A Caveolar Complex between the Cationic Amino Acid Transporter 1 and Endothelial Nitric-oxide Synthase May Explain the “Arginine Paradox”*

(Received for publication, August 27, 1997, and in revised form, October 8, 1997)

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Immunohistochemistry of porcine pulmonary artery endothelial cells (PAEC) with antibodies specific for caveolin, endothelial nitric-oxide synthase (eNOS), and the arginine transporter (CAT1) demonstrates that all of these proteins co-localize in plasma membrane caveolae. When incubated with solubilized PAEC plasma membrane proteins, eNOS-specific antibody immunoprecipitates CAT1-mediated arginine transport. These results document the existence of a caveolar complex between CAT1 and eNOS in PAEC that provides a mechanism for the directed delivery of substrate arginine to eNOS. Direct transfer of extracellular arginine to membrane-bound eNOS accounts for the “arginine paradox” and explains why caveolar localization of eNOS is required for optimal nitric oxide production by endothelial cells.

Pulmonary endothelial cells are a rich source of nitric oxide (NO), a nitrogen-centered free radical with multiple and unique physiologic and bioregulatory activities. Pulmonary endothelial cells generate NO from arginine via the catalytic action of a NADPH-requiring, Ca2⁺/calmodulin-dependent NO synthase (referred to as eNOS, ecNOS, or type III NOS) that is membrane-associated (1–3). In endothelial cells, eNOS-mediated formation of NO from arginine is dependent upon an adequate and continuing supply of arginine (4–8). Several studies have shown that the half-saturating arginine concentration for eNOS is less than 10 μM (9–11). We (12) and others (13–16) have reported intracellular arginine concentrations that range from 0.1 to 0.8 mm in cultured endothelial cells. Consequently, eNOS should be saturated in these cells, and therefore increasing the extracellular arginine should not increase NO production any further. However, a number of in vitro and in vivo studies indicate that NO production by vascular endothelial cells under physiological conditions can be increased by extracellular arginine, despite a saturating intracellular arginine concentration (4–8, 17). Furthermore, a recent report by Arnal et al. (18) demonstrates that the intracellular concentration of arginine in endothelial cells can be varied over 100-fold without changing NO production. This observation, i.e. that extracellular arginine administration seems to drive NO production even when intracellular levels of arginine are available in excess, has been termed the “arginine paradox” and cannot be explained based on the available data (19). One paradigm that would explain this observation is that in endothelial cells the intracellular arginine is sequestered in one or more pools that are poorly, if at all, accessible to eNOS, whereas extracellular arginine transported into the cell is preferentially delivered to eNOS. Under this paradigm, a plasma membrane arginine transporter must be in close spatial alignment with or directly linked to the eNOS protein.

Arginine transport is mediated by several independent transport activities in mammalian cells (20–22). The distribution and relative contribution of each of these transport activities to the total arginine uptake by a particular cell type varies widely due to cell-specific expression of the corresponding genes. Arginine transport into endothelial cells has been investigated by several laboratories (23–26). Transport into porcine pulmonary artery endothelial cells (PAEC) is mediated by only two agencies (26–28). System y⁺ has been extensively characterized in PAEC (28) and is responsible for 60–80% of total carrier-mediated arginine uptake (26, 27). In 1991, two laboratories independently documented that the native biologic function of the previously cloned murine ecotropic retroviral receptor was System y⁺ transport activity (29, 30). The mRNA and corresponding protein, termed CAT1, are expressed in a wide variety of cells, with the notable exception of liver (29–32).

Woodard et al. (33) documented the expression of CAT1 within the plasma membrane of PAEC by immunohistochemistry. Interestingly, that study illustrated that the CAT1 transporter protein was not uniformly distributed over the cell surface but instead was concentrated in randomly distributed clusters within the plasma membrane. The CAT1 transporter-containing clusters could be dispersed by nocodazole-induced disruption of the microtubule network, but they reformed within a few hours after removal of the drug. Recently, Kizhatil and Albritton have confirmed the cytoskeletal association of CAT1 and demonstrated that the clusters are required for retrovirus infectivity. With regard to micro-domains within the plasma membrane, considerable information has been published regarding the presence of plasma membrane regions referred to as caveolae (34–38). This specialized membrane region contains one of a family of structural proteins called caveolins, as well as numerous signaling proteins and a high

* This work was supported by Grants DK-28374 (to M. S. K.) and HL-52136 (to E. R. B.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: NO, nitric oxide; PAEC, pulmonary artery endothelial cell(s); eNOS, endothelial nitric-oxide synthase; CAT1, cationic amino acid transporter 1 (System y⁺); FITC, fluorescein isothiocyanate; LM, light microscopy.

2 K. Kizhatil and M. Albritton (1997) J. Virol. 71, 7145–7156.
cholesterol content. Within endothelial cells a significant portion of eNOS is localized to caveolae (9, 39–42). For example, Garcia-Cardena et al. (41) showed that caveolin and membrane-bound eNOS co-localize in lung microvascular endothelial cells and that antibodies against one could be used to immunoprecipitate the other, strongly suggesting that eNOS is complexed with caveolin (41). Interestingly, for reasons that were previously not known, caveolar localization optimizes the ability of eNOS to produce NO (9, 42). We hypothesize that in PAEC the CAT1 transporter-containing clusters represent plasma membrane caveolae. We also propose that co-localization of CAT1-mediated arginine transport and eNOS would provide an efficient mechanism for delivery of substrate for NO synthesis, perhaps even in a direct manner. The following experiments document co-localization of CAT1 and eNOS within PAEC caveolae.

**MATERIALS AND METHODS**

**Cell Culture and Immunohistochemistry**—PAEC were obtained from the main pulmonary artery of 6–7-month-old pigs and were cultured for 3–7 passages as described by Block et al. (12). Cells were cultured on glass coverslips to a density of approximately 70% and then subjected to immunohistochemistry using the incubation conditions and methodology described by Woodard et al. (33). Mouse monoclonal antibodies against caveolin and eNOS were obtained from Transduction Laboratory (Lexington, KY). Production and characterization of a rabbit polyclonal antisemur against a predicted extracellular loop of the murine CAT1 arginine transporter has been described previously (33). FITC-labeled goat anti-rabbit IgG (Sigma) was used to detect anti-CAT1, whereas Texas Red-labeled goat anti-mouse IgG (ICN Pharmaceuticals, Inc., Aurora, OH) was used to detect anti-caveolin and anti-eNOS.

**Analysis by Deconvolution Microscopy**—Fluorescently stained cells were analyzed by deconvolution microscopy as described originally by Agard et al. (43). Three-dimensional light microscopy (LM) data collection and computational removal of out-of-focus information used an integrated, cooled CCD-based, fluorescence LM data collection, processing, and visualization workstation described in detail elsewhere (Ref. 44; Applied Precision, Inc., Mercer Island, WA). Three-dimensional data sets were processed as has been described previously (43, 45). LM images were viewed using an integrated modeling program (PRISM) specially designed for analyzing complex three-dimensional biological structures (46).

**Immunodepletion of CAT1 Transport Activity**—Plasma membrane vesicles were prepared by sucrose gradient centrifugation as described by Teitel (47) and modified by Bhat and Block (48, 49). Plasma membrane proteins were solubilized by the method described by Fafournoux et al. (50). The solubilized proteins in the supernatant were precipitated by incubation with 20% polyethylene glycol (PEG-8000) at 4 °C for 20 min. Immunodepletion of CAT1 transporter was performed using the protocol of Tamarappoo et al. (51). Briefly, a 1-ml aliquot of goat anti-mouse IgG covalently linked to agarose beads (Sigma) was incubated for 1 h with 20 μg of monoclonal eNOS antibody (Transduction Laboratories) on ice and centrifuged, after which the supernatant was discarded. The agarose beads were then washed once with STAB buffer (20% glycerol, 2 mM EDTA, 2 mM diithiothreitol, 0.2% sodium cholate, 0.25% asolectin, and 10 mM HEPES, pH 7.4) and mixed with solubilized proteins resuspended in STAB buffer. After incubation for 1 h on ice, the beads were centrifuged, the supernatant was removed, and the proteins were reconstituted into proteoliposomes.

**Reconstitution and Assay of Amino Acid Transport**—Reconstitution of proteins into proteoliposomes was performed following the protocol of Fafournoux et al. (50) and transport assays were performed as described previously (28). Briefly, plasma membrane vesicles or proteoliposomes (20 μg/30 μl) were added to 270 μl of external solution containing 140 mM NaSCN, 1 mM MgSO4, 10 mM HEPES-Tris, pH 7.4, and 50 μM [3H]-arginine or 50 μM [3H]-glutamine. After incubation for 3 min at 37 °C, reactions were terminated by the addition of 5 ml of ice-cold 140 mM NaCl (stop solution) followed by filtration through glass fiber Whatman GF/C filters presoaked in 0.3% polyethylenimine to decrease the nonspecific absorption of [3H]-arginine or glutamine. The filters were washed four times with 5 ml of stop solution, dried, and counted using liquid scintillation spectrometry. Zero time blank values (membrane vesicles or proteoliposomes added after stop solution) were subtracted from all experimental values.

**RESULTS AND DISCUSSION**

Localization of CAT1 in several cell types revealed localized transporter-containing clusters within the plasma membrane when analyzed by epifluorescence microscopy (33). Fig. 1 illustrates this staining pattern analyzed at much greater resolution before (Fig. 1A) and after (Fig. 1B) the use of deconvolution microscopy technology (43–46). As reported previously, the CAT1 transporter is not uniformly distributed over the entire cell surface. Instead, the cell surface has discrete regions that contain a high transporter content. Staining with the anti-CAT1 transporter antibody is completely inhibited by preadsorption of the antibody with the corresponding peptide antigen (Fig. 1C), whereas incubation of the antibody with nonantigenic peptide sequences from within the CAT1 transporter does not block cell staining (33).

Immunohistochemistry of the PAEC with antibodies specific for caveolin also documented intensely staining plasma membrane-associated clusters, consistent with proposed caveolae structure and localization (Fig. 2A). Co-staining of the PAEC with anti-CAT1 (Fig. 2B) and anti-caveolin antibodies resulted in significant overlap with regard to localization of the two proteins (Fig. 2C). It is clear from these results that the majority of the clusters containing the arginine transporter coincide with caveolae in the PAEC plasma membrane. Furthermore, in PAEC we have confirmed, as reported previously by others (41, 42), the co-localization of caveolin and eNOS by immunohistochemistry (data not shown).

The presence of the CAT1 arginine transporter in plasma membrane caveolae raised the possibility that CAT1 and eNOS may be co-localized in a caveolar complex to facilitate NO synthesis. Therefore, PAEC were subjected to immunohistochemistry to test for co-localization of CAT1 and eNOS. A significant portion of the detectable plasma membrane-associated eNOS was clustered within random regions over the cell.
surface (Fig. 3A). As mentioned above, these eNOS-containing micro-domains have been identified as caveolae, and the eNOS present can be co-immunoprecipitated with anti-caveolin antibodies (41, 42). The CAT1 arginine transporter-containing clusters were similar in distribution (Fig. 3B), and when the stained regions were analyzed for co-localization of CAT1 and eNOS, a significant degree of overlap was clearly revealed (Fig. 3C).

Co-localization of the CAT1 arginine transporter and eNOS within caveolae is consistent with the proposal that these membrane micro-domains are a site for concentrating proteins involved in signaling (34–38). However, the present observations also raise the intriguing possibility that the CAT1 arginine transporter and eNOS are physically associated. Such a complex might provide a mechanism for directed delivery or even channeling of newly acquired extracellular arginine to eNOS for NO synthesis. Selective delivery of transported arginine to membrane-bound eNOS could explain the arginine paradox discussed above (19). As a more direct test for a complex between CAT1 and eNOS, PAEC plasma membrane arginine transport activity was detergent solubilized and subjected to immunoprecipitation with anti-eNOS antibody (50). Using the proteins in the immunoprecipitate supernatant to reconstitute proteoliposomes (51) provided an assay to check for anti-eNOS-dependent immunodepletion of arginine transport. Immunodepletion with control mouse IgG caused no loss of reconstitutable arginine transport, whereas the anti-eNOS monoclonal antibody caused immunoprecipitation of 73% of the Na⁺-independent arginine transport (Table I). To establish that the anti-eNOS did not result in immunodepletion of arginine transport in a nonspecific manner, glutamine transport was monitored in the reconstituted proteoliposomes after immunoprecipitation with control and anti-eNOS IgG (Table I). No immunodepletion of glutamine transport was observed. These data document that a protein-protein association exists between the CAT1 arginine transporter and membrane-bound eNOS in PAEC.

Caveolae are abundant in lung endothelial cells, and they have been implicated in transcytosis, potocytosis, and signal transduction (34–38). García-Cardena et al. (41) have recently reported a protein-protein interaction between eNOS and caveolin, a caveolar coat protein found in endothelial cells. It is likely that this eNOS-caveolin interaction is responsible for positioning eNOS adjacent to other caveolar proteins such as CAT1 to form a highly efficient signal transduction cascade and to optimize production of the vital signaling molecule NO. Our results demonstrate that the CAT1 arginine transporter is localized to plasma membrane caveolae of PAEC and that treatment of PAEC plasma membrane vesicles with an antibody directed against eNOS depletes CAT1-mediated arginine

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**Fig. 2.** Caveolin and CAT1 amino acid transporter are co-localized in plasma membrane clusters in PAEC. Using the methods described under “Materials and Methods,” the PAEC were subjected to immunohistochemistry with antibodies specific for CAT1 (A), caveolin (B), or both (C). Co-localization of the proteins was assayed by using simultaneously a rabbit polyclonal antibody against CAT1 (15) detected by FITC-labeled goat anti-rabbit IgG and a mouse monoclonal antibody against caveolin detected by Texas Red-labeled goat anti-mouse IgG (C). Staining from three independent experiments was analyzed by deconvolution microscopy and shown to be reproducible.

**Fig. 3.** CAT1 amino acid transporter and eNOS are co-localized in the PAEC plasma membrane. By the same methodology outlined in Fig. 1, PAEC were used to detect the plasma membrane localization of the cationic amino acid transporter CAT1 (A), eNOS (B), or both (C) by immunohistochemistry. The cells were stained using a rabbit polyclonal antibody against CAT1 detected by a goat anti-rabbit IgG linked to FITC and a mouse monoclonal antibody against eNOS detected by a goat anti-mouse IgG linked to Texas Red. Staining was analyzed by deconvolution microscopy, and cells from three independent experiments documented the reproducibility of the results.
A CAT1 and eNOS Complex in Endothelial Caveolae

The saturable, Na+-dependent arginine transport activity in PAEC vesicles has been characterized previously and is mediated primarily by the CAT1 transporter given that neither b0, y, nor y L are detectable in these assays (28) and that these cells contain CAT1, but little or no CAT2 or CAT2a mRNA (unpublished results). After solubilization of CAT1 transport activity (50), the protein fraction was subjected to immunodepletion (51) and then incubated with either control mouse IgG or anti-eNOS IgG bound to anti-mouse IgG immobilized on agarose beads. After pelleting the beads, proteins remaining in the supernatant were reconstituted into proteoliposomes to test for immunodepletion of saturable, Na+-dependent arginine transport activity (28). As a control for transporter specificity by immunodepletion, the reconstituted proteoliposomes also were assayed for Na+-dependent 50 μg glutamine transport to demonstrate that not all amino acid transporters were precipitated by the eNOS antibody.

| Immunoprecipitation | Transport velocity | Control |
|---------------------|-------------------|---------|
| prior to reconstitution | pmol · mg⁻¹ · protein · 3 min⁻¹ | % |
| Arginine transport | | |
| No antibody | 3511 ± 50 | 100 |
| Mouse IgG | 4054 ± 44 | 100 |
| Anti-eNOS IgG | 1113 ± 17* | 27 |
| Glutamine transport | | |
| Mouse IgG | 4026 ± 176 | 100 |
| Anti-eNOS IgG | 4368 ± 706 | 109 |

* p < 0.001 versus no antibody or non-immune mouse IgG, n = 3 independent experiments.

transport. Taken together, these results document the existence of a caveolar complex between the CAT1 arginine transporter and eNOS in PAEC.

Association of the CAT1 arginine transporter and eNOS in PAEC provides a mechanism for the directed delivery of substrate to eNOS and, for mammalian cells, represents the first example of a functional complex between a plasma membrane transport protein and an enzyme. Such directed delivery of extracellular arginine to eNOS would account for the arginine paradox described earlier (19) and would also explain the observation by Liu et al. (9) that caveolar localization of eNOS is required for optimal NO production by eNOS.

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