Cardiac-Specific Overexpression of Caveolin-3 Induces Endogenous Cardiac Protection by Mimicking Ischemic Preconditioning

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Background—Caveolae, lipid-rich microdomains of the sarcolemma, localize and enrich cardiac-protective signaling molecules. Caveolin-3 (Cav-3), the dominant isoform in cardiac myocytes, is a determinant of caveolar formation. We hypothesized that cardiac myocyte–specific overexpression of Cav-3 would enhance the formation of caveolae and augment cardiac protection in vivo.

Methods and Results—Ischemic preconditioning in vivo increased the formation of caveolae. Adenovirus for Cav-3 increased caveolar formation and phosphorylation of survival kinases in cardiac myocytes. A transgenic mouse with cardiac myocyte–specific overexpression of Cav-3 (Cav-3 OE) showed enhanced formation of caveolae on the sarcolemma. Cav-3 OE mice subjected to ischemia/reperfusion injury had a significantly reduced infarct size relative to transgene-negative mice. Endogenous cardiac protection in Cav-3 OE mice was similar to wild-type mice undergoing ischemic preconditioning; no increased protection was observed in preconditioned Cav-3 OE mice. Cav-3 knockout mice did not show endogenous protection and showed no protection in response to ischemic preconditioning. Cav-3 OE mouse hearts had increased basal Akt and glycogen synthase kinase-3β phosphorylation comparable to wild-type mice exposed to ischemic preconditioning. Wortmannin, a phosphoinositide 3-kinase inhibitor, attenuated basal phosphorylation of Akt and glycogen synthase kinase-3β and blocked cardiac protection in Cav-3 OE mice. Cav-3 OE mice had improved functional recovery and reduced apoptosis at 24 hours of reperfusion.

Conclusions—Expression of caveolin-3 is both necessary and sufficient for cardiac protection, a conclusion that unites long-standing ultrastructural and molecular observations in the ischemic heart. The present results indicate that increased expression of caveolins, apparently via actions that depend on phosphoinositide 3-kinase, has the potential to protect hearts exposed to ischemia/reperfusion injury. (Circulation. 2008;118:1979-1988.)

Key Words: caveolae ■ caveolins ■ heart ■ myocardial ischemia

The concept that an organ can develop tolerance to subsequent ischemic stress was initially suggested by studies of traumatic injury. In 1986, Murry et al found that nonlethal injury, called ischemic preconditioning (IPC), could protect the heart from lethal injury. Subsequent work has shown that IPC is a highly effective way to protect multiple organs from ischemic injury. Many studies have evaluated individual signaling molecules and pathways in IPC, but no single, unifying intervention explains the temporal/spatial conundrum of IPC. An emerging idea in signal transduction emphasizes the role of multiprotein complexes organized in discrete microenvironments in cell regulation and pathophysiology, such organization might explain the temporal/spatial conundrum of IPC.

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Caveolae, cholesterol- and sphingolipid-enriched invaginations of the plasma membrane, a subset of lipid/membrane signaling and spatial 3-dimensionality (ie, simultaneous activation of numerous parallel pathways) of IPC. An emerging idea in signal transduction emphasizes the role of multiprotein complexes organized in discrete microenvironments in cell regulation and pathophysiology, such organization might explain the temporal/spatial conundrum of IPC.

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rafts, are 1 such microenvironment.6–8 Caveolins, structural proteins essential for caveolae formation, are present in 3 isoforms:8 caveolin (Cav)-1 and Cav-2 are expressed in multiple cell types, and Cav-3 is found primarily in striated (skeletal and cardiac) muscle and certain smooth muscle cells.10 Caveolins have scaffolding domains that anchor and regulate the function of proteins that modulate a variety of cellular processes11 and signal transduction.3,5 Caveolins can function as scaffolds for multiple, interacting signaling molecules, thereby providing temporal and spatial regulation of cellular signal transduction.5

Disruption of caveolae attenuates protection of adult cardiac myocytes from ischemic damage,12 and Cav-3 knockout mice, which lack cardiac myocyte caveolae, are resistant to pharmacological preconditioning.13 Such findings imply that myocyte caveolae are a prerequisite for protection against ischemia/reperfusion injury. Because Cav-3 expression is essential for the formation of caveolae in cardiac myocytes,14 we hypothesized and provide evidence that cardiac myocyte–specific overexpression of Cav-3 increases the formation of caveolae and enhances protective signaling from ischemia, thus identifying cell-specific expression of caveolins and caveolae as a novel approach to achieve such protection.

**Methods**

**Antibodies**

Sources of antibodies included the following: polyclonal antibody to Cav-1, Abcam Inc (Cambridge, Mass) and Cell Signaling Technology (Danvers, Mass); polyclonal antibody to Cav-2, Abcam; monoclonal and polyclonal antibody to Cav-3, BD Transduction, Abcam, and Santa Cruz Biotechnology (Santa Cruz, Calif); monoclonal inducible nitric oxide synthase (NOS), polyclonal neuronal NOS, and polyclonal antibodies to phospho-eNOS (Ser1177) and (Thr495), Cell Signaling Technology; polyclonal antibody to inhibitor of apoptosis protein-1, Abcam; monoclonal antibody to Fas, Upstate; and monoclonal to GAPDH, IMGENEX (San Diego, Calif).

**Animals**

Animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals (National Academy of Science), and protocols were approved by the VA San Diego Healthcare System Institutional Animal Care and Use Committee. Animals were kept on a 12-hour light-dark cycle in a temperature-controlled room with ad libitum access to food and water.

Transgenic (TG) mice with cardiac myocyte–specific overexpression of Cav-3 (Cav-3 OE) were produced in a C57BL/6 background. Whole hearts or cells were fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer for 2 hours, postfixed in 1% OsO4 in 0.1 mol/L cacodylate buffer (1 hour), and embedded as monolayers in 1% BSA) to remove nonmyocytes, and cardiac myocytes were incubated at 37°C in 5% CO2.

**Plasmid and Recombinant Adenovirus Production**

A mouse Cav-3 cDNA (455 bp) was generated, cloned, and cotransfected with pJM170 (containing E1 region deletion). Plaques were expanded in HEK293 cells transformed with adenovirus E1. Adenovirus containing LacZ (Adv.LacZ) served as control. Cardiac myocytes were treated with Adv.LacZ or Adv.Cav-3 for 16 to 24 hours.

**Echocardiography**

Echocardiography was performed in mice anesthetized with isoflurane using an echocardiograph and L15/MHz transducer (Sonos 5500, Philips Medical Systems, Andover, Mass) as described previously.16

**In Vivo Ischemia/Reperfusion**

Mice anesthetized with pentobarbital (80 mg/kg) were mechanically ventilated, and ischemia was produced by occluding the left coronary artery with a 7-0 silk suture on a tapered BV-1 needle (Ethicon, Johnson & Johnson, New Brunswick, NJ) for 30 minutes.18 After 30 minutes of occlusion, the ligature was released, and the heart was reperfused for 2 or 24 hours. IPC was induced by occlusion of the left coronary artery for 5 minutes, followed by 15 minutes of reperfusion just before ischemia. Some Cav-3 OE mice were treated with 5-hydroxydecanoate (5-HD; a mitochondrial KATP channel inhibitor; Sigma, St Louis, Mo; 10 mg/kg IV) 10 minutes before

**Sucrose Density Membrane Fractionation**

Whole hearts were fractionated with sucrose density gradients as reported.16 Fractions 4 through 6 were buoyant membrane fractions (BFs) enriched in caveolins and proteins associated with caveolins. Fractions 9 through 12 were defined as nonbuoyant fractions (non-BFs).

**Immunoblot**

Whole tissue or cell lysates were separated by SDS-PAGE with 10% polyacrylamide precast gels (Invitrogen, Carlsbad, Calif) and transferred to polyvinylidene difluoride membranes by electroelution. Membranes were blocked in 20 mmol/L TBS Tween (1%) containing 4% BSA and incubated with primary antibody overnight at 4°C. Blots were visualized using secondary antibodies conjugated with horseradish peroxidase from Santa Cruz Biotechnology and enhanced chemiluminescence reagent from GE Healthcare (Waukesha, Wis).

**Adult Cardiac Myocytes**

Cardiac myocytes were isolated from male Sprague-Dawley rats as described.12 Myocytes were plated in 4% FBS on laminin (2 μg/cm2)-coated plates for 1 hour. Plating media was changed to serum-free media (1% BSA) to remove nonmyocytes, and cardiac myocytes were incubated at 37°C in 5% CO2.
ischemia or wortmannin (a phosphoinositide 3-kinase [PI3K] inhibitor; 15 μg/kg) 15 minutes before ischemia.

Infarct Size
The area at risk (AAR) was determined by staining with 1% Evans blue (1.0 mL; Sigma).18 The heart was immediately excised and cut into 1-mm slices (McIlwain tissue chopper, Brinkmann Instruments, West Bury, NY). The left ventricle was counterstained with 1% 2,3,5-triphenyltetrazolium chloride (Sigma). Images were analyzed by Image-Pro Plus (Media Cybernetics, Bethesda, Md), and infarct size was determined by planimetry. Cardiac troponin I levels in the serum were measured with a high-sensitivity mouse cardiac troponin-I ELISA kit (Life Diagnostics, West Chester, Pa).

Cardiac Function
Mice underwent surgery as described in the ischemia/reperfusion protocol and were allowed to recover for 24 hours. After 24 hours, mice were anesthetized with pentobarbital (80 mg/kg), and cardiac catheterization was performed with a high-fidelity 1.4F microtip pressure transducer (SPR-671, Millar Instruments Inc, Houston, Tex). The catheter was advanced via the right carotid artery into the left ventricle after measuring mean arterial pressure. Parameters were determined by an algorithm from EMKA Technologies (Falls Church, Va).

Apoptosis
For terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assays, AAR was removed from the mice after 24 hours of reperfusion. The tissue was cut and fixed in 3.7% formalin, and sections (5 μm) were used for TUNEL assays with the Apoptosis Detection Kit (R&D Systems, Minneapolis, Minn) according to the manufacturer’s instructions. Real-time polymerase chain reaction analysis of gene expression of proapoptotic and antiapoptotic genes was performed on total RNA isolated from the heart after 24 hours of reperfusion with the RNeasy Mini Kit (Qiagen Inc, Valencia, Calif) as described previously.18 Immunoblots for Fas and inhibitor of apoptosis-1 were performed.

Statistical Analysis
Data analysis was performed by observers blinded to experimental groups. Group size to determine the primary outcome variable of infarct size was established by power analysis. The power analyses suggest that a sample size of 7 mice per experimental group was sufficient, assuming α=0.05, 2 tailed at 90% power with a hypothetical mean difference of 20%. We performed statistical analysis with Prism 4.0 (GraphPad) by the unpaired Student t test or 1-way ANOVA followed by a posthoc test with Bonferroni correction for multiple comparisons. All data are expressed as mean±SEM. Statistical significance was defined as *P<0.05.

All authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
IPC Modulates Membrane Caveolae and Cav-3 Expression
We assessed the effect of IPC on cardiac membrane caveolae by subjecting mice to IPC and performing electron microscopy. Representative electron microscopy images show that IPC increases formation of caveolae (arrows in Figure 1A). To verify these morphological findings with biochemical techniques, hearts from IPC and control animals were fractionated on a discontinuous sucrose gradient and analyzed for protein and cholesterol content and for distribution of caveolin. IPC increases the total protein and cholesterol content in fractions 4 through 6 (BFs), which are enriched in caveolin17 (Figure 1B and 1C), and increases the amount of Cav-3, but not Cav-1, in BF (Figure 1D and 1E). These findings are consistent with evidence that the formation of cardiac myocyte caveolae is dependent on Cav-3.14,19
Adenoviral Overexpression of Cav-3 in Adult Cardiac Myocytes In Vitro Increases Caveolar Formation and Phosphorylation of Survival Kinases

Cardiac myocytes incubated with a Adv.Cav-3 have increased expression of caveolae (Figure 2A) and increased levels of phosphorylated protein kinase B (Akt) and glycogen synthase kinase-3β (GSK3β), 2 enzymes associated with IPC-induced cardiac protection3 (Figure 2B).

Cardiac Myocyte–Specific Cav-3 OE Mice Have Increased Myocyte Caveolae but Unaltered NOS Expression and Activity

Using an α-myosin heavy chain promoter, we generated mice with cardiac myocyte–specific overexpression of Cav-3 (supplementary Figure IA). Thirty-nine mouse lines were generated, 5 of which were TG positive (supplementary Figure IB). Two of these 5 lines were propagated, one of which had an 8-fold elevation in Cav-3 mRNA expression compared with TG-negative (TGneg) animals (Figure 3A). We developed and characterized this line and refer to it as the Cav-3 OE. Cav-3 OE mice have increased Cav-3 protein expression without a change in Cav-1 and Cav-2 expression (Figure 3B and 3C, red pixels). The increased protein expression results in an increased number of caveolae in cardiac myocytes (Figure 3D); increased expression of Cav-3 was not observed in lung, brain, liver, kidney, or skeletal muscle of these mice (data not shown).

Because caveolins negatively regulate activity of NOS isoforms,11,20 we quantified basal NOS expression and activity in the Cav-3 OE mice. Given the well-known interaction of NOS with caveolins, we were surprised to find similar expression of NOS and phosphorylated endothelial NOS (eNOS) isoforms and similar NOS activity in the whole hearts of TGneg and Cav-3 OE mice (Figure 4A and 4B). To determine whether activity of NOS in caveolin-enriched membrane fractions may be altered by cardiac myocyte–specific caveolin-3 overexpression, we subjected TGneg and Cav-3 OE hearts to sucrose density fractionation and assessed NOS activity in BF and non-BF using a [3H]-arginine assay. No difference in NOS activity was observed in BF and non-BF from Cav-3 OE hearts.

Figure 2. Cav-3 adenovirus increases caveolae expression in adult cardiac myocytes (ACMs). A, ACMs incubated with Adv.Cav-3 for 72 hours increased the caveola (C) number. B, ACMs exposed to no virus or to Adv.LacZ or Adv.Cav-3 for 72 hours were lysed and immunoblotted (left). Adv.Cav-3–treated ACMs have increased expression of Cav-3 protein and increased phosphorylated Akt and GSK3β vs control or LacZ-treated ACMs (n = 4 for Akt and GSK3β, n = 6 for Cav-3) (right). *P < 0.05 vs Adv.LacZ.

Figure 3. Cardiac myocyte–specific Cav-3 OE mice: caveolin and caveolae. A, Real-time polymerase chain reaction analysis of Cav-3 mRNA expression in 2 founder lines. Line 2 had 8-fold increased Cav-3 mRNA expression vs line 1. Data are represented relative to TGneg and normalized to GAPDH expression (n = 4). B, Immunoblot of Cav-1, -2, and -3 in whole-heart homogenates from Cav-3 OE and TGneg mice (left). Densitometry was normalized to expression of GAPDH and showed a significant increase in Cav-3 protein in whole-heart homogenates from Cav-3 OE vs TGneg mice (right); *P = 0.011; n = 7 for TGneg, n = 9 for Cav-3 OE. C, Immunohistochemistry showed increased Cav-3 (red pixels) in sarcolemma of cardiac myocytes from Cav-3 OE vs TGneg mice. Bar = 10 μm. D, Electron microscopy shows increased caveolae (C) in cardiac myocytes from Cav-3 OE vs TGneg mice.
non-BF in TGNeg versus Cav-3 OE mice (Figure 4C). Rat cerebellum homogenate was used as a positive control ([3H]-citrulline, 42 000 cpm/μL sample).

Cav-3 OE Mice Are Protected From Ischemia/Reperfusion Injury

Cav-3 OE mice exposed to 30 minutes of cardiac ischemia and 2 hours of reperfusion have a substantial reduction in infarct size compared with TGNeg mice (23.4±3.0% versus 43.0±3.9% AAR; n=11; P<0.001; Figure 5A), even though these mice show no differences in preocclusion hemodynamics (heart rate, mean arterial pressure, and rate-pressure product; supplementary Table I) or in the cardiac AAR (supplementary Figure II). This “endogenous protection” in Cav-3 OE mice is similar to that produced in TGNeg mice subjected to IPC (26.3±2.4% risk area; n=8; P<0.05) and

Figure 4. NOS expression and activity. A, Immunoblot analysis of basal expression of NOS and phosphorylated eNOS in TGNeg and Cav-3 OE mice (left). Densitometry was normalized to GAPDH (right). No differences were observed in expression of any NOS isoforms. Data are from 5 mice per group. B, Basal NOS activity was measured in TGNeg and Cav-3 OE murine whole-heart homogenates. No differences in basal NOS activity were observed between groups. C, Hearts from TGNeg and Cav-3 OE mice were homogenized in triton-X and fractionated on a discontinuous sucrose density gradient to separate BF (caveolae) and non-BF (noncaveolar membrane). NOS activity was measured in BF and non-BF with a [3H]-arginine assay. No difference in NOS activity was observed in the 2 separate fractions in TGNeg vs Cav-3 OE mice. Data are from 6 mice per group. iNOS indicates inducible NOS; and nNOS, neuronal NOS.

Figure 5. Cardiac protection in Cav-3 OE mice. Mice were subjected to ischemia/reperfusion injury. A, Infarct size (percent of AAR) was reduced by IPC in control animals; however, Cav-3 OE mice were protected to similar levels with and without IPC. Cav-3 knockout mice could not be protected with IPC. Treatment of Cav-3 OE mice with 5-HD (10 mg/kg IV), a mitochondrial KATP channel inhibitor, abolished protection. TGNeg treated with 5-HD had an infarct size similar to that of controls. Group sizes are indicated on the individual bars in parentheses. *P<0.05, **P<0.001 vs TGNeg mice; #P<0.05 vs Cav-3 OE+5-HD. B, Serum cardiac troponin-I, a marker of cardiac myocyte damage, was measured after 2 hours of reperfusion. Group sizes are indicated on the individual bars in parentheses. *P<0.06, **P<0.001 vs TGNeg mice; ##P<0.01 vs Cav-3 OE+5-HD.
GSK3β/H11021

B–D, To determine the role of PI3K/Akt/mTOR signaling in this endogenous protection, Cav-3 OE mice were treated with wortmannin, a PI3K inhibitor, or dimethyl sulfoxide (vehicle). Wortmannin treatment resulted in decreased basal phosphorylation of Akt and GSK3β compared with vehicletreated Cav-3 OE OE (Figure 6A), signaling molecules involved in cardiac protection.21 The level of basal elevation in phosphorylated Akt and GSK3β was attenuated by pretreatment with 5-HD, a mitochondrial ATP-sensitive potassium (KATP) channel inhibitor (38.1±4.4% risk area; n=8; Figure 5A). Akin to the results with 5-HD, IPC does not protect Cav-3 OE knockout mice from ischemic damage (Figure 5A). Cardiac troponin I confirmed the infarct size measurements (Figure 5B).

Role of Survival Kinases in Endogenous Protection

Hearts excised from Cav-3 OE mice had an ∼3-fold increase in basal phosphorylation of Akt and GSK3β (P<0.05; n=6; Figure 6A), signaling molecules involved in cardiac protection.21 The level of basal elevation in phosphorylated Akt and GSK3β in the hearts of Cav-3 OE mice was comparable to the elevation seen after IPC in vivo (Figure 6A). To determine the role of PI3K/Akt/GSK3β in this endogenous protection, Cav-3 OE mice were treated with wortmannin, a PI3K inhibitor, or dimethyl sulfoxide (vehicle). Wortmannin reduced the phosphorylation of both Akt and GSK3β in Cav-3 OE mice relative to vehicle control (Figure 6B). Vehicle-treated Cav-3 OE mice showed a cardiac-protected phenotype (as in Figure 5A) with reduced infarct size and cardiac troponin I (23.3±2.0% risk area; n=7; Figure 6C and 6D). Wortmannin attenuated the endogenous cardiac protective effect observed in vehicle-treated Cav-3 OE mice (45.0±3.0% risk area; n=7; Figure 6C and 6D).

Cav-3 OE Mice Have Preserved Ultrastructure After Ischemia/Reperfusion Injury

We also used electron microscopy analysis to examine the AAR after ischemia/reperfusion (30 minutes/2 hours) in Cav-3 OE and TGneg groups (Figure 7). After injury, the ischemia/reperfusion TGneg groups displayed highly disorganized patterns of cardiac myocytes and their mitochondria. In addition, the sarcolemma exhibited evidence of damage, including disrupted Z lines and myofibrillar stretching. Mitochondria were swollen and contained amorphous matrix densities, indicating that injury was in an irreversible phase22 (Figure 7C). In contrast, hearts of Cav-3 OE mice subjected to ischemia/reperfusion showed limited ultrastructural change relative to sham, in particular, no mitochondrial swelling, myofibril stretching, or Z-line deformation (Figures 7D).

Cav-3 OE Mice Have Preserved Cardiac Function and Reduced Apoptosis After Ischemia/Reperfusion

We assessed cardiac function of TGneg and Cav-3 OE mice during cardiac catheterization after 30 minutes of ischemia and 24 hours of reperfusion. Left ventricular systolic function (dp/dtmax) was greater and cardiac troponin I levels were significantly lower in the Cav-3 OE mice (Figure 8A and 8B). Cav-3 OE mice also had reduced apoptosis, assayed by TUNEL-positive cells (arrows in Figure 8C, left), as the percent of total nuclei after 24 hours of reperfusion (Figure 8C, right). Hearts of Cav-3 OE animals had decreased proapoptotic and increased antiapoptotic gene (Figure 8D) and protein (Figure 8E) expression.

Total-body overexpression of Cav-3 results in cardiomyopathy in 6-month-old mice.23 Accordingly, we assessed morphology, echocardiography, and hemodynamics in 6- to 9-month-old cardiac myocyte–specific Cav-3 OE mice. We found that the ratios of heart weight to tibia length were similar in Cav-3 OE and age-matched TGneg mice (supplemental Table II). In addition, echocardiographic parameters, heart rate, mean arterial pressure, and rate-pressure product were similar between Cav-3 OE and TGneg mice (supplementary Table II).

Discussion

Numerous mechanisms have been proposed to explain the ability of an organ to develop tolerance to subsequent lethal ischemia/reperfusion injury.24 IPC, an intervention that was first shown >20 years ago to prevent such injury, has lacked a unifying hypothesis to account for the diverse pathways by which it affects the heart and other organs. We show here that IPC alters the morphology and composition of the plasma membrane of cardiac myocytes, increasing the number of caveolae. Moreover, we find that the protein Cav-3 is both necessary and sufficient to protect the heart from ischemia/reperfusion injury. Spatial organization of signaling mole-
cules within caveolar microdomains and the interaction of signaling molecules with caveolins thus help to determine the protection of the heart from ischemia/reperfusion injury. The current data imply that expression of Cav-3 and caveolae represents a unifying mechanism for IPC.

Caveolae were first identified by electron microscopy in the 1950s by Palade and Yamada in endothelium and epithelium, respectively, and in the sarclemma of cardiac myocytes in 1975 by McNutt. Early research on myocardial ischemia focused on ischemia-induced changes in the sarclemma and provided evidence that caveolae in cardiac myocytes can be rapidly affected by perturbation of oxygen tension and tonicity. Despite such results, a role for caveolae and caveolins in the setting of IPC has not been explored.

An emerging concept emphasizes the organization of signaling molecules in multiprotein complexes, “signa-somes,” that form and dissociate under basal and stimulated conditions. Caveolins play an integral role in the dynamics of these multiprotein complexes in caveolae by interacting with a wide range of signaling molecules that include multiple G-protein–coupled receptors, Gs subunits of heterotrimeric G proteins, Src kinases, PI3K, eNOS, protein kinase C isoforms, extracellular signal-regulated kinase 1 and 2 (ERK1/2), and superoxide dismutase. Many of these proteins can bind to the scaffolding domain of caveolin and be regulated by caveolin. A number of proteins that bind caveolin have been overexpressed in cardiac myocytes and shown to produce tolerance to myocardial ischemia/reperfusion injury; these include adenosine receptors, α, adrenergic receptors, protein kinase C isoforms, mitogen-activated protein kinases, eNOS, and proteins involved in the scavenging of free radicals. Overexpression of heat shock proteins (αB crystallin, heat shock protein 60 to 10 complex, and H11 kinase) also confers protection from ischemic damage; conceivably, caveolins contribute to such interactions. In silico analysis of the protein sequence of αB crystallin reveals a putative caveolin binding motif (aa167-wvcyqypggy), suggesting future avenues of investigation.

Caveolar microdomains are enriched in cholesterol. We show that IPC increases caveolar microdomains and total cholesterol primarily in BFs enriched in caveolin. The mechanism by which IPC increases total cholesterol is not known. Class B scavenger receptors (CD36) localize to caveolae and regulate cholesterol homeostasis. Coexpression of CD36 and caveolin has been shown to enhance the uptake of cholesterol. It is possible that the increase in cholesterol helps drive caveolar formation, or alternatively, an increase in caveolar formation may drive the influx of cholesterol. Defining this distinction will be an interesting avenue for future investigation.

Figure 7. Electron micrograph of AAR from the hearts of TG<sub>NP</sub> and Cav-3 OE mice after ischemia/reperfusion. A and B, No tissue swelling or structural changes were seen in sham groups from TG<sub>NP</sub> and Cav-3 OE mice. C, After ischemia/reperfusion in TG<sub>NP</sub> mice, myofibrils were dis- tended, Z lines were irregular and unclear, and mitochondria were swollen and contained amorphous matrix densities. D, Cav-3 OE mice had fewer damaged myocytes after ischemia/reperfusion. Myofi-brils had well-arranged Z lines and orga-nized mitochondria. Bar=100 nm.
signaling via enhanced receptor-effector coupling or enhanced receptor affinity.\(^{11,48}\) On the basis of data showing that infusion of a peptide with the scaffolding domain of caveolin sequence can protect the heart from ischemia/reperfusion injury, Young et al\(^{49}\) proposed that the scaffolding domain of caveolin peptide produces ischemic tolerance by enhancing release of endothelium-derived NO.\(^{49}\) Additional studies showed that ischemia/reperfusion injury activates a redistribution of Cav-3 and a downregulation of Cav-1 association with ERK,\(^{50}\) whereas IPC leads to a translocation of eNOS to caveolae.\(^{51}\) The present results, in which overexpressed Cav-3 in cardiac myocytes yields no change in basal expression of NOS isoforms or in NO generation, lead us to conclude that the ischemic tolerance produced by Cav-3 expression in myocytes does not result from changes in NO production and that cardiac myocyte eNOS is not a major source of cardiac NO. The latter idea is supported by evidence that reexpression of Cav-1 exclusively within the endothelium of Cav-1 knockout mice rescues cardiac defects.\(^{52}\)

The phenotype of cardiac myocyte–specific Cav-3 OE mice is strikingly different from that of mice that have a total-body overexpression of Cav-3 in brain, fat, liver, lung, and spleen, as well as smooth, skeletal, and cardiac muscle.\(^{53}\) Such mice develop a muscular dystrophy phenotype at 3 to 4 weeks of age and after 6 months show cardiac degeneration, fibrosis, and reduced cardiac NOS activity and cardiac function.\(^{23}\) In contrast, cardiac myocyte–specific overexpression of Cav-3 does not result in cardiomyopathy in 6- to 8-month-old mice or a reduction in NOS activity. We conclude that increased cardiac myocyte expression of Cav-3 is not responsible for the late-appearing cardiac changes observed in the total-body Cav-3 OE mice.

**Conclusions**

The present results show that IPC increases the number of plasma membrane caveolae in cardiac myocytes and that Cav-3 OE mice are protected from ischemic injury in a manner that mimics the cardiac protection produced by IPC.
The ability to recapitulate or block the alterations of the plasma membrane produced by IPC by the respective over-expression or knockout of Cav-3 implies that Cav-3 is necessary and sufficient for IPC-induced cardiac protection. This protection may depend on PI3K and mitochondrial K\textsubscript{ATP} channels. Our results define a molecular mechanism to explain aspects of sarcolemmal ultrastructure, caveolae, and IPC that have been incompletely understood for many years. Cardiac myocyte–targeted overexpression of Cav-3 may provide a novel means to protect the heart from ischemia/reperfusion injury. More generally, our results imply that cell type–selective expression of caveolins may offer a means to augment mechanisms of preservation of the heart and perhaps other organs.

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Disclosures

Drts Tsutsumi, Patel, Head, Insel, Patel, and Roth have a patent pending with the University of California, San Diego: “Molecular Scaffolds for Treating Cardiac Injury.” The other authors report no conflicts.

References


**CLINICAL PERSPECTIVE**

Myocardial ischemia/reperfusion injury is a major cause of morbidity and mortality in several important clinical scenarios, including acute myocardial infarction, cardiac arrest, percutaneous coronary artery intervention, and cardiac surgery. More than 20 years ago, researchers showed in a landmark *Circulation* article that nonlethal ischemic stress to the heart, called ischemic preconditioning (IPC), protects against subsequent lethal myocardial ischemia/reperfusion injury. Many studies in the field have evaluated signaling molecules and pathways involved in IPC, but no single, unifying intervention explains the temporal efficiency (ie, rapid coupling of plasma membrane to intracellular signaling) and spatial 3-dimensionality (ie, simultaneous activation of numerous parallel pathways) of IPC. As such, no clinical interventions other than tissue plasminogen activator have come to fruition. An emerging idea in signal transduction emphasizes the role of multiprotein complexes organized in discrete microenvironments; such organization might explain the temporal/spatial efficiency of IPC. Caveolae, lipid-enriched invaginations of the plasma membrane, are 1 such microenvironment. We investigated a role for caveolae and a structural protein component of caveolae, caveolin-3, in IPC. We show that an IPC stimulus to the heart increases caveola number in the cardiac myocyte and that cardiac myocyte-specific overexpression of caveolin-3 mimics IPC and renders the heart resistant to myocardial ischemia/reperfusion injury. In addition, using caveolin-3 knockout mice, we show that caveolae are both necessary and sufficient for cardiac protection from myocardial ischemia/reperfusion injury. Given that caveolin-3 scaffolds many signaling molecules, our findings provide a unifying mechanism and unite long-standing ultrastructural and molecular observations in the ischemic heart. These results suggest that caveolae are novel targets for cardiac protection.
Cardiac-Specific Overexpression of Caveolin-3 Induces Endogenous Cardiac Protection by Mimicking Ischemic Preconditioning

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Supplementary Figure 1 Generation of a cardiac myocyte specific caveolin-3 (Cav-3) transgenic mouse.  (a) Schematic of Cav-3 construct.  Full-length cDNA for mouse Cav-3 (~489bp) was cloned into a vector containing the α-myosin heavy chain promoter (αMHC) to facilitate cardiac myocyte-specific expression of Cav-3.  (b) PCR products using transgenic (TG) primers (from (a) for positive control vector DNA, negative control, and tail snip genomic DNA of TG-positive and TG-negative (TGneg) mice were run on an agarose gel.  Five mice were Cav-3 TG positive (Cav-3 OE).
Supplementary Figure 2 Area at risk (AAR) as a percent of the left ventricle (LV) was no different among the groups.
Table 1. Hemodynamics

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Pre-occlusion</th>
<th>Ischemia 30 min</th>
<th>Reperfusion 120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heart rate, beats · min⁻¹</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGneg</td>
<td>457 ± 7</td>
<td>453 ± 8</td>
<td>431 ± 17</td>
<td>430 ± 19</td>
</tr>
<tr>
<td>Cav-3 OE</td>
<td>440 ± 7</td>
<td>442 ± 9</td>
<td>429 ± 12</td>
<td>440 ± 11</td>
</tr>
<tr>
<td>TGneg + 5-HD</td>
<td>439 ± 13</td>
<td>427 ± 10</td>
<td>424 ± 18</td>
<td>416 ± 10</td>
</tr>
<tr>
<td>Cav-3 OE + 5-HD</td>
<td>465 ± 11</td>
<td>439 ± 12</td>
<td>425 ± 10*</td>
<td>413 ± 11*</td>
</tr>
<tr>
<td>Cav-3 OE + Wortmannin</td>
<td>435 ± 7</td>
<td>441 ± 9</td>
<td>427 ± 10</td>
<td>418 ± 11</td>
</tr>
<tr>
<td>Cav-3 OE + Vehicle</td>
<td>448 ± 12</td>
<td>442 ± 12</td>
<td>428 ± 14</td>
<td>426 ± 12</td>
</tr>
<tr>
<td>Cav-3 KO</td>
<td>438 ± 7</td>
<td>428 ± 12</td>
<td>414 ± 13</td>
<td>406 ± 14</td>
</tr>
<tr>
<td>IPC</td>
<td>452 ± 13</td>
<td>444 ± 15</td>
<td>430 ± 12</td>
<td>428 ± 15</td>
</tr>
<tr>
<td>Cav-3 OE + IPC</td>
<td>443 ± 20</td>
<td>428 ± 15</td>
<td>441 ± 23</td>
<td>425 ± 13</td>
</tr>
<tr>
<td>Cav-3 KO + IPC</td>
<td>450 ± 21</td>
<td>422 ± 20</td>
<td>412 ± 23</td>
<td>417 ± 13</td>
</tr>
<tr>
<td><strong>Mean arterial pressure, mmHg</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGneg</td>
<td>74 ± 2</td>
<td>71 ± 2</td>
<td>67 ± 3</td>
<td>59 ± 2*§</td>
</tr>
<tr>
<td>Cav-3 OE</td>
<td>75 ± 2</td>
<td>72 ± 2</td>
<td>67 ± 2*</td>
<td>69 ± 2</td>
</tr>
<tr>
<td>TGneg + 5-HD</td>
<td>72 ± 2</td>
<td>69 ± 2</td>
<td>61 ± 1*†</td>
<td>57 ± 1*§</td>
</tr>
<tr>
<td>Cav-3 OE + 5-HD</td>
<td>73 ± 2</td>
<td>71 ± 2</td>
<td>62 ± 2*</td>
<td>58 ± 2*§</td>
</tr>
<tr>
<td>Cav-3 OE + Wortmannin</td>
<td>75 ± 2</td>
<td>73 ± 2</td>
<td>62 ± 2*</td>
<td>59 ± 2*§</td>
</tr>
<tr>
<td>Cav-3 OE + Vehicle</td>
<td>72 ± 2</td>
<td>71 ± 2</td>
<td>69 ± 1</td>
<td>66 ± 2</td>
</tr>
<tr>
<td>Cav-3 KO</td>
<td>75 ± 2</td>
<td>73 ± 2</td>
<td>61 ± 3*†</td>
<td>58 ± 3*§</td>
</tr>
<tr>
<td>IPC</td>
<td>71 ± 1</td>
<td>69 ± 1</td>
<td>71 ± 1</td>
<td>66 ± 1*</td>
</tr>
<tr>
<td>Cav-3 OE + IPC</td>
<td>72 ± 3</td>
<td>70 ± 3</td>
<td>65 ± 2</td>
<td>66 ± 2</td>
</tr>
<tr>
<td>Cav-3 KO + IPC</td>
<td>73 ± 2</td>
<td>69 ± 3</td>
<td>63 ± 1*</td>
<td>56 ± 1*§†</td>
</tr>
<tr>
<td><strong>Rate-pressure product, beats · min⁻¹ · mmHg · 10⁻³</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGneg</td>
<td>33.7 ± 1.1</td>
<td>32.4 ± 1.5</td>
<td>28.8 ± 1.9</td>
<td>25.3 ± 1.8*</td>
</tr>
<tr>
<td>Cav-3 OE</td>
<td>33.0 ± 1.0</td>
<td>31.7 ± 0.7</td>
<td>28.8 ± 0.8*</td>
<td>30.3 ± 0.8</td>
</tr>
<tr>
<td>TGneg + 5-HD</td>
<td>31.8 ± 1.5</td>
<td>29.4 ± 1.1</td>
<td>26.0 ± 1.2*</td>
<td>23.9 ± 0.9*§</td>
</tr>
<tr>
<td>Cav-3 OE + 5-HD</td>
<td>33.9 ± 1.3</td>
<td>31.3 ± 1.5</td>
<td>26.5 ± 1.1*</td>
<td>23.8 ± 1.0*§</td>
</tr>
<tr>
<td>Cav-3 OE + Wortmannin</td>
<td>32.4 ± 1.0</td>
<td>32.1 ± 1.1</td>
<td>26.5 ± 0.9*</td>
<td>24.7 ± 1.2*</td>
</tr>
<tr>
<td>Cav-3 OE + Vehicle</td>
<td>32.1 ± 1.0</td>
<td>31.3 ± 0.8</td>
<td>29.6 ± 1.0</td>
<td>28.0 ± 0.9*</td>
</tr>
<tr>
<td>Cav-3 KO</td>
<td>32.9 ± 0.9</td>
<td>31.3 ± 1.3</td>
<td>25.2 ± 1.0*</td>
<td>23.7 ± 1.4*§</td>
</tr>
<tr>
<td>IPC</td>
<td>32.0 ± 1.1</td>
<td>30.7 ± 1.3</td>
<td>30.4 ± 0.8</td>
<td>28.3 ± 1.2</td>
</tr>
<tr>
<td>Cav-3 OE + IPC</td>
<td>32.0 ± 2.1</td>
<td>30.0 ± 1.9</td>
<td>28.8 ± 1.7</td>
<td>27.9 ± 1.3</td>
</tr>
<tr>
<td>Cav-3 KO + IPC</td>
<td>32.7 ± 1.7</td>
<td>27.5 ± 1.8</td>
<td>25.7 ± 1.2*</td>
<td>23.5 ± 0.6*§</td>
</tr>
</tbody>
</table>

Data are mean ± SEM.  n=6-11 per group.  Abbreviations are: TGneg, transgenic-negative; Cav-3 OE, caveolin-3 overexpression; 5-HD, 5-hydroxydecanoate; Cav-3 KO, caveolin-3 knockout; IPC, ischemic preconditioning.  * Significantly (p<0.05) different from baseline (intragroup comparison).  § Significantly (p<0.05) different from Cav-3 OE (intergroup comparison).  † Significantly (p<0.05) different from IPC (intergroup comparison).
Table 2. Morphology, echocardiography and hemodynamics

<table>
<thead>
<tr>
<th></th>
<th>TGneg mice</th>
<th>Cav-3 OE mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Weight/Tibia Length (mg/mm)</td>
<td>6.3 ± 0.5</td>
<td>5.9 ± 0.4</td>
</tr>
<tr>
<td>Echocardiography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AWd (mm)</td>
<td>0.79 ± 0.04</td>
<td>0.86 ± 0.01</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>3.62 ± 0.14</td>
<td>3.68 ± 0.11</td>
</tr>
<tr>
<td>PWd (mm)</td>
<td>0.86 ± 0.02</td>
<td>0.90 ± 0.01</td>
</tr>
<tr>
<td>AWs (mm)</td>
<td>1.24 ± 0.07</td>
<td>1.28 ± 0.07</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>2.17 ± 0.12</td>
<td>2.31 ± 0.12</td>
</tr>
<tr>
<td>PWs (mm)</td>
<td>1.32 ± 0.05</td>
<td>1.20 ± 0.05</td>
</tr>
<tr>
<td>FS (%)</td>
<td>39.9 ± 3.3</td>
<td>37.5 ± 2.3</td>
</tr>
<tr>
<td>Hemodynamics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>431 ± 14</td>
<td>436 ± 16</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>72 ± 4</td>
<td>72 ± 2</td>
</tr>
<tr>
<td>RPP (beats · min⁻¹ · mmHg · 10^3)</td>
<td>31.2 ± 2.2</td>
<td>31.6 ± 1.6</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. n = 9-10. Abbreviations are: TGneg, transgenic-negative; Cav-3 OE, caveolin-3 overexpression; AWd and AWs, anterior wall thickness in diastole and systole, respectively; LVIDd and LVIDs, left ventricular internal cavity diameter in diastole and systole, respectively; PWd and PWs, posterior wall thickness in diastole and systole, respectively; FS, fractional shortening; HR, heart rate; MAP, mean arterial pressure; RPP, rate-pressure product.