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Caveolin-1 Detergent Solubility and Association with Endothelial Nitric Oxide Synthase Is Modulated by Tyrosine Phosphorylation

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Caveolin-1 and endothelial nitric oxide synthase (eNOS) are associated within endothelial caveolae. We have shown previously that eNOS is translocated to the detergent-insoluble, cytoskeletal fraction of bovine aortic endothelial cells (BAEC) in response to bradykinin (BK)-stimulation or tyrosine phosphatase inhibition. In the present study, we have examined whether caveolin-1 is similarly translocated in response to these or other stimuli. Exposure of BAEC to the eNOS-activating agonists, BK, histamine, or ATP produces transient increases in the amounts of detergent-insoluble caveolin-1. Increases in insolubility are blocked by tyrosine kinase inhibitors and are potently mimicked by tyrosine phosphatase inhibitors. Increased insolubility is accompanied by an increased association of caveolin-1 with eNOS and inhibition of eNOS catalytic activity. Agonist-activation of eNOS in endothelial cells thus appears to involve tyrosine phosphorylation-dependent changes in the interaction of eNOS with caveolin-1. Increased interaction of eNOS with caveolin-1 may deactivate the enzyme subsequent to its activation by Ca^{2+}/calmodulin.

Caveolae are small, bulb-shaped invaginations of the plasma membrane that are especially abundant in endothelial cells. These membrane microdomains were initially identified because of their function in the transport processes of endocytosis, potocytosis, and transcytosis. More recently, it has been recognized that caveolae also function as plasma membrane signal transduction centers that compartmentalize hormone receptors with downstream effectors (1-3). A principal protein component of caveolae is caveolin (4), a 21-24 kDa integral membrane protein of undefined function. Three homologous but distinct caveolin proteins are known (termed caveolins-1, -2, and -3) that have different but overlapping tissue distributions (3). The form of caveolin expressed in endothelial cells is caveolin-1. Caveolin-1 exists in two isoforms that are derived from the use of two alternate translational start sites, methionine at position 1 and an internal methionine at position 32 (5). These two isoforms are designated caveolin-1α (24 kDa) and caveolin-1β (21 kDa), respectively. Caveolin-1α and caveolin-1β can both form SDS-resistant, high molecular mass homooligomers comprised of up to 14 subunits (6,7). Caveolin-1 can also be purified from many cell types as part of a heterooligomeric complex with various signaling proteins including G protein subunits and nonreceptor tyrosine kinases (8-10). It has been suggested, therefore, that caveolin-1 homooligomeric complexes may serve as docking sites for sequestration of caveolin-interacting signaling molecules at the cytoplasmic face of the plasma membrane (6). Caveolin-1 is not static in the plasma membrane, however, but cycles dynamically between caveolae and the Golgi apparatus (11,12).

Endothelial nitric oxide synthase (eNOS), like caveolin-1, is a membrane-associated protein, localized predominately in the plasmalemmal caveolae and Golgi of endothelial cells (13-19). Recently, we have shown that eNOS translocates to the Triton X-100-insoluble, cytoskeletal fraction of bovine aortic endothelial cells (BAEC) upon stimulation of BAEC with bradykinin (BK) or the tyrosine phosphatase inhibitor, phenylarsine oxide (PAO) (20). In the present study, we have investigated whether caveolin-1, known to be associ-

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Abbreviations: eNOS, endothelial nitric oxide synthase; BK, bradykinin; PAO, phenylarsine oxide; BAEC, bovine aortic endothelial cells; BLMVEC, bovine lung microvascular endothelial cells.

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ated with eNOS in BAEC (19,21), is translocated together with eNOS following either agonist-stimulation or tyrosine phosphatase inhibition. We have also determined whether tyrosine phosphorylation-dependent changes in eNOS or caveolin-1 detergent-solubilities represents movement of the proteins from the Golgi to caveolae or *vice versa*. In addition, we have examined whether tyrosine phosphorylation regulates eNOS-caveolin-1 complex formation or eNOS catalytic activity.

MATERIALS AND METHODS

Materials. Monoclonal (clone #3) and polyclonal anti-eNOS antibodies, monoclonal (clone #2297) and polyclonal anti-caveolin-1 antibodies, PY20 anti-phosphotyrosine monoclonal antibody, and PY20 agarose were purchased from Transduction Laboratories (Lexington, KY). Geldanamycin, Protein A/Protein G agarose, and immunoprecipitin were obtained from Life Technologies Inc. BK, PAO, histamine, ATP, sodium orthovanadate, monoclonal β-COP antibody, and whole anti-mouse IgG came from Sigma Chemical Co. PP1 was obtained from Calbiochem. ECL detection kits were purchased from Amersham. Protein molecular weight standards, protein assay kit, and peroxidase-conjugated anti-IgG antibodies were purchased from Bio-Rad.

Cell culture. BAEC were passaged from primary cultures. Bovine lung microvascular endothelial cells (BLMVEC) were obtained from Cell Systems Incorporated (Kirkland, WA). BAEC cultures were maintained as described previously (20). BLMVEC were maintained in CS-C complete media on dishes coated with attachment factor from Cell Systems Incorporated. Cells were subcultured at 3-5 day intervals and used for experiments in passage four through passage six. Serum-containing medium was replaced by serum-free medium 24 hours prior to each experiment.

Treatment of endothelial cells with agonists and tyrosine phosphatase inhibitors. Confluent BAEC or BLMVEC in 100 mm culture dishes were treated with BK (10 μM), histamine (10 μM), ATP (1 mM), PAO (10 μM), or Na_3VO_4 (1mM) for 0,0.5,1,2,5, or 10 minutes at 37° C and prepared for immunoprecipitation and analysis of detergent-solubility as described previously (20). In some experiments, eNOS activity of cell lysates was determined by arginine-to-citrulline conversion assay as described previously (22).

Immunoprecipitation. One ml aliquots of the RIPA lysates were precleared for 1 hour at 4° C with 20 μl immunoprecipitan prior to addition of either anti-eNOS (clone 3, monoclonal), anti-caveolin-1 (polyclonal), or anti-phosphotyrosine antibody (PY20, monoclonal) (0.5 μg). Immunoprecipitation and immunoblotting were carried out as described previously (20). Antibodies used for immunoblotting were anti-eNOS (polyclonal), anti-caveolin-1 (clone 2297, monoclonal), and anti-phosphotyrosine (PY20, monoclonal) antibody.

Analysis of eNOS and caveolin detergent solubilities. Triton X-100-soluble and Triton X-100-insoluble fractions of cell lysates (1.5 ml) were separated by centrifugation at 12,000 $\times$ *g* and analyzed as described previously (20).

Isolation of caveolae. Caveolae membranes were isolated from BAEC and BLMVEC cultures essentially as described by Song *et al.* (23) except that all solutions used in the isolation were buffered at pH 7.5.

RESULTS AND DISCUSSION

To determine the percent of detergent-soluble and detergent-insoluble caveolin-1 in BAEC under basal conditions, cells were lysed in ice-cold buffer containing 1% Triton X-100 and detergent-soluble (supernatant) and insoluble (pellet) fractions were separated by centrifugation. Relative amounts of caveolin-1 in soluble and insoluble fractions were quantitated by immunoblotting with anti-caveolin-1 antibody and densitometric scanning of immunoblots. Suprisingly, 95.5 $\pm$ 3.7% of caveolin-1α and -1β in BAEC was found to be detergent-soluble. Similar results were also obtained with BLMVEC. The detergent-solubility of caveolin-1 in cultured endothelial cells, therefore, appears to differ significantly from that of caveolin-1 in caveolae prepared directly from lung endothelium or in caveolae from other types of cultured cells. Caveolin-1 in caveolae isolated from lung has been reported to be insoluble in 1% Triton X-100 (9,24). Caveolin-1 in cultured fibroblasts, adipocytes, and epithelial cells is also predominately detergent-insoluble (1-3,8).

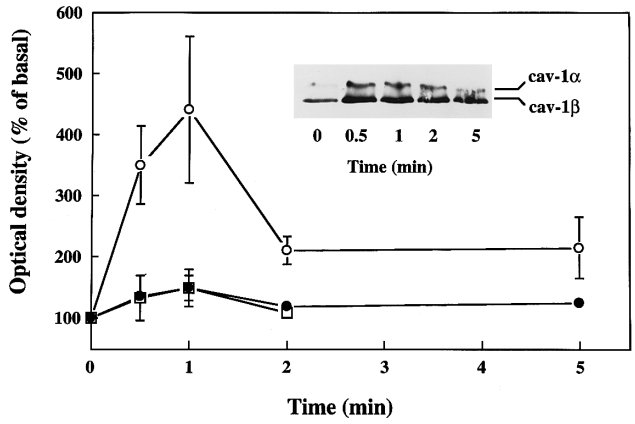

FIG. 1. BK-stimulated alteration of the detergent-solubility of caveolin-1 in BAEC. Following pretreatment with or without geldanamycin (1 μg/ml) or PP1 (10 μM), cells were stimulated with BK (10 μM) for 0,0.5,1,2, and 5 min and lysed in buffer containing 1% Triton X-100. Triton X-100-insoluble (pellet) and Triton X-100-soluble (supernatant) fractions were separated by centrifugation at 12,000*g*. Proteins in insoluble fractions at various time points were subjected to polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with anti-caveolin-1 antibody. Shown is a representative autoradiogram obtained in the absence of inhibitors. Also shown is densitometric analysis of three autoradiograms each for the control (○ — ○), plus geldanamycin (● — ●), and plus PP1 (□ — □) conditions (mean ± S.E.M., n = 3).

BAEC were treated with BK (10 μM) for various times and the amounts of caveolin-1 and eNOS in detergent-insoluble fractions at various time points were then quantitated by immunoblotting and densitometry. BK stimulated a transient (4-fold) increase in the amount of detergent-insoluble caveolin-1α and -1β (Fig. 1). When eNOS detergent-solubility was assessed in the same experiments, solubility changes of eNOS paralleled those of caveolin-1. To determine if the BK-stimulated increases in detergent-insolubilities of caveolin-1 were dependent on tyrosine phosphorylation,

we utilized the tyrosine kinase inhibitors, geldanamycin and PP1. Geldanamycin has been shown previously to be a potent inhibitor of the abl, erbB, fps, and Src family tyrosine kinases (25). PP1 has been shown to be highly selective for Src tyrosine kinases relative to other known tyrosine kinases (26). BAEC were treated with geldanamycin (1μg/ml) for 1 h or PP1 (10 μM) for 15 min prior to determining the time-course of BK-stimulated changes in the detergent-solubility of caveolin-1. Pretreatment with either geldanamycin or PP1 almost completely blocked the BK-stimulated changes in caveolin-1 detergent-solubility (Fig. 1). BK-stimulated solubility changes of caveolin-1, therefore, appear to depend on tyrosine phosphorylation and may occur through a signaling cascade involving a Src family tyrosine kinase. Results similar to those shown in Fig. 1 were also obtained following BK stimulation of BLMVEC. In addition, similar results (in both magnitude and time-dependence) were also obtained when BAEC were stimulated by either histamine (10 μM) or ATP (1 mM).

BAEC were treated with the tyrosine phosphatase inhibitor, PAO (10 μM) for various times and detergent-soluble and -insoluble fractions were prepared. The relative contents of caveolin-1 in the both soluble and insoluble fractions for various time points were quantitated by immunoblotting and densitometry. As shown in Fig. 2, caveolin-1α and -1β, which were predominately detergent-soluble under basal conditions, became almost entirely detergent-insoluble following 10 min of PAO treatment. In the same experiments, similar PAO-induced changes in solubility were also observed for eNOS. PAO effects were specific for caveolin-1 and eNOS because no changes were detected in the total amounts of insoluble protein. Data comparable to those shown in Fig. 2 were also obtained for BLMVEC. Geldanamycin pretreatment (1 μg/ml for 1 h) reduced the PAO-induced (10 μM for 10 min) translocation of caveolin-1 by 86 ± 12% (mean ± S.E.M., n = 3). Furthermore, a structurally different tyrosine phosphatase inhibitor, sodium orthovanadate (1 mM) stimulated a similar redistribution of caveolin-1 and eNOS except that the magnitude of the response was less. These data, together with the geldanamycin and PP1 inhibition of BK-stimulated translocation shown in Fig. 1, suggest that detergent-solubilities in endothelial cells of both caveolin-1 and eNOS are regulated by tyrosine phosphorylation. Caveolin-1 detergent-solubility has been a subject of considerable interest to many researchers (1-3,27). That solubility may be altered by agonist-stimulation and by tyrosine phosphorylation, however, has not been reported previously.

We next investigated whether agonist-activation of eNOS involves a transient change in the subcellular localization of the eNOS-caveolin-1 complex. Our rationale for investigation of this possibility is that movement to a different subcellular location could explain the change in detergent-solubility. Alternatively, agonist-stimulated changes in solubility may reflect a transient association of eNOS and caveolin-1 with caveolae cytoskeletal proteins such as actin, myosin II, annexin II, or dystrophin which have been shown previously to reside in caveolae (9). Indeed, agonist-stimulated increases in insolubility of a protein in Triton X-100 is commonly interpreted as a translocation to the cytoskeleton. This is the interpretation that is given, for example, for the increases in insolubility of c-Src, phosphoinositide 3-kinase, protein kinase C, phospholipase C, and other proteins reported to occur in platelets in response to thrombin-stimulation (28-32). To determine whether changes in detergent-solubilities of ca-

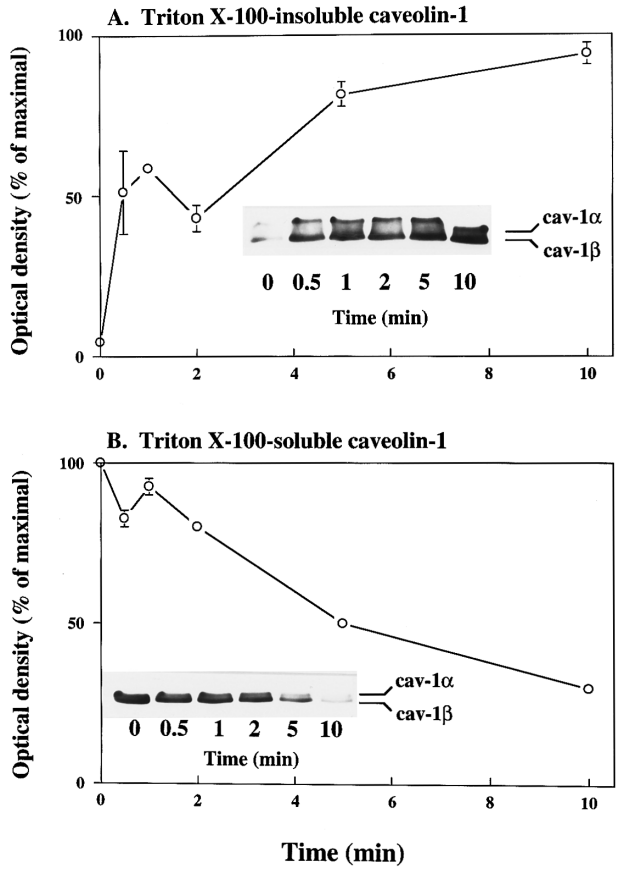

FIG. 2. PAO-stimulated alteration of the detergent-solubility of caveolin-1 in BAEC. Cells were treated with PAO (10 μM) for 0,0.5,1,2,5, and 10 min and lysed in buffer containing 1% Triton X-100. Triton X-100-insoluble (pellet) and Triton X-100-soluble (supernatant) fractions were separated by centrifugation at 12,000*g*. Proteins in both insoluble and soluble fractions at various time points were subjected to polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with anti-caveolin-1 antibody. Shown is a representative autoradiogram and densitometric analysis of three autoradiograms (mean ± S.E.M., n = 3). *Panel A,* Triton X-100-insoluble caveolin-1; *Panel B,* Triton X-100-soluble caveolin-1.

veolin-1 and eNOS represents movement of the proteins from Golgi to caveolae (or *vice versa*) we isolated caveolae membranes from BAEC following either no treatment or treatment with BK (10 μM, 1 min) or PAO (10 μM, 10 min). Caveolae were purified by a modification of the detergent-free method of Song *et al.* (23). To verify that our isolation procedure effectively separated caveolae membranes from Golgi membranes, we have used an antibody to β-COP to probe for the presence of Golgi vesicles in gradient fractions. β-COP is a peripheral Golgi membrane protein that is present in a membrane-bound (~20%) form and in a cytosolic complex (~80%). The membrane-bound form is associated with both Golgi cisternae and Golgi vesicles in the trans-Golgi network (33-37). To confirm that β-COP remains associated with Golgi vesicles during the lysis and homogenization of cells, we centrifuged the homogenate at 100,000 $\times$ *g* and quantitated the amount of particulate and soluble β-COP by immunoblotting with anti-β-COP antibody. Approximately 20% of β-COP immunoreactivity was found in the particulate fraction indicating that the protein remains associated with Golgi under these isolation conditions.

BAEC homogenates were fractionated on a discontinuous sucrose gradient and the distribution of total protein, β-COP, caveolin-1, and eNOS in gradient fractions were determined. Very little protein was found in the upper, low density fractions of the sucrose gradient (Fig. 3B). However, when the caveolin-1 content of fractions was determined by immunoblotting with anti-caveolin-1 antibody, ~95% of caveolin-1 was found in fractions 3 and 4, a position corresponding to the interface between the 5% and 35% sucrose layers (Fig. 3A). The fractions of the gradient that contain caveolae are thus identified as fractions 3 and 4. These two fractions were uncontaminated by Golgi membranes because no β-COP immunostaining could be detected in either fraction even after very long exposure times of the X-ray film used to detect the chemiluminescent signal on the blot. Golgi membranes were identified as being in fraction 6 because this was the only fraction containing all three of the Golgi-associated proteins caveolin-1, eNOS, and β-COP. These data suggest that almost all caveolin-1 in BAEC is localized in caveolae. Therefore, our finding that BAEC caveolin-1 is 95% Triton X-100-soluble cannot be explained by a lack of caveolae in BAEC resulting from the reported tendency of endothelial cells to lose caveolae during maintenance in culture (38). When the eNOS contents of sucrose gradient fractions were quantitated by immunoblotting, approximately 35% of the enzyme was found in the caveolae fractions with the remainder being found in the lower, high density fractions of the gradient containing Golgi-associated and cytosolic eNOS (Fig. 3A). Similar results to those shown in Fig. 3 were also obtained with BLMVEC. Furthermore, when caveolae membranes

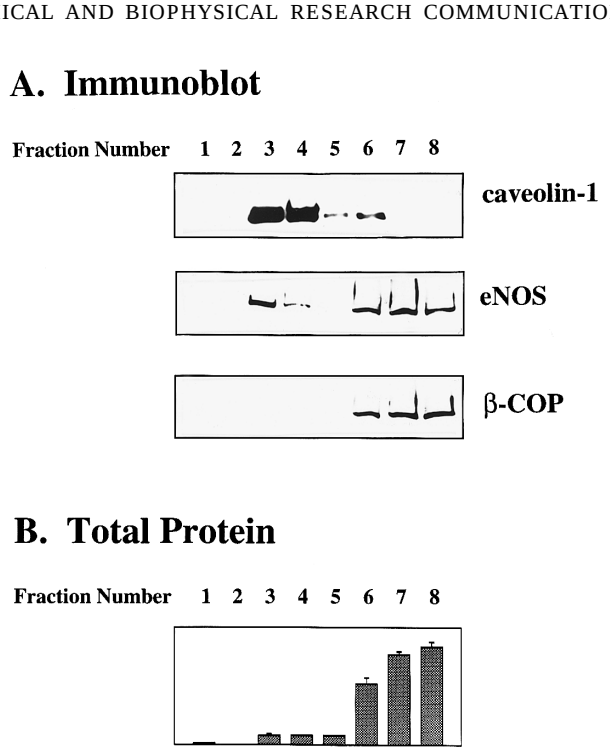

FIG. 3. Distribution of total protein, caveolin-1, eNOS, and β-COP from BAEC in sucrose density gradient fractions. BAEC homogenates were fractionated in a discontinuous sucrose density gradient, eight fractions were collected from the top of each tube, and aliquots of each fraction were analyzed by immunoblotting for the content of caveolin-1, eNOS, β-COP, and total protein.

were prepared from BAEC or BLMVEC following stimulation with BK or PAO, no change in the caveolae content of either caveolin-1 or eNOS was detected indicating that solubility changes of the two proteins do not represent a change in subcellular localization.

To determine whether BK or tyrosine phosphatase inhibition alters eNOS-caveolin-1 complex formation, BAEC were either treated or not treated with BK (10 μM, 1 min) or PAO (10 μM, 10 min), cells were lysed in RIPA buffer (which solubilizes 100% of both eNOS and caveolin-1), and equal amounts of eNOS were immunoprecipitated from the various conditions with anti-eNOS antibody. Precipitated proteins were separated by gel electophoresis, transferred to nitrocellulose, and immunoblotted with anti-caveolin-1 antibody. Only caveolin-1α (and not -1β) was coprecipitated with eNOS. The amount of caveolin-1α that was coprecipitated was increased by 108 $\pm$ 28% and 311 $\pm$ 59% (mean $\pm$ S.E.M., n = 5) by BK and PAO treatment, respectively (Fig. 4). Furthermore, a 1 h treatment of BAEC with geldanamycin decreased the basal level of eNOS associated with caveolin-1α by 52 $\pm$ 9% (n = 7). Pretreatment with geldanamycin also significantly blocked any subsequent BK- or PAO-stimulated increases in association (n = 3). The extent or the stability of the eNOS-caveolin-1 association in BAEC thus

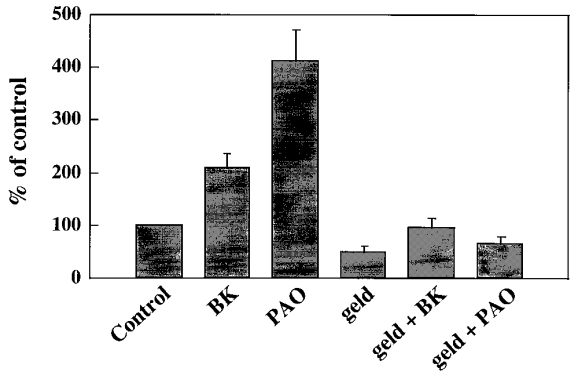

FIG. 4. Effect of BK, PAO, and geldanamycin treatment of BAEC on the amount of caveolin-1α coimmunoprecipitated by anti-eNOS antibody. BAEC were either not treated (*control*) or treated with BK (10 μM, 1 min) (*BK*), PAO (10 μM, 10 min) (*PAO*), geldanamycin (1 μg/ml, 1 h) (*geld*), geldanamycin (1 μg/ml, 1 h) then BK (10 μM, 1 min) (*geld + BK*), or geldanamycin (1 μg/ml, 1 h) then PAO (10 μM, 10 min) (*geld + PAO*). Cell lysates were immunoprecipitated with anti-eNOS antibody, precipitated proteins were separated by gel electrophoresis, transferred to nitrocellulose, and probed with anti-caveolin-1 antibody. Relative amounts of caveolin-1α precipitated in each condition were quantitated by densitometry of immunoblots. Values shown represent mean ± S.E.M. from three to seven experiments.

appears to be regulated, at least in part, by agonist-stimulated tyrosine phosphorylation.

Tyrosine phosphorylation-dependent changes in eNOS-caveolin-1 asociation could be due to direct tyrosine phosphorylation of one or both of the proteins. However, in the present study, neither protein appeared to be tyrosine-phosphorylated in response to BK-stimulation or tyrosine phosphatase inhibition. As reported previously (20), we have been unable to detect phosphotyrosine in eNOS by anti-phosphotyrosine immunoblotting of anti-eNOS immunoprecipitates. Similar negative results have also been obtained with BLMVEC. Michel *et al.* (39) and Corson *et al.* (40) have also failed to detect phosphotyrosine in eNOS from unstimulated or stimulated BAEC. Low levels of phosphotyrosine, however, are not always detected on immunoblots by all anti-phosphotyrosine antibodies (41). This may explain the discrepency between the results of ourselves and others (20,39,40) and those of García-Cardeña (21) who have reported that eNOS in BAEC is tyrosine-phosphorylated, albeit to a small extent. These authors, however, also report that the phosphotyrosine content of eNOS is not changed following BK-stimulation. Caveolin-1 is tyrosine-phosphorylated in v-Src-transformed cells (42) but has only been shown to be tyrosine-phosphorylated in normal cells in the case of insulin-treated 3T3-L1 adipocytes (43). In the present study, BAEC were either treated or not treated with BK (10 μM, 1 min) or PAO (10 μM, 10 min), caveolin-1 was immunoprecipitated, and immunoprecipitates were probed with anti-phosphotyrosine antibody. No tyrosine phosphate was detected for caveolin-1α or -1β under basal conditions or after treatment with BK or PAO.

Tyrosine phosphorylation-dependent association of caveolin-1 and eNOS may be due to phosphorylation of a protein within the eNOS-caveolin-1-cytoskeletal complex that is neither caveolin-1 or eNOS. Phosphorylation of this protein could, in turn, stabilize the eNOS-caveolin-1 interaction. Support for this conclusion is provided by the results of immunoprecipitation experiments using anti-phosphotyrosine antibody. Tyrosine-phosphorylated proteins in BAEC and BLMVEC were immunoprecipitated with anti-phosphotyrosine antibody (the same antibody that fails to react with either caveolin-1 or eNOS on immunoblots of anti-caveolin-1 or anti-eNOS immunoprecipitates). Anti-phosphotyrosine immunoprecipitates were then probed with either anti-caveolin-1 or anti-eNOS antibodies. As shown in Fig. 5, both caveolin-1α and eNOS from BAEC were coprecipitated by the anti-phosphotyrosine antibody even though the antibody does not appear to react with either protein directly. When the experimental protocol was changed and immunoprecipitation was replaced with anti-phosphotyrosine-agarose affinity purification, similar results were obtained. Only caveolin-1α (and not -1β) was precipitated or affinity purified. Caveolin-1α and eNOS thus appear to be associated with a tyrosine-phosphorylated protein (or proteins) that allows for their coprecipitation with anti-phosphotyrosine antibody. Caveolin-1β, on the other hand, appears not to have a tyrosine-phosphorylated protein partner. These results are interesting in light of the fact that when BAEC lysates were immunoprecipitated by anti-

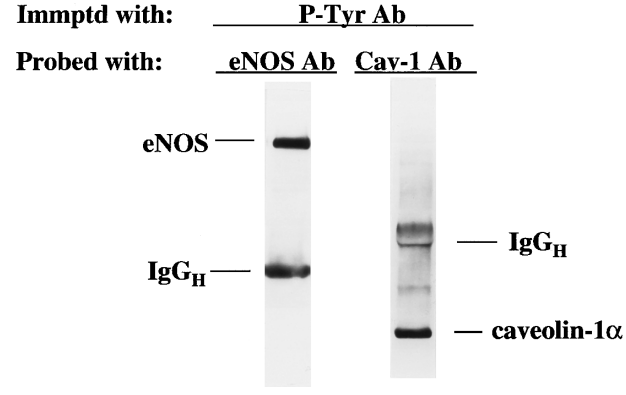

FIG. 5. Coimmunoprecipitation of eNOS and caveolin-1α from BAEC lysates by anti-phosphotyrosine antibody. Cell lysates were immunoprecipitated with anti-phosphotyrosine antibody. Precipitated proteins were separated by gel electrophoresis, transferred to nitrocellulose, and immunoblotted with either anti-eNOS or anti-caveolin-1 antibodies. Results are representative of three experiments.

eNOS antibody, only caveolin-1α (and not -1β) was coimmunoprecipitated. Furthermore, BK (10 μM, 1 min) and PAO (10 μM, 10 min) treatment increased the amount of both caveolin-1 and eNOS coprecipitated by the anti-phosphotyrosine antibody even though neither agent altered the phosphotyrosine content of caveolin-1 or eNOS directly.

Based on results obtained in *in vitro* binding assays and in a yeast two-hybrid system we have recently reported that caveolin-1 and eNOS interact directly. Furthermore, direct interaction significantly inhibits eNOS activity.[2] In the present study, therefore, we examined whether the PAO-induced, 4-fold increase in eNOS-caveolin-1 association shown in Fig. 4, is accompanied by a decrease in eNOS catalytic activity. BAEC were either treated or not treated with PAO (10 μM, 10 min), cells were lysed, and eNOS activity was determined by arginine-to-citrulline conversion assay in the presence of excess cofactors, Ca^{2+}, and calmodulin. In these experiments, PAO treatment of BAEC reduced the maximal activity of eNOS in cell homogenates by 83 $\pm$ 3.5% (mean $\pm$ S.E.M., n = 3). The inhibition was significantly reversed, however, when homogenates were subjected to extensive sonication. This likely explains the discrepency between the results of the present study and those of our previous study (20) in which samples were extensively sonicated and no effect of PAO on eNOS activity was detected. Additional support for the hypothesis that association of eNOS with caveolin-1 may inhibit eNOS catalytic activity in BAEC is provided by experiments in which particulate and cytosolic fractions of BAEC were prepared by lysis of cells in non-detergent buffer and separation of the two fractions by centrifugation at 100,000 $\times$ *g*. eNOS activity in the two fractions was determined by arginine-to-citrulline conversion assay. Amounts of eNOS and caveolin-1 protein were quantitated by immunoblotting and densitometry. Caveolin-1 protein was found only in the particulate fraction whereas 15% of the eNOS protein was found in the cytosolic fraction. The relative activity of cytosolic eNOS, which is not complexed with caveolin-1, was 4-fold greater than that of the particulate eNOS, which exists, at least in part, in a complex with caveolin-1.

In summary, the results of the present study domonstrate several novel and previously unrecognized features of caveolin-1 in cultured endothelial cells. First, caveolin-1 is predominately detergent-soluble in cultured endothelial cells. Second, detergent-solubility is modulated by agonist-stimulated tyrosine phosphorylation. Third, tyrosine phosphorylation-dependent increases in caveolin-1 detergent-insolubility are accompanied by an increased association of the protein with eNOS and inhibition of eNOS catalytic activity. Agonist-activation of eNOS in endothelial cells thus appears to involve tyrosine phosphorylation-dependent changes in the interaction of eNOS with caveolin-1. Increased interaction of eNOS with caveolin-1 may deactivate the enzyme subsequent to its activation by Ca^{2+}/calmodulin.

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REFERENCES

1. Anderson, R. G. W. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10909–10913.
2. Lisanti, M. P., Scherer, P. E., Tang, Z., and Sargiacomo, M. (1994) *Trends Cell Biol.* **4,** 231–235.
3. Parton, R. G. (1996) *Curr. Opin. Cell. Biol.* **8,** 542–548.
4. Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y-S., Glenney, J. R., and Anderson, R. G. W. (1992) *Cell* **68,** 673–682.
5. Scherer, P. E., Tang, Z., Chun, M., Sargiacomo, M., Lodish, H. F., and Lisanti, M. P. (1995) *J. Biol. Chem.* **270,** 16395–16401.
6. Sargiacomo, M., Scherer, P. E., Tang, Z., Kübler, E., Song, K. S., Sanders, M. C., and Lisanti, M. P. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 9407–9411.
7. Li, S., Song, K. S., Koh, S. S., Kikuchi, A., and Lisanti, M. P. (1996) *J. Biol. Chem.* **271,** 28647–28654.
8. Sargiacomo, M., Sudol, M., Tang, Z., and Lisanti, M. P. (1993) *J. Cell Biol.* **122,** 789–807.
9. Lisanti, M. P., Scherer, P. E., Vidugiriene, J., Tang, Z., Hermanowski-Vosatka, A., Tu, Y.-H., Cook, R. F., and Sargiacomo, M. (1994) *J. Cell Biol.* **126,** 111–126.
10. Chang, W-J., Ying, T-s., Rothberg, K. G., Hooper, N. M., Turner, A. J., Gambliel, H. A., De Gunzburg, J., Mumby, S. M., Gilman, A. G., and Anderson, R. G. W. (1994) *J. Cell Biol.* **126,** 127–138.
11. Kurzchalia, T. V., and Parton, R. G. (1996) *FEBS Lett.* **389,** 52–54.
12. Dupree, P., Parton, R. G., Raposo, G., Kurzchalia, T. V., and Simons, K. (1993) *The EMBO Journal* **12,** 1597–1605.
13. Morin, A. M., and Stanboli, A. (1993) *J. Neurosci. Res.* **36,** 272–279.
14. Stanboli, A., and Morin, A. M. (1994) *Neurosci. Lett.* **171,** 209–212.
15. O'Brien, A. J., Young, H. M., Povey, J. M., and Furness, J. B. (1995) *Histochemistry* **103,** 221–225.
16. Sessa, W. C., García-Cardeña, G., Liu, J., Keh, A., Pollock, J. S., Bradley, J., Thiru, S., Braverman, I. M., and Desai, K. M. (1995) *J. Biol. Chem.* **270,** 17641–17644.
17. Shaul, P. W., Smart, E. J., Robinson, L. J., German, Z., Yuhanna, I. S., Ying, Y., Anderson, R. G. W., and Michel, T. (1996) *J. Biol. Chem.* **271,** 6518–6522.
18. García-Cardeña, G., Oh, P., Liu, J., Schnitzer, J. E., and Sessa, W. C. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 6448–6453.

19. Feron, O., Belhassen, L., Kobzik, L., Smith, T. W., Kelly, R. A., and Michel, T. (1996) *J. Biol. Chem.* **271,** 22810–22814.
20. Venema, V. J., Marrero, M. B., and Venema, R. C. (1996) *Biochem. Biophys. Res. Commun.* **226,** 703–710.
21. García-Cardeña, G., Fan, R., Stern, D. F., Liu, J., and Sessa, W. C. (1996) *J. Biol. Chem.* **271,** 27237–27240.
22. Venema, R. C., Sayegh, H. S., Arnal, J.-F., and Harrison, D. G. (1995) *J. Biol. Chem.* **270,** 14705–14711.
23. Song, K. S., Li, S., Okamoto, T., Quilliam, L. A., Sargiacomo, M., and Lisanti, M. P. (1996) *J. Biol. Chem.* **271,** 9690–9697.
24. Schnitzer, J. E., McIntosh, D. P., Dvorak, A. M., Liu, J., and Oh, P. (1995) *Science* **269,** 1435–1439.
25. Uehara, Y., Hori, M., Takeuchi, T., and Umezawa, H. (1986) *Mol. Cell. Biol.* **6,** 2198–2206.
26. Hanke, J. H., Gardner, J. P., Dow, R. L., Changelian, P. S., Brissette, W. H., Weringer, E. J., Pollok, B. A., and Connelly, P. A. (1996) *J. Biol. Chem.* **271,** 695–701.
27. Kurzchalia, T. V., Hartmann, E., and Dupree, P. (1995) *Trends Cell Biol.* **5,** 187–189.
28. Grondin, P., Plantavid, M., Sultan, C., Breton, M., Mauco, G., and Chap, H. (1991) *J. Biol. Chem.* **266,** 15705–15709.
29. Zhang, J., Fry, M. J., Waterfield, M. D., Jaken, S., Liao, L., Fox, J. E. B., and Rittenhouse, S. E. (1992) *J. Biol. Chem.* **267,** 4686–4692.
30. Fox, J. E. B., Lipfert, L., Clark, E. A., Reynolds, C. C., Austin, C. D., and Brugge, J. S. (1993) *J. Biol. Chem.* **268,** 25973–25984.
31. Dash, D., Aepfelbacher, M., and Siess, W. (1995) *J. Biol. Chem.* **270,** 17321–17326.
32. Banno, Y., Nakashima, S., Ohzawa, M., and Nozawa, Y. (1996) *J. Biol. Chem.* **271,** 14989–14994.
33. Allan, V. J., and Kreis, T. E. (1986) *J. Cell Biol.* **103,** 2229–2239.
34. Ktistakis, N. T., Roth, M. G., and Bloom, G. S. (1991) *J. Cell Biol.* **113,** 1009–1023.
35. Donaldson, J. G., Kahn, R. A., Lippincott-Schwartz, J., and Klausner, R. D. (1991) *Science* **254,** 1197–1199.
36. Sugai, M., Chen, C-H., and Wu, H. C. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 8903–8907.
37. Duden, R., Griffiths, G., Frank, R., Argos, P., and Kreis, T. E. (1991) *Cell* **64,** 649–665.
38. Schnitzer, J. E., Siflinger-Birnboim, A., Del Vecchio, P. J., and Malik, A. B. (1994) *Biochem. Biophys. Res. Commun.* **199,** 11–19.
39. Michel, T., Li, G. K., and Busconi, L. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 6252–6256.
40. Corson, M. A., James, N. L., Latta, S. E., Nerem, R. M., Berk, B. C., and Harrison, D. G. (1996) *Circ. Res.* **79,** 984–991.
41. Kamps, M. P. (1991) *Methods Enzymol.* **201,** 101–110.
42. Glenney, J. R., Jr. (1989) *J. Biol. Chem.* **264,** 20163–20166.
43. Mastick, C. C., Brady, M. J., and Saltiel, A. R. (1995) *J. Cell Biol.* **129,** 1523–1531.