Protection of adult rat cardiac myocytes from ischemic cell death: role of caveolar microdomains and δ-opioid receptors

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Patel, Hemal H., Brian P. Head, Heidi N. Petersen, Ingrid R. Niesman, Diane Huang, Garrett J. Gross, Paul A. Insel, and David M. Roth. Protection of adult rat cardiac myocytes from ischemic cell death: role of caveolar microdomains and δ-opioid receptors. Am J Physiol Heart Circ Physiol 291: H344–H350, 2006. First published February 24, 2006; doi:10.1152/ajpheart.01100.2005.—The role of caveolar microdomains and δ-opioid receptors (DOR) in the protection of adult rat cardiac myocytes from ischemic cell death is investigated. Ischemic preconditioning (IPC), a beneficial experimental intervention for preventing myocardial ischemia, protected cardiac myocytes in a caveolae-dependent manner. We determined protein expression and localization of δ-OR (DOR) using immunohistochemistry, caveolar fractionation, and immunoprecipitations. DOR colocalized in fractions with caveolin-3 (Cav-3), a structural component of caveolae in muscle cells, and could be immunoprecipitated by a Cav-3 antibody. Immunohistochemistry confirmed plasma membrane colocalization of DOR with Cav-3. Cardiac myocytes were subjected to simulated ischemia (2 h) or an ischemic preconditioning (IPC) protocol (10 min ischemia, 30 min recovery, 2 h ischemia) in the presence and absence of methyl-β-cycloexetrin (MβCD, 2 mM), which blocks cholesterol and disrupts caveolae. We also assessed the cardiac protective effects of SNC-121 (SNC), a selective DOR agonist, on cardiac myocytes with or without MβCD and MβCD pre-loaded with cholesterol. Ischemia, simulated by mineral oil layering to inhibit gas exchange, promoted cardiac myocyte cell death (trypan blue staining), a response blunted by SNC (P < 0.01) or by use of the IPC protocol (P < 0.01). MβCD treatment, which disrupted caveolae (as detected by electron microscopy), fully attenuated the protective effects of IPC or SNC, resulting in cell death comparable to that of the ischemic group. By contrast, SNC-induced protection was not abrogated in cells incubated with cholesterol-saturated MβCD, which maintained caveolae structure and function. These findings suggest a key role for caveolae, perhaps through enrichment of signaling molecules, in contributing to protection of cardiac myocytes from ischemic damage.

opiods; caveolae; G proteins; ischemia; myocytes

MYOCARDIAL INFARCTION (MI) remains a major cause of death in industrialized countries. Ischemic preconditioning (IPC) is a beneficial experimental intervention for preventing MI, whereby exposure of the heart to brief periods of ischemia results in protection from subsequent prolonged ischemic injury. The signal transduction pathways that mediate IPC are diverse and complex (11). Putative triggers, mediators, and effectors have been described, but there is an imprecise understanding as to how these factors interact to produce cardiac protection.

Opioid receptor stimulation has provided a model to study how extracellular molecules can produce cardiac protection. Opioid peptides are expressed in the heart (16, 49) and the δ-opioid receptor (DOR), the dominant opioid receptor in the heart, facilitates acute IPC (36), thus suggesting a role in IPC for endogenously released opioid peptides. In addition, agonists or antagonists of DOR induce or attenuate cardiac protection, respectively (3, 12, 36–38). Although acute and delayed cardiac protection elicited by opioids has been characterized, mechanistic information regarding expression of opioid receptor subtypes and the role of their subcellular localization on signaling, in particular in IPC, is lacking.

Recent data emphasize a potentially important role for signaling microdomains such as lipid rafts, and a subset of lipid rafts, caveolae, cholesterol- and sphingolipid-enriched 50- to 100-nm invaginations of the plasma membrane (28, 52) as sites that localize G protein-coupled receptors (GPCR), heterotrimeric G proteins, and G protein-regulated effector molecules in a confined region via lipid-protein and protein-protein interaction (17, 24). The latter interaction occurs in caveolae via caveolins, structural proteins that contain scaffolding domains; such localization facilitates coordinated, precise, and rapid regulation of cell function (21, 23, 25–27, 44, 50). There are three isoforms of caveolin: caveolin-1 (Cav-1) and -2 are expressed in multiple cell types, whereas caveolin-3 (Cav-3) is found exclusively in striated (i.e., skeletal and cardiac) myocytes and certain smooth muscle cells, in which it forms oligomeric complexes (43, 47). Compartmentation and spatial organization of GPCR signaling molecules in caveolae appears to play a key role in physiological and pharmacological responses (6, 9, 21, 27, 45). Little is known, however, regarding opioid receptor localization in caveolae or how caveolae might impact cardiac myocyte function during ischemia. In the present study, we therefore sought to 1) characterize the expression, localization, and cardiac protective role of DOR in isolated adult rat cardiac myocytes and 2) determine whether intact caveolae are important for cardiac protection produced by DOR activation.

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Materials. Antibodies to DOR were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cav-3 (monoclonal) antibody was obtained from BD Biosciences (San Jose, CA). SNC-121 was obtained from Tocris. Unless otherwise stated, all other chemicals and reagents were obtained from Sigma.

Preparation of adult rat ventricular myocytes. Cardiac myocytes were isolated from adult Sprague-Dawley rats (250–300 g, male). All animal use protocols were approved by the University of California, San Diego, Institutional Animal Care and Use Committee. The investigations conform with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Animals were heparinized (1,000–2,000 units ip) 5 min before being anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and the hearts were removed and placed in ice-cold cardioplegic (20 mM KCl) heart media solution (in mmol/l: 112 NaCl, 5.4 KCl, 1 MgCl2, 9 NaH2PO4, and 11.1 d-glucose; supplemented with 10 HEPES, 30 taurine, 2 DL-carnitine, and 2 creatine, pH 7.4). The hearts were retrograde perfused on a Langendorff apparatus with Na2CO3 (pH 11.0), homogenized with detergent-free methods (41, 43). Serum-free medium (1% BSA) to remove all nonmyocytes, and freshly isolated myocytes. Before experiments. Additionally, experiments were performed on 280,000 cardiac myocytes were plated on precoated laminin (2 μg/cm²) glass coverslips, grown for 24 h, and fixed with 4% buffered paraformaldehyde for 10 min at room temperature. The fixed cells were quenched with 100 mM glycine (pH 7.4) for an additional 5 min to remove aldehyde groups, permeabilized in buffered Triton X-100 (0.1%) for 10 min, and then blocked with 1% BSA-PBS-0.05% Tween for 20 min. Cells were incubated with primary antibodies (1:100) in 1% BSA-PBS-Tween (0.05%) for 24 h at 4°C. Excess antibody was removed by treatment with PBS-Tween (1%) three times at 5-min intervals. The cells were then incubated with FITC or Alexa-conjugated [F(ab)] secondary antibody (1:250) for 1 h. To remove excess secondary antibody, the cells were washed three times at 5 min intervals with PBS-Tween (0.1%) and incubated with 4,6-diamidino-2-phenylindole (1:5,000) diluted in PBS for 20 min. Cells were then washed for 10 min with PBS and mounted in gelvatol for microscopic imaging.

Deconvolution image analysis. Deconvolution images were obtained as previously described (1, 2). Images (3 cells each from 4 separate myocyte isolations) were captured with a DeltaVision deconvolution microscope system (Applied Precision, Issaquah, WA). The system includes a Photometrics charge-coupled device mounted on a Nikon TE-200 inverted epifluorescence microscope. In general, between 20 and 50 optical sections spaced by 0.2 μm were taken. Exposure times were set such that the camera response was in the linear range for each fluorophore. Lenses included ×100 (numerical aperture (NA) 1.4), ×60 (NA 1.4), and ×40 (NA 1.3). The data sets were deconvolved and analyzed using SoftWorx software (Applied Precision) on a Silicon Graphics Octane workstation. When applicable, image quantitation was performed with the Data Inspector program in SoftWorx. Maximal projection volume views or single optical sections are shown as indicated. Colocalization was assessed by CoLocalizer Pro 1.3 analysis software.

Electron microscopy. Cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h at room temperature, postfixed in 1% OsO4 in 0.1 M cacodylate buffer (1 h), and embedded as monolayers in LX-112 (Ladd Research, Williston, VT), as described previously (7). Sections were stained in uranyl acetate and lead citrate and observed with an electron microscope (JEOL 1200 EX-II or Philips CM-10). Caveolae were quantitated on random images per micrometer of membrane.

Simulated ischemia in isolated adult cardiac myocytes. Isolated cardiac myocytes were plated on laminin-coated 24-well plates and allowed to incubate for 24 h or were used freshly isolated. Cells were then washed in PBS at room temperature and subjected to various experimental conditions. “Ischemia/hypoxia” (“simulated ischemia”) was induced by layering mineral oil (0.5 ml for 120 min) over a thin film of media covering the cells followed by 60 min of “reperfusion” in normal media in the 24-well plate. “IPC” was performed by a 10-min period of incubation of cells under mineral oil followed by a 30-min recovery period before the 120-min “ischemic insult” and reperfusion. In other experiments, various concentrations of the DOR-selective agonist SNC-121 were administered 15 min before the ischemic insult. Cell death was quantified by counting trypan blue-stained cells and expressed as a percentage of the total cells counted. All experiments were carried out at 37°C (myocytes isolated from n = 6–9 animals/group).

To determine the impact of intact caveolae on cardiac protective events, we used methyl-β-cyclodextrin (MβCD), which removes cellular cholesterol and thereby disrupts cholesterol-rich caveolae (5, 14). Cells were incubated in MβCD (1 mM)-containing media for 1 h.
before the ischemia, IPC, or SNC-121 treatment protocols. Control experiments were conducted using MβCD preloaded with cholesterol, which results in a cyclodextrin not capable of removing cellular cholesterol (5, 33).

Statistical analysis. Statistical analyses were performed by one-way ANOVA followed by the Bonferroni post hoc test. All data are expressed as means ± SE. Statistical significance was defined as $P < 0.05$.

RESULTS

Effects of IPC and exogenous opioid stimulation on cardiac protection in isolated cardiac myocytes. We exposed cardiac myocytes to simulated ischemia-reperfusion (SI/R) and determined cell death by trypan blue staining. A preconditioning protocol (10 min of mineral oil layering followed by 30 min recovery in maintenance media) before sustained SI/R decreased cell death compared with SI/R alone (35 ± 3 vs. 59 ± 3, $P < 0.05$, Fig. 1). Treatment with the DOR agonist SNC-121 (0.1–1 μM) 15 min before sustained SI/R resulted in a concentration-dependent reduction in cell death compared with SI/R alone (Fig. 1). We obtained similar results for the preconditioning protocol in freshly isolated cells (data not shown).

Biochemical characterization of caveolin distribution in cardiac myocytes. Subcellular fractionation after Na2CO3 buffer treatment of cardiac myocytes was performed on a discontinuous sucrose gradient (42). Immunoblot analysis of the resulting fractions demonstrated enrichment of Cav-3, the muscle-specific marker of caveolae (47), in BF 4 and 5 (Fig. 2A), which also predominantly localized DOR (Fig. 2B). Confirmation of these results was obtained by immunoprecipitation; an antibody to Cav-3 immunoprecipitated DOR and an antibody to DOR immunoprecipitated Cav-3, implying an association between Cav-3 and DOR (Fig. 2C) in the same structural complex. The localization of DOR to caveolae and their association with Cav-3 was further confirmed by immunohistochemistry. Cav-3 and DOR localized to the plasma membrane and in a transverse pattern along the length of the myocyte (78.5 ± 6.3% colocalization), a pattern consistent with T tubules; the proteins colocalized predominantly at the membrane, as seen most readily in the overlap and mask images, which assess localization of overlapping pixels (Fig. 2D).

Effect of MβCD treatment on the expression of caveolae of cardiac myocytes. We treated cardiac myocytes with MβCD, an agent that disrupts caveolae by depletion of cholesterol (5, 14, 30). Electron microscopy revealed disruption of cardiac myocyte caveolae with increasing concentrations of MβCD but absence of such disruption by treatment with cholesterol-

Fig. 1. In vitro cardiac protection: role of opioid receptors. Adult rat cardiac myocytes were subjected to simulated ischemia. Control cells were incubated in culture media for the duration of the experiment. Simulated ischemia-reperfusion significantly increased trypan blue-positive cells, indicative of cell death. Ischemic preconditioning (PC) and SNC-121 (SNC) pretreatment for 15 min reduced cell death induced by simulated ischemia. $P < 0.05$ vs. basal (+) and vs. control ischemia (+).

Fig. 2. Biochemical and immunohistochemical evaluation of caveolin distribution and interaction with δ-opioid receptor (DOR). A: fractionated cardiac myocytes (CM) demonstrate a buoyant [fractions 4 and 5, buoyant fractions (BF)] localization of caveolin-3 (Cav-3) and heavier membrane fractions [fractions 9–12, heavy fractions (HF)] with less Cav-3 enrichment. IB, immunoblot. B: DOR are found in BF where Cav-3 is enriched. WCL, whole cell lysate. C: Cav-3 immunoprecipitates or detergent-solubilized WCL show that Cav-3 and DOR specifically interact. IP, immunoprecipitate. D: whole cells were costained with antibodies for Cav-3 and DOR. Cav-3 stains predominantly the cell membrane and also inside the cell in a pattern consistent with the distribution of T tubules. DOR stains predominantly the sarcolemmal membrane with Cav-3 (78.5 ± 6.3% colocalization, $n = 12$ myocytes from 4 rats).
loaded MβCD, which is unable to deplete membrane cholesterol (Fig. 3A; see Refs. 5 and 33). Quantification revealed that MβCD treatment reduced the number of caveolae (per μm of membrane), an effect not observed with cholesterol-loaded MβCD (Fig. 3B).

Role of caveolae in IPC- and opioid-induced cardiac protection in isolated myocytes. Cytoprotection by IPC to blunt simulated ischemia was attenuated in cardiac myocytes pretreated with MβCD for 1 h (Fig. 4A) and was not altered by cholesterol-loaded MβCD, which is not able to deplete cholesterol. Similar results were observed in experiments performed on freshly isolated myocytes (data not shown). Cytoprotection produced by the DOR agonist SNC-121 was also attenuated by MβCD, but treatment with cholesterol-loaded MβCD did not blunt SNC-121-induced cytoprotection (Fig. 4B).

**DISCUSSION**

In the present study, we show that compartmentation of opioid receptors contributes to cardiac protection; DOR, the opioid receptor subtype thought to mediate cardiac protection, localizes to caveolae, and this localization appears to be critical for such protection. The results imply the necessity of having intact caveolar microdomains to achieve cardiac protection by either preconditioning or a receptor agonist. Our combined use of subcellular fractionation with immunoblot analysis, immunofluorescence, and immunoprecipitation provides strong complementary evidence that Cav-3 organizes opioid receptors in caveolae of adult cardiac myocytes. The ability of MβCD treatment to attenuate cytoprotection in response to agonist treatment in cardiac myocytes implies a role for intact caveolae in IPC. Importantly, MβCD preloaded with cholesterol, which is unable to deplete membrane cholesterol and therefore does not disrupt caveolae, did not hinder the ability of SNC-121 to protect cardiac myocytes from ischemia-induced cell death.

Caveolins function as scaffolds to compartmentalize signaling molecules in caveolae, providing a mechanism for temporal and spatial regulation of signal transduction and cross-talk among signaling molecules (40, 50). Such interactions may contribute to regulation of cell death. For example, infusion of a peptide comprising the scaffolding domain of Cav-1 in isolated-perfused rat hearts produces cardiac protection (53). In prostate cancer, the cav-1 gene can be downregulated by c-myc, and maintaining high levels of cav-1 results in suppression of c-myc-induced apoptosis and sustains active Akt (48). Such data suggest multiple mechanisms whereby caveolins may have a role in apoptosis and perhaps necrosis.

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**Fig. 3.** Disruption of cardiac myocyte caveolae by methyl-β-cyclodextrin (MβCD). Control cardiac myocytes or cells treated with MβCD (1 and 2 mM, 1 h) were scraped and fixed for electron microscopic (EM) analysis. Random sections were taken for each treatment group and quantified as the no. of caveolae/μm membrane. A: representative EM membrane images of control and MβCD-treated (1 and 2 mM) cells. There are fewer caveolae in the MβCD-treated groups compared with control. Cardiac myocytes treated with cholesterol-loaded MβCD expressed a similar number of caveolae as did the control group. Cho, cholesterol loaded. B: bar graph representing caveolae/membrane length in control and MβCD-treated cells (1 and 2 mM). *P < 0.05 vs. control group.
In the heart, overexpression of Cav-3 in neonatal rat cardiac myocytes inhibits myocyte hypertrophy (19), which contributes to adverse cardiac remodeling and the progression of cardiomyopathy (10). Moreover, transgenic mice that overexpress L-type Ca\(^{2+}\) channels show decreased inotropic response to \(\beta\)-adrenergic stimulation linked to a deficiency in protein kinase C signaling secondary to suppression of Cav-3 (31). In contrast, Cav-1 and Cav-3 double knockout mice show a reduction in caveolae and develop severe cardiomyopathy (29). Dissociation of caveolin from caveolae has been associated with aging and myocardial ischemia-induced heart failure (32). Collectively, these data, as well as the current findings, suggest that increased expression of caveolins may be beneficial in the ischemic heart, and interventions that decrease their expression may be detrimental.

We show that cholesterol depletion can disrupt caveolae formation and impair cardiac protection; this finding would appear to be contradictory to cholesterol reduction produced by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (e.g., statins), which inhibit the rate-limiting enzyme in cholesterol synthesis and produce cardiac protection. We believe this is likely because of the inability of statins to alter membrane caveolae in cardiac myocytes but more importantly because of “pleiotropic” actions of statins that are not necessarily related to plasma cholesterol reduction (8, 20, 51). In preliminary studies (data not shown), we have found that statins do not appear to alter the distribution of Cav-3 in cardiac myocyte sucrose density fractions, suggesting that statins may not alter caveolae in cardiac myocytes. Statins have been shown to have other effects that would influence cardiac protection, such as attenuation of oxidant stress (18), activation of protein kinase C (46), and inactivation of glycogen synthase kinase-3B (4). In addition, another response to statins, prevention of cardiac hypertrophy, is thought to occur via cholesterol-independent mechanisms (22).

Limitations of the study include the use of a treatment protocol whereby SNC-121 was not washed out; such a protocol differs from IPC. We used this protocol to mimic in vivo pharmacological studies in which an opioid is given as a preconditioning stimulus and ischemia-reperfusion is induced shortly thereafter (13). In addition, one of the original protocols using simulated ischemia in isolated cardiac myocytes used isotonic or hypotonic media for reperfusion (15), whereas more recent studies do not incorporate a reperfusion protocol (34, 35). In most of our studies, we used cells that were allowed to stabilize for 24 h after isolation with induction of ischemia by mineral oil layering and performed reperfusion using normal media that resulted in a sufficient “death response” in the simulated ischemia group. We undertook key confirmatory studies with freshly isolated myocytes and obtained similar results to those of the 24-h cultures (data not shown).

In conclusion, the present study provides evidence for a mechanism utilized by DOR to protect cardiac myocytes from ischemic damage. The findings emphasize the importance of the cellular distribution and spatial organization of signaling components in caveolae in cardiac myocytes and suggest that cardiac pathophysiology can be impacted by subcellular organization of GPCRs in general, and perhaps DOR in particular. Based on evidence that disruption of caveolae blunts cardiac protection produced by DOR, the results provide a mechanistic link between receptor compartmentation in caveolae, opioid receptor stimulation, and cardiac protection.

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![Graph](http://example.com/graph.png)

**Fig. 4.** Effect of caveolar disruption on ischemic preconditioning (IPC) and opioid-induced cytoprotection. A: rat cardiac myocytes were subjected to simulated ischemia (mineral oil layering) or to preconditioning (IPC, 10 min ischemia with 30 min recovery) in the presence and absence of MβCD (2 mM for 1 h). Cells treated with MβCD could no longer be preconditioned. *P < 0.05 vs. respective ischemia group. B: isolated cells were subjected to ischemia or pretreated with SNC-121 (1 mM) to induce protection in the presence and absence of MβCD and cholesterol-loaded MβCD. SNC-121 did not provide protection in MβCD-treated cells but did in cholesterol-loaded MβCD-treated cells. *P < 0.05 vs. respective ischemia group.
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