Codistribution of NOS and caveolin throughout peripheral vasculature and skeletal muscle of hamsters

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Segal, Steven S., Suzanne E. Brett, and William C. Sessa. Codistribution of NOS and caveolin throughout peripheral vasculature and skeletal muscle of hamsters. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H1167–H1177, 1999.—In isolated cell systems, nitric oxide synthase (NOS) activity is regulated by caveolin (CAV), a resident caveolae coat protein. Because little is known of this interaction in vivo, we tested whether NOS and caveolin are distributed together in the intact organism. Using immunohistochemistry, we investigated the localization of constitutive neuronal (nNOS) and endothelial (eNOS) enzyme isoforms along with caveolin-1 (CAV-1) and caveolin-3 (CAV-3) throughout the systemic vasculature and peripheral tissues of the hamster. The carotid artery, abdominal aorta, vena cava, femoral artery and vein, feed artery and collecting vein of the cheek pouch retractor muscle, capillaries and muscle fibers of retractor and crmamster muscles, and arterioles and venules of the cheek pouch were studied. In endothelial cells, eNOS and CAV-1 were present throughout the vasculature, whereas nNOS and CAV-3 were absent except in capillaries, which reacted for nNOS. In smooth muscle cells, nNOS and CAV-1 were also expressed systemically, whereas eNOS was absent; CAV-3 was present in the arterial but not the venous vasculature. Both nNOS and CAV-3 were located at the sarcolemma of skeletal muscle fibers, which were devoid of eNOS and CAV-1. These immunolabeling patterns suggest functional interactions between eNOS and CAV-1 throughout the endothelium, regional differences in the modulation of nNOS by caveolin isoforms in vascular smooth muscle, and modulation of nNOS by CAV-3 in skeletal muscle.

The constitutive production of NO from L-arginine is mediated by two primary isoforms of nitric oxide synthase (NOS) (36). Endothelial NOS (type III, eNOS) has been found in endothelial cells throughout the cardiovascular system (2, 32). The expression of neuronal NOS (type I; nNOS) was first observed in the brain (6) and has since been found in skeletal muscle fibers (19, 27) and vascular smooth muscle cells (5). The enzymatic activity of constitutive NOS is stimulated by a rise in intracellular Ca²⁺ concentration, which promotes the binding of calmodulin to the enzyme and disrupts the association between NOS and caveolin (17, 26). Conversely, the allosteric binding of caveolin to NOS suppresses NO production in endothelial cells (15) as well as in muscle fibers (42). Caveolin-1 (CAV-1) has been found to be expressed in both endothelial cells (16) and caveolin-3 (CAV-3) in skeletal muscle (42). The interaction between NOS and caveolin proteins has been studied extensively in isolated cell systems (15–17, 26), which have provided insight into the molecular mechanisms underlying the regulation of NO production. Nevertheless, little is known of how these respective enzymatic and regulatory proteins are distributed in the intact organism.

The purpose of this study was to examine the systemic distribution of CAV-1, CAV-3, eNOS, and nNOS expression throughout a full spectrum of blood vessels and in peripheral tissues of the hamster using immunohistochemistry. We reasoned that if caveolin modulates the constitutive activity of NOS under physiological conditions, then these proteins should be found together systematically. To investigate this relationship, representative samples were taken of conduit arteries and veins, resistance arteries and collecting veins of the cheek pouch retractor muscle, and microvessels within several tissues that have been established as microcirculatory preparations (3, 10, 28). On the basis of collective findings from several laboratories (16, 17, 26, 42), we hypothesized that CAV-1 would be found codistributed with eNOS in endothelium and CAV-3 with nNOS in skeletal muscle.

METHODS

All procedures were approved by the Animal Care and Use Committee of The John B. Pierce Laboratory and were performed in accord with the Guide for the Care and Use of Laboratory Animals of the National Research Council.

Vessel and Tissue Specimens

Male golden hamsters (80–110 g; Charles River Laboratories, Kingston, NY) were anesthetized with pentobarbital sodium (65 mg/kg ip). A tracheostomy was performed (PE-190

NITRIC OXIDE (NO) is implicated in the regulation of numerous cellular functions throughout the systemic circulation. The pervasive nature of its influence includes the regulation of smooth muscle tone (14), microvascular resistance (18) and permeability (22), platelet aggregation (33) and leukocyte adhesion (21), and remodeling of the vascular wall (35). Physical exercise promotes blood flow to the periphery and the delivery of oxygen to active skeletal muscle, where NO can govern mitochondrial respiration (20, 40) as well as force production (1, 19). Both of these processes influence the magnitude and distribution of blood flow through the systemic circulation (34).
field, NJ). Serial sections were collected on poly-L-lysine using a microtome (model 500, Bright's Instruments, Fair-

Immunohistochemistry

sectioned (typically within 48 h). Hamsters were exsanguinated or given an intravenous overdose of pentobarbital.

Approximate in vivo dimensions, and then covered with

muscle, and cheek pouch. Tissue specimens were rinsed in

vein of the retractor muscle, cremaster muscle, retractor muscle, and cheek pouch. Tissue specimens were rinsed in deionized water, permanently mounted with Biome-

dia gel/mount (Electron Microscopy Sciences, Fort Washing-

Control Experiments

For each antibody, the specificity of immunolabeling was evaluated by omitting the primary antiserum (i.e., incubation with secondary antibody and ABC alone) and omitting both primary and secondary antisera (incubation with ABC alone). Additional positive controls (data not shown) included incubation with antibodies to von Willebrand factor (polycystic: 2.0 µg/ml; Dako, Carpinteria, CA) as an endothelial cell marker and to smooth muscle α-actin (monoclonal: 6.9 µg/ml; Sigma) as a smooth muscle cell marker. To further test the specificity of the nNOS antibody, it was incubated (undiluted) with excess nNOS protein (0.5 µg/ml; purified from HEK-293 cells expressing rat nNOS cDNA). This preabsorbed antibody was then diluted to working concentrations and applied to serial sections as described above.

Data analysis. Sections processed for DAB staining were visualized through a Nikon E800 microscope with Plan Fluor infinity objectives (magnification, numerical aperture: ×20, 0.50; ×40, 0.75; ×60, 0.85) using differential interference contrast with Köhler illumination. Relative staining intensity (−, if absent; +, if apparent; ++, if definite; ++++, if bold) and localization (endothelial, myocyte) were evaluated by a trained observer who developed the working conditions for these experiments. Evaluations of specific vessels (Table 1) and tissues (Table 2) were based on at least eight samples from as many hamsters. The apparent codistribution of protein isoforms was based on the similarity of staining in serial sections.

Photomicrography

Photomicrographs were acquired digitally from the E800 microscope using a Spot Camera (Diagnostics Instruments, Sterling Heights, MI) coupled to a Matrox (Dorval, PQ, Canada) Millennium II frame grabber (resolution, 1,200 × 1,024).

Table 1. Summary of vessel immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Carotid EC</th>
<th>Abdominal Aorta EC</th>
<th>Vena Cava EC</th>
<th>Femoral Artery EC</th>
<th>Femoral Vein EC</th>
<th>Feed Artery EC</th>
<th>Collecting Vein EC</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>−</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td>nNOS</td>
<td>−</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CAV-1</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CAV-3</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Absolute staining intensity is defined in Table 1. *Arteriolar SMC staining; staining with CAV-3 antibody was absent from venules.

Table 2. Summary of tissue immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Retractor MF</th>
<th>Cremaster MF</th>
<th>Cheek Pouch EC</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>−</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>nNOS</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>CAV-1</td>
<td>−</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>CAV-3</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

DAB chromagen staining with primary antibodies for eNOS, nNOS, CAV-1, and CAV-3. Tissue subheadings are divided based on striated muscle fiber (MF) staining, capillary (CAP) staining, and endothelial cell (EC) or smooth muscle cell (SMC) staining. Relative staining intensity is defined in Table 1. *Arteriolar SMC staining; staining with CAV-3 antibody was absent from venules.
1,600 pixels) housed in a Pentium-based personal computer. Images were arranged for display using Adobe Photoshop (v. 4.01).

RESULTS

Summary data are presented for vessels in Table 1 and for tissues in Table 2.

Systemic Distribution of NOS and Caveolin Isoforms

eNOS was present in endothelial cells of all conduit vessels, microvessels, and capillaries. CAV-1 was present in endothelial and smooth muscle cells throughout the vasculature, including capillaries. nNOS was present in smooth muscle throughout the vasculature, in the periphery of skeletal muscle fibers, and in capillaries of skeletal muscle. CAV-3 was present in smooth muscle cells of the arterial vasculature and the periphery of skeletal muscle fibers, yet was strikingly absent from smooth muscle cells throughout the venous vasculature.

Codistribution of NOS and Caveolin Isoforms According to Cell Type

In endothelial cells, eNOS and CAV-1 were present throughout arterial and venous vasculature; however, neither nNOS nor CAV-3 was apparent. In smooth muscle, nNOS and CAV-1 were present systemically, with no staining for eNOS. Whereas smooth muscle of arteries and arterioles showed CAV-3 to be present, it was distinctly absent from smooth muscle of veins and venules. In skeletal muscle fibers, nNOS and CAV-3 were codistributed near the sarcolemma, with no evidence of eNOS or CAV-1. In capillaries supplying muscle fibers, both eNOS and nNOS were found intermittently with CAV-1.
Expression of NOS and Caveolin Isoforms in Specific Vessels and Tissues

Conduit vessels. In the carotid artery (not shown), abdominal aorta (Fig. 1), and femoral artery (Fig. 2), endothelial cells labeled for eNOS and CAV-1, whereas the surrounding smooth muscle cells labeled for CAV-1, nNOS, and CAV-3 (Figs. 1 and 2). In the femoral vein (Fig. 2) and vena cava (Fig. 3), endothelial cells labeled for eNOS and CAV-1. Venous smooth muscle cells were positive for nNOS and CAV-1 but did not label for eNOS or CAV-3 (Table 1).

Microvessels. In the feed artery of the retractor muscle (Fig. 4) and arterioles of the cheek pouch (Fig. 5), endothelial cells labeled for eNOS and CAV-1. The surrounding smooth muscle cells labeled for CAV-1, nNOS, and CAV-3. In the collecting vein and venules (Figs. 4 and 5), smooth muscle cells labeled for CAV-1 and nNOS but not for CAV-3. These patterns of distribution reflect those observed for conduit arteries and veins, respectively.

Skeletal muscle. Striated muscle fibers examined in cross section labeled at or near the sarcolemma for both nNOS and CAV-3 in the retractor muscle (Fig. 6) as well as the cremaster muscle (not shown). In contrast, there was no evidence of immunoreactivity to eNOS or to CAV-1 in these myocytes. In both muscles, intermittent capillaries labeled for nNOS as well as eNOS and CAV-1. The codistribution of nNOS with eNOS was apparent in some but not all of the labeled capillaries.

Controls. Endothelial cells in all locations routinely labeled for von Willebrand's factor as did smooth muscle cells for α-actin (data not shown). No staining was found following incubation with secondary antibody + ABC (Fig. 7) or with ABC alone. Preabsorption of the nNOS antibody eliminated staining for nNOS in vessels and tissues (Fig. 7). Further evidence for the
selectivity of immunolabeling included 1) the expression of nNOS in capillaries (Fig. 6) but not arterial or venous endothelium (Figs. 1–5); 2) the absence of CAV-3 throughout the endothelium and from venous but not arterial smooth muscle (Figs. 1–5); and 3) the absence of CAV-1 in striated (Fig. 6) but not smooth muscle (Figs. 1–5).

**DISCUSSION**

Immunohistochemistry was used to investigate the location of constitutive isoforms of NOS and caveolin throughout the systemic vasculature and in tissues routinely used to study the microcirculation of the hamster. Without exception, at least one isoform of NOS was codistributed with at least one isoform of caveolin. CAV-1 and eNOS were found together in endothelial cells from arteries to veins and throughout the microcirculation, whereas CAV-3 codistributed with nNOS in arterial and arteriolar smooth muscle cells, as well as in skeletal muscle fibers. These findings support the hypothesis that NOS and caveolin proteins are constitutively coexpressed throughout the peripheral vasculature. However, CAV-3 was distinctly absent from venous smooth muscle, whereas CAV-1 was absent from skeletal muscle. In capillaries supplying muscle fibers, the presence of eNOS was complimented intermittently by that of nNOS, yet only the CAV-1 isoform of caveolin was present. These findings argue against the hypothesis that CAV-1 is exclusively colocalized with eNOS or that CAV-3 is always found with nNOS (16, 17, 26, 42). Nevertheless, based on in vitro studies that show nNOS and eNOS to interact similarly with both isoforms of caveolin (15, 17, 42), we suggest that the constitutive regulation of NO production by caveolin (in conjunction with other modulators) is of physiological importance throughout the intact organism.

**Localization and Interaction of NOS and Caveolin Isoforms**

The goals of this study were to determine in which endothelial cells and myocytes NOS and caveolin iso-
forms are expressed physiologically, and whether there was consistent evidence for codistribution of respective proteins. In earlier studies (32), a monoclonal antibody to eNOS was developed and used to show that eNOS was present throughout the endothelium of arteries, veins, and microvessels of bovine and human tissues. Electron microscopy revealed the protein to be associated primarily with the plasma membrane and cytoplasmic vesicles, although a cytosolic component was also evident. In arterioles, venules, and capillaries of guinea pig submucosa, immunoreactivity for eNOS was found in endothelial cells but not in smooth muscle cells (29). Electron microscopy located the staining to membranes of the Golgi apparatus, to intracellular vesicles, and to microdomains of the plasma membrane and rough endoplasmic reticulum (29). Further research has confirmed these observations while revealing how the association of NOS with caveolin can regulate the production of NO (2, 16).

In microvascular endothelial cells cultured from bovine lung, eNOS was found to be colocalized with caveolin on discrete regions of plasma membrane that were determined to be caveolae; association of these proteins was present to a lesser extent in the perinuclear region (16). A similar pattern has recently been documented in vascular and endocardial endothelial cells of the rat heart (2), where much of the staining was associated with the Golgi apparatus, as first described for eNOS in bovine aortic endothelial cells (38). This pattern of labeling suggests that eNOS may “traffic” from the Golgi apparatus to specialized regions of the plasma membrane (16). In turn, caveolin has been shown to cycle between plasma and Golgi membranes along microtubules, acting as a molecular “chaperone” for eNOS (8).

Caveolae are specialized regions of the plasma membrane that are not only rich in caveolin but also contain a host of molecules involved in signal transduction...
initiated at the cell surface (30). Such “targeting” of eNOS to the surface of endothelial cells provides an effective mechanism for producing NO in response to hemodynamic forces and luminal receptor activation. Both myristoylation and palmitoylation are necessary to direct eNOS to Golgi membranes (23, 38), whereas palmitoylation is required for targeting to caveolae (16, 39). A rise in intracellular Ca\(^{2+}\) concentration enables calmodulin to disrupt the association of eNOS from the caveolin complex (26); as intracellular Ca\(^{2+}\) concentration returns to basal levels, the interaction between eNOS and caveolin is restored (12). In turn, the turnover of palmitate suggests that eNOS cycles between Golgi membranes within the cell and caveolae at the cell surface (16). Indeed, prevention of eNOS targeting into caveolae or Golgi membranes reduces NO release upon stimulation of the enzyme (24, 38).

The presence of biologically active eNOS in different subcellular compartments suggests that each may be regulated differentially in response to different modes of stimulation. Nevertheless, as shown in vitro, neither acylation nor targeting to caveolae is required for eNOS to interact with caveolin (15, 17, 24). A similar interaction for nNOS and CAV-3 occurs, with either isoform of NOS able to be regulated by either isoform of caveolin (15, 42). Thus present findings in the hamster suggest that the signaling pathways for NOS regulation that have been defined in isolated cell systems (above) may well apply to those of intact tissues.

**Coincident Expression of Protein Isoforms**

More than a single isoform of each protein is often expressed in individual cells. Both CAV-1 and CAV-3 were present in smooth muscle cells throughout the arterial vasculature (Figs. 1–3 and 5), as previously found in rat cardiac myocytes (11). As expected from previous studies (11, 13, 41), CAV-3 was not observed in

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**Fig. 5.** Light micrographs of serial oblique sections of hamster cheek pouch arteriole (in center) and venule (at lower right corner of each panel except eNOS) labeled for eNOS, CAV-1, nNOS, and CAV-3. Endothelial cells labeled for eNOS and CAV-1, revealing cell borders. Surrounding smooth muscle cells in arteriole labeled for CAV-1, nNOS, and CAV-3. Smooth muscle cells in venule labeled for CAV-1 and nNOS but not CAV-3 (arrow). Scale bar = 20 µm.
endothelial cells, which routinely labeled for CAV-1 (Table 1). The absence of CAV-3 from postcapillary smooth muscle (i.e., venules and veins; Figs. 2–5) was particularly striking in light of its clear presence in precapillary smooth muscle (i.e., arteries and arterioles; Figs. 1, 2, 4, and 5), as well as skeletal muscle (Fig. 6 and Ref. 42) and cardiac myocytes (11, 41).

In Northern and Western blot analyses of human skeletal muscle homogenates, nNOS message and protein were highly expressed, with lesser expression of eNOS (27); unfortunately, the cell types actually containing the respective isoforms were not identified. With the use of immunohistochemistry, both nNOS and eNOS have been localized to discrete neural populations of the brain (6, 9), whereas nNOS has been identified in a subpopulation (~5%) of endothelial cells in the rabbit aorta (25) and in capillary cultures (31) that also contained eNOS. These observations are consistent with our findings in skeletal muscle, where at least some capillaries labeled for both NOS isoforms (Fig. 6). It is unlikely that “pericapillary nerves” are responsible for this nNOS labeling. However, we cannot exclude the possibility that pericytes contain nNOS (e.g., as found in smooth muscle cells) and thereby may have contributed to our capillary staining. Nevertheless, the similarity of staining we observed for von Willebrand’s factor (data not shown) supports the interpretation that capillary endothelial cells do contain nNOS. As shown by others (13, 19), nNOS in skeletal muscle otherwise appeared restricted to the periphery of myocytes (Fig. 6). Whereas only eNOS was present in the endothelium of the other vessels we studied (Figs. 1–5), the expression of this isoform has been found within striated myocytes (11, 20).

Before the identification of nNOS in the sarcolemma of rats (19) and human (13) skeletal muscle fibers (and see Fig. 6), eNOS was taken to be the only major isoform associated with cell membranes (37). In more
recent studies of murine skeletal muscle, nNOS was found localized to the sarcolemma with dystrophin, a muscle-specific plasma membrane marker, with a similar relationship apparent for cardiac and aortic smooth muscle cells of the rat (41). Immunoprecipitation of nNOS with dystrophin suggested a physical association such that dystrophin complexes enriched in caveolae were coated with CAV-3 (41). The mechanism of targeting nNOS to the myocyte membrane is distinct from the acylation of eNOS (12, 16). Instead, nNOS interacts with α1-syntrophin in the membrane cytoskeleton of the dystrophin complex, mediated by an NH2-terminal extension of the protein that is not found in other NOS isoforms (7). Analogous to CAV-1 in endothelial cells, CAV-3 is proposed to act as a scaffolding protein within myocyte caveolae, recruiting proteins that interact with caveolin to the membrane to enable rapid and effective coupling of membrane events to cellular responses (30, 41).

Functional Implications

In the rat heart, the subcellular localization of eNOS favored the plasma membrane of postcapillary endothelial cells and the Golgi apparatus in precapillary endothelial cells (2). Such heterogeneity of protein targeting may convey subtle differences to the regulation of NO production in endothelial cells according to the particular requirements of the local milieu; e.g., flow control in arterioles (18) and transvascular macromolecule transport in postcapillary venules (22). In turn, the presence of both constitutive isoforms of NOS in capillary endothelial cells (Fig. 6) suggests a unique role for (and regulation of) NO production in capillaries of skeletal muscle (40). In biopsies of human quadriceps muscle, immunohistochemical staining for eNOS was restricted to microvascular endothelial cells (13). Whereas staining for nNOS was most apparent at the periphery of muscle fibers, punctate nNOS staining

![Fig. 7. Light micrographs of cross sections of hamster abdominal aorta (A and C) and retractor muscle (B and D). Sections in A and B incubated with nNOS antibody preabsorbed with nNOS protein. Sections in C and D incubated with secondary antibody + ABC. Scale bar = 30 µm.](http://ajpheart.physiology.org/Downloadedfrom/10.220.32.247)
was also present throughout fiber cross sections, with an intracellular pattern similar to that for mitochondrial enzymes (13). Such intracellular localization of nNOS is consistent with a role for NO in governing respiration (20, 40) as well as force production (1, 19).

When the present findings are taken in light of previous studies, it is now apparent that NO can be generated physiologically from vascular smooth muscle cells (5), endothelial cells (32), and skeletal muscle (4, 19). Therefore, studies that have inhibited all constitutive NOS activity (e.g., by treating an entire animal or tissue with an L-arginine analog), or that have measured NO production and release from an intact tissue (4) [or its homogenate (27)], overlook the subtleties of NO generation from the respective cellular “elements” of a tissue. This raises the question of where NO production that has previously been attributed to arterial (14) and arteriolar (18) endothelial cells, skeletal muscle capillaries (40), or venular endothelium (21, 22) may actually occur in vivo. Refining experimental approaches to these questions should provide valuable new insight into the regulation and action(s) of NO in the intact system.

Summary and Conclusion

Immunohistochemical studies of the constitutive expression of NOS have focused on where eNOS (2, 29, 32), nNOS (5, 6, 19, 27), or both [(13, 31) and present study] are localized in particular tissues and cells. The corresponding expression of caveolin has received relatively less attention (2, 11, 41). In isolated cell systems, studies have focused on elucidating the molecular interaction and biochemical regulation of NOS isoforms with and by caveolin isoforms (11, 15, 38). These efforts have provided fundamental insight into a signaling pathway that is central to many physiological processes, not only for the cells involved, but also for homeostasis of the organism as a whole. Nevertheless, there has been a paucity of information concerned with whether (and if so, where) NOS and caveolin may systematically interact in vivo. In the present study NOS and caveolin were consistently codistributed in endothelial cells and myocytes throughout the hamster, albeit with differential expression of respective isoforms. In turn, findings that eNOS and nNOS are inhibited similarly by CAV-1 or CAV-3 (15, 42) imply that the regulation of NOS by caveolin occurs throughout the intact system, regardless of which of the respective isoforms are present. Regional heterogeneity in the distribution of one and/or the other type of NOS or caveolin protein may reflect differences not only in the influence of the local milieu on gene expression but also in the subtleties of physiological control effected through a common signaling pathway.

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