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Endothelial Nitric Oxide Synthase Targeting to Caveolae

SPECIFIC INTERACTIONS WITH CAVEOLIN ISOFORMS IN CARDIAC MYOCYTES AND ENDOTHELIAL CELLS*

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The endothelial isoform of nitric oxide synthase (eNOS) modulates cardiac myocyte function and is expressed in the particulate subcellular fraction. We have previously shown that eNOS is targeted to plasmalemmal caveolae in endothelial cells. Caveolae, specialized domains of the plasma membrane, may serve to sequester signaling proteins; a family of transmembrane proteins, the caveolins, form a key structural component of these microdomains. Caveolae in cardiac tissues contain the muscle-specific isoform caveolin-3, and caveolae in endothelial cells contain the widely expressed isoform caveolin-1, which shares limited sequence identity with caveolin-3. Our immunohistochemical analyses of rat cardiac muscle used isoform-specific caveolin antibodies to reveal prominent caveolin-3 staining in myocyte sarcolemmal membranes and at intercalated discs, whereas caveolin-1 staining was prominent in the vascular endothelium. Caveolin or eNOS antibodies were utilized to immunoprecipitate cardiac myocyte or cultured aortic endothelial cell lysates, which then were analyzed in immunoblots. In endothelial cells, we found that eNOS is quantitatively immunoprecipitated by antibodies to caveolin-1. In cardiac myocyte lysates, nearly all the eNOS is immunoprecipitated instead by antibodies to caveolin-3 and, conversely, eNOS antiserum immunoprecipitated primarily caveolin-3. These studies establish expression of eNOS in cardiac myocyte caveolae and document tissue-specific and quantitative associations of eNOS with caveolin. These findings may have important implications for the regulation of eNOS by caveolin isoforms and by other signaling proteins targeted to caveolae.

Nitric oxide is an important determinant of cardiac function (see review in Ref. 1). Studies of cardiac myocytes in culture, as well as investigations in animal models and in human subjects, have identified a key role for endogenous nitric oxide synthesis in the modulation of receptor-mediated autonomic responses controlling contractility and heart rate. Cardiac myocytes were recently discovered robustly to express the isoform of nitric oxide synthase that was originally characterized in endothelial cells (2). Indeed, the endothelial isoform of nitric oxide synthase, termed eNOS[1] or NOS3, has recently been discovered in diverse nonendothelial tissues, including blood platelets and hippocampal neurons, in addition to cardiac myocytes (see review in Ref. 3). However, an understanding of the cell-specific molecular regulation of eNOS has been more challenging to delineate in these less tractable cellular systems, and cultured endothelial cells have thus served as the model system for characterizing the complex intracellular pathways regulating eNOS.

In both endothelial cells and in cardiac myocytes, eNOS is found predominantly in the particulate subcellular fraction (2, 4–6), and, in both cell types, the enzyme appears to participate in the response to transmembrane signaling events initiated by diverse G protein-coupled receptors. However, the same NOS isoform expressed in different tissues may show important differences in cell-specific targeting and regulation. For example, the neuronal isoform of NOS (NOS1 or nNOS) is expressed in both brain and skeletal muscle and in both tissues can be found in the particulate subcellular fraction (7, 8). In neuronal tissues, nNOS is targeted to the post-synaptic density by highly specific protein-protein interactions with PSD-95 (7). By contrast, nNOS in skeletal muscle is found in the cytoskeleton by virtue of its interactions with dystrophin (8). In the present studies, we apply paradigms established for the subcellular targeting of eNOS in endothelial cells to the study of this enzyme isoform in cardiac myocytes, and we will describe important similarities as well as striking differences in eNOS targeting in these two cell types.

eNOS appears to undergo a complex series of covalent modifications that affect its subcellular targeting (3). In endothelial cells, *N*-myristoylation (6) and thiopalmitoylation (9) of eNOS represent the key determinants for its subcellular localization, in that dual acylation is required for efficient eNOS targeting. We have further shown that eNOS is specifically targeted to plasmalemmal caveolae consequent to enzyme palmitoylation (10). It also appears that the targeting of eNOS to endothelial cell caveolae can be dynamically regulated, in that agonists such as bradykinin promote depalmitoylation and subcellular translocation of the enzyme (9, 11). Although cardiac myocytes are also known to contain caveolae, these cells express a distinct muscle-specific caveolin isoform (caveolin-3) which has

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[1] The abbreviations used are: eNOS, endothelial isoform of nitric oxide synthase; NO, nitric oxide; nNOS, neuronal isoform of NOS; BAEC, bovine aortic endothelial cells; ARVM, adult rat ventricular myocytes; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

only limited similarity (~60% sequence identity) to the more ubiquitous caveolin-1 isoform that is present in endothelial cells (12). Since caveolae may play a key role in the targeting and sequestration of signaling proteins, we designed experiments to explore the possibility of eNOS targeting to plasmalemmal caveolae in cardiac myocytes.

For many years and in many cell types, including cardiac myocytes and endothelial cells, the appearance of small flask-shaped invaginations of the plasma membrane, termed caveolae, has been described (13). More recently, the regulatory properties and biochemical constituents of caveolae have been studied intensively (see reviews in Refs. 13 and 14). Caveolae appear to play a role in the compartmentation of a number of plasma membrane-linked signal transduction pathways, including those mediated by muscarinic cholinergic (15) and β-adrenergic agonists (16). Caveolae are characterized by a distinctive lipid composition and appear to be devoid of phospholipids but contain abundant cholesterol and glycosphingolipids. The principal structural proteins in caveolae are the caveolins, recently identified as a family of palmitoylated integral membrane proteins essential for caveolar structure (17). Caveolins are 20–24-kDa oligomeric membrane proteins, and the known caveolin isoforms have distinct tissue-specific patterns of expression. Caveolin-1 is found in a variety of cell types, including endothelium, whereas caveolin-2 is expressed primarily in adipocytes (18); caveolin-3 is expressed predominantly in striated muscle but its relative abundance and immunohistochemical pattern of expression have yet not been determined (12). Additionally, there appear to be important differences between the various caveolin isoforms in the nature of their direct interactions with and regulation of G-protein α subunits (12, 18, 19).

In this work, we provide evidence that eNOS is targeted to caveolae in cardiac myocytes, where it associates predominantly with caveolin-3, and, to a lesser extent, with caveolin-1. In endothelial cells, in which caveolin-3 is not found, eNOS associates quantitatively and specifically with caveolin-1. Thus, in two distinct cell types, cardiac myocytes and endothelial cells, eNOS is targeted to plasmalemmal caveolae, but this targeting is mediated by interactions with distinct cell-specific caveolin isoforms.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Cellular Lysates —Purified adult rat ventricular myocytes (ARVM) were prepared as previously reported (20). This technique includes multiple washes and density gradient sedimentation steps through a bovine serum albumin cushion, as well as differential cell attachment to laminin-coated plates in order to minimize non-myocyte cell contamination, and yield a cell population containing 90–95% cardiac myocytes. In this study, freshly isolated cardiac myocytes were used 3 to 4 h after isolation. Bovine aortic endothelial cells (BAEC) were purchased from Cell Systems and cultured as described (21); cells were used between passages 4 and 10.

Cardiac myocytes and aortic endothelial cells were harvested by scraping in phosphate-buffered saline, pelleted by centrifugation, and then resuspended in a hypotonic buffer containing 50 mM Tris-HCl (pH 7.4), 0.1 mM EGTA, 2 mM β-mercaptoethanol, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM phenymethysulfonyl fluoride). Immunoprecipitation protocols were essentially identical to previously reported methods from our laboratory (22), as outlined below. Because of the marked quantitative difference in eNOS expression between ARVM and BAEC, a 10-fold greater quantity of ARVM cellular protein lysate was utilized for these immunoprecipitations. Cell suspensions were diluted 4-fold in "CHAPS buffer," containing 50 mM Tris-HCl (pH 7.4), 20 mM CHAPS, 125 mM NaCl, 2 mM dithiothreitol, 0.1 mM EGTA, 4 μM tetrahydrobiopterin, 1 mM L-arginine and protease inhibitors as above, then lysed by sonication (three 10-s burst, output power ≈ 1) using a Branson sonifier 450 (Branson Ultrasonic Corp., Danbury, CT); cell debris was discarded following a 1000 × *g* centrifugation. This solubilized cell lysate was then used for immunoprecipitation.

Immunoprecipitation—Aliquots of cell lysates were incubated with the mouse anti-caveolin-1 and/or anti-caveolin-3 IgG1 (Transduction Laboratories) at a final concentration of 4 μg/ml or with a previously characterized eNOS-specific rabbit antipeptide antiserum (final dilution of 1:100) that we raised against eNOS and have extensively characterized (6). The isoform specificity and lack of cross-reactivity of the caveolin antibodies have been established by the manufacturer and are consistent with our findings that the caveolin-3 antibody does not recognize caveolin-1 immunoprecipitated from endothelial cells.[2] Negative controls for the mouse monoclonal antibodies used nonimmune idiotype-specific purified mouse myeloma IgG1 (Zymed); for the polyclonal rabbit antiserum, negative controls used preimmune serum obtained from these rabbits or, alternatively, used the cognate immunogen peptide (final concentration 20 μg/ml) added to the eNOS immune serum to block protein immunoprecipitation, as specified in the text. After 1 h at 4 °C, samples were submitted to an ultracentrifugation step (100,000 × *g*, 1 h at 4 °C) in order to precipitate particulate material; ~80% of the eNOS originally present in the cell lysate is recovered following solubilization with CHAPS (5). Protein A-Sepharose beads (Sigma, 50 μl of a 50% slurry) were added to the supernatant for a further incubation of 1 h at 4 °C. When anti-caveolin and nonimmune murine IgG1 were used, an additional step of 30 min at 4 °C in the presence of a rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories) was used before adding the Protein A-Sepharose beads; gentle agitation was maintained during each incubation. Bound immune complexes were washed 6 times with CHAPS buffer and then once with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl supplemented with 4 μM tetrahydrobiopterin, and 1 mM L-arginine. Immunoprecipitated proteins were eluted by boiling for 5 min in Laemmli sample buffer.

SDS-PAGE and Immunoblotting—Denatured immunoprecipitated proteins were loaded and separated on 12% SDS-polyacrylamide gels (Mini-Protean II, Bio-Rad) and transferred to a polyvinylidene difluoride membrane (Bio-Rad). After blocking with 5% non-fat dry milk in Tris-buffered saline with 0.1% (v/v) Tween 20 (TBST; Sigma), membranes were incubated with the specified primary antibody (Transduction Laboratories) for 1 h in TBST containing 1% non-fat dry milk. After 6 washes (10 min each), the membranes were incubated for 1 h with a horseradish peroxidase-labeled goat anti-mouse immunoglobulin secondary antibody (Jackson ImmunoResearch Laboratories) at a 1:10,000 dilution in TBST containing 1% non-fat dry milk. After 5 additional washes in TBST, the membranes were rinsed once in TBST, incubated with a chemiluminescent reagent according to the manufacturer's protocols (Renaissance, DuPont NEN), and exposed to x-ray film.

Immunohistochemistry—Cryostat sections of heart tissue were fixed in buffered 2% paraformaldehyde for 5 min, followed by 10 min in 100% methanol. After rinsing, immunostaining was performed by sequential application of primary antibody (mouse anti-caveolin-1 or anti-caveolin-3, 5 μg/ml, Transduction Laboratories), goat anti-mouse IgG (1/50, Steinberg Monoclonals, Inc.), mouse peroxidase-anti-peroxidase complex (1/100, Steinberg Monoclonals, Inc.), followed by labeling with the chromogen diaminobenzidine (Sigma) and H_2O_2. The slides were washed with water, counterstained with hematoxylin, dehydrated, and mounted for light microscopy. For negative controls, mouse myeloma IgG (Sigma) was used as the primary antibody, and the samples were processed identically.

RESULTS

In order to determine the distribution of caveolin-1 and caveolin-3 in our cardiac preparations, we performed immunohistochemical analyses in sections of ventricular muscle tissue from adult rat hearts. With caveolin-3 antibody staining of heart tissue, we detected a strong and specific signal in cardiac myocytes (Fig. 1*A*), with prominent linear staining of sarcolemma and intercalated disks (Fig. 1*C*). In contrast, staining with the anti-caveolin-1 antibody revealed a very strong signal in the microvasculature as well as the endothelium of coronary vessels (Fig. 1*B*). No staining is detectable when the primary antibody was omitted or when nonspecific mouse IgG1 was used (Fig. 1*D*). The pattern of caveolin immunohistochemistry shows striking parallels with the pattern of expression of eNOS seen in cardiac and endothelial tissues (2). Furthermore, subcellular fractionation of caveolae (23) in cardiac myocyte ly-

[2] O. Feron, unpublished observations.

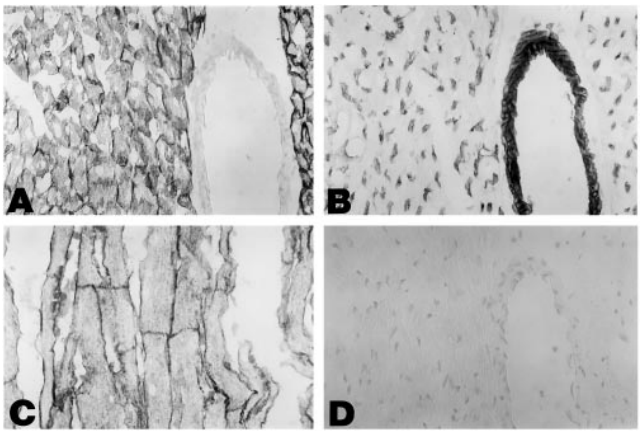

FIG. 1. **Immunohistochemical detection of caveolin-1 and caveolin-3 in ventricular myocardium.** Shown are photomicrographs of adult rat cardiac tissue stained *in situ* with caveolin isoform-specific antibodies, as described; magnifications are × 200 in *A*, *B*, and *D*, and × 400 in *C*. *A*, caveolin-3 antibody staining: ventricular myocytes *in situ* show specific immunostaining, but there is no caveolin-3 staining of vascular endothelium. *B*, caveolin-1 immunostaining: prominent staining is noted in a large coronary vessel, as well as within endothelium of the cardiac microvasculature (evident as multiple small punctate stained structures among the myocytes). *C*, caveolin-3 antibody staining in the longitudinal plane: cardiac myocytes show prominent caveolin-3 staining of the sarcolemma and of intercalated disks. *D*, nonimmune idiotype-specific mouse myeloma IgG1: there is no staining when this nonimmune IgG1 is used as primary antibody.

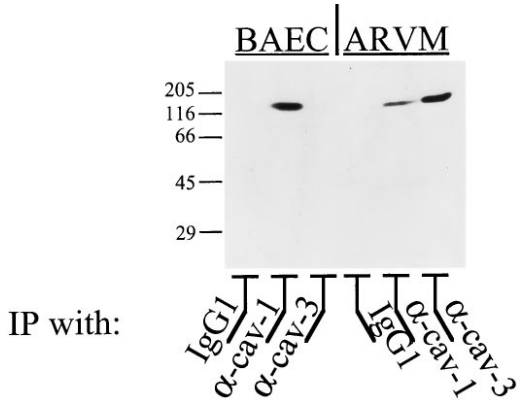

FIG. 2. **Co-immunoprecipitation of eNOS by antibodies to caveolin isoforms from aortic endothelial cell and ventricular myocyte lysates.** Cell lysates were prepared from BAEC or ARVM and immunoprecipitated with either an anti-caveolin-1 antibody (*cav-1*) or an anti-caveolin-3 (*cav-3*) antibody or with a nonimmune IgG1 control antibody (*IgG1*). Immunoprecipitates were then resolved by denaturing SDS-PAGE, blotted, and then probed with an anti-eNOS antibody; an autoradiogram exposed following immunodetection with a chemiluminescence reagent (see text) is shown here. In order to detect the lower abundance caveolin isoforms, 3-fold more caveolin-1 immunoprecipitate than caveolin-3 immunoprecipitate was loaded in the myocyte lane and, conversely, 3-fold more caveolin-3 immunoprecipitate than caveolin-1 immunoprecipitate was loaded in the BAEC lane. The experiment shown is representative of three independent experiments.

sates showed that eNOS co-migrates with caveolin-3 as well as caveolin-1 by analysis of immunoblots of gradient fractions (data not shown). Taken together, these observations suggest that eNOS may co-localize with caveolins-3 and -1 in cardiac tissues, and we therefore devised experimental approaches to explore further the intracellular associations of eNOS and caveolins in cardiac myocytes and in endothelial cells.

We performed a series of co-immunoprecipitation and immunoblotting experiments to investigate interactions between caveolins and eNOS. Cell lysates were solubilized under nondenaturing conditions for immunoprecipitation, using protocols we had previously used to explore eNOS oligomerization (22). Cardiac myocyte or aortic endothelial cell lysates were prepared and solubilized with CHAPS, then immunoprecipitated either with caveolin-1 and/or caveolin-3 antibodies, or, alternatively with anti-eNOS antiserum. After performing denaturing SDS-PAGE and electroblotting, the immunoblots were probed with the complementary antibodies, *i.e.* either directed against eNOS following the caveolin immunoprecipitation or with caveolin-1 or caveolin-3 antibodies following immunoprecipitations performed with the eNOS antiserum. This experimental approach, with appropriate controls, should thus detect co-immunoprecipitation of eNOS and the caveolins in cardiac and endothelial cells.

The first series of experiments explored the pattern seen when endothelial cell or cardiac myocyte lysates are immunoprecipitated using either caveolin-1 or caveolin-3 antibodies, after which the precipitates are electroblotted and then probed using the eNOS antibody. As can be seen in Fig. 2, in endothelial cells, only the caveolin-1 antibody co-immunoprecipitates the 135-kDa eNOS band; neither nonimmune IgG nor the caveolin-3 antibody precipitate eNOS in endothelial cells. By contrast, in cardiac myocytes, the caveolin-3 antibody clearly and specifically co-immunoprecipitates eNOS; the caveolin-1 antibody only weakly precipitates eNOS in the myocyte lysates even when a relatively greater quantity of myocyte protein is analyzed in the caveolin-1 immunoblot (Fig. 2).

In order to explore the proportion of cellular eNOS that is associated with caveolin isoforms in these two cell types, the next experiments determined the fraction of the cellular eNOS that could be immunoprecipitated by the antibodies to caveolin-1 and/or caveolin-3. We therefore analyzed eNOS immunoblots of the supernatants remaining *after* pelleting the protein A-bound immunocomplexes that had been previously precipitated from the solubilized cell lysates by the caveolin antibodies (as analyzed in Fig. 2). As shown in Fig. 3, only a very small fraction of the initial eNOS protein remains in the supernatant following immunoprecipitation from endothelial cell lysates with the caveolin-1 antibody, indicating that the majority of eNOS is associated with caveolin-1 in these endothelial cells. The supernatants of both the caveolin-3 and control nonimmune IgG1 immunoprecipitations from endothelial lysates contained essentially identical amounts of eNOS, providing further confirmation that endothelial cells do not express caveolin-3. In cardiac myocyte lysates, following immunoprecipitation with both caveolin-1 and caveolin-3 antibodies, no residual eNOS could be detected in the supernatant (Fig. 3). The caveolin-3 antibody alone was able to immunoprecipitate most of the eNOS present in the ARVM lysates, since only a small residual signal for eNOS could subsequently be detected in the corresponding supernatant. By contrast, the caveolin-1 antibody consistently immunoprecipitated a relatively smaller fraction of the total eNOS signal from cardiac myocyte lysates, but when both caveolin-1 and caveolin-3 antibodies were combined for immunoprecipitation, there was no residual eNOS signal remaining in the postimmune myocyte lysate.

A complementary series of experiments further established the co-immunoprecipitation of eNOS with the caveolin isoforms. Fig. 4 shows the results of immunoprecipitating endothelial or myocyte lysates with eNOS antiserum followed by immunoblotting with caveolin-1 (Fig. 4*A*) and caveolin-3 (Fig. 4*B*) antibodies. Immunoprecipitations performed using the eNOS antiserum did not substantively affect the total pool of caveolin present in these lysates (data not shown): the eNOS postimmune supernatant contained essentially the same quantity of caveolin as the control, suggesting that the eNOS antiserum immunoprecipitates only a small fraction of the total

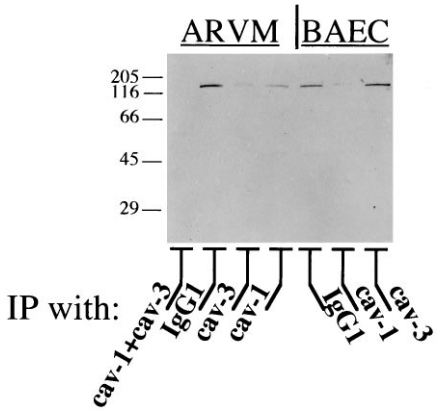

FIG. 3. **Quantitative association of eNOS with caveolins in cardiac myocytes and endothelial cells.** Samples of endothelial cell (BAEC) or ventricular myocyte (ARVM) lysates were immunoprecipitated with either an anti-caveolin-1 antibody (*cav-1*) or an anti-caveolin-3 antibody (*cav-3*) or both (*cav-1 + cav-3*) or a nonimmune IgG1 (*IgG1*), as detailed under "Experimental Procedures." After centrifugation of the protein A-bound immunocomplexes, the remaining supernatants were evaporated to a residual volume of 50 μl and then loaded onto 12% SDS-polyacrylamide gels and then analyzed by immunoblotting using the eNOS antibody, as described in the text.

cellular caveolin pool. Following immunoprecipitation from cardiac myocyte lysates using the eNOS antiserum, both caveolin-3 and caveolin-1 are detected by immunoblotting with their respective antibodies; the caveolin-3 signal is considerably stronger than for caveolin-1, especially considering that three times as much protein was loaded in the lane for the caveolin-1 immunoprecipitation (Fig. 4). By contrast, in endothelial cells, only caveolin-1 (Fig. 4*A*) but not caveolin-3 (Fig. 4*B*) can be precipitated by the eNOS antibody. The association of eNOS with the caveolins is highly specific: no caveolin immunoreactivity whatsoever is detected when preimmune serum is used, and, further, the immunoprecipitation of caveolins by the eNOS antiserum is completely blocked by incubating the immune serum with the immunogen peptide (Fig. 4). Thus, in both myocytes and endothelial cells, the eNOS antiserum co-immunoprecipitates caveolin-1, but caveolin-3 could be precipitated by the eNOS antiserum only in cardiac myocytes.

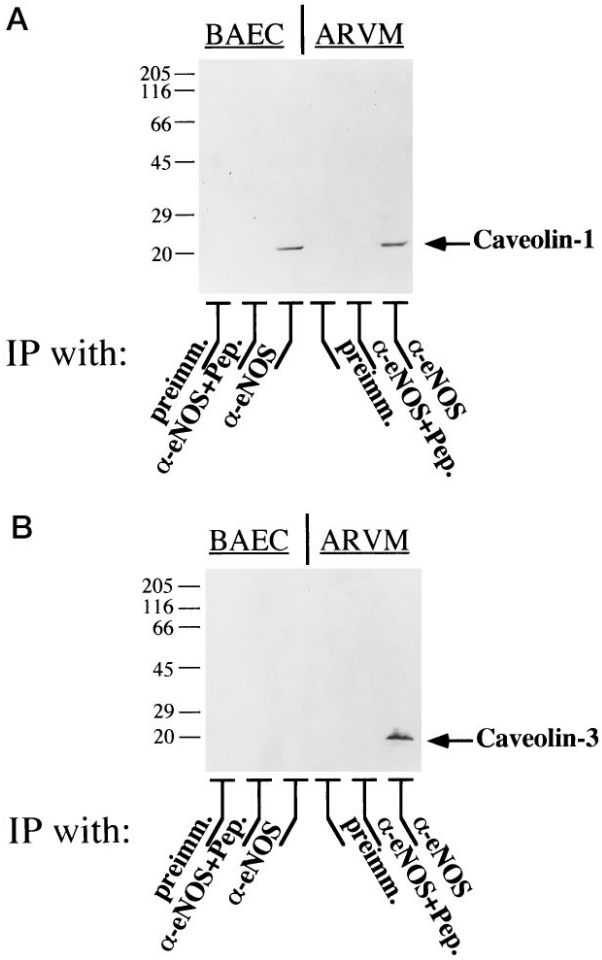

FIG. 4. **Co-immunoprecipitation of caveolin-1 and caveolin-3 by eNOS antiserum.** In this experiment, representative of three independent experiments, samples of endothelial cell (BAEC) or ventricular myocyte (ARVM) lysates were immunoprecipitated with rabbit preimmune serum (*preimm.*), eNOS antiserum in the presence of the cognate immunogen peptide (20 μg/ml) (*α-eNOS + Pep.*), or eNOS antiserum alone (*α-eNOS*), as detailed under "Experimental Procedures." Immunoprecipitates were then resolved by SDS-PAGE, blotted, and then probed with the caveolin-1 antibody (*A*) or the caveolin-3 antibody (*B*). Because of the difference in levels of caveolin isoform expression between the two tissues, a 3-fold greater quantity of eNOS immunoprecipitate was loaded in the lanes corresponding to ARVM than in those corresponding to BAEC in *A* and, conversely, 3-fold more eNOS immunoprecipitates were loaded in the BAEC lanes than in those of ARVM in *B*.

DISCUSSION

Over the past several years, the expression and regulation of eNOS has been extensively characterized in endothelial cells, where the enzyme functions as a key determinant of blood pressure homeostasis and platelet function (3). More recent studies have established that this same eNOS isoform is present also in cardiac myocytes, where it subserves important roles in modulating β-adrenergic and muscarinic cholinergic signal transduction (2, 24). In endothelial cells, we have observed the targeting of eNOS to plasmalemmal caveolae (10); the present studies significantly extend these observations to explore the localization and specific interactions of eNOS with distinct caveolin isoforms expressed in cardiac myocytes and endothelial cells. Caveolins, the essential structural proteins in caveolae, are themselves characterized by distinct tissue-specific patterns of expression, and show isoform-specific patterns of regulation.

Our immunohistochemical analyses of adult rat heart *in situ* document caveolin-3 expression in cardiac myocytes, with particularly robust staining at the sarcolemma and at the intercalated disks that demarcate the cellular borders within the cardiac syncitium. The co-localization of caveolin-3 and eNOS in caveolae at these cellular boundaries may facilitate both eNOS activation by cell surface receptors as well as NO release at the cell surface for intercellular signaling. Caveolin-3 immunoreactivity is not seen in the vascular endothelium, consistent with its proposed distribution as a muscle-specific isoform. The smaller proportion of eNOS precipitated by caveolin-1 antibodies in cardiac myocyte lysates may derive from the small fraction (5–10%) of non-myocyte cells present in these primary isolates. Alternatively, there may be a very low level of caveolin-1 expression in the myocytes themselves. By contrast, in endothelial cells, caveolin-1 appears to be the major isoform, and caveolin-3 is not expressed. From all these data, we conclude that caveolin-1 and caveolin-3 are the predominant isoforms expressed in endothelial cells and cardiac myocytes, respectively, and, within each cell type, essentially all of the cellular eNOS can be precipitated by antibodies directed against their corresponding caveolin isoform.

Caveolae are being intensively studied as domains for the compartmentation of plasma membrane-linked signal transduction pathways, including receptors, G-proteins, protein kinases, and ion channels (13). Targeting of eNOS to plasmalemmal caveolae in endothelial cells is dependent upon eNOS

palmitoylation (10), a post-translational modification that may be dynamically regulated by agonists in diverse cell types (9). We have recently found that eNOS expressed in cardiac myocytes contains the consensus sequences and sites for enzyme myristoylation and palmitoylation and likely also undergoes enzyme acylation.[3] The targeting of eNOS to plasmalemmal caveolae in both cardiac myocytes and endothelial cells may reflect intrinsic features of the signal transduction pathways linked to NO generation in these cells. The mechanism(s) by which muscarinic cholinergic receptor activation leads to eNOS activation in cardiac myocytes, particularly during concomitant β-adrenergic receptor stimulation, remains poorly characterized. It has been shown, however, that muscarinic cholinergic receptors, as well as β-adrenergic receptors, may translocate to caveolae upon agonist binding (15, 16). Since several proteins involved in Ca^{2+}-dependent signaling processes (including inositol 1,4,5-trisphosphate-dependent calcium release channels and Ca^{2+}-ATPase) are localized to caveolae (25), it seems plausible that the targeting of these signaling molecules to caveolae may facilitate the calcium-calmodulin dependent activation of eNOS.

Our studies have used co-immunoprecipitation approaches to show that nearly all of the eNOS in endothelial cells is associated with caveolin-1; conversely, most of the eNOS in cardiac myocytes is associated with caveolin-3. Two distinct hypotheses are suggested by the finding of eNOS-caveolin co-immunoprecipitation: perhaps eNOS and caveolin interact directly through stable protein-protein associations, or, alternatively, these proteins may co-exist within a larger, stable, heteromeric detergent-solubilized complex. Differentiating between these possibilities is not as straightforward as it might seem. In the first place, these hypotheses are not mutually exclusive. For example, it has recently been reported that caveolins can be isolated as multimeric complexes comprising 14–16 caveolin monomers, and it is possible that these caveolin complexes might directly interact with G-protein α subunits (12, 26). It seems plausible that similar high molecular weight oligomers of caveolin and eNOS may be maintained under our experimental conditions; the presence of large membrane aggregates in our experiments is quite unlikely since the CHAPS-solubilized eNOS remains in the supernatant after prolonged ultracentrifugation of the cell lysates and consistently yields nearly quantitative recovery of eNOS in the soluble preparation. Direct analyses of the molecular size of the detergent-protein complex immunoprecipitated by the eNOS or caveolin antibodies, which are confounded by the effects of detergent binding on the hydrodynamic properties of the complex, are ongoing. Exploration of the direct interactions, if any, between eNOS and caveolin isoforms may provide further insights into the tissue-specific and quantitative associations of eNOS and caveolin isoforms documented in these studies.

Although the number of known mammalian nitric oxide synthase isoforms has remained at three since the original identification and characterization of NOS cDNAs, an increasingly large number of different tissues have been discovered to synthesize NO. Considering the great diversity of tissues in which NO is synthesized and the wide spectrum of physiological roles subserved by this small number of genetically distinct isoforms, it seems likely that important cell-specific regulatory pathways will be discovered. One example is the differential subcellular targeting of nNOS in skeletal muscle and in neurons by virtue of cell-specific protein-protein associations (7, 8). The present studies have shown that eNOS associates with caveolins differentially expressed in cardiac myocytes and endothelial cells, raising a potentially important mechanism for the cell-specific regulation of eNOS mediated by caveolin isoforms and by other signaling proteins targeted to caveolae.

[3] L. Belhassen, unpublished observations.

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