Caveolin-1 and caveolin-3 form heterooligomeric complexes in atrial cardiac myocytes that are required for doxorubicin-induced apoptosis

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Volonte D, McTiernan CF, Drab M, Kasper M, Galbiati F. Caveolin-1 and caveolin-3 form heterooligomeric complexes in atrial cardiac myocytes that are required for doxorubicin-induced apoptosis. Am J Physiol Heart Circ Physiol 294: H392–H401, 2008. First published November 2, 2007; doi:10.1152/ajpheart.01039.2007—Caveolae are 50- to 100-nm invaginations of the plasma membrane. Caveolins are the structural protein components of caveolar membranes. The caveolin gene family is composed of three members: caveolin-1, caveolin-2, and caveolin-3. Caveolin-1 and caveolin-2 are coexpressed in many cell types, including adipocytes, endothelial cells, epithelial cells, and fibroblasts. In contrast, caveolin-3 expression is essentially restricted to skeletal and smooth muscle cells as well as cardiac myocytes. While the interaction between caveolin-1 and caveolin-2 has been documented previously, the reciprocal interaction between endogenous caveolin-1 and caveolin-3 and their functional role in cell types expressing both isoforms have yet to be identified. Here we demonstrate for the first time that caveolin-1 and caveolin-3 are coexpressed in mouse and rat cardiac myocytes of the atria but not ventricles. We also found that caveolin-1 and caveolin-3 can interact and form heterooligomeric complexes in this cell type. Doxorubicin is an effective anticancer agent, but its use is limited by the possible development of cardiotoxicity. Using caveolin-1- and caveolin-3-null mice, we show that both caveolin-1 and caveolin-3 expression are required for doxorubicin-induced apoptosis in the atria through activation of caspase 3. Together, these results bring new insight into the functional role of caveolae and suggest that caveolin-1/caveolin-3 heterooligomeric complexes may play a key role in chemotherapy-induced cardiotoxicity in the atria.

Caveolae are 50- to 100-nm invaginations of the plasma membrane. Caveolin is the structural protein component of caveolar membranes. The caveolin gene family consists of three members: caveolin-1, caveolin-2, and caveolin-3 (28, 33). Caveolin-1 and caveolin-2 are coexpressed in a variety of cell types, while caveolin-3 is a muscle-specific isoform, being mostly expressed in skeletal and cardiac myocytes as well as smooth muscle cells (8, 9). Smooth muscle cells express caveolin-1 and caveolin-2 in addition to caveolin-3, making them a cell type that coexpresses all three caveolin isoforms (32). To date, caveolin-1 expression has not been shown in differentiated skeletal muscle cells and cardiac myocytes in vivo. Caveolins act as scaffolding proteins to concentrate and regulate signaling molecules within caveolar membranes. Direct interaction with caveolin-1 results in the inhibition of a number of signaling molecules, such as G protein α-subunit, Ras, nitric oxide synthase (NOS), protein kinase C (PKC), and protein kinase A (PKA) (14, 19, 24). However, caveolin-1 is also capable of promoting certain signaling pathways. In fact, caveolin-1 has been shown to stimulate the estrogen receptor and the insulin receptor signaling (30, 43). Caveolin-3 is most closely related to caveolin-1. Caveolin-3 and caveolin-1 are ~65% identical and 85% similar based on protein sequence homology (33). Caveolin-3 has been shown to interact with different signaling molecules. For example, Gq/11, Gβγ, c-Src, and Src-like kinases (Lyn) all cofractionated with caveolin-3 in C2C12 cells (32), suggesting a role of muscle cell caveolae in the transduction events mediated by these molecules. Also, caveolin-3 has been reported to directly interact with neuronal NOS (nNOS), the NOS isoform expressed in skeletal muscle (13, 35). Interaction with caveolin-3 results in the inhibition of nNOS enzymatic activity in vitro (13, 35).

Although expression of both caveolin-3 and caveolin-2 in ventricular cardiac myocytes as well as caveolin-1 in cardiac endothelial and smooth muscle cells of blood vessels have been well documented (17, 25, 26), expression of caveolin-1 in ventricular cardiac myocytes remains controversial (15, 23, 26). In addition, expression of caveolin-1 in atrial cardiac myocytes has yet to be reported. Finally, the reciprocal interaction between endogenous caveolin-1 and caveolin-3 in muscle cells and their functional relationship remain to be explored. Here we investigated the hypothesis that caveolin-1, in addition to caveolin-3, is expressed in atrial cardiac myocytes and that the two caveolin isoforms form functional heteromeric complexes that control apoptotic signaling.

MATERIALS AND METHODS

Materials. Antibodies and their sources were as follows: anti-caveolin-3 IgG (mouse MAb 26) and anti-caveolin-1 IgG (mouse MAb 2297) were from Becton Dickinson Biosciences (San Jose, CA). Anti-caveolin-3 IgG (goat PAb N-18) and anti-caveolin-1 IgG (rabbit PAb H-97) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase 3 IgG (rabbit PAb AP113E) was from Assay Designs (Ann Arbor, MI). Anti-MF-20 IgG (mouse MAb) was from Developmental Studies Hybridoma Bank at the University of Iowa. Anti-rododolysin IgG (rabbit PAb) was a gift from Dr. Marilyn G. Farquhar (University of California-San Diego, La Jolla, CA).

Animals. The animal protocols described in this article were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Male Wistar rats (350–
400 g; 6 mo of age) were used for immunoperoxidase and immunofluorescence staining. Generation of cardiac myocytes was performed on 1- to 2-day-old Sprague-Dawley rats. Male and female C57BL/6 mice (20–30 g; 10–16 wk of age) were used for immunoperoxidase and immunofluorescence staining. Male C57BL/6 wild-type (WT), caveolin-1-null, and caveolin-3-null mice (20–22 g; 8 wk of age) were used for terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assays.

Immunoperoxidase staining. Male Wistar rats (n = 5) and male and female C57BL/6 mice (n = 6) were killed, and hearts were routinely processed for embedding in paraffin. Immunohistochemistry was performed by indirect immunoperoxidase techniques using dewaxed 4-μm-thick paraffin sections on silane-coated glass slides. The sections were microwaved in 0.01 mol/l sodium citrate buffer (pH 6.0) twice for 5 min each at 850 W. After being washed with PBS, the sections were treated with 0.3% hydrogen peroxide and dissolved in methanol for 30 min at room temperature (RT). Blocking was performed with 3% normal goat or horse serum for 30 min (RT) before incubation with primary antibodies. Slides were washed three times for 10 min each in PBS, pH 7.4, and detection of signals was performed with the Vectastain ABC Elite Kit (Vector Laboratories, Burlingham, CA) according to the manufacturer’s instructions. Peroxidase activity was visualized with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Vector Laboratories). Controls for immunospecificity were included in all experiments by omission of the primary antibody and its replacement by PBS and matching concentrations of normal rabbit serum (unpublished results).

Immunofluorescence. Cells grown on glass coverslips were washed three times with PBS and fixed for 30 min at RT with 2% paraformaldehyde in PBS. Fixed cells were rinsed with PBS and permeabilized with 0.1% Triton X-100–0.2% bovine serum albumin (BSA) for 10 min. Cells were then incubated with the primary antibody for 1 h at room temperature. After three washes with PBS, cells were incubated with the secondary antibody for 1 h at RT: lissamine rhodamine B sulfonyl chloride-conjugated goat anti-rabbit antibody (or donkey anti-goat antibody) (5 μg/ml) and fluorescein isothiocyanate-conjugated goat anti-mouse antibody (or donkey anti-mouse antibody) (5 μg/ml). Slides were mounted with slow-Fade antifade reagent (Molecular Probes, Eugene, OR).

Generation of cardiac myocytes. Hearts from Sprague-Dawley rats were removed, and atria (left and right pooled) and biventricles were carefully dissected. Atrial and ventricular cardiac myocytes were prepared by sequential collagenase digestion and preplating on uncoated tissue culture-grade plastic to deplete fibroblasts as previously described (7). Cardiac myocyte-enriched fractions were plated onto glass coverslips or tissue culture-grade plates precoated with Promecin (Biosource International, Camarillo, CA). Cardiac myocytes were cultured in DMEM-F-12 containing 5% horse serum, 0.1 mmol/l sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% glutamine, 1% nonessential amino acids, 1% sodium selenite, 2% BSA, and 0.1% sodium selenite. Medium was changed every 2 days.

Preparation of caveola-enriched membrane fractions. Cells were scraped into 2 ml of MES-buffered saline containing 1% (vol/vol) Triton X-100. Homogenization was carried out with 10 strokes of a loose-fitting Dounce homogenizer. The homogenate was adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose prepared in MES-buffered saline and placed at the bottom of an ultracentrifuge tube. A 5–30% linear sucrose gradient was formed above the homog eneate and centrifuged at 45,000 rpm for 16–20 h in a SW60 rotor (Beckman Coulter, Fullerton, CA). A light-scattering band confined to the 15–20% sucrose region was observed that contained endogenous caveolin-1 and caveolin-3 but excluded most other cellular proteins. From the top of each gradient, 1-ml gradient fractions were collected to yield a total of 11 fractions. Fractions 4–6, representing caveolar membranes, and fractions 9–11, representing noncaveolar membranes, were pooled together. An equal amount of protein from each of the two groups was separated by SDS-PAGE and subjected to immunoblot analysis.

Velocity gradient centrifugation. Samples were dissociated in MES-buffered saline (25 mM MES, pH 6.5, 0.15 M NaCl) containing 60 mM octyl glucoside. Solubilized material was loaded atop a 5–40% linear sucrose gradient and centrifuged at 50,000 rpm (34,000 g) for 10 h in a SW60 rotor (Beckman). Gradient fractions were collected from the top and subjected to immunoblot analysis. Molecular mass standards for velocity gradient centrifugation were as described previously (12).

TUNEL assay. WT, caveolin-1-null, and caveolin-3-null mice were treated intraperitoneally with doxorubicin (20 mg/kg). Mice were killed 4 days after doxorubicin treatment. Atria were then extracted, and formalin-fixed and paraffin-embedded sections were subjected to TUNEL assay to detect apoptosis with an ApopTag Red In Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA) according to the manufacturer’s instructions. Labeling indexes were obtained by counting TUNEL-positive nuclei from 50 randomly chosen fields and expressed as percentage of total nuclei. Coo staining with DAPI was performed to detect total nuclei. Sections treated with DNase I were used as positive controls for TUNEL staining.

RESULTS

Caveolin-1 is expressed in cardiac myocytes of the atria, but not ventricles. To investigate the possible expression of caveolin-1 in cardiac myocytes in vivo, whole mouse (4 mo old) heart sections were incubated with an antibody probe specific for caveolin-1, and caveolin-1 expression was evaluated with peroxidase-based staining. Figure 1A illustrates that caveolin-1 was expressed in both the ventricles and the atria, although the expression levels in the atria appeared more intense. To better understand the pattern of expression of caveolin-1, mouse heart sections were examined at a higher magnification. As shown in Fig. 1, B–D, while caveolin-1 appeared to be expressed only in endothelial cells of blood vessels in the ventricles, caveolin-1 was found in both endothelial cells and cardiac myocytes in the atria.

To confirm these results, we performed immunoperoxidase staining on sections derived from mouse atria, using antibody probes specific for caveolin-1, podocalyxin, a vascular endothelium marker (16, 18), and caveolin-3, a well-known marker of cardiac myocytes (1). We found that podocalyxin was expressed only in endothelial cells of blood vessels (Fig. 2B) and caveolin-3 only in cardiac myocytes (Fig. 2C). In contrast, caveolin-1 was expressed both in endothelial cells and cardiac myocytes of mouse atria (Fig. 2A).

Caveolin-2 is known to form heterooligomeric complexes with caveolin-1 in a variety of cell types. To examine whether caveolin-2 was coexpressed together with caveolin-1 and caveolin-3 in atrial cardiac myocytes, we performed immunoperoxidase staining on sections derived from rat atria, using antibody probes specific for caveolin-1, caveolin-2, and caveolin-3. We found that caveolin-2 (Fig. 2E) was coexpressed...
Coexpression of caveolin-1 and caveolin-3 at plasma membrane of atrial cardiac myocytes in vivo. We next evaluated the precise localization of caveolin-1 and caveolin-3 within cardiac myocytes in vivo by performing immunofluorescence analysis on rat atrial and ventricular sections, using anti-caveolin-1 and anti-caveolin-3. Our results showed that both proteins were coexpressed at the plasma membrane of atrial cardiac myocytes in vivo, indicating a possible role in signaling processes specific to atrial cells.

Conclusions: The expression of caveolin proteins in atrial cardiac myocytes is essential for maintaining proper cardiac function. Further research is needed to understand the specific roles of caveolin-1 and caveolin-3 in atrial cardiac myocytes and their potential implications for cardiovascular disease.
anti-caveolin-3 IgGs. Figure 3A shows that the cardiac myocyte marker caveolin-3, but not caveolin-1, was expressed in ventricular cardiac myocytes. The expression of caveolin-1 was limited to blood vessels in these sections (Fig. 3A), as further demonstrated by colocalization with podocalyxin (Fig. 3C). In contrast, caveolin-1 and caveolin-3 were coexpressed in atrial cardiac myocytes and colocalized at the plasma membrane (Fig. 3B). Caveolin-1 also stained blood vessels of the atria (Fig. 3, B and D). Thus these results directly support the data of Figs. 1 and 2.

Differentiated skeletal muscle cells express caveolin-3 but not caveolin-1 (38, 39). In contrast, only caveolin-1 is expressed in undifferentiated myogenic precursor cells, which do not differentiate to multinucleated myotubes (38, 39). To rule

Fig. 3. Caveolin-1 and caveolin-3 are coexpressed in atrial cardiac myocytes. Rat ventricular (A and C) and atrial (B and D) sections and differentiated skeletal muscle cells (E) were double stained with anti-caveolin-1 and anti-caveolin-3 IgGs (A, B, and E) and anti-caveolin-1 and anti-podocalyxin IgGs (C and D). Expression of these proteins was detected with fluorescent secondary antibodies. Magnification: ×30 (A–D), ×40 (E).
Caveolin-1 and caveolin-3 are coexpressed and colocalize in primary cultures of atrial cardiac myocytes. We next examined whether caveolin-1 and caveolin-3 were expressed in primary cultures of neonatal rat cardiac myocytes derived from the atria. Figure 4A shows that both caveolin isoforms were expressed in this cell type, as demonstrated by immunoblotting analysis using antibody probes specific for caveolin-1 and caveolin-3. We also demonstrated by RT-PCR analysis that the mRNAs of both isoforms were expressed in rat atrial cardiac myocytes in culture (Fig. 4B). In contrast, caveolin-3, but not caveolin-1, protein and mRNA were found in ventricular cardiac myocytes (Fig. 4, A and B, respectively).

To further demonstrate that the two caveolin isoforms were coexpressed only in cardiac myocytes derived from the atria, but not ventricles, we performed immunofluorescence analysis on neonatal rat atrial and ventricular cardiac myocyte cultures, using antibody probes specific for caveolin-1, caveolin-3, MF-20 (which labels sarcomeric myosin heavy chains), and connexin 40 (a marker of atrial cardiac myocytes) (31, 36, 41).

As expected, both atrial and ventricular cardiac myocytes resulted positive for MF-20, while only atrial cardiac myocytes were connexin 40 positive (Fig. 5A). Figure 5A also shows that only atrial cardiac myocytes expressed both caveolin-1 and caveolin-3. Ventricular cardiac myocytes were caveolin-1 negative and caveolin-3 positive (Fig. 5A). Identical results were obtained with either mouse monoclonal or rabbit polyclonal caveolin-1/3 antibodies. Interestingly, we demonstrate in Fig. 5B that caveolin-1 and caveolin-3 were colocalized at the plasma membrane and, to a lesser extent, into vesicles in the cytoplasm of atrial cardiac myocytes. This result suggests that the two caveolin isoforms may be expressed in the same domains in this cell type.

Caveolin-1 and caveolin-3 are targeted to caveolae in atrial cardiac myocytes. We next evaluated whether the two caveolin isoforms were enriched into detergent-resistant domains by examining their Triton insolubility properties. Caveolae are enriched in cholesterol and saturated sphingolipids (19). As a consequence, caveolins are insoluble at low temperature in the detergent Triton X-100 in a variety of cell types. As shown in Fig. 6A, caveolin-1 and caveolin-3 were enriched in the Triton-insoluble fraction, suggesting that the two caveolin isoforms may be expressed within the same domains in atrial cardiac myocytes.

Given the localization of both caveolin-1 and caveolin-3 in the Triton-insoluble fraction, we next evaluated whether the two caveolin isoforms were targeted to caveolar membranes, using a well-established procedure to isolate caveolae based on their detergent resistance and low buoyant density (12, 39). As shown in Fig. 6B, both caveolins were enriched into caveolar membranes, indicating that the two caveolin isoforms are localized within the same microdomains in atrial cardiac myocytes.

Flotillin-1 is a member of a new family of proteins associated with caveolae (2, 10). Flotillin-1 has been shown to coimmunoprecipitate with caveolin-1 and localize to Triton-insoluble/cholesterol-enriched domains (37). We next examined whether the expression of two caveolin isoforms, i.e., caveolin-1 and caveolin-3, within the same cell type may affect the localization of flotillin-1 by sucrose density centrifugation. We show in Fig. 6B that flotillin-1 remained localized to lipid raft/caveolar fractions in atrial cardiac myocytes, suggesting that coexpression of caveolin-1 and caveolin-3 does not displace flotillin-1 from its known cellular localization.

Caveolin-1 and caveolin-3 are part of the same protein complex in cardiac myocytes. The fact that both caveolin isoforms were 1) colocalized at the plasma membrane, 2) Triton insoluble, and 3) enriched into caveolar membranes in atrial cardiac myocytes indicates that caveolin-1 and caveolin-3 may be part of the same protein complex. To investigate this possibility, we next evaluated the interaction between the two caveolin isoforms by examining whether either caveolin-1 or caveolin-3 can coimmunoprecipitate the other isoform in atrial cardiac myocytes. Figure 7A illustrates that caveolin-1 successfully coimmunoprecipitated caveolin-3 and caveolin-3 successfully coimmunoprecipitated caveolin-1. Together, these data indicate that caveolin-1 and caveolin-3 are part of the same protein complex in neonatal rat atrial cardiac myocytes.

Oligomerization properties of caveolin-1 and caveolin-3 in cardiac myocytes are identical. Caveolin proteins possess the ability to form functional oligomeric complexes in an isoform-specific manner (27). These oligomers act as building blocks in the construction of caveolar membranes. Caveolin-1 can form either homo-oligomeric complexes or hetero-oligomeric complexes together with caveolin-2 composed of 14–16 caveolin monomers (29). Caveolin-3 has been shown to form homo-oligomeric complexes, while caveolin-2 is also capable of making dimeric complexes (28, 33). However, the ability of caveolin-1 and caveolin-3 to form oligomeric complexes in cell types where the two caveolin isoforms are endogenously expressed remains to be determined. Thus we evaluated the oligomeric state of both caveolin-1 and caveo-
lin-3 in cultured atrial cardiac myocytes, using a well-established velocity gradient centrifugation system (12). We demonstrate in Fig. 7B that both caveolin-1 and caveolin-3 formed high-molecular-mass oligomers of 200–400 kDa (fractions 6–8), which are representative of 14–16 caveolin monomers. Thus either caveolin-1 or caveolin-3 maintains the ability to oligomerize in a cell type where both caveolin isoforms are expressed.
Caveolin-1 and caveolin-3 expression are required for doxorubicin-induced apoptosis. Doxorubicin has been widely used for the treatment of a variety of solid tumors and hematologic malignancies. However, the downside associated with administration of doxorubicin is the induction of cardiotoxicity. Since caveolae were reported previously to be mediators of apoptotic signaling (11, 20, 44), in order to gain mechanistic insight into the functional role of caveolin-1 and caveolin-3 in atrial cardiac myocytes we investigated their ability to modulate doxorubicin-induced apoptosis. Toward this end, we took advantage of null mouse models lacking either caveolin-1 or caveolin-3 expression.

WT, caveolin-1-null, and caveolin-3-null mice were injected intraperitoneally with doxorubicin (20 mg/kg). PBS-injected mice were used as controls. Four days after doxorubicin injection, mice were killed and atria were extracted and analyzed. We show in Fig. 8, A and B, that treatment with doxorubicin induced apoptosis in WT atria, as demonstrated by a dramatic increase in TUNEL-positive nuclei compared with PBS-injected atria (0.82 ± 0.1% vs. 0.03 ± 0.002%). In contrast, the number of TUNEL-positive nuclei was significantly reduced in either caveolin-1-null (0.21 ± 0.06%) or caveolin-3-null (0.19 ± 0.05%) mice (Fig. 8, A and B). To gain mechanistic insight into caveolin-1/-3-mediated apoptosis, we looked at activation of caspase 3, a well-known mediator of apoptotic signaling. We demonstrate in Fig. 8C that doxorubicin-promoted activation of caspase 3 was significantly prevented in both caveolin-1- and caveolin-3-null mice compared with WT mice, suggesting that these caveolin isoforms mediate apoptosis in atrial cardiac myocytes through a caspase 3-dependent pathway.

DISCUSSION

In the present study, we demonstrated for the first time that caveolin-1 is expressed in mouse and rat atrial cardiac myocytes. Interestingly, we did not detect caveolin-1 expression in ventricular cardiac myocytes. The expression of caveolin-1 in atrial cells did not prevent the expression of the muscle-specific caveolin isoform, i.e., caveolin-3. In fact, endogenous caveolin-1 and caveolin-3 were found in atrial cardiac myocytes both in vivo and in vitro. The localization of the two caveolin isoforms within atrial cardiac myocytes was virtually identical, as demonstrated by their colocalization at the plasma membrane and in vesicles in the cytoplasm. In contrast, caveolin-3 was expressed in both atrial and ventricular cardiac myocytes. Our data contribute to the explanation of why caveolin-1 expression was not detected in rat cardiac myocytes in previous studies (23, 26): the expression of caveolin isoforms was examined only in cardiac myocytes from the ventricle, which do not express caveolin-1, and not the atrium, which are caveolin-1 positive. These studies, together with the present report, are in contrast with recent data showing caveolin-1 expression in ventricular cardiac myocytes in culture (15, 22). Although this discrepancy needs to be further addressed, one possible explanation for the detection of caveolin-1 in certain

Fig. 6. Triton insolubility properties and caveolar localization of caveolin-1 and caveolin-3. A: Triton insolubility. Atrial cardiac myocytes were solubilized with 1% Triton X-100. Equal volumes of Triton-soluble and Triton-insoluble fractions were subjected to Western blotting analysis using antibody probes specific for caveolin-1 and caveolin-3. B: caveolar localization. Atrial cardiac myocyte caveolar membranes were separated from the bulk of cellular membranes and cytosolic proteins by equilibrium sucrose density gradient centrifugation (see MATERIALS AND METHODS for details). In this fractionation scheme, immunoblotting with anti-caveolin IgGs can be used to track the position of caveolae-derived membranes within the bottom-loaded sucrose gradient.

Fig. 7. Caveolin-1 and caveolin-3 are part of the same protein complex and form oligomeric complexes in atrial cardiac myocytes. A: cell lysates of atrial cardiac myocytes were immunoprecipitated (IP) with anti-caveolin-1 IgGs or anti-caveolin-3 IgGs. Immunoprecipitates were then subjected to SDS-PAGE and immunoblotting (Western blot [WB]) analysis using antibody probes specific for caveolin-3 and caveolin-1, respectively. Immunoprecipitations with irrelevant IgGs (anti-GFP IgGs) were used as controls. B: after extraction in a buffer containing 60 mM octyl glucoside, the solubilized material of atrial cardiac myocytes was loaded atop a 5–40% linear sucrose gradient and centrifuged. Gradient fractions were collected from the top and subjected to immunoblot analysis. Molecular mass standards for velocity gradient centrifugation are as indicated.
cultures of ventricular cardiac myocytes is that conditions that lead to oxidative stress, such as exposure of in vitro cell cultures to atmospheric oxygen levels, may promote overtime expression of caveolin-1. This possibility is consistent with the notion that oxidative stress upregulates caveolin-1 expression in fibroblasts and epithelial cells (6, 40). More recently, Chow and colleagues (4) concluded from their immunofluorescence studies that caveolin-1 was expressed in mouse left ventricular cardiac myocytes. However, the authors failed to use appropriate controls, i.e., markers of cardiac myocytes and endothelial cells, to rule out the possibility that the detected caveolin-1 expression was limited to endothelial cells of blood vessels. In fact, caveolin-1 expression in their ventricular sections appeared mainly restricted to areas surrounding nuclei that are located at the edges of cardiac myocytes, where endothelial cells are expected to be found.

Both caveolin-1-null and caveolin-3-null mice have been shown to develop cardiac defects. More specifically, caveolin-3-null mice develop cardiac myocyte hypertrophy, mild cardiomyopathy, and hyperactivation of the p42/44 mitogen-activated protein (MAP) kinase pathway (42). Lack of caveolin-1 in mice results in the development of cardiac hypertrophy with hyperactivation of the p42/44 MAP kinase pathway in cardiac fibroblasts (5). Apoptosis has not been described in the heart of these mouse models. Interestingly, although activation of p42/44 MAP kinase in cardiac fibroblasts was associated with lack of caveolin-1 and considered one of the potential molecular explanations for the cardiac phenotype of caveolin-1-null mice (5), the molecular mechanisms underlying these defects remain unknown. Our data showing that mouse atrial cardiac myocytes express caveolin-1 imply that lack of caveolin-1 in this cell type may disrupt yet unidentified signaling pathways.

Fig. 8. Caveolin-1 and caveolin-3 modulate doxorubicin-induced apoptosis in atrial cardiac myocytes. Wild-type, caveolin-1-null (Cav-1 KO), and caveolin-3-null (Cav-3 KO) mice (n = 3) were injected intraperitoneally with doxorubicin (DOX, 20 mg/kg). PBS-injected mice were used as controls. Four days after injection, atria were subjected to terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) analysis (A and B) and immunoblotting using antibody probes specific for caveolin-1, caveolin-3, and caspase 3 (C). Representative fields of TUNEL analysis are shown in A: TUNEL-positive nuclei are shown in red; DAPI staining is shown in blue. Quantification of TUNEL analysis is shown in B. Values represent means ± SE (n = 50 for each experimental point). *P < 0.01. Arrowhead in C indicates caspase 3 activation.
pathways and contribute to the cardiac defect observed in caveolin-1-null mice.

We also demonstrated that caveolin-1 and caveolin-3 were part of the same protein complex, as shown by the ability of caveolin-1 to communoprecipitate caveolin-3 and vice versa, suggesting that the two caveolin isoforms form heterooligomeric complexes in atrial cardiac myocytes. Consistent with our data, it was recently shown that exogenously expressed caveolin-3 in L6 myoblasts (which express endogenous caveo-

lin-1 but not caveolin-3) colocalizes and communoprecipitates with caveolin-1 (3).

To date, the smooth muscle cell is the only well-established cell type known to express endogenous caveolin-1 and caveo-

lin-3 (32), but the functional significance of their coexpression remains unknown. In this study, we asked whether caveolin-1 and caveolin-3 play similar or distinct roles in atrial cardiac myocyte signaling by evaluating the apoptotic response of doxorubicin-treated caveolin-1-null and caveolin-3-null mice. Interestingly, we found that both caveolin-1 and caveolin-3 expression are required for doxorubicin-induced apoptosis in the atria. While apoptotic nuclei were observed in 0.82% of atrial cells in doxorubicin-treated WT mice, apoptosis was limited to 0.21% of atrial cells in caveolin-1-null mice, which express caveolin-3, and 0.19% of atrial cells in caveolin-3-null mice, which express caveolin-1. Thus it appears that caveolin-1 and caveolin-3 synergistically regulate apoptosis in atrial cells. Since we demonstrated that the two caveolin isoforms coexist as heterooligomeric complexes in atrial cardiac myocytes, these results suggest that their localization within the same cellular microdomain is required to achieve a full apoptotic response in this cell type.

However, caveolin-1 differentially regulates apoptosis in cell types where caveolin-3 is not expressed. On one hand, caveolin-1 has been shown to promote ceramide-induced apoptosis in diploid fibroblasts (44). Moreover, Lisanti and colleagues (20) have demonstrated that caveolin-1 sensitizes fibroblasts and epithelial cells to staurosporine-induced programmed cell death and that caveolin-1 antisense cells are resistant to staurosporine-induced apoptosis. In addition, we have shown (11) that transgenic expression of caveolin-1 in mouse embryonic fibroblasts sensitizes these cells to stauros-

porine-induced apoptosis. On the other hand, caveolin-1 has been shown to act as a suppressor of c-myc-induced apoptosis in LNCaP cells, a human epithelial prostate cancer-derived cell line (34), and we have shown that caveo-

lin-1 protects against oxidative stress-induced apoptosis in 3T3 (40) and MCF-7 (6) cells. We can conclude that, depending on the presence or absence of caveolin-3 and the type of stimulus, caveolin-1 may have either pro- or antiapoptotic properties.

Because antitumor therapy with doxorubicin and other antichelation agents is limited by the possible development of cardiotoxicity on chronic administration, our findings propose caveo-

lin-1/caveolin-3 heterooligomeric complexes as potential novel therapeutic targets for limiting anthracycline-induced side ef-

fects in cardiac cells.

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REFERENCES

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lin isoforms: differential complex formation between caveolins in fibro-


4. Chow AK, Cen J, El-Yazbi AF, Crawford BD, Hsu CH, Cho WJ, 


11. Galbiati F, Volonte D, Minetti C, Chu JB, Lisanti MP. Phenotypic behavior of caveolin-3 mutations that cause autosomal dominant limb girdle muscular dystrophy (LGMD-1C). Retention of LGMD-1C caveo-


