody or with a polyclonal anti-$P^{Ser1179}$eNOS antibody. After three washes with TBST, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies and then washed again three times with TBST. Protein bands were localized by chemiluminescence using the Super Signal West Pico Kit (Pierce).

ENDOCYTOTIC VESICLES TRAFFICKING

We used the water-soluble membrane dye *N*-(3-triethylaminopropyl)-4-(*p*-dibutylaminostyryl) pyridinium dibromide (FM 1-43; Molecular Probes) to label plasmalemma-derived vesicles, as previously described [Niles and Malik, 1999] and therefore to quantify endocytosis. BAE-1 cells grown on glass-bottom dishes were incubated at 37°C, 5% CO_2 for 15 min in PBS containing 5 μg/ml FM 1-43, 20 mM HEPES, 2 mM Ca^{2+}, 2 mM Mg^{2+} (PBS buffer) for control condition and in PBS buffer plus VS-1 for experimental condition. Before observation cells were washed three times in ice-cold dye-free PBS buffer containing fixed BSA concentration (6 mg/ml, fraction V, 99% pure, endotoxin free; Sigma) to remove all unincorporated styryl probe from the external surface of endothelial cells. To investigate the involvement of heparan sulfate proteoglycans in VS-1-dependent processes, we pretreated BAE-1 cells with 2 U/ml Heparinase III from *Flavobacterium heparinum* (Sigma) for 6 h at 37°C, 5% CO_2. After two washes with PBS, cells were incubated for 15 min with PBS buffer plus VS-1 and we proceeded as described above. To investigate the role of the PI3K pathway in signaling the formation of vesicles, we pretreated BAE-1 cells with the PI3K

inhibitor Wortmannin (100 nM; Sigma) for 20 min and then we incubated in PBS buffer with either 100 nM Wortmannin or VS-1. For each experiment we randomly acquired three fields/sample and then analyzed vesicles by ImageJ Software. Endocytosys quantification was performed on the *Z*-axis reconstruction (i.e., average of the slices) of the stack sequences, in order to analyze the amount of intracellular-stained vesicles in the endocytosis quantification. We applied the "analyze particles" plugin, counting the number in the different conditions of particles/cell/field above a predefined threshold selected to be approximately fourfold brighter with respect to the background.

TRANSFERRIN INTERNALIZATION

Control or heparinase pretreated (6 h at 37°C, 5% CO_2) BAE-1 cells, grown on glass-bottom dishes, were incubated with 20 μg/ml Alexa Fluor 488 transferrin (Invitrogen) for 5 min at 37°C, 5% CO_2. After two washes with PBS, the transferrin internalization was evaluated under confocal microscope.

STATISTICAL ANALYSIS

All values are presented as the mean ± SE. All data were subjected to ANOVA followed by the Bonferroni correction for post hoc tests. Significance was accepted at a P level <0.05.

RESULTS

VS-1 INDUCES ENDOCYTOTIC VESICLES FORMATION

The role of VS-1 in the endocytotic process was determined by incubating BAE-1 cells monolayers wih the styryl pyridinium dye (5 μg/ml) for 15 min at 37°C and then rinsing with dye-free buffer to visualize plasmalemma-derived endocytotic vesicles. In these experiments cells exhibited the punctate distribution of fluorescence characteristic of vesicles (Fig. 1, panel A).

We observed that VS-1 induced a significant increase in the FM 1-43 derived punctate fluorescence (Fig. 1, panel B), indicating a marked stimulation of the endocytotic process.

To quantify these results we randomly acquired three fields/sample in each experiment and we applied the "analyze particles" plugin of ImageJ Software, counting the number of particles/cell/field above a predefined threshold (see the Methods Section), in control condition and after VS-1 stimulation. The bar graph in panel B summarizes this set of experiments: in the VS-1-treated samples the number of bright spots/cell/field was significantly increased with respect to control cells (VS-1: 18,953 ± 3,077, n = 41; control: 9,110 ± 1,449, n = 36, $P < 0.01$).

HEPARINASE AND WORTMANNIN ABOLISH THE VS-1-DEPENDENT VESICLES TRAFFICKING

As the molecular properties of VS-1 (amphipathic helical structures) resemble those of the polycationic peptides (named "cell penetrating peptides" or CPPs), we hypothesized that the first contact of VS-1 with the cell surface takes place through proteoglycans: internalization mechanisms mediated by heparan sulfate proteoglycans (HSPGs) interaction and involving different routes of endocytosis have indeed been described for several CPPs [Morris et al., 2008].

To verify this mechanism HSPGs were selectively removed from the cell surface by treating cells with heparinase III (2 U/ml): in this condition VS-1 strongly reduces its ability to stimulate endocytosis (Fig. 2), suggesting the fundamental requirement for the binding to HSPGs to initiate its intracellular cascade. In these experiments endocytotic process was quantified as described in the previous section and the number of bright spots/cell/field in the different conditions was expressed as percent increase above control (Fig. 2, panel B). To point out the lack of unspecific effects of heparinase on endocytosis machinery, we looked at the transferrin internalization mechanism in heparinase-treated BAE-1 cells. For these experiments we used the Alexa Fluor 488 transferrin on live cells. The results (Fig. 2, panel C) show that clathrin-mediated endocytosis is preserved in heparinase-treated cells, highlighting the specific role of HSPGs in mediating the VS-1 interaction with plasma membrane and therefore in triggering its intracellular cascade.

In a recent report we showed that in BAE-1 cells VS-1 induced a PI3K-dependent NO release [Gallo et al., 2007]. To investigate the role of the PI3K pathway also in the VS-1-dependent endocytotic vesicles formation, we performed the FM 1-43 experiments in the presence of the PI3K inhibitor Wortmannin (100 nM). As shown in Figure 2, Wortmannin strongly reduces the VS-1-dependent endocytosis.

THE VS-1-INDUCED ENDOCYTOSIS INVOLVES CAVEOLIN 1 TRAFFICKING

Different endocytotic mechanisms (macropinocytosis and clathrin- and caveolin-dependent endocytosis) have been described for different CPPs [Morris et al., 2008]. As VS-1 stimulates NO production [Gallo et al., 2007], we hypothesized that the endocytotic process triggered by this endogenous peptide was caveolae dependent. To verify this hypothesis we followed the cellular localization of caveolin 1 (Cav1) by immunofluorescence experiments in different conditions (Fig. 3). In these experiments confocal images were analyzed by applying a Laplacian operator, to enhance edges and therefore better distinguish between plasma membrane bound and internalized Cav1. When cells were incubated in VS-1-free medium (Fig. 3, panel A) Cav1 was, as expected, clearly localized at the plasma membrane. In the presence of VS-1, Cav1 staining appeared significantly reduced at the plasma membrane, while was almost confined in cytoplasmic multivesicular-like structures (Fig. 3, panel B). In agreement with the FM 1-43 endocytosis experiments described in the previous section, both heparinase and Wortmannin were able to revert the Cav1 internalization induced by VS-1 (Fig. 3, panels C and D). These data suggest that the proteoglycans–PI3K-dependent endocytotic process activated by VS-1 in BAE-1 cells involves caveolar trafficking.

VS-1 INDUCES A PI3K AND PROTEOGLYCAN-DEPENDENT eNOS PHOSPHORYLATION

In a previous work we showed a PI3K-dependent increase in NO production in VS-1-treated BAE-1 cells [Gallo et al., 2007]. In the present report we observed that VS-1 induces a HSPGs–PI3K-dependent endocytosis of caveolae. Taken together, these results

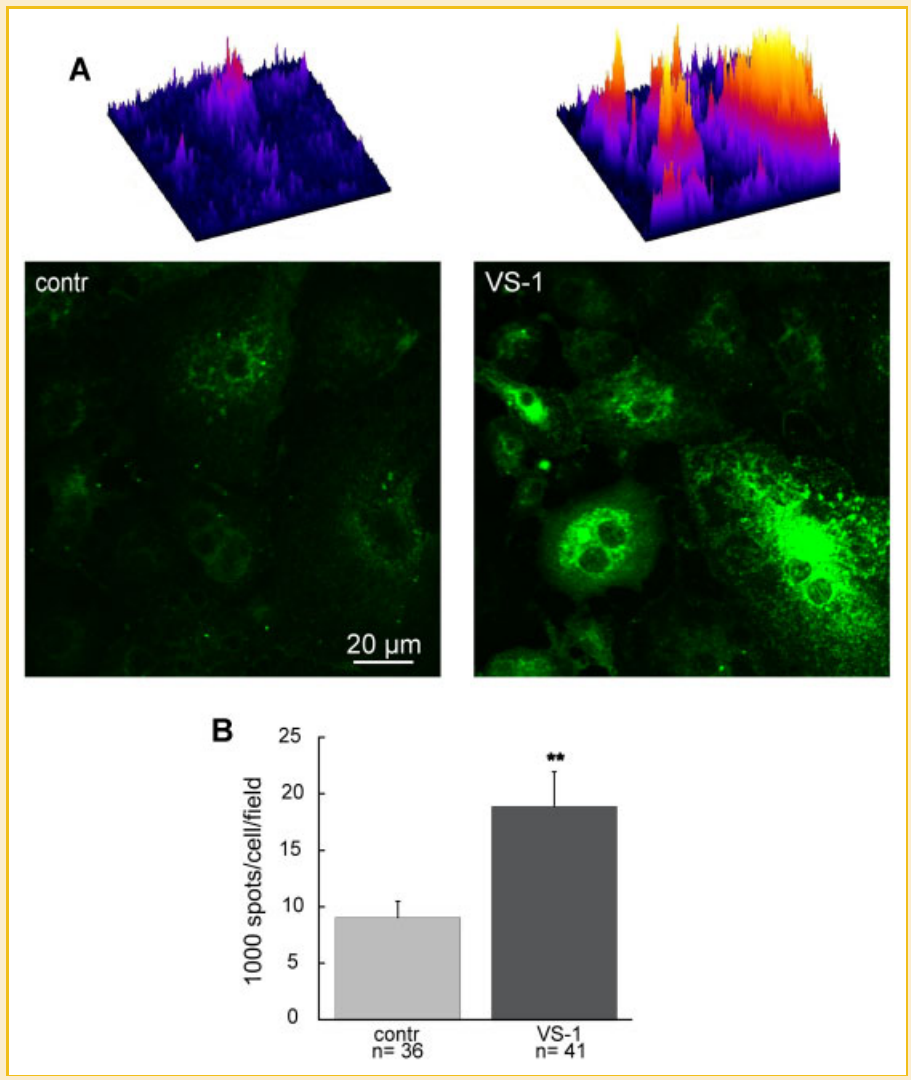

Fig. 1. Effect of VS-1 on endocytotic vesicles formation in BAE-1 cells. Panel A: BAE-1 cells incubated with the water-soluble styryl pyridinium membrane dye (FM 1-43). The punctate fluorescence distribution indicates the increase in the vesicles formation consequent to VS-1 (100 nM) stimulation. The upper graphs (surface plots) display three-dimensional graphs of the intensities of pixels in a pseudo-color image. The height and the color represent the pixel intensity. In panel B, the bar graph shows the number of particles/cell/field above a predefined threshold (selected to be approximately fourfold brighter with respect to the background) in control (9,110 ± 1,449, n = 36 fields) and VS-1 stimulated BAE-1 cells (18,953 ± 3,077, n = 41 fields). Magnification: 60×. Scale bar: 20 μm. $^{**}P < 0.01$.

suggest a critical role for both proteoglycans and endocytosis of caveolae in the VS-1-dependent NO production.

Therefore, the following step of our study was to examine the eNOS activation mechanism involved in the VS-1-dependent pathway and the role carried out by both PI3K and HSPGs in this process. For this purpose we performed Western blot and immunofluorescence experiments for eNOS and $P^{Ser1179}$eNOS in different conditions.

Our results show that VS-1 (100 nM) induces a significant and time-dependent increase in the level of $P^{Ser1179}$eNOS (Fig. 4, panel A). We also investigated on the VS-1-dependent eNOS activation by following the eNOS/Cav1 colocalization in control condition and in the presence of the peptide. Dissociation of eNOS from Cav1 has been indeed shown as a marker of eNOS activation [Minshall et al., 2003]. We observed that VS-1 strongly reduces the eNOS/Cav1 colocalization at plasma membrane detected in control condition (Fig. 4, panel B).

Moreover, the increase in $P^{Ser1179}$eNOS induced by VS-1 was strongly reduced by Wortmannin (Fig. 4, panel C).

The VS-1-dependent increase in $P^{Ser1179}$eNOS was also blocked by heparinase, as we observed in both immunofluorescence (Fig. 5) and Western blot experiments (Fig. 6, panel A).

In these experiments (Fig. 5) confocal images analysis was performed by applying the colocalization finder plugin of ImageJ, to highlight the eNOS/PeNOS colocalization pixels (green spots) on a grayscale eNOS image (panel A), and to display correlation diagrams for the eNOS and $P^{Ser1179}$eNOS RGB images (panel B). The colocalization results clearly show that heparinase is able to revert the increase in $P^{Ser1179}$eNOS level induced by VS-1, as also shown by Western blot technique (Fig. 6, panel A).

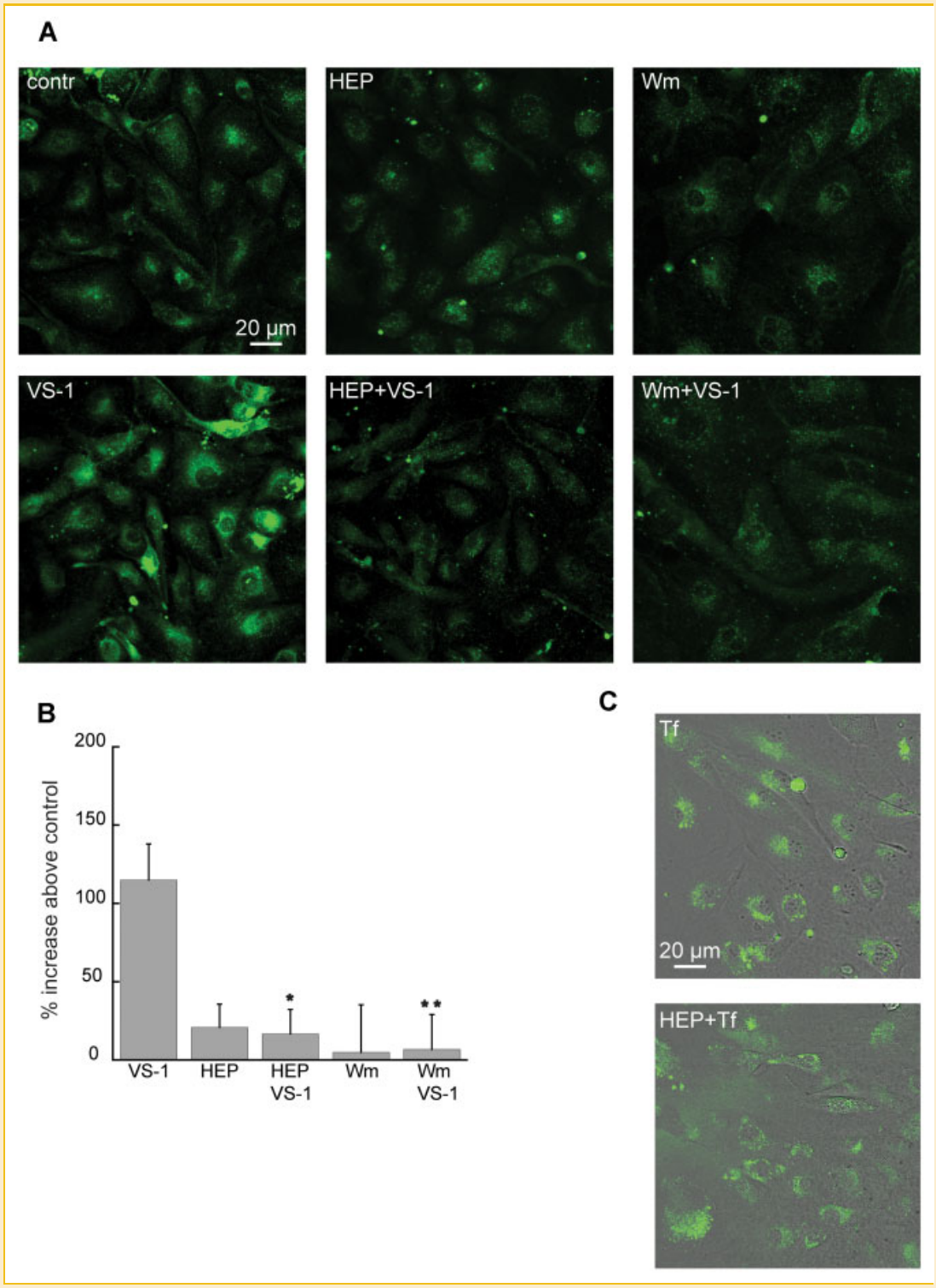

Fig. 2. Heparinase III and Wortmannin inhibit VS-1-dependent endocytotic process in BAE-1 cells. BAE-1 cells incubated with the water-soluble styryl pyridinium membrane dye (FM 1-43) in different conditions. Panel A: BAE-1 cells were treated with 2 U/ml HEP to remove HSPGs from cell surface, or with 100 nM Wortmannin in order to block PI3K. In these conditions, VS-1 significantly reduced the endocytotic process. In panel B, the bar graph represents the percentage of increase above control of spots/cell/field in VS-1-stimulated BAE-1 cells ($115 \pm 23\%$, $n = 20$), in HEP-treated cells ($21 \pm 15\%$, $n = 7$), in HEP + VS-1-treated cells ($17 \pm 15\%$, $n = 7$), in Wm-treated cells ($5 \pm 30\%$, $n = 14$), and in Wm + VS-1-treated cells ($7 \pm 22\%$, $n = 14$). Panel C: the clathrin-mediated endocytosis of Alexa Fluor 488-Transferrin (Tf, top panel) is unaffected by HEP treatment (bottom panel). Magnification: 60×. Scale bar: 20 μm. $^{*}P < 0.05$.

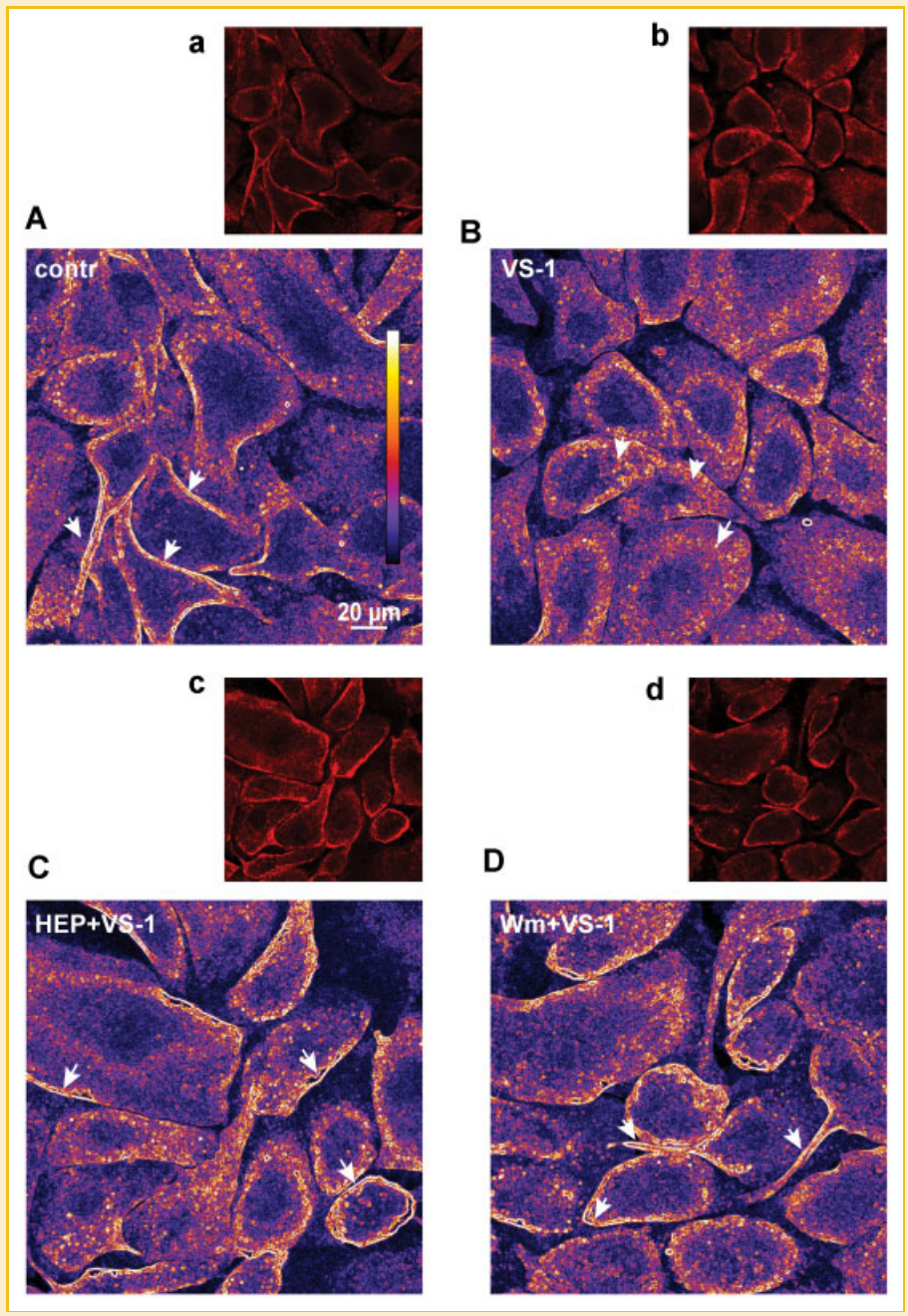

Fig. 3. Caveolin 1 trafficking is involved in the VS-1-dependent endocytotic process in BAE-1 cells. Immunofluorescence staining for Cav1 in BAE-1 cells in different conditions. Confocal images were analyzed by applying the Laplacian operator of the ImageJ Software, to enhance edges corresponding to plasma membrane bound and to multivesicular-like bound Cav1 pools. Panel A shows the typical plasma membrane associated Cav1 as indicated by the arrows. In VS-1-treated BAE-1 cells (panel B), Cav1 is internalized in vesicular-like structures indicating a caveolae-mediated endocytotic process. HSPGs depletion and PI3K blockade both restore the plasma membrane Cav1 association (panels C and D). Panels a–d represent the original fluorescence micrographs. Images are representative of three experiments. Scale bar: 20 μm. Magnification: 60×.

CAVEOLAE DISRUPTING ABOLISHES THE VS-1-INDUCED eNOS PHOSPHORYLATION

To strengthen the proposed pathway of a VS-1-dependent caveolae internalization and later eNOS phosphorylation, we evaluated the level of VS-1-induced $P^{Ser1179}$eNOS in the presence of MβCD (5 mM, 30 min; Fig. 6, panel B), by Western blot experiments: the lack of VS-1-dependent eNOS phosphorylation after caveolae destabilization strongly supports the obligatory requirement of caveolae integrity to allow the VS-1 intracellular pathway activation.

DISCUSSION

The present study reveals the first step of the signaling pathway activated by the cardio-inhibitory and vasodilator peptide VS-1,

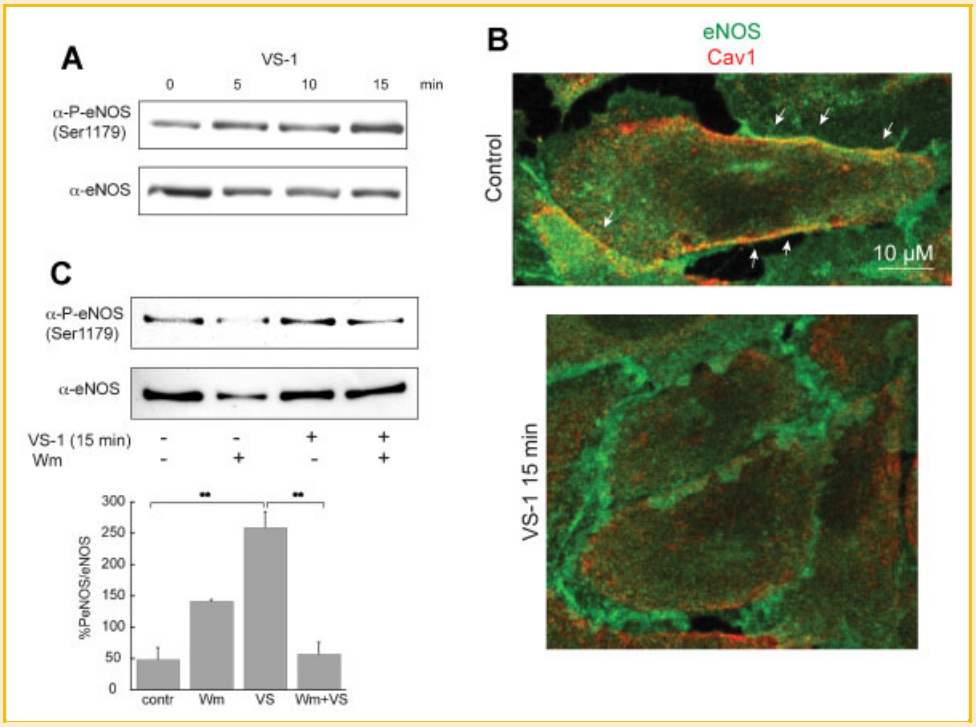

Fig. 4. VS-1 stimulates a PI3K and Cav1-dependent eNOS activation in BAE-1 cells. Panel A: Western blot experiment showing that VS-1 induces a time-dependent increase in the level of $P^{Ser1179}$eNOS. Panel B: confocal immunofluorescence imaging on BAE-1 cells showing the colocalization (yellow spots indicated by arrows) of eNOS/Cav1 at plasma membrane in control condition, while the eNOS/Cav1 colocalization is strongly decreased in VS-1-treated BAE-1 cells. Two-color immunofluorescence was carried out using Alexa Fluor 488 antimouse for total eNOS combining with Cy3 antirabbit for Cav1. Magnification: 100×. Scale bar: 10 μm. Panel C: upper panel: representative Western blot experiment showing that VS-1-induced $P^{Ser1179}$eNOS is strongly reduced by Wm (100 nM). Lower panel: $P^{Ser1179}$eNOS/eNOS ratio of densitometric values from Western blots. Data are given as % values ± SE ($^{**}P < 0.01$). Blots and immunofluorescence images are representative of a minimum of three experiments.

identifying a proteoglycans–PI3K-dependent caveolae endocytosis as the initiating factor for its intracellular cascade.

The premise of our working hypothesis grounded on the biochemical feature of VS-1, which is structurally characterized by amphipathic properties and interactions with mammalian and microbial membranes [Kang and Yoo, 1997; Maget-Dana et al., 2002].

As it was recently shown that endocytosis plays a mayor role in the signaling of different basic and amphipathic exogenous peptides, that is, Antp, R9, and Tat [Richard et al., 2005; Duchardt et al., 2007], we supposed a similar mechanism for VS-1.

The results presented in Figure 1 clearly show that in BAE-1 cells VS-1 strongly increases the number of endocytotic vesicles. Moreover, VS-1 induces Cav1 displacement from the plasma membrane of BAE-1 cells (Fig. 3), suggesting caveolae-mediated endocytosis as the form involved in this process. Interestingly, internalization of CgA1–78 has been recently observed in HUVEC, with respect to its protective role against TNF-induced vascular leakage [Ferrero et al., 2004].

Given the critical requirement for the surface HSPGs for endocytosis of cationic peptides [Poon and Gariépy, 2007], a receptorial-like role for HSPGs in the VS-1 pathway was very likely.

HSPGs, which are the mayor components of extracellular matrix, act as an anchor for many external molecules and pathogens at the host cell surface. In particular, HSPGs of the syndecans subfamily are membrane spanning proteoglycans composed of a polysaccharide side chain (HS), that because of its high negative charge binds a plethora of proteins, and a core protein, that may recruit specific interacting partners activating pathways [Bishop et al., 2007].

Along with these knowledge, we observed that HSPGs removal by treatment of BAE-1 cells with heparinase completely abolished the VS-1-dependent endocytosis (Fig. 2). Moreover, heparinase also reverted the VS-1-induced displacement of Cav1 from the plasma membrane to cytoplasm (Fig. 3, panel C). This latter result strengthens our hypothesis of a caveolae-dependent endocytotic process.

We also observed that VS-1-induced vesicles trafficking (Fig. 2) and Cav1 displacement (Fig. 3, panel D) were both suppressed by Wortmannin. PI3K is widely reported to have an important role in membrane budding and fission in endothelial cells [Li et al., 1995; Niles and Malik, 1999], and recently a mechanism of eNOS activation involving caveolae-mediated endocytosis, Src, Akt, and PI3K pathway in endothelial cells has been shown [Maniatis et al., 2006].

The PI3K pathway could therefore represent the central core of the VS-1-activated cellular signaling, as its blockade inhibits the endocytotic process (Fig. 2), the Cav1 trafficking (Fig. 3, panel D), and the eNOS phosphorylation mechanism (Fig. 4, panel C).

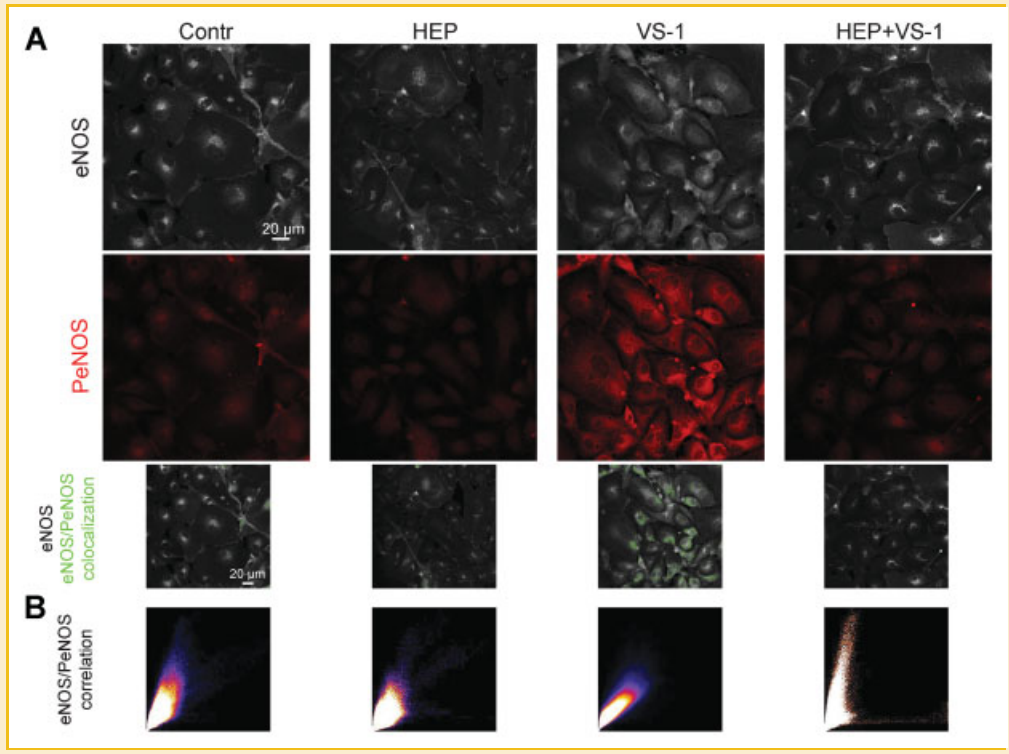

Fig. 5. Proteoglycans removal abolishes the VS-1-dependent eNOS activation in BAE-1 cells. Panel A: confocal immunofluorescence imaging on BAE-1 cells showing that eNOS phosphorylation ($P^{Ser1179}$) is blocked by heparinase III (2 U/ml). The upper and middle panels show, respectively, eNOS (gray) and $P^{Ser1179}$eNOS (red) staining, while the lower panels represent confocal immunofluorescence analysis performed by applying the colocalization finder plugin of ImageJ Software, to highlight the eNOS/$P^{Ser1179}$eNOS colocalization pixels (green spots) on a grayscale eNOS image. Magnification: 60×. Scale bar: 20 μm. Panel B: correlation diagrams clearly evidence eNOS/$P^{Ser1179}$eNOS colocalization in the different experimental conditions shown in panel A. Immunofluorescence images are representative of a minimum of three experiments.

In agreement to our results, the membrane remodeling and actin filament dynamics during endocytotic traffic seem to be strictly related to the PI3K/eNOS pathway, as the guanosine triphosphatase dynamin regulates vesicle scission and interact with both PI3K and eNOS with its consequent activation [Cao et al., 2003; Schafer, 2004].

Furthermore, NO has been widely reported as an important regulator of vesicles trafficking in the cardiovascular system, as *S*-nitrosylation of dynamin accelerates the cleavage of vesicles from the plasma membrane [Wang et al., 2006].

PI3K/Akt-mediated Ser1179 phosphorylation represents a common pathway among the multiple regulatory mechanisms affecting eNOS activity. This mechanism has been linked to several typical high-affinity membrane receptor-dependent stimulations. In this perspective, the originality of our results is the evidence for a novel machinery leading to eNOS Ser1179 phosphorylation, and the highlight on the role of HSPGs and caveolae in triggering the whole process, as the VS-1-dependent increase in eNOS Ser1179 phosphorylation was blocked by both heparinase treatment (Figs. 5 and 6, panel A) and MβCD-induced caveolae destabilization (Fig. 6, panel B).

The involvement of HSPGs in the Akt-eNOS activation has been recently proposed in endothelial cells also with regard to tyrosine kinase receptors signaling. Interestingly, syndecan-4 null mice ($S4^{-/-}$) have an increased arterial blood pressure and $S4^{-/-}$ endothelial cells show decreased eNOS phosphorylation [Partovian et al., 2008].

As HSPGs and extracellular matrix also seem to participate in the mechanosensing that mediates NO production in response to shear stress [Florian et al., 2003], our results enhance the relevance of the HSPGs–NO axis in the control of the vasomotor tone.

In conclusion, we have elucidated the mechanism responsible for the physiological properties of VS-1 on endothelial cells, in particular with respect to its ability to activate the intracellular PI3K–eNOS pathways in the absence of a typical high-affinity membrane receptor.

This is, to our knowledge, the first example of an endogenous and physiological ''cell penetrating peptide'' exerting modulatory properties on cell functions.

Moreover, as VS-1 takes part in the protective modulation of the cardiovascular activity because of its ability to counteract the adrenergic signal, elucidations on its signaling pathway represent important findings in the cardiovascular basic science.

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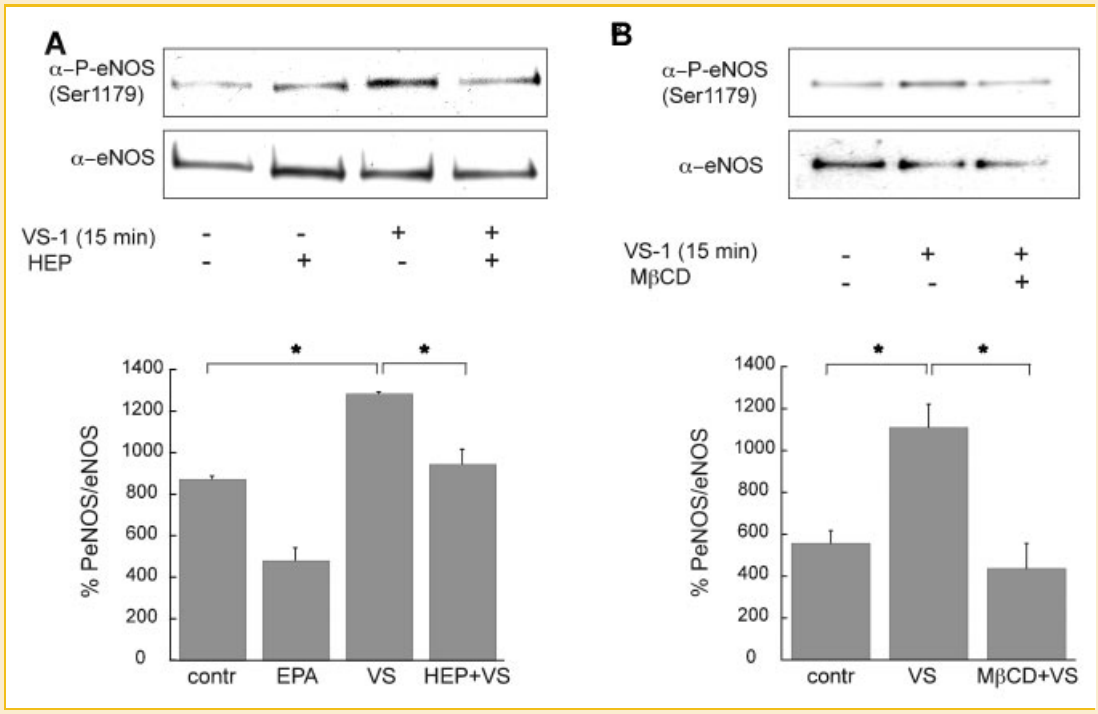

Fig. 6. Both proteoglycans and caveolae are required to allow the VS-1-dependent eNOS phosphorylation. Panel A: upper panel: typical Western blot experiment showing that VS-1-induced $P^{Ser1179}$eNOS is reduced by HEP (2 U/ml). Lower panel: $P^{Ser1179}$eNOS/eNOS ratio of densitometric values from Western blots. Data are given as % values ± SE. $^{*}P < 0.05$. Panel B: upper panel: typical Western blot experiment indicating that VS-1-induced $P^{Ser1179}$eNOS is reduced by MβCD (5 mM, 30 min). Lower panel: $P^{Ser1179}$eNOS/eNOS ratio of densitometric values from Western blots. Data are given as % values ± SE. $^{*}P < 0.05$. Blots are representative of a minimum of three experiments.

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