Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 304 (2003) 22–25
BBRC
www.elsevier.com/locate/ybbrc

Expression of caveolin-3 in rat aortic vascular smooth muscle cells is determined by developmental state☆

Donald D. Doyle, Judy Upshaw-Earley, Eric Bell, and H. Clive Palfrey*

Department of Neurobiology, Pharmacology, and Physiology, University of Chicago, Chicago, IL 60637, USA

Received 14 March 2003

Abstract

The presence of caveolin-3 was reinvestigated in smooth muscle cells in situ and in culture. Immunocytochemistry of aortic strips showed that both caveolins-1 and -3 are present, but that caveolin-3 is considerably less abundant and detected in only a fraction of the cells. Cultured smooth muscle cells of passage 4 or greater still expressed caveolin-1 but lacked caveolin-3, likely due to de-differentiation. Cultures exposed to redifferentiation-inducing medium were found to re-express caveolin-3. The return of caveolin-3 protein in these cells was accompanied by a fourfold increase in cav-3 mRNA, as detected by quantitative PCR, suggesting that the loss of protein in untreated cells was due to transcriptional rather than translational inhibition. These results suggest that caveolin-3 has a specialized role associated with the differentiated state in certain types of smooth muscle cells.

Keywords: Caveolin-3; Differentiation state; Caveolae; Cell division; Smooth muscle

Caveolins are proteins which coat plasmalemmal invaginations in many cell types [1]. Three major isoforms of caveolin exist, of which caveolin-1 (cav-1) is the most widely expressed. The caveolin-3 (cav-3) isoform is found predominantly in muscle cells [2,3], but is also expressed in some glial cells [4]. It is present in high concentration in adult striated muscle, where caveolin-1 levels are low or absent, but the disposition of caveolin-3 in smooth muscle cells is less certain. Low levels were detected in intestinal muscle by Western blotting [5], but the same investigators failed to detect cav-3 in rat aorta. In contrast, other investigators [6] detected low levels of cav-3 in freshly isolated smooth muscle cells from rat uterus, as well as vascular and visceral smooth muscle cells from both rat and ferret. In addition, Segal et al. [7] presented evidence of cav-3 in hamster arterioles.

Vascular smooth muscle cells in vivo transform between a contractile and synthetic state [8], which may underlie some of the etiology of atherosclerosis. Isolated vascular smooth muscle cells in primary culture quickly adopt a synthetic phenotype and after passaging cannot be induced to revert to a contractile phenotype [9]. Thyberg et al. [10] reported that primary cultures of smooth muscle cells derived from rat aorta show a redistribution of caveolae toward the cell interior as a function of passage number (passages 1–5), while caveolin levels as measured by immunoblotting did not change. However, these investigators detected only a single protein band and thus apparently only measured cav-1 in these cells. In the present work we reinvestigate the existence of cav-3 in vascular smooth muscle cells in culture relative to its existence in vivo and find that its distribution is variable, both within the tissue and between tissue and cultured cells.

Materials and methods

Materials. Female Holtzman Sprague–Dawley, 225–250 g, were obtained from Harlan, (indianapolis, IN). A polyclonal antibody was made commercially to the N-terminal residues 2–18 of the rat cav-3 sequence (QCB, Hopkinton, MA). Polyclonal and monoclonal antibodies to cav-1 and -3 were obtained from Transduction Laboratories (Louisville, KY). Primary cultures of rat aortic smooth muscle cells

☆ This work was supported by National Institutes of Health Grant HL54302.
* Corresponding author. Fax: 1-773-702-3774.
E-mail address: hpalfrey@midway.uchicago.edu (H.C. Palfrey).

0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)00528-X

were gifts from Dr. Ken Byron, Loyola University Medical Center, Maywood, IL, and Dr. Thomas Lincoln, University of Alabama, Birmingham, AL. A smooth muscle cell differentiation-inducing additive (MCDB 131 medium) obtained from Fisher contained insulin, transferrin, selenous acid, linoleic acid, and BSA.

Confocal immunofluorescence microscopy. To obtain left ventricle and thoracic aorta tissues for cryosectioning, rats (Harlan, Indianapolis, IN) were anesthetized with isofluorane. These tissues were quickly removed to ice-cold physiological saline, cut into pieces, and then frozen in isopentane cooled by liquid nitrogen. Sectioning was done at −20 °C on a Leica 1850CM Cryostat. Left ventricle sections (7 μm) and aorta sections (10 μm) on poly-L-lysine coated coverslips were fixed in 4% paraformaldehyde in phosphate buffer, and then the aldehyde groups were quenched by 100 mM ammonium chloride. To prevent non-specific binding, sections were blocked with 10% normal goat serum while being permeabilized in 0.2% Triton X-100 and 0.02% Tween detergents. Sections were incubated overnight at 4 °C in anti-cav-1 and anti-cav-3, paired as polyclonal/monoclonal or vice versa, both at 1:30 or, as a control, in mouse and rabbit non-immune IgGs at similar concentrations. Incubation in the fluorescently tagged secondary antibodies Alexa 488 and Alexa 660 (both 1:400) was done for 90 min at room temperature. To quantify the signal intensity due to each antibody in each section, aorta and ventricle, images were processed using MetaMorph software, version 5 (Universal Imaging). Background within the image was subtracted from each image by reading the intensity of a tissue-free region on the section. Each section was thresholded manually. Thresholding manually is one method of discarding noise judged not to be signal. A comparison between thresholding and, another approach, subtracting the intensity of the IgG controls (which was very light), gave similar readings. Then the mean intensity of specified regions, nine in the aorta section and four in ventricle, was read.

RNA isolation. Total RNA was isolated from either tissue or cell culture using the RNAqueous-4PCR kit from Ambion (Austin, TX). Instructions included with the kit were followed except for the following modifications. Four units of DNAse (supplied with kit) was added per column eluate and samples were subsequently incubated at 37 °C for 1 h. DNAse was inactivated with DNAse Inactivation Reagent (supplied with the kit). RNA was ethanol precipitated overnight and then quantified at 260 nm.

RT-PCR. Reverse transcription and PCR components were obtained from Ambion and used to manufacturer's specifications. Briefly, 1 μg RNA was mixed with dNTPs (500 μM each), random decamer primers (5 μM), and H_2O to 16 μl. RNA was denatured at 70 °C for 3 min and then placed on ice for 1 min. Ten times RT buffer, 10 U RNase Inhibitor, and 25 U M-MLV RT were added to the reaction and then incubated for 1 h at 42 °C and then for 10 min at 95 °C. The RT reaction mixture was ethanol precipitated overnight and then quantified at 260 nm. For a 50 μl PCR, 0.5μg template was mixed on ice with dNTPs (200 μM each), 10× PCR buffer + 15 mM $MgCl_2$, 2 U of SuperTaq Plus Polymerase, forward and reverse primers (400 nM each), and H_2O to 50 μl. Samples were incubated for 2 min at 94 °C, then cycled 35 times (1 min at 94 °C, 1 min at 54 °C, and 1 min at 72 °C), and then extended for 5 min at 72 °C. Products were visualized in a TAE agarose gel stained with ethidium bromide. Primers were: Cav-1 (forward) 5′-CTA CAA GCC CAA CAA CAA GGC-3′ and (reverse) 5′-AGG AAG CTC TTG ATG CAC GGT-3′; and Cav-3 (forward) 5′-GGA CAT TGT GAA GGT GGA TTT-3′ and (reverse) 5′-GCA CTG GAT CTC AAT CAG GTA-3′. Reactions were carried out in a MJ Research MiniCycler model PTC-150.

Real time PCR for caveolin-1 and caveolin-3. All primers and probes were obtained from Megabases (Evanston, IL) and the probes were labeled with 6-FAM and quenched with QSY 7. Caveolin-1 (forward: 5′-CGC GCA CAC CAA GGA AAT-3′, reverse: 5′-CCT TCT GGT TCC GCA ATC A-3′, probe: 5′-CAA CCG CGA CCC CAA GCA TCT C-3′) and caveolin-3 (forward: 5′-CCT GCA TTA AGA GCT ACC TGA TTG A-3′, reverse: 5′-TCG CAG CAC CAC CTT AAT GTT-3′, probe: 5′-CAA CCC GCT CTT TGC CGC ACT G-3′) primer/probe sets were designed using Primer Express (Applied Biosystems). Rodent GAPDH sequences from Applied Biosystems were used for the housekeeping gene. Samples were prepared as 50 μl reactions containing template, forward and reverse primers (900 nM), 6-FAM and QSY 7 labeled probe (250 nM), 2× TaqMan Universal PCR Master Mix (Applied Biosystems), and water to 50 μl. Samples were analyzed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) under the following conditions: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and then 1 min at 60 °C. The amount of reverse transcribed RNA in the unknown samples was calculated from the threshold cycle (C_t) values in combination with the 5-point standard curves that were simultaneously generated from reverse transcribed whole heart RNA. All unknown samples fit on the standard curves. Unknown samples were assayed in sets of five and standards were done in triplicate. Samples were normalized with GAPDH and then compared to calculate relative changes of cDNA.

Preparation of tissue and cellular SDS extracts. Tissue and cell pellets were weighed and then directly dissolved in hot SDS sample buffer to minimize proteolysis prior to immunoblotting. Freshly excised rat aorta was stripped of adventitia on ice and minced with scissors, and the pieces were collected by centrifugation at 1000*g* for 10 min. Smooth muscle cells in primary culture were washed with PBS, collected by scraping, and pelleted. Enriched ventricular myocytes were isolated as previously described [11]. Protein in extracts was estimated as 0.15 g/g wet weight of pellet.

Results and discussion

Cav-3 and cav-1 were detected by confocal immunofluorescence microscopy in sections of acutely excised rat aorta (Fig. 1). The intensity of cav-3 staining in aortic smooth muscle cells was found to be significantly less than that for cardiac myocytes in a section of rat ventricle processed side-by-side on the same coverslip, while cav-1 staining in aortic cells more closely approximated cav-1 staining in non-myocytes of the ventricle (data not shown). In order to quantify the difference in concentrations of the caveolin isoforms in the two different tissues, the intensity of the signal in the aorta was amplified until it was approximately the same as that seen in the ventricle (Fig. 1). This procedure required an amplification of nearly 8 for cav-3, although less than 2 for cav-1. Both cav-1 and cav-3 proteins were detected in immunoblots of extracts of acutely excised aortic smooth muscle sheets which had been stripped of adventitia (Fig. 2). Again cav-3 levels in aortic extracts were substantially less than those for similarly processed ventricular myocyte samples. Of the two anti-cav-3 antibodies used here, a commercial monoclonal antibody detected cav-3 in both tissue sections and immunoblots from smooth muscle, whereas our own polyclonal antibody made against C-terminal residues 2–18 detected cav-3 only on immunoblots, despite the fact that this antibody readily detects cav-3 in rat heart atrial myocyte sections. This difference may be due to epitope masking of the cav-3 N-terminal domain in smooth muscle cells and may explain previous failures to identify cav-3 in this tissue [5].

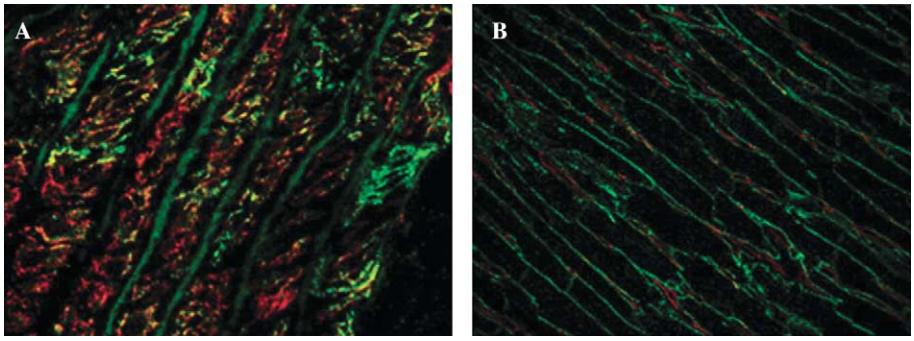

Fig. 1. Localization of cav-1 and cav-3 in freshly excised rat aorta and ventricle by confocal immunofluorescence microscopy. Cross-sections of rat aorta (A) and ventricle (B) were mounted side-by-side on a single coverslip. The slip was dual-labeled with polyclonal anti-cav-1 (red) and monoclonal anti-cav-3 (green) antibodies and fluorescently labeled secondary antibodies. In order to quantify the relative concentration of protein in the two sections, the intensity of the cav-3 signal after background correction in the aortic section was amplified 7.9 times so that its intensity was approximately equal to the intensity in the unamplified ventricle specimen. For cav-1 the degree of amplification was 1.94.

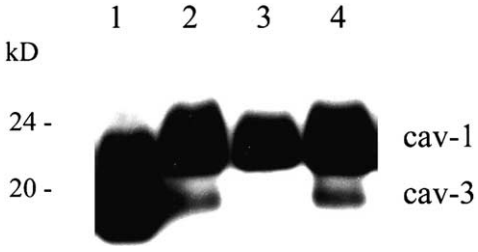

Fig. 2. Proteins (100 μg) extracted from (1) freshly isolated ventricular cells enriched in cardiac myocytes; (2) freshly excised aorta stripped of adventitia; and primary cultures of aortic smooth muscle cells (passage 10) either (3) not treated or (4) treated with DM, were separated by gel electrophoresis and probed with anti-cav-1 and anti-cav-3 antibodies.

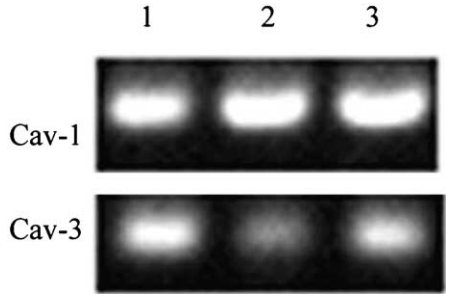

Fig. 3. RT-PCR analysis of primary untreated rat aortic smooth muscle cells (RASMC) (2), RASMC treated with differentiation medium (DM) (3), and whole heart RNA (1) as a positive control. Primers were directed against sequences of caveolin-1 and caveolin-3 described in Materials and methods.

In contrast to tissue sections, cav-3 was not detected by immunoblot of whole cell extracts of primary cultures of aortic smooth muscle cells (Fig. 2). The lack of detectable cav-3 in aortic smooth muscle primary cultures was found at the earliest stage examined (passage 4) and in cells derived by two different laboratories. As cultured smooth muscle cells can revert to a synthetic-like state, we grew passage 10 cells on laminin in the presence of a smooth muscle cell differentiation-inducing additive in the differentiation medium (DM) to promote redifferentiation. In DM-stimulated cells cav-3 protein returned to a level comparable to that found in vivo aorta. These results suggest that the levels of cav-3 in smooth muscle are regulated and may correlate with the differentiated vs. synthetic state of the cells. By contrast, cells not exposed to DM or cells grown to passage 17 before induction did not show increased cav-3 expression (data not shown).

To determine whether the lack of cav-3 protein expression in cultured cells was due to the lack of mRNA we performed RT-PCR (Fig. 3). Message was detected for cav-3 just as it was for cav-1 in untreated cells in culture within 35 cycles (Table 1). Visually it appears as if the cav-3 level in DM-stimulated cells is greater than

Table 1
Real time PCR results measuring the relative amounts of caveolin-1 and caveolin-3 cDNA in primary untreated rat aortic smooth muscle cells (RASMC) and rat aortic smooth muscle cells treated with a differentiation medium (DM)

	cDNA (μg)			cDNA (normalized)		cDNA (relative)	
	Cav-1	Cav-3	GAPDH	Cav-1	Cav-3	Cav-1	Cav-3
RASMC	7.23	0.38	1.35	5.36	0.28	1.00	1.00
RASMC DM	11.89	1.32	1.03	11.56	1.28	2.16	4.59

Values for the caveolin isoforms were normalized to GAPDH. Values for the treated cells were divided by the values for the untreated cells to obtain relative values of differential expression. This experiment is representative of three separate experiments which gave similar results.

that in untreated cells. In order to better quantify message levels we performed real-time PCR. In DM-stimulated cells cav-3 message levels increased fourfold over unstimulated levels, while cav-1 levels increased twofold (Table 1), suggesting that the loss of protein in untreated cells was dependent upon transcriptional events, as it has been reported for other contractile- and synthetic-state-associated proteins [12].

The role of cav-3 in smooth muscle cells is poorly understood. A requirement for cav-3 for the fusion of C2C12 cells in the differentiation process of myotube formation has been demonstrated in skeletal muscle [13]. In light of the present results we speculate that since the cav-3 protein disappears from smooth muscle cells in primary culture, cav-3 expression may be related to the growth status of these cells. Previously we have demonstrated a molecular association of dystrophin with cav-3 in mammalian adult heart myocytes [11], while others have shown a similar relationship in skeletal muscle [14]. We postulated a linkage between cav-3 in caveolae and dystroglycan in the surface membrane in heart myocytes, mediated by dystrophin [11]. Such an arrangement may be facilitated by the rosette pattern of caveolae found in cardiac muscle [15], a pattern which brings a portion of the caveola in sufficiently close apposition to the bulk sarcolemma for such an association to occur. Rosette configurations are also seen in skeletal and smooth muscle cells [10,16]. The loss of cav-3 in the synthetic state may be associated with the need to dismantle caveolar–cytoskeletal associations in order for cell division and other functions to take place.

References

[1] B. Razani, M.P. Lisanti, Caveolins and caveolae: molecular and functional relationships, Exp. Cell Res. 271 (2001) 36–44.
[2] M. Way, R.G. Parton, M-caveolin, a muscle-specific caveolin-related protein, FEBS Lett. 378 (1996) 108–112.
[3] K.S. Song, P.E. Scherer, Z. Tang, T. Okamoto, S. Li, M. Chafel, C. Chu, D.S. Kohtz, M.P. Lisanti, Expression of caveolin-3 in skeletal, cardiac, and smooth muscle cells. Caveolin-3 is a component of the sarcolemma and co-fractionates with dystrophin and dystrophin-associated glycoproteins, J. Biol. Chem. 271 (1996) 15160–15165.
[4] T. Ikezu, H. Ueda, B.D. Trapp, K. Nishiyama, J.F. Sha, D. Volonte, F. Galbiati, A.L. Byrd, G. Bassell, H. Serizawa, W.S. Lane, M.P. Lisanti, T. Okamoto, Affinity-purification and characterization of caveolins from the brain: differential expression of caveolin-1, -2, and -3 in brain endothelial and astroglial cell types, Brain Res. 804 (1998) 177–192.
[5] M. Voldstedlund, J. Vinten, J. Tranum-Jensen, cav-p60 expression in rat muscle tissues. Distribution of caveolar proteins, Cell Tissue Res. 306 (2001) 265–276.
[6] M.J. Taggart, P. Leavis, O. Feron, K.G. Morgan, Inhibition of PKCα and ρA translocation in differentiated smooth muscle by a caveolin scaffolding domain peptide, Exp. Cell Res. 258 (2000) 72–81.
[7] S.S. Segal, S.E. Brett, W.C. Sessa, Codistribution of NOS and caveolin throughout peripheral vasculature and skeletal muscle of hamsters, Am. J. Physiol. 277 (1999) H1167–H1177.
[8] N.F. Worth, B.E. Rolfe, J. Song, G.R. Campbell, Vascular smooth muscle cell phenotypic modulation in culture is associated with reorganisation of contractile and cytoskeletal proteins, Cell Motil. Cytoskeleton 49 (2001) 130–145.
[9] K. Hayashi, M. Takahashi, W. Nishida, K. Yoshida, Y. Ohkawa, A. Kitabatake, J. Aoki, H. Arai, K. Sobue, Phenotypic modulation of vascular smooth muscle cells induced by unsaturated lysophosphatidic acids, Circ. Res. 89 (2001) 251–258.
[10] J. Thyberg, J. Roy, P.K. Tran, K. Blomgren, A. Dumitrescu, U. Hedin, Expression of caveolae on the surface of rat arterial smooth muscle cells is dependent on the phenotypic state of the cells, Lab. Invest. 77 (1997) 93–101.
[11] D.D. Doyle, G. Goings, J. Upshaw-Earley, S.K. Ambler, A. Mondul, H.C. Palfrey, E. Page, Dystrophin associates with caveolae of rat cardiac myocytes: relationship to dystroglycan, Circ. Res. 87 (2000) 480–488.
[12] K. Hayashi, M. Takahashi, K. Kimura, W. Nishida, H. Saga, K. Sobue, Changes in the balance of phosphoinositide 3-kinase/protein kinase B (Akt) and the mitogen-activated protein kinases (ERK/p38MAPK) determine a phenotype of visceral and vascular smooth muscle cells, J. Cell Biol. 145 (1999) 727–740.
[13] F. Galbiati, D. Volonte, J.A. Engelman, P.E. Scherer, M.P. Lisanti, Targeted down-regulation of caveolin-3 is sufficient to inhibit myotube formation in differentiating C2C12 myoblasts. Transient activation of p38 mitogen-activated protein kinase is required for induction of caveolin-3 expression and subsequent myotube formation, J. Biol. Chem. 274 (1999) 30315–30321.
[14] E.M. McNally, E. de Sa Moreira, D.J. Duggan, C.G. Bonnemann, M.P. Lisanti, H.G. Lidov, M. Vainzof, M.R. Passos-Bueno, E.P. Hoffman, M. Zatz, L.M. Kunkel, Caveolin-3 in muscular dystrophy, Hum. Mol. Genet. 7 (1998) 871–877.
[15] E. Page, H. Iida, D.D. Doyle, 2001, Cell physiology amd cell biology of myocardial cell caveolae, in: E. Page, H.A. Fozzard, R.J. Solaro (Eds.), Handbook of Physiology, vol. 1: The Heart, Oxford University Press, Oxford, 2000, pp. 145–167.
[16] P. Rahkila, T.E. Takala, R.G. Parton, K. Metsikko, Protein targeting to the plasma membrane of adult skeletal muscle fiber: an organized mosaic of functional domains, Exp. Cell Res. 267 (2001) 61–72.