JCI The Journal of Clinical Investigation

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J Clin Invest. 2004;113(5):668-675. https://doi.org/10.1172/JCI20410.

Article Cardiology

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Smooth muscle cell–extrinsic vascular spasm arises from cardiomyocyte degeneration in sarcoglycan-deficient cardiomyopathy

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Vascular spasm is a poorly understood but critical biomedical process because it can acutely reduce blood supply and tissue oxygenation. Cardiomyopathy in mice lacking γ-sarcoglycan or δ-sarcoglycan is characterized by focal damage. In the heart, sarcoglycan gene mutations produce regional defects in membrane permeability and focal degeneration, and it was hypothesized that vascular spasm was responsible for this focal necrosis. Supporting this notion, vascular spasm was noted in coronary arteries, and disruption of the sarcoglycan complex was observed in vascular smooth muscle providing a molecular mechanism for spasm. Using a transgene rescue strategy in the background of sarcoglycan-null mice, we replaced cardiomyocyte sarcoglycan expression. Cardiomyocyte-specific sarcoglycan expression was sufficient to correct cardiac focal degeneration. Intriguingly, successful restoration of the cardiomyocyte sarcoglycan complex also eliminated coronary artery vascular spasm, while restoration of smooth muscle sarcoglycan in the background of sarcoglycan-null alleles did not. This mechanism, whereby tissue damage leads to vascular spasm, can be partially corrected by NO synthase inhibitors. Therefore, we propose that cytokine release from damaged cardiomyocytes can feed back to produce vascular spasm. Moreover, vascular spasm feeds forward to produce additional cardiac damage.

Introduction

Sarcoglycan is a multimember transmembrane complex found in all muscle types and is a component of the dystrophin glycoprotein complex (DGC). Sarcoglycan has a complex mechanosignaling role for the maintenance of striated muscle cells (1). In striated muscle, sarcoglycan interacts with dystrophin and dystroglycan connecting the intracellular cytoskeleton to the ECM and contributing to the structural integrity of muscle cells (2–4). Dystrophin, taken together with sarcoglycan, dystroglycan, syntrophins, and dystrobrevins, plays an important role in anchoring diverse signaling proteins to the plasma membrane (5). Sarcoglycan is thought to stabilize the linkages between dystroglycan and dystrophin on the intracellular surface and between dystroglycan and laminin-2 on the extracellular surface.

Mice with null mutations in γ-sarcoglycan, δ-sarcoglycan, or β-sarcoglycan develop cardiomyopathy that is characterized by focal degeneration. δ-Sarcoglycan– and β-sarcoglycan–null mice display disruption of the vascular smooth muscle (VSM) sarcoglycan complex (6–8). In contrast, mice lacking α-sarcoglycan develop muscular dystrophy but not cardiomyopathy (9). In α-sarcoglycan mutant mice, the VSM sarcoglycan complex remains intact. Therefore, it was reasoned that VSM sarcoglycan complex disruption promotes cardiomyopathy (7). Consistent with this, microvascular filling defects were found in β- or δ-sarcoglycan mutant mice, but not α-sarcoglycan mutant mice (6, 7). Moreover, long-term treatment with calcium channel antagonists reduced vasospasm and slowed cardiomyopathy progression (10).

Nonstandard abbreviations used: α-myosin heavy chain (α-MHC); dystrophin glycoprotein complex (DGC); Evans Blue Dye (EBD); N_G-nitro-L-arginine methyl ester hydrochloride (L-NAME); NO synthase (NOS); sulfonylurea receptor (SUR); vascular smooth muscle (VSM).

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* **113**:668–675 (2004). doi:10.1172/JCI200420410.

The sarcoglycan complex varies in composition in different muscle tissues. In mice, the major sarcoglycan complex type found in skeletal and cardiac muscle consists of α-, β-, γ-, and δ-sarcoglycans (11). In addition, ζ- and ε-sarcoglycan are expressed in a subset of cardiac and skeletal muscle sarcoglycan complexes performing an as yet unclear function, which may include substituting for other subunits or acting in discreet locations of cells, such as at the neuromuscular junction (11, 12). In contrast, the arterial VSM sarcoglycan complex consists of β-, δ-, ε-, and ζ-sarcoglycan (12, 13).

We examined the role of the VSM sarcoglycan complex as a direct mediator of vascular spasm in sarcoglycan-mediated cardiomyopathy by generating a series of tissue-specific transgenes to express δ-sarcoglycan or γ-sarcoglycan in the background of mice lacking δ-sarcoglycan (*dsg*$^{-/-}$) (8) or γ-sarcoglycan (*gsg*$^{-/-}$) (14), respectively. Using the α-myosin heavy chain (α-MHC) gene promoter (15) to drive expression exclusively in cardiomyocytes, we showed that cardiomyocyte sarcoglycan restoration is sufficient to correct VSM vasospasm. Additionally, rescue of cardiomyocyte sarcoglycan expression corrected the focal degenerative process that leads to cardiomyopathy. Alternatively, using the SM22α promoter (16) to drive expression of sarcoglycan exclusively in arterial VSM, we showed that restoration of the VSM sarcoglycan complex was not sufficient to ameliorate vascular spasm, nor does it correct cardiac or skeletal muscle pathology. We postulate that the mechanism underlying VSM cell-extrinsic spasm involves cytokines released from damaged cardiomyocytes. We previously noted that eNOS was upregulated in regions of cardiomyocyte damage (17), and we now postulate that gradients of NO may create substrate for vascular spasm. We treated mice with a broad spectrum inhibitor of NOS and showed a reduction in vascular spasm. As cardiomyocyte damage occurs in response to a variety of stimuli including infarction, toxic, metabolic, and genetic defects, damage-responsive vascular

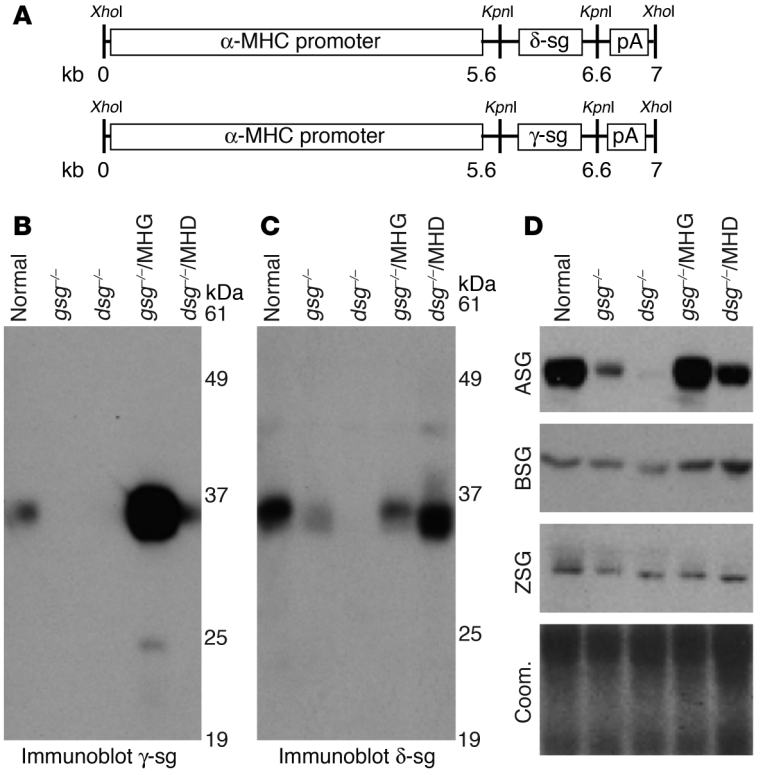

Figure 1

Construction and expression of cardiomyocyte-specific sarcoglycan transgenes. (**A**) Full-length murine γ-sarcoglycan (γ-sg) or δ-sarcoglycan (δ-sg) cDNA was ligated into a vector containing the 5.6-kb murine α-MHC promoter (15) to create MHG or MHD, respectively. Transgenic mice with the MHG transgene were crossed with γ-sarcoglycan–null (*gsg*$^{-/-}$) mice to generate *gsg*$^{-/-}$/MHG mice. Transgenic mice with the MHD transgene were crossed with δ-sarcoglycan–null (*dsg*$^{-/-}$) mice generating *dsg*$^{-/-}$/MHD mice. pA, polyadenylation signals. (**B**) Immunoblot of whole heart extracts from normal, *gsg*$^{-/-}$, *dsg*$^{-/-}$, *gsg*$^{-/-}$/MHG, and *dsg*$^{-/-}$/MHD animals at 12 weeks of age using γ-sarcoglycan Ab showed γ-sarcoglycan expression was absent in the hearts of *gsg*$^{-/-}$ and *dsg*$^{-/-}$ animals, but was restored in transgenic animals. Quantitative Western blot analysis determined the level of γ-sarcoglycan expression in *gsg*$^{-/-}$/MHG animals to be sevenfold above normal. (**C**) δ-Sarcoglycan expression was restored to normal levels by expression of the MHD transgene in *dsg*$^{-/-}$/MHD hearts. Expression of δ-sarcoglycan from the MHD transgene also resulted in recovery of γ-sarcoglycan to normal levels (last lane). (**D**) Immunoblots for the remaining sarcoglycan subunits showed that α-sarcoglycan (ASG) expression is recovered in hearts with either γ- or δ-sarcoglycan transgene expression. β-Sarcoglycan protein (BSG) is increased to normal levels, and ζ-sarcoglycan protein (ZSG) levels are not significantly different in transgenic hearts. Loading control is shown for **B**, **C**, and **D** . Coom., Coomassie blue.

spasm may be a broad mediator of cardiovascular pathology and a target for therapeutic intervention.

Methods

Transgenic constructs. The MHG transgene was generated with the α-MHC promoter (15) and full-length murine γ-sarcoglycan amplified from a mouse skeletal muscle cDNA. This resulting PCR product was ligated to *Kpn*I-digested pMHC. The pMHC plasmid contained the 5,571 bp mouse α-MHC promoter (15) and SV40 polyadenylation signal sequence. The transgene MHD was generated by PCR amplification of mouse δ-sarcoglycan from mouse skeletal muscle cDNA. The resulting PCR product was ligated to TOPO TA vector (Invitrogen Corp., Carlsbad, California, USA). The mouse δ-sarcoglycan insert was ligated to pMHC linearized using *Kpn*I.

SMG was generated using the SM22α promoter (16). The p-441SM22α-luc (a generous gift from M. Strobeck and M. Parmacek, University of Pennsylvania, Philadelphia, Pennsylvania, USA) was digested with *Xho*I and *Hind*III. The MCKgsg vector (18) was digested with *Xho*I and *Hind*III, and the SM22α promoter was ligated 5′ of mouse γ-sarcoglycan. The bovine growth hormone termination and polyadenylation signal sequence from pcDNA3 (Invitrogen Corp.) was previously added at the *Xba*I site. SMD was generated by using δ-sarcoglycan, amplified by PCR from mouse skeletal muscle cDNA. This PCR product was ligated into TOPO TA and the resultant vector digested with *Hind*III and *Bgl*II and ligated in pBluescript II KS (Stratagene, La Jolla, California, USA). The SM22α promoter was cloned as above 5′ to the δ-sarcoglycan coding sequence. The bovine growth hormone polyadenylation signal sequence from pcDNA3 (Invitrogen Corp.) was added at the *Xba*I site. All constructs were verified by sequencing.

MHG and MHD transgene fragments were liberated from their respective vectors by digestion with *Xho*I. SMG and SMD transgenes were digested with *Xho*I and *Not*I. Transgene fragments were isolated, purified, and injected after dialyzing against injection buffer (10 mM Tris, pH 7.5, 5 mM NaCl, 0.1 mM EDTA) (19).

Animals. γ-Sarcoglycan–null and δ-sarcoglycan–null mice were described previously (8, 14). Mice were derived in the C57Bl/6 strain following ten generations of heterozygote matings with C57Bl/6 control mice (The Jackson Laboratory, Bar Harbor, Maine, USA). Transgenes were injected into fertilized oocyte pronuclei generated from a cross between C57Bl/6/C3H females and C57Bl/6 males (19). The MHC and SMG transgenic mice were bred to *gsg*$^{-/-}$ mice through two generations to generate transgene-positive, genomic-null mice (*gsg*$^{-/-}$/MHG or *gsg*$^{-/-}$/SMG). Similarly, MHD and SMD transgenic mice were each bred to *dsg*$^{-/-}$ mice through two generations to generate transgene-positive, genomic-null mice (*dsg*$^{-/-}$/MHD or *dsg*$^{-/-}$/SMD). Subsequent genotyping was performed by PCR using transgene-specific primers, and phenotypic assessment was performed in the sarcoglycan-null mice (*gsg*$^{-/-}$ or *dsg*$^{-/-}$) with or without the transgene. All comparisons were made to littermate controls. Animals were housed, treated, and handled in accordance with the guidelines set forth by the University of Chicago's Institutional Animal Care and Use Committee, the Animal Welfare Act regulations, and the NIH Guide for the Care and Use of Laboratory Animals.

Immunoblot analysis. Whole protein lysates were prepared from mouse hearts at 12 weeks of age. Protein was extracted with lysis buffer: 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10% glycerol, 1% Triton X-100, 50 μM PMSF, plus complete protease inhibitor (Roche Molecular Biochemicals, Mannheim, Germany). After quantitation of protein content, 50 μg was separated on 10% polyacrylamide-SDS gels and blotted to Immobilon P membrane (Millipore Corp., Bedford, Massachusetts, USA). Membranes were blocked in 5% milk in Tris-buffered saline with 0.1% Tween 20 and incubated with polyclonal γ-sarcoglycan Ab (1:1,000) (20), poly-

clonal δ-sarcoglycan Ab (1:2,000) (8), polyclonal ζ-sarcoglycan Ab (1:1,000) (12), polyclonal β-sarcoglycan Ab (1:2,000) (21), or monoclonal α-sarcoglycan Ab (NCL-ASG, 1:200; Novocastra, Newcastle-Upon-Tyne, United Kingdom). Goat anti-rabbit or goat anti-mouse Ab's conjugated to HRP (1:10,000; Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA) were used as secondary Ab's, and blots were developed with ECL PLUS and imaged with Kodak film and/or chemiluminescent phosphorimaging. Duplicate gels were run and stained with Coomassie brilliant blue to assess loading equivalence.

Histology. Mice of each genotype were sacrificed at 4, 12, 26, and 52 weeks of age, and tissues were fixed in saline-buffered 10% formalin. Sections from heart and skeletal muscle were stained with Masson's trichrome or H&E.

Vital staining with Evans blue dye. Evans blue dye (EBD; 20 mg/ml) (Sigma-Aldrich, St. Louis, Missouri, USA) was dissolved in sterile PBS. Eighteen hours before sacrifice, a subset of mice that did not undergo surgical manipulation was given intraperitoneal injections at 100 μg EBD/g body weight (14).

Immunofluorescence. Mice were sacrificed and hearts were frozen in liquid nitrogen–cooled isopentane. Frozen sections, 7–8 μm, were fixed in ice-cold methanol. Slides were rinsed with PBS and blocked with 5% FBS in PBS. Sections were incubated overnight at 4°C with polyclonal anti-γ sarcoglycan Ab, polyclonal anti-δ sarcoglycan Ab, and monoclonal anti–smooth muscle α-actin Ab (Sigma-Aldrich). Following washes with PBS, goat anti-rabbit antibody was conjugated with Pacific Blue (1:5,000; Molecular Probes Inc., Eugene, Oregon, USA) or Cy3 (1:10,000, Jackson ImmunoResearch Laboratories

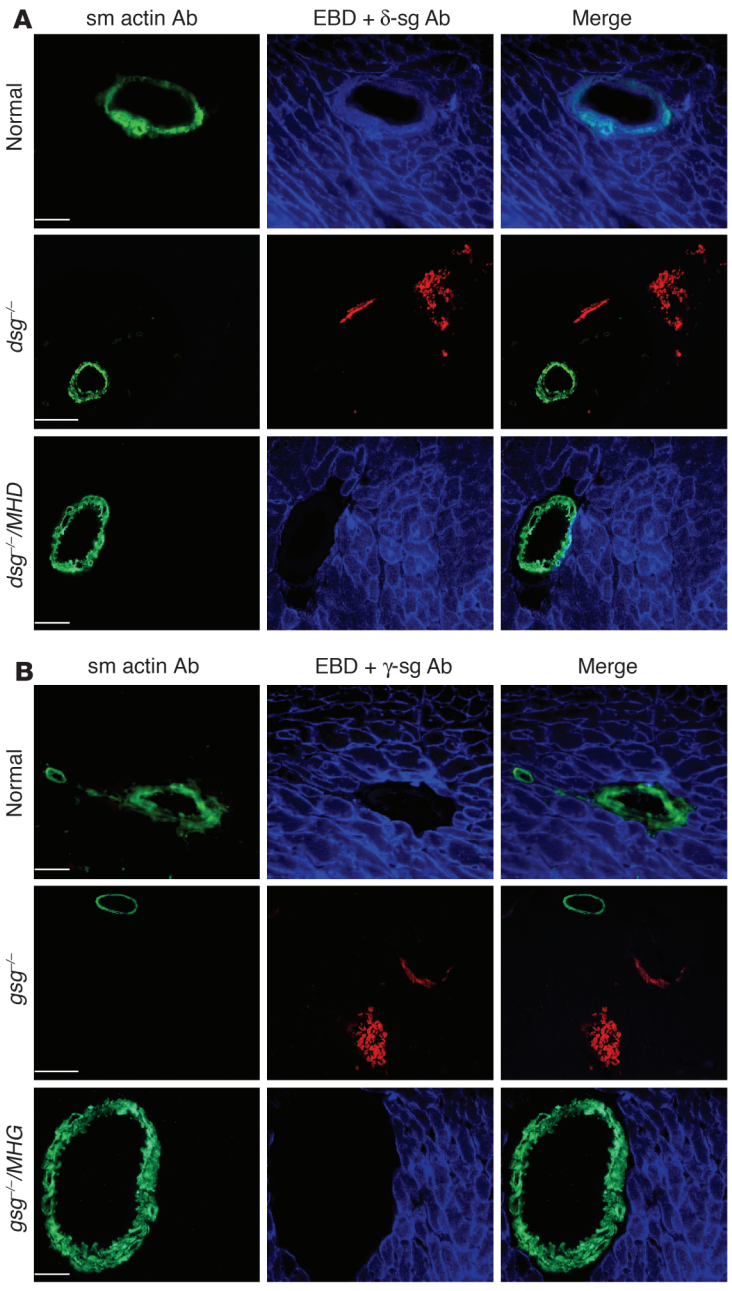

Figure 2
Cardiomyocyte-specific transgenes rescue sarcoglycan at the membrane and restore membrane-permeability defects. Immunofluorescence microscopy was performed on sections of hearts containing coronary vessels from normal, *gsg*$^{-/-}$, *dsg*$^{-/-}$, *gsg*$^{-/-}$/MHG, and *dsg*$^{-/-}$/MHD mice at 26 weeks of age. In the left column, smooth muscle α-actin (sm actin) staining coronary VSM is shown in green; in the middle column, EBD staining is shown in red and sarcoglycan staining is shown in blue. The third column shows a merged view. (**A**) δ-Sarcoglycan Ab is present at the membrane of cardiomyocytes of normal and *dsg*$^{-/-}$/MHD hearts but not *dsg*$^{-/-}$ hearts. EBD uptake (red) is found in the cardiomyocytes of *dsg*$^{-/-}$ animals but is never found in normal or *dsg*$^{-/-}$/MHD cardiomyocytes. δ-Sarcoglycan is normally expressed in coronary arteries. Transgene expression in *dsg*$^{-/-}$/MHD does not induce δ-sarcoglycan expression in the coronary artery tree (bottom row). (**B**) γ-Sarcoglycan Ab localizes γ-sarcoglycan in the membrane of normal and *gsg*$^{-/-}$/MHG cardiomyocytes. EBD uptake (red) is seen in *gsg*$^{-/-}$ cardiomyocytes but is never seen in *gsg*$^{-/-}$/MHG or normal cardiomyocytes. Scale bars: *dsg*$^{-/-}$ and *gsg*$^{-/-}$ are 100 μm; others are 20 μm.

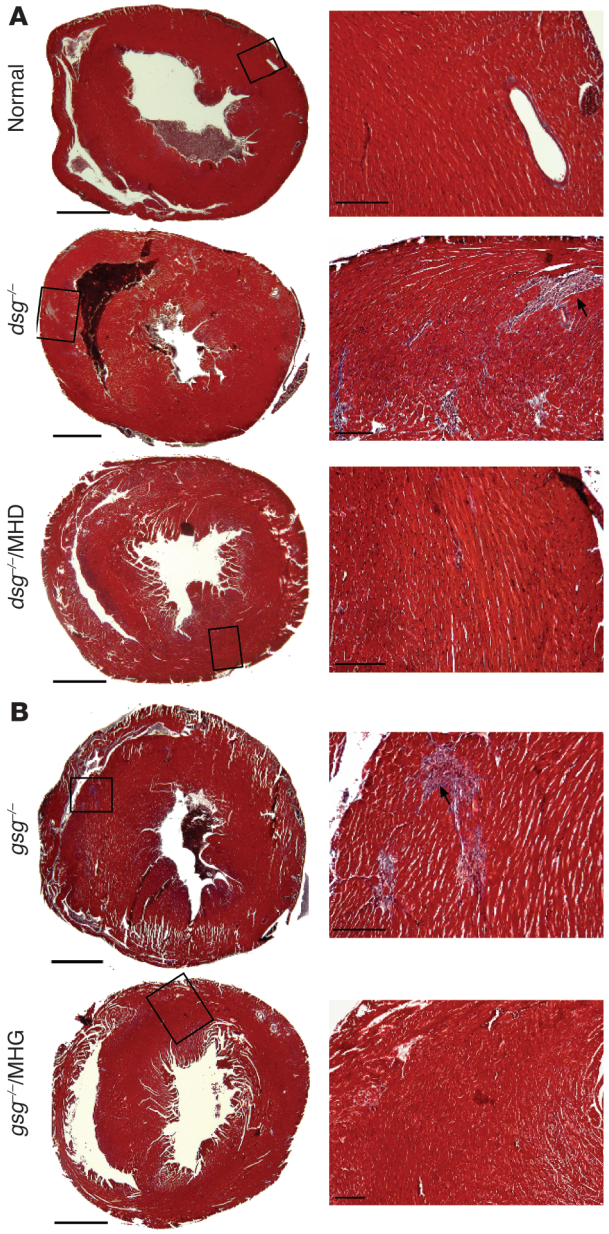

Figure 3
Focal areas of fibrosis and necrosis are eliminated with cardiomyocyte-specific sarcoglycan expression. Shown is Masson's trichrome staining of midventricular cross sections of hearts from 26-week-old animals. (**A**) Areas of fibrosis and necrosis are seen throughout *dsg*$^{-/-}$ hearts; arrow in high-power view shows an area in right ventricle. Evidence of ventricular wall thickening is seen in the low-power view. Expression of δ-sarcoglycan specifically in cardiomyocytes (*dsg*$^{-/-}$/MHD) eliminates foci of fibrosis and necrosis. Transgenic hearts are indistinguishable from normal hearts. (**B**) Foci of fibrosis, necrosis, and inflammation are also seen in *gsg*$^{-/-}$ hearts, shown here in the intraventricular septum. In contrast, exclusive expression of γ-sarcoglycan in cardiomyocytes eliminates areas of degeneration. Scale bars: left column, 1 mm; right column, 100 μm.

Inc.), and goat anti-mouse antibody conjugated with FITC (1:2,500; Jackson ImmunoResearch Laboratories Inc.) for 1 hour at room temperature. Following another series of PBS washes, coverslips were mounted with Vectashield containing DAPI media (Vector Laboratories, Burlingame, California, USA).

Microvascular filling. Microvascular filling experiments were performed essentially as previously described (6, 7, 10, 22, 23). This method has been validated in an animal model of Prinzmetal-like vascular spasm where it was corroborated with electrocardiographic evidence of vascular spasm (22). Animals were anesthetized with methoxyflurane (Metofane; Schering Plough, Omaha, Nebraska, USA), and a midsternotomy exposed the free wall of the heart. Freshly prepared Microfil red (Flowtech Inc., Carver, Massachusetts, USA) was injected into the apex of the heart with a 26-gauge needle. Pressure was applied by hand, and the heart was excised approximately 1 minute after initiation of injection. Hearts were immediately placed in ice-cold saline-buffered formalin and fixed overnight. Hearts were dehydrated by 24-hour incubations in successively increasing concentrations of ethanol. A final incubation in methyl salicylate cleared the dehydrated tissue, leaving the plastic cast of the coronary vessels. Hearts were examined and scored for evidence of stenoses. This assessment was performed blinded to genotype. Vessels found to be filled with Microfil were scored for presence or absence of focal narrowings, representing areas of focal vasospasm. Where indicated, an intraperitoneal miniosmotic pump (Alza Corp., Mountain View, California, USA) containing L-NAME (N_G-nitro-L-arginine methyl ester hydrochloride) was implanted so that mice received 0.2 mg/g/day. Mice were treated for 6 days with L-NAME prior to microvascular filling studies.

Blood pressure monitoring. The unanesthetized, ambulatory arterial blood pressures of three to six animals (6 and 12 months of age) per genotype were analyzed by implantable pressure-sensing catheter-connected radiofrequency transmitters as described (22). Catheterization of the left carotid artery was performed with the implantable TA11-PAC20 transmitter (Data Sciences International, St. Paul, Minnesota, USA). Animals were then allowed to recover 24–72 hours prior to recording. Pressure recordings of 24–72 hours from each animal were analyzed by Dataquest A.R.T. Analysis (Data Sciences International). Average systolic, diastolic, pulse pressure, and mean arterial pressures were calculated by averaging the 10-second waveform averages calculated by the analysis program for each animal, followed by averaging of the animals of each genotype studied. Statistical tests were performed using InStat3 (GraphPad Software for Science Inc., San Diego, California, USA).

Results

Cardiomyocyte-specific replacement of γ-sarcoglycan or δ-sarcoglycan. To determine the mechanism of coronary artery vascular spasm in sarcoglycan-mediated cardiomyopathy, we generated mice that expressed sarcoglycan solely in the cardiomyocytes but not in skeletal or VSM. We used the α-MHC promoter because this promoter results in sustained ventricular expression from late embryonic stages through adulthood (15). Full-length δ-sarcoglycan was placed under the control of the α-MHC promoter to generate the MHD transgene (Figure 1A). Mice harboring the MHD transgene were bred to mice null for δ-sarcoglycan to create mice with (*dsg*$^{-/-}$/MHD) and without the transgene (*dsg*$^{-/-}$). Southern blot analysis demonstrated a copy number of eight for MHD (data not shown). Similarly, full-length γ-sarcoglycan was placed under the control of α-MHC to generate the MHG transgene (Figure 1A). MHG transgenic mice were bred to γ-sarcoglycan–null (*dsg*$^{-/-}$) mice to generate mice with cardiomyocyte-specific γ-sarcoglycan expression (*gsg*$^{-/-}$/MHG).

Immunoblotting with sarcoglycan-specific Ab's against whole heart extracts showed expression in the hearts of transgenic animals (Figure 1, B and C). Quantitative immunoblotting of γ-sarcoglycan expression in *gsg*$^{-/-}$/MHG determined expression to be between fivefold and sevenfold higher than normal (data not shown). We previously examined the effect of overexpression of γ-sarcoglycan on striated muscle and found that levels between 30-fold and 50-fold over normal are required for striated muscle toxicity (18). Thus, the level of γ-sarcoglycan expression produced from MHG is not expected to produce pathology. Quantitative immunoblotting to determine the level of expression produced from *dsg*$^{-/-}$/MHD indicated that δ-sarcoglycan was approximately twofold to threefold normal level. Expression of γ-sarcoglycan or δ-sarcoglycan in cardiomyocytes restored the sarcoglycan complex in *gsg*$^{-/-}$/MHG and *dsg*$^{-/-}$/MHD hearts (Supplemental Figure 1; supplemental material available at http://www.jci.org/cgi/content/full/113/5/668/DC1). Expression levels of the remaining sarcoglycans (α, β, and ζ) were increased in transgene-rescued mice compared with *gsg*$^{-/-}$ and *dsg*$^{-/-}$ mice (Figure 1D).

Expression of sarcoglycan subunits at the cardiomyocyte membrane of MHG and MHD hearts. Transgenic expression of δ- and γ-sarcoglycan produced sarcoglycans correctly targeted to the plasma membrane. Figure 2 shows sections from hearts stained with Ab's against smooth muscle actin (green) and Ab's specific to either γ- or δ-sarcoglycan (blue). In normal hearts, δ-sarcoglycan is expressed in both cardiomyocytes and VSM (Figure 2A, top row, merged image). Figure 2A shows that the expression of δ-sarcoglycan is restored to the cardiomyocyte membrane in *dsg*$^{-/-}$/MHD transgenic hearts, similar to what is seen in normal hearts. γ-Sarcoglycan is normally only expressed in striated muscle (Figure 2A). In *gsg*$^{-/-}$/MHG hearts (Figure 2B) expression of γ-sarcoglycan (blue) is restored.

Mice were injected with EBD, a small molecular tracer dye to which normal cardiac and skeletal myocytes are impermeable. Mutations that disrupt the sarcoglycan complex alter membrane permeability so that EBD uptake can be seen scattered throughout heart and skeletal muscle. These membrane-permeability defects indicate impending or ongoing cell degeneration. Microscopically, EBD uptake is seen as red fluorescence in cardiomyocytes of *gsg*$^{-/-}$ and *dsg*$^{-/-}$ hearts (Figure 2). In Figure 2, EBD uptake is seen in immediate proximity to a smooth muscle actin containing coronary vessels. EBD uptake was never seen in *dsg*$^{-/-}$/MHD or *gsg*$^{-/-}$/MHG transgenic hearts, indicating restoration of membrane permeability and correction of the underlying defect. In contrast, skeletal muscle of *dsg*$^{-/-}$/MHD and *gsg*$^{-/-}$/MHG mice showed complete absence of δ-sarcoglycan or γ-sarcoglycan. These data indicate that the transgenic constructs successfully rescued the acute membrane damage seen in the hearts of δ- and γ-sarcoglycan–deficient animals.

Cardiomyocyte sarcoglycan expression corrects focal cardiac degeneration. Masson trichrome staining was performed on both *dsg*$^{-/-}$/MHD and *gsg*$^{-/-}$/MHG paraffin-embedded hearts. The histopathologic process in both *dsg*$^{-/-}$ and *gsg*$^{-/-}$ mutant hearts is one of focal degeneration accompanied by fibrosis. In Figure 3A, fibrosis (arrow) seen in the *dsg*$^{-/-}$ heart is corrected by the presence of the MHD transgene. Phenotypically, *dsg*$^{-/-}$/MHD hearts were indistinguishable from normal hearts. Similarly, the patchy fibrosis seen in *gsg*$^{-/-}$ mutant hearts (Figure 3B) was eliminated in *gsg*$^{-/-}$/MHG transgenic hearts. Cardiac pathology in sarcoglycan mutant mice can be fully corrected with cardiomyocyte-transgene rescue of sarcoglycan expression.

Cardiomyocyte expression corrects vascular spasm. The focal nature of fibrosis in sarcoglycan mutant hearts is consistent with the presence of microinfarction. It was suggested that vascular spasm was responsible for cardiomyopathy in sarcoglycan mutations (7, 10, 23). Supporting this suggestion, disruption of the VSM sarcoglycan complex and microvascular filling defects were noted in mice in δ-sarcoglycan–null hearts (7). We analyzed *dsg*$^{-/-}$/MHD and *gsg*$^{-/-}$/MHG using microvascular filling to evaluate whether vasospasm arises as a VSM cell-extrinsic process. Figure 4 (top) shows examples of vasospasm with focal narrowings and microvascular filling defects in both *dsg*$^{-/-}$ and *gsg*$^{-/-}$ mutant hearts. Notably, *gsg*$^{-/-}$ coronary vessels had an equivalent frequency of vascular spasm to coronary vessels in strain-matched *dsg*$^{-/-}$ hearts (Table 1). Microvascular filling of transgene-rescued hearts showed no evidence of vasospasm in either *dsg*$^{-/-}$/MHD or *gsg*$^{-/-}$/MHG transgenic hearts. Representative photomicrographs of microvascular filling experiments in *dsg*$^{-/-}$/MHD and *gsg*$^{-/-}$/MHG hearts show smoothly tapered, well-filled coronary arterial vessels and filling of capillaries (Figure 4, bottom). Restoration of γ-sarcoglycan or δ-sarcoglycan specifically in cardiomyocytes was sufficient to eliminate vasospasm in these transgenic hearts.

Cardiomyopathy occurs despite expression of γ-sarcoglycan or δ-sarcoglycan in VSM. To evaluate the role of the sarcoglycan complex in VSM, we specifically restored VSM sarcoglycan expression. We used the SM22α gene (16) to generate transgenes expressing δ-sarcoglycan (SMD) or γ-sarcoglycan (SMG) in arterial smooth muscle (Figure 5A). In the case of γ-sarcoglycan, we expressed γ-sarcoglycan in smooth muscle to determine whether γ-sarcoglycan can substitute for δ-sarcoglycan in VSM. Founder mice carrying the SMD or SMG transgenes were bred to mice lacking δ-sarcoglycan or γ-sarcoglycan, respectively. The copy number for the *gsg*$^{-/-}$/SMG transgene was approximately 19, while for *dsg*$^{-/-}$/SMD, it was approximately 2 (data not shown). Figure 5B shows that δ-sarcoglycan or γ-sarcoglycan expression was restricted to VSM in *dsg*$^{-/-}$/SMD and *gsg*$^{-/-}$/SMG (shown in blue). Smooth muscle actin Ab staining is shown in green and colocalized with δ- and γ-sarcoglycan expression. No evidence of cardiomyocyte membrane δ-sarcoglycan or γ-sarcoglycan expres-

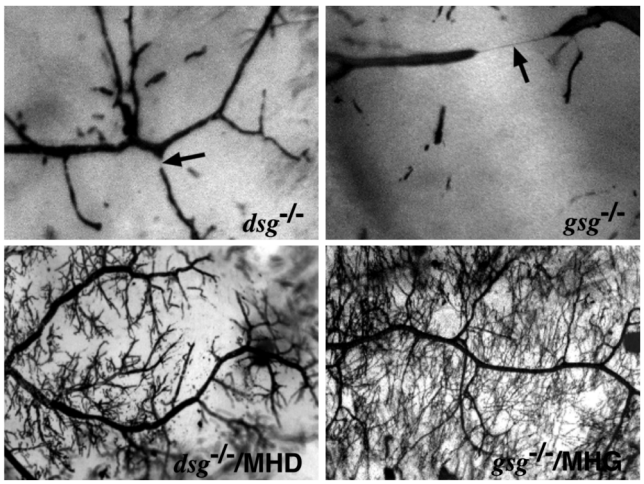

Figure 4
Vascular spasm is present in *dsg*$^{-/-}$ and *gsg*$^{-/-}$ but not in *dsg*$^{-/-}$/MHD or *gsg*$^{-/-}$/MHG animals. Microvascular filling defects were seen in *dsg*$^{-/-}$ and *gsg*$^{-/-}$ animals at similar frequencies. Examples of focal narrowings are shown (arrows). In contrast, no focal narrowings were ever seen in *dsg*$^{-/-}$/MHD and *gsg*$^{-/-}$/MHG animals. Well-filled vascular trees with smoothly tapered vessels are shown at lower power (bottom row).

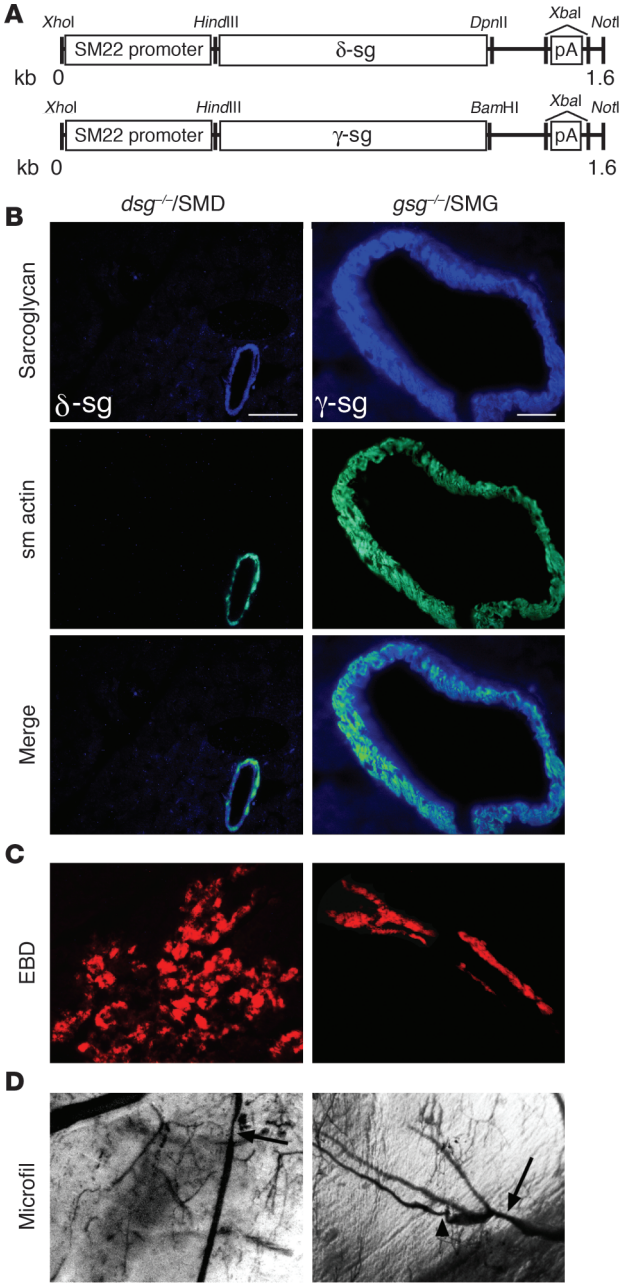

Figure 5
VSM sarcoglycan expression does not prevent vascular spasm. (**A**) Full-length δ-sarcoglycan was placed under the control of the smooth muscle–specific SM22α 441-bp promoter (16). Mice harboring the transgene were bred with *dsg*$^{-/-}$ mice, creating *dsg*$^{-/-}$/SMD mice. Similarly, full-length γ-sarcoglycan was placed behind the SM22α promoter, and *gsg*$^{-/-}$/SMG mice were generated. (**B**) Expression of δ-sarcoglycan, left column, is seen in the VSM of *dsg*$^{-/-}$/SMD coronary arteries. δ-Sarcoglycan, in blue, colocalizes with smooth muscle α-actin (green) in coronary vessels, as seen in the merged image. Expression of γ-sarcoglycan (blue), right column, is found in coronary VSM of *gsg*$^{-/-}$/SMG mice and colocalizes with smooth muscle α-actin (green). Cardiomyocytes of *gsg*$^{-/-}$/SMG mice do not express γ-sarcoglycan. Although γ-sarcoglycan is not normally found in VSM, we generated *gsg*$^{-/-}$/SMG to determine if γ-sarcoglycan could substitute for δ-sarcoglycan in VSM. (**C**) EBD uptake (red) is found in cardiomyocytes of *dsg*$^{-/-}$/SMD and *gsg*$^{-/-}$/SMG mice, similar to that which is seen in *dsg*$^{-/-}$ and *gsg*$^{-/-}$ mice, indicating that cardiomyocyte membrane defects persist despite correction of VSM sarcoglycan expression. (**D**) Restoration of coronary VSM sarcoglycan does not prevent evidence of coronary vasospasm. Microvascular filling experiments (Microfil) demonstrate areas of focal narrowing (arrows) in *dsg*$^{-/-}$/SMD (left) and *gsg*$^{-/-}$/SMG (right) coronary arteries. An ectatic area of a *gsg*$^{-/-}$/SMG coronary vessel is also seen (arrowhead), as is often identified in *gsg*$^{-/-}$ and *dsg*$^{-/-}$ hearts (not shown). Scale bars: **B** and **C**, *gsg*$^{-/-}$/SMG, 20 μm; *dsg*$^{-/-}$/SMD, 50 μm.

sion was seen in hearts of *dsg*$^{-/-}$/SMD and *gsg*$^{-/-}$/SMG animals, respectively. In both *dsg*$^{-/-}$/SMD and *gsg*$^{-/-}$/SMG hearts, EBD uptake was evident (Figure 5C). Therefore, restoration of VSM sarcoglycan expression was insufficient to prevent cardiomyocyte damage. Supporting this, both *dsg*$^{-/-}$/SMD and *gsg*$^{-/-}$/SMG hearts develop cardiac degeneration (Supplemental Figure 2). The cardiac pathology is identical to that which is seen in *dsg*$^{-/-}$ and *gsg*$^{-/-}$ hearts; that is, it is relatively mild at 8 to 12 weeks and progresses to more extensive and widespread lesions at 6 months to 1 year.

Microvascular-filling experiments in *dsg*$^{-/-}$/SMD and *gsg*$^{-/-}$/SMG transgenic hearts revealed ready evidence of vascular spasm (arrows, Figure 5D). Quantitation of these microvascular filling experiments showed that microvascular filling defects were equally present in VSM sarcoglycan-rescued mice as they were in sarcoglycan-null mice, as shown in Table 1. Therefore, it is the absence of δ- or γ-sarcoglycan in cardiomyocytes, and not in VSM, that promotes vascular spasm.

To evaluate VSM, we measured blood pressures from *dsg*$^{-/-}$, *gsg*$^{-/-}$, *dsg*$^{-/-}$/MHD, *gsg*$^{-/-}$/MHG, *dsg*$^{-/-}$/SMD, *gsg*$^{-/-}$/SMG, and strain-matched normal control mice at 1 year of age. These mice have fully developed histopathology and would be most likely to show systemic hemodynamic changes. Blood pressure was not altered in sarcoglycan mutant or transgene-rescued mice (Supplemental Figure 3).

Regional NO release influences vascular tone. The mechanism by which primary degeneration in cardiomyocytes can lead to vascular spasm may relate, in part, to cytokine release elicited from degenerating cardiomyocytes or infiltrating inflammatory cells. In sarcoglycan-mediated cardiomyopathy, regional or focal degeneration occurs. The earliest cellular pathologic signs relate to abnormal membrane permeability evidenced by uptake of EBD. The subsequent cytokine release that accompanies cellular degeneration may affect neighboring vessels in a paracrine fashion. Supporting this, we imaged EBD uptake and vasospasm simultaneously. An example of this is shown in Figure 6, where a large focus of EBD uptake can be seen upon gross examination as blue staining in juxtaposition to a vascular stenotic lesion.

We noted previously that sarcoglycan-null cardiomyocytes with EBD uptake exhibit marked upregulation of eNOS and NO (17). Because regions of cardiomyocyte degeneration in sarcoglycan-null animals typically constitute less than 5% of the myocardium, an increase in eNOS is not detected in whole cell lysates. We hypothesized the focal upregulation of eNOS may paradoxically contribute to vascular pathology. To evaluate this possibility, we pretreated six mice (n = 4, *gsg*$^{-/-}$; n = 2 *dsg*$^{-/-}$) with L-NAME, an inhibitor of NOS. Blinded analysis found five focal arterial narrowings in 24 filled coronary artery branches (20.8%) in the saline-treated group (n = 4 each of *gsg*$^{-/-}$ and *dsg*$^{-/-}$) compared with two focal arterial narrowings of 21 filled branches (9.5%) in the L-NAME–treated group. Thus, NOS inhibition can reduce the frequency of coronary artery vasospasm in sarcoglycan mutant mice supporting the concept that cytokine release from damaged cardiomyocytes predisposes to vascular spasm.

Table 1
Quantitation of microvascular filling experiments

Genotype	*n*	Hearts positive (%)	Vessels positive (%)	*P* vs. null
dsg$^{-/-}$/MHD	12	0	0	0.005
dsg$^{-/-}$	12	58	25	N/A
dsg$^{-/-}$/SMD	9	56	19	1
gsg$^{-/-}$/MHG	10	0	0	0.04
gsg$^{-/-}$	12	42	18	N/A
gsg$^{-/-}$/SMG	11	55	18	0.68

Hearts were examined for presence or absence of focal narrowings of coronary arterial trees. Filled vessels were scored for focal narrowings. All hearts were scored blinded to genotype and age. Hearts positive indicates percentage of hearts scored with at least one vessel positive for focal narrowing. Vessels positive indicates percentage of total filled vessels scored positive for stenosis. *P* versus null determined using two-sided *P* value of Fisher's exact test comparing percentage of hearts positive for stenosis of transgenic animals to nontransgenic sarcoglycan-null animals.

Discussion

Vascular spasm is an important contributor to cardiac pathology. In myocardial infarction, there is acute coronary artery occlusion and oxygen deprivation to the tissue normally supplied by the occluded vessel. In Prinzmetal variant angina or primary coronary artery vascular spasm there is damage to cardiac tissue in the presence of minimal to no atherosclerotic disease. In Prinzmetal vasospasm, VSM is unusually hyperreactive, leading to spasm and tissue infarction (24, 25). Using a model of focal degeneration that leads to cardiomyopathy, we now demonstrate that vascular spasm arises from a cardiomyocyte-intrinsic process. Mice lacking either γ-sarcoglycan or δ-sarcoglycan display progressive focal cardiomyocyte degeneration that ultimately leads to reduced cardiac function, irregular heart rhythms, and death. This model of cardiomyopathy closely parallels what is seen in humans with sarcoglycan and dystrophin gene mutations (26–29).

We previously described vascular spasm in mice lacking sulfonylurea receptor 2 (SUR2), a model for Prinzmetal variant angina and primary vascular spasm (22). We now compared vasospasm in SUR2 mutant mice with vasospasm in mice lacking sarcoglycan. Cell-intrinsic vasospasm, as it occurs in SUR2 mutant mice, is characterized by approximately double the frequency of focal arterial narrowings than that seen in hearts that lack sarcoglycan. Moreover, vasospasm as it occurs in SUR2 mutant mice is such that transient cardiomyocyte injury/infarction can be detected on a surface ECG. Similar ECG changes were not seen in sarcoglycan mutant mice (data not shown) and may reflect a slower time course of vascular spasm. While every SUR2 heart examined had evidence for vascular spasm as found by the microvascular filling technique, only a fraction of sarcoglycan mutant hearts showed filling defects. Thus, VSM cell-intrinsic spasm differs qualitatively and quantitatively from VSM cell-extrinsic spasm.

Cytokine release accompanies cardiomyocyte degeneration. Liberated cytokines may derive from damaged cardiomyocytes or may derive from the inflammatory infiltrate that accompanies striated muscle damage. Invading macrophages and lymphocytes are commonly noted in sarcoglycan mutant muscle (8, 14) and may serve as a source of NO. In sarcoglycan mutant hearts, eNOS is focally upregulated coincident with EBD uptake into cardiomyocytes (17), suggesting that the degenerating myocyte is the major source of NO. Concomitant with eNOS upregulation, NO is focally increased as well (17). We find that gradients of NO dispersed throughout the myocardium likely have a pathologic effect on neighboring VSM because we now show that inhibition of NO reduced vascular spasm. It should be noted that NO inhibition produced only a partial reduction of vascular spasm suggesting that additional factors contribute to cell-extrinsic vasospasm. There is precedent for the involvement of NO in vascular spasm because a paradoxical response to NO has been described in human patients with Prinzmetal or atheromatous vascular disease (30, 31). In sarcoglycan mutant hearts, the paradoxical response to NO arises as a cardiomyocyte-driven, VSM cell-extrinsic mechanism.

Inhibition of vascular spasm is a therapeutic target to slow cardiomyopathy progression. Vascular spasm, as it occurs in sarcoglycan-deficient cardiomyopathy, likely contributes to the pathogenic process. Supporting this, the calcium channel antagonist verapamil was used to inhibit vascular spasm in the BIO 14.6 cardiomyopathic

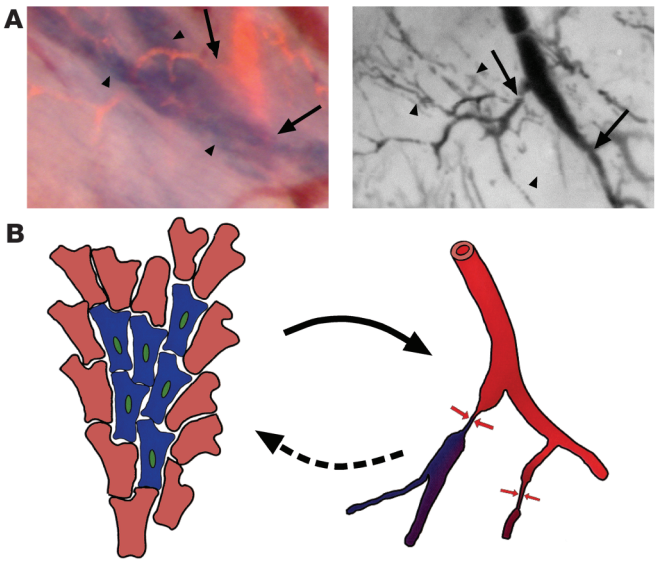

Figure 6
Vasospasm occurs as a consequence of focal cardiomyocyte degeneration in sarcoglycan deficiency. (**A**) Evidence for focal cardiomyocyte damage leading to coronary vasospasm. EBD uptake appears pale blue at gross examination and is seen in a heart from a γ-sarcoglycan–deficient animal (left, demarcated by arrowheads). Microvascular filling in the same heart (left, orange, and right) shows evidence of vasospasm with focal narrowings (arrows) and poor perfusion of the area that has taken up EBD. (**B**) Diagram of cardiomyocyte degeneration and vasospasm. Disruption of the sarcoglycan complex through gene mutation causes loss of the sarcoglycan complex at the membrane in cardiomyocytes and impairs the function of the dystrophin glycoprotein mechanosignaling complex. The early marker of degeneration in cardiomyocytes, EBD uptake (blue cardiomyocytes in schematic), indicates the active disruption and degenerative changes in the cardiomyocyte due to loss of sarcoglycan in cardiomyocyte sarcolemma. Coronary vessels, in proximity to the areas of local cardiomyocyte degeneration, undergo vasospasm (red arrows, right) due to local changes in the surrounding myocardium (solid arrow). Transient ischemia downstream of these events may lead to further degeneration within the myocardium (dashed arrow).

hamster and in mice mutant for δ-sarcoglycan (10, 23, 32). The BIO14.6 hamster model harbors a large deletion in the δ-sarcoglycan gene and displays a similar phenotype to humans and mice with sarcoglycan gene mutations (32). Verapamil is effective in reducing vascular spasm since it acts directly on VSM calcium channels. Calcium channel antagonists that lack negative inotropic action, however, are likely to be better tolerated in cardiomyopathic hearts. In the sulfonylurea mutant model of Prinzmetal vasospasm, the dihydropyridine calcium channel antagonist nifedipine was effective in suppressing vascular spasm (22). Therefore, in animal models inhibition of vasospasm has been achieved at many levels and may prove useful clinically in human disease.

Finally, cardiomyocyte degeneration is common to many forms of cardiac pathology including myocardial infarction, myocarditis, and idiopathic dilated cardiomyopathy. Some forms of hypertrophic cardiomyopathy have been associated with microvascular disease and focal degeneration occurring within the myocytes (33, 34). The mechanism of VSM cell-extrinsic vascular spasm may occur in any of these pathologic states if the paracrine pathway for vascular spasm is simply one that derives from cardiac degeneration itself. Studies on human subjects with myocardial infarction are consistent with vascular spasm, but further investigation is warranted to identify additional cytokines that mediate this effect and to establish whether this process can be exploited therapeutically.

Acknowledgments

This work was supported by NIH grant HL-61322, the Muscular Dystrophy Association, and the Burroughs Wellcome Fund (to E.M. McNally). E.M. McNally is an Established Investigator of the American Heart Association. M.T. Wheeler is supported by an American Heart Association Predoctoral Award (Midwest Affiliate) and the Medical Scientist Training Program. M.J. Allikian is supported by NIH grant HL-68472. A. Heydemann is supported by NIH grant HL-10432.

Received for publication October 28, 2003, and accepted in revised form December 23, 2003.

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1. Hack, A.A., Groh, M.E., and McNally, E.M. 2000. Sarcoglycans in muscular dystrophy. *Microsc. Res. Tech.* **48**:167–180.
2. Ozawa, E., Noguchi, S., Mizuno, Y., Hagiwara, Y., and Yoshida, M. 1998. From dystrophinopathy to sarcoglycanopathy: evolution of a concept of muscular dystrophy. *Muscle Nerve.* **21**:421–438.
3. Chan, Y.M., Bonnemann, C.G., Lidov, H.G., and Kunkel, L.M. 1998. Molecular organization of sarcoglycan complex in mouse myotubes in culture. *J. Cell Biol.* **143**:2033–2044.
4. Yoshida, M., et al. 2000. Biochemical evidence for association of dystrobrevin with the sarcoglycan-sarcospan complex as a basis for understanding sarcoglycanopathy. *Hum. Mol. Genet.* **9**:1033–1040.
5. Blake, D.J., Weir, A., Newey, S.E., and Davies, K.E. 2002. Function and genetics of dystrophin and dystrophin-related proteins in muscle. *Physiol. Rev.* **82**:291–329.
6. Durbeej, M., et al. 2000. Disruption of the β-sarcoglycan gene reveals pathogenetic complexity of limb-girdle muscular dystrophy type 2E. *Mol. Cell.* **5**:141–151.
7. Coral-Vazquez, R., et al. 1999. Disruption of the sarcoglycan-sarcospan complex in vascular smooth muscle: a novel mechanism for cardiomyopathy and muscular dystrophy. *Cell.* **98**:465–474.
8. Hack, A.A., et al. 2000. Differential requirement for individual sarcoglycans and dystrophin in the assembly and function of the dystrophin-glycoprotein complex. *J. Cell Sci.* **113**:2535–2544.
9. Duclos, F., et al. 1998. Progressive muscular dystrophy in α-sarcoglycan-deficient mice. *J. Cell Biol.* **142**:1461–1471.
10. Cohn, R.D., et al. 2001. Prevention of cardiomyopathy in mouse models lacking the smooth muscle sarcoglycan-sarcospan complex. *J. Clin. Invest.* **107**:R1–R7.
11. Liu, L.A., and Engvall, E. 1999. Sarcoglycan isoforms in skeletal muscle. *J. Biol. Chem.* **274**:38171–38176.
12. Wheeler, M.T., Zarnegar, S., and McNally, E.M. 2002. ζ-sarcoglycan, a novel component of the sarcoglycan complex, is reduced in muscular dystrophy. *Hum. Mol. Genet.* **11**:2147–2154.
13. Straub, V., et al. 1999. ε-sarcoglycan replaces alpha-sarcoglycan in smooth muscle to form a unique dystrophin-glycoprotein complex. *J. Biol. Chem.* **274**:27989–27996.
14. Hack, A.A., et al. 1998. γ-sarcoglycan deficiency leads to muscle membrane defects and apoptosis independent of dystrophin. *J. Cell Biol.* **142**:1279–1287.
15. Subramaniam, A., et al. 1991. Tissue-specific regulation of the α-myosin heavy chain gene promoter in transgenic mice. *J. Biol. Chem.* **266**:24613–24620.
16. Solway, J., et al. 1995. Structure and expression of a smooth muscle cell-specific gene, SM22 α. *J. Biol. Chem.* **270**:13460–13469.
17. Heydemann, A., Hadhazy, M.R., Huber, J.M., Wheeler, M.T., and McNally, E.M. 2004. Functional nitric oxide mislocalization in cardiomyopathy. *J. Mol. Cell. Cardiol.* In press.
18. Zhu, X., et al. 2001. Overexpression of γ-sarcoglycan induces severe muscular dystrophy. Implications for the regulation of sarcoglycan assembly. *J. Biol. Chem.* **276**:21785–21790.
19. Zhu, X., Hadhazy, M., Wehling, M., Tidball, J.G., and McNally, E.M. 2000. Dominant negative myostatin produces hypertrophy without hyperplasia in muscle. *FEBS Lett.* **474**:71–75.
20. McNally, E.M., et al. 1996. Mutations that disrupt the carboxyl-terminus of γ-sarcoglycan cause muscular dystrophy. *Hum. Mol. Genet.* **5**:1841–1847.
21. Bonnemann, C.G., et al. 1996. Genomic screening for β-sarcoglycan gene mutations: missense mutations may cause severe limb-girdle muscular dystrophy type 2E (LGMD 2E). *Hum. Mol. Genet.* **5**:1953–1961.
22. Chutkow, W.A., et al. 2002. Episodic coronary artery vasospasm and hypertension develop in the absence of $Sur2_{KATP}$ channels. *J. Clin. Invest.* **110**:203–208. doi:10.1172/JCI200215672.
23. Factor, S.M., Minase, T., Cho, S., Dominitz, R., and Sonnenblick, E.H. 1982. Microvascular spasm in the cardiomyopathic Syrian hamster: a preventable cause of focal myocardial necrosis. *Circulation.* **66**:342–354.
24. Pepine, C.J. 1995. Effect of calcium antagonists in variant or Prinzmetal angina. *Can. J. Cardiol.* **11**:952–956.
25. Lanza, G.A., and Maseri, A. 2000. Coronary artery spasm. *Curr. Treat. Options Cardiovasc. Med.* **2**:83–90.
26. Politano, L., et al. 2001. Evaluation of cardiac and respiratory involvement in sarcoglycanopathies. *Neuromuscul. Disord.* **11**:178–185.
27. Barresi, R., et al. 2000. Disruption of heart sarcoglycan complex and severe cardiomyopathy caused by β sarcoglycan mutations. *J. Med. Genet.* **37**:102–107.
28. van der Kooi, A.J., et al. 1998. The heart in limb girdle muscular dystrophy. *Heart.* **79**:73–77.
29. Tsubata, S., et al. 2000. Mutations in the human δ-sarcoglycan gene in familial and sporadic dilated cardiomyopathy. *J. Clin. Invest.* **106**:655–662.
30. Ludmer, P.L., et al. 1986. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *N. Engl. J. Med.* **315**:1046–1051.
31. Dzau, V.J., and Cooke, J.P. 1996. Pathophysiology of vasospasm. In *Vascular medicine: a textbook of vascular biology and diseases*. J. Loscalzo, M.A. Creager, and V.J. Dzau, editors. Lippincott, Williams & Wilkins. Boston, Massachusetts, USA. 371–389.
32. Nigro, V., et al. 1997. Identification of the Syrian hamster cardiomyopathy gene. *Hum. Mol. Genet.* **6**:601–607.
33. Krams, R., et al. 1998. Decreased coronary flow reserve in hypertrophic cardiomyopathy is related to remodeling of the coronary microcirculation. *Circulation.* **97**:230–233.
34. Maron, B.J., Wolfson, J.K., Epstein, S.E., and Roberts, W.C. 1986. Intramural ("small vessel") coronary artery disease in hypertrophic cardiomyopathy. *J. Am. Coll. Cardiol.* **8**:545–557.