Caveolin-3 Peptide Protects Cardiomyocytes from Apoptotic Cell Death via Preserving Superoxide Dismutase Activity and Inhibiting Caspase-3 Activation under Hypoxia-reoxygenation

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Abstract

Recent progress suggests that caveolins are cardiac protective molecules in regulating apoptotic cell death during myocardial ischemia-reperfusion injury. To understand the mechanisms of caveolins in cardiac protection, we investigated the expressions of caveolin-1(cav-1), caveolin-2 (cav-2) and caveolin-3 (cav-3) proteins in the isolated cultured hypoxia-reoxygenated cardiomyocytes. Neonatal rat cardiomyocytes were subjected to 4 h hypoxia and 24 h reoxygenation by exposed to 1% [O2] and replaced with normal air respectively. The expression of cav-3, instead of cav-1 and cav-2, was down-regulated by hypoxia-reoxygenation. Pre-treatments of superoxide dismutase and catalase (SOD/CAT) or peroxynitrite decomposition catalyst FeTMPyP prevented the down-regulation of cav-3. We then investigated the effects of extraneous free radicals on regulating the expression of cav-3 by incubating with xanthine/xanthine oxidase (X/XO), H2O2, 1-[N, N-di-(2-aminoethyl) amino] diazen-1-ium-1, 2-diolate (DETA/NO, NO donor) or 3-morpholinosydnonimine (SIN-1, peroxynitrite donor) respectively. Treatments of X/XO, H2O2, DETA/NO and SIN-1 significantly induced the down-regulation of cav-3 protein. The results indicate that the productions of free radicals contribute to the down-regulation of cav-3 protein in the hypoxia-reoxygenated cardiomyocytes. Moreover, our data showed that cav-3 peptide significantly enhanced the expression and the activity of SOD, inhibited O2− production and caspase 3 activity, and reduced the rates of early and late apoptotic cell death, whereas cav-1 peptide had no effect on SOD, but slightly reduced O2− production and decreased the rates of apoptotic cell death in the hypoxia-reoxygenated cardiomyocytes. Taken together, we conclude that caveolin-1 and caveolin-3 could ameliorate free radicals-induced oxidative injury via diverse mechanisms in hypoxia-reoxygenated cardiomyocytes.
1. Introduction

Recent progress in membrane biology has proposed the roles of membrane microdomains such as caveolae in many cellular events including apoptotic cell death. Several isoforms of proteins, caveolins-1, 2, 3 with size ~ 22 kDa, are found to reside in the flask-shaped caveolae. Caveolins are involved in signal transduction by regulating the functions of caveolae-associated signaling molecules such as protein kinase C, and tyrosine kinase-associated receptors as well as endothelial nitric oxide synthase (eNOS) [1-5]. The proposed apoptotic promoting or inhibitory effects of caveolin-1 are cell-type specific. Over-expression of caveolin-1 is associated with induction of apoptotic cell death in macrophages, fibroblasts and epithelial cells [7-8]. For example, tumor necrosis factor-α (TNF α) receptor co-localizes with caveolae domain, and the TNF α-induced apoptosis is dependent on caveolae [6]. On the contrary, caveolin-1 is a suppressor of c-myc-induced apoptosis in human epithelial prostate cancer cells [9]. Caveolin-1, -2 and -3 are found in adult cardiomyocytes and only caveolin-3 specifically resident in myocytes. Caveolin-1 and -3 dissociations from caveolae to cytosol have been previously demonstrated in the aging and infarct rat hearts, implicating that caveolins may participate in the process of ischemic myocardial injury [10].

Caveolin-1 and -3 appears to play diverse roles in cardiovascular diseases. Early studies suggest that over-expression of caveolin-3 is sufficient to induce severe cardiomyopathy [11,12]. Recent studies indicate that caveolin-1 and caveolin-3 have cardiac protective effects via interacting with various cellular signal molecules during myocardial ischemia-reperfusion injury and ischemic preconditioning [13-17]. Expression of caveolin-3 is necessary and sufficient for cardiac protection in ischemic heart, which is dependent with phosphoinositide 3-kinase [17]. Both caveolin-1 and caveolin-3 co-localize with matrix metalloproteinase-2 in cardiomyocytes [18]. Under ischemic preconditioning, caveolin-1 and caveolin-3 can interact with proapoptotic p38MAPKα and anti-apoptotic p38MAPKβ respectively in the hearts, and those interactions function as a molecular switch for the conversions of ischemia-reperfusion–induced cell death signals into precondition-induced survival signals [13].

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are important mediators in the myocardial ischemia-reperfusion injury [19-21]. Superoxide anions (O2-) can induce the release of caveolin-1 from plasma membrane, inhibit the trafficking of newly synthesized caveolin-1 to membrane microdomains and decrease caveolae formation and eNOS-caveolin-1 interactions in endothelial cells [22]. Nitric oxide (NO) is a free radical which has a variety of cardiovascular functions including modulations of endothelial function, blood flow, blood pressure, vascular tone, and immune system, etc., but excessive NO could inhibit cardiac contractility, impair mitochondrial respiration and trigger apoptosis [23-27]. The interactions of caveolin-3 and nitric oxide synthases in cardiomyocytes are previously described [28-29]. Decreased caveolin-3 was accomplished with increased eNOS activity in chronic hypoxic myocardia [30]. NO can rapidly react with O2- to form peroxynitrite (ONOO-), leading to apoptotic cell death in myocardial ischemia-reperfusion injury.

Activation of caspase apoptotic pathway is a critical cellular signal pathway in myocardial ischemia-reperfusion injury [31-32]. A potential role for caveolae in the regulation of caspase signal pathway has been proposed [33]. Caspase 3 locates at the plasmalemmal surface in close proximity to caveolin-1 of the caveolar fractions isolated from cardiac endothelial cells [33]. Disruption of caveolar structure alters the profile of staurosporine-induced caspase 3 activity. Caveolin-1 peptide has been reported to attenuate ploymorphonuclear neutrophil (PMN)-induced cardiac contractile dysfunction in ischemia-reperfused myocardia [34].
However, there are many unanswered questions in the interactions of free radicals and caveolins in myocardial ischemia-reperfusion injury. For example, it is unknown yet whether O$_2^-$, NO and ONOO$^-$ could affect the expression of caveolins in the cardiomyocytes. It is also of interest to address whether the mechanisms of cardiac protections of caveolin-1 and -3 are associated with the inhibition of caspase 3 pathway in myocardial ischemia-reperfusion injury. In this study, we tested the hypothesis that caveolins can be target molecules of free radicals in myocardial ischemia-reperfusion injury. We investigated the expressions of caveolin-1(cav-1), caveolin-2 (cav-2) and caveolin-3 (cav-3), the expression and activity of superoxide dismutase (SOD), the activation of caspase-3, the production of O$_2^-$, and the rates of apoptotic cell death in the cardiomyocytes after exposed to hypoxia-reoxygenation.

2. Materials and methods

2.1 Cell Culture

Neonatal rat cardiomyocytes were cultured following the previous method with minor modification [35]. The rats were obtained from the Laboratory Animal Unit of the University of Hong Kong. Animal housing, care, and application of experimental procedures were in accordance with the institutional guidelines and approved by the University Committee on the Use of Live Animals in Teaching and Research for the University of Hong Kong. In brief, after anesthesia, the hearts from the rats at 2–3 day were minced and dissociated with 0.06% trypsin (Sigma). The cells were incubated on 100-mm culture dish for 15 min at 37 °C with 95% air/5% CO$_2$ and 100% relative humidity in a CO$_2$ incubator. Non-attached viable cells were collected and incubated in DMEM supplemented with 10% fetal calf serum, penicillin (50 units/ml) and streptomycin (50 μg/ml) for 6 hours, followed by incubation in the same media supplemented with 10$^{-6}$ mol/L cytosine arabinoside for 48 hours to reduce the rate of non-cardiomyocytes. Then the cardiomyocytes were replaced with fresh medium without cytosine arabinoside under the same culture condition for 2 days and used in the following experiments. The purity of the isolated cardiomyocytes was identified to be more than 90%. The isolated cardiomyocytes were cultured in high glucose DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin and incubated at 37 °C with 100% relative humidity in a CO$_2$ incubator for 3 days.

2.2 Hypoxia and reoxygenation treatment

To mimic ischemia, the cells were incubated with DMEM supplemented with 1% FCS and placed in an air-tight hypoxic chamber where normal air was pumped out with a vacuum pump and replaced by 1% O$_2$/5% CO$_2$ balanced with nitrogen gas 37 °C for 4 hours. After that, the media were replaced by DMEM supplemented with 5% FCS and the gas was removed and replaced by normal air plus 5% CO$_2$ for 24 hours. Corresponding control cells were incubated under normoxia (ambient atmosphere). The oxygen concentration in the chamber was monitored and controlled to 1% [O$_2$] with PA-10A paramagnetic O$_2$ analyzer (Stable Systems International Inc.).

2.3 Drug Administration

In hypoxia-reoxygenation experiments, non-selective NOS inhibitor N$^G$-nitro-L-arginine methyl ester (L-NNAME, 100 μmol/L, Sigma), superoxide dismutase/catalase (SOD/catalase, 100U/ml each, sigma) and peroxynitrite decomposition catalyst 5,10,15,20-tetrakis(N-methyl-4'-pyridyl)porphinato iron (III) chloride (FeTMPyP, 5 μmol/L, Sigma) were respectively added into the culture medium prior to hypoxia-reoxygenation experiments.

In a separate designed oxidative stress experiment, the cultured cardiomyocytes were challenged with exogenous O$_2^-$ by incubating with xanthine (50, 100 μmol/L, Sigma) and xanthine oxidase (0.025, 0.05 U/ml, Sigma), H$_2$O$_2$ (0.005%, 0.01%), NO donor intramolecular salt 1-[N, N-di-(2-aminoethyl) amino] diazen-1-ium-1, 2-dioolate (DETA/NO, 5, 10 μmol/L, Sigma) or peroxynitrite donor 3-morpholinosydnonimine (SIN-1, 5, 10 μmol/L, Sigma).

In caveolin peptide experiments, before hypoxia-reoxygenation treatment, the
The cardiomyocytes were pretreated with different synthetic cell-permeable peptides including caveolin-1 scaffolding domain peptide (cav-1 peptide, DGIWKAFTETTVKRYFYR), caveolin-2 scaffolding domain peptide (cav-2 peptide, DKVWICSHALFEISKYVMYK), caveolin-3 scaffolding domain peptide (cav-3 peptide, DGVRVVSYTFTVSKYWCYR), the caveolin scrambled control peptide (control peptide, NRDPKHLNDDVVKIDFEDVIAEPG THSF) with Antennapedia internalization sequence (RQIKIWFQNRRMKWKK) at the concentration of 5 μmol/L. All of the peptides were synthesized and provided by Dr. Yang Wang from University of Auckland, whose current address is The University of Hong Kong.

2.4 Western blot analysis

The cardiomyocytes were harvested in lysis buffer containing 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1.0 mM sodium EDTA, 1.0 mM sodium EGTA, 1.0 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 250 mM sucrose, supplemented with protease inhibitors (10 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin). After sitting on ice for 15 min, the cells were homogenized and centrifuged at 1,000 rpm for 15 min at 4 °C. Protein concentration was determined using the Bradford assay [39]. Proteins (25 μg) were separated by 15% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.01% Tween 20, and incubated with the antibodies including anti-caveolin-1 (Santa Cruz), anti-caveolin-2 (BD Transduction), anti-caveolin-3 (Santa Cruz), anti-caspase-3 (Calbiochem), anti-CuZn-SOD (Calbiochem) and β-actin (Sigma). Blots were washed, incubated with goat anti-mouse IgG conjugated to horseradish peroxidase, developed by incubation with enhanced chemiluminescence western blot detection reagents (Amersham) and autovisualized by exposure to X-ray films.

2.5 Superoxide dismutase activity

The total activity of superoxide dismutase (SOD) was determined by BIOXYTECH SOD-525 (Oxis Research). The SOD was pretreated with 1,4,6-trimethyl-2-vinylpyridinium at 37°C for 1min that eliminated mercaptans like reduced glutathione for avoiding the interference during measurement. By adding the reagent 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene, SOD was mediated the autoxidation to produce a chromophore with maximum absorbance at 525nm. During the experiment (300sec), the 525 nm absorbance change was performed and the ratio of the autoxidation rates in the presence (Vs) and in the absence (Vc) of SOD was obtained. From the Vs/Vc ratio, the activity of SOD was calculated and expressed as SOD unit/ mg protein.

2.6 Superoxide detection

The cardiomyocytes (2 × 10⁵ cells) were plated on 24-well plates with a 12-mm glass coverslip precoated with poly-L-lysine (10 μg/mL). Hydroethidine (HEt, Polyscience), a reduced derivative of ethidium bromide, can cross cell membrane and be oxidized by superoxide (O₂⁻) specifically, yielding red fluorescent ethidium bromide that tightly binds to DNA [36-37]. To detect O₂⁻ production quantitatively, the cardiomyocytes were incubated with 1 μg/ml HEt for 15 min in dark at 37 °C. The cells were collected by pepteting without washing and then analyzed with flow cytometer (Coulter, EPICS XC) with a laser emitting excitation light at 488nm was calibrated the red (FL-2) channel to detect the emission 575 nm generated by oxidation of HEt dye.

2.7 Caspase cleavage assay

Caspase activity was measured as previously described [38]. In brief, cells were washed with PBS and suspended in 500 µl of lysis buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 10 mM EGTA and 10 mM digitonin) at 4°C for 30 min. Lysates were centrifuged at 7200g for 10 min, and the supernatant containing 50 μg of protein was incubated with caspase-3 substrate II Ac-DEVD-AMC (50 μM, Calbiochem) at 37°C for 30 min. The cleavage activity of caspase-3 was measured with Hitachi F-3010 fluorescence spectrophotometer (Ex380 nm/Em 460 nm). The protein concentration of the supernatant was determined by Bradford method [39]. The enzyme...
activity was expressed as fluorescent units per minute per milligram of protein.

2.8 Apoptotic cell death assay
Annexin V is a Ca\(^{2+}\) -dependent phospholipids binding protein with high affinity for phosphatidylserine (PS). Annexin V-staining technique is a reliable method to detect PS exposure on the cell surface, an early apoptotic cell death [40], and is therefore suitable to detect early apoptotic cells. When annexin V is simultaneously applied with propidium iodide (PI), it can differentiate necrotic cells from apoptotic cells. Double staining for Annexin-V/FLUOS/PI was performed as our previous described [41]. In brief, after hypoxia-reoxygenation treatment, the cardiomyocytes (1x10^6 cells/ml) were washed with PBS and resuspended in binding buffer (10 mmol/l HEPES/NaOH, pH 7.4, 140 mmol/l NaCl, 2.5 mmol/l CaCl\(_2\)). Annexin V-FLUOS (Boehringer Mannheim) and PI (Sigma) were respectively added to a final concentration of 1 \(\mu\)g/ml in cell suspension. After incubated in the dark at 23\(^\circ\)C for 20 min, the cell populations with Annexin-V/PI staining were accounted with a flow cytometer (Coulter, EPICS XC).

2.9 Statistical Analysis
All data are expressed as Mean ± SD. Student’s unpaired t-test was used to assess the statistical significance of differences. P<0.05 was considered as statistically difference.
B

C

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3. Results

3.1 Expressions of cav-1, 2, 3 in cardiomyocytes under oxidative stress

We first investigated the expression of cav-1, -2, -3 proteins in the hypoxia-reoxygenated cardiomyocytes. The normoxic and hypoxia-reoxygenated cardiomyocyte lysates were examined by standard western blot analysis. As showed in Figure 1, the expressions of cav-1, cav-1 and cav-3 were detectable in the cardiomyocytes under normoxic condition. Exposure to 4 h hypoxia and 4 h hypoxia plus 24 h reoxygenation had no significant influence on the expressions of cav-1 and cav-2 proteins. However, the exposure to 4 h hypoxia plus 24 h reoxygenation resulted in the down-regulation of cav-3 expression in the cardiomyocytes. To address whether free radicals contribute to the down-regulation of cav-3 expression, L-NAME, SOD/CAT and FeTMPyP were added into the cultured media prior to hypoxia-reoxygenation treatment. Both SOD/CAT
and FeTMPyP prevented the down-regulation of cav-3 protein whereas L-NAME had no effect on the expression of cav-3 protein. To further identify whether the productions of \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), NO and ONOO\(^-\) can affect the expression of cav-3 in the cardiomyocytes, we designed the experiments to investigate the effects of xanthine/xanthine oxidase (X/XO), hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), DETA/NO and SIN-1 on the expression of cav-3. As showed in Figure 2, the exogenous production of \( \text{O}_2^- \) from the reaction of xanthine (50, 100 µmol/L) and xanthine oxidase (0.025, 0.05 U/ml) significantly induced the down-regulation of cav-3 expression. In the meantime, the treatments of \( \text{H}_2\text{O}_2 \) (0.005%, 0.01%) and SIN-1 (5, 10 µmol/L) also revealed to induce the down-regulation of cav-3 expression. Low concentration of NO donor DTEA-NO (5 µmol/L) had no significant effect on the expression of cav-3 protein whereas high dose (10 µmol/L) down-regulated the expression of cav-3 in the cardiomyocytes. These results indicate that cav-3 is a target protein of oxidative injury in the cardiomyocytes.

**Figure 2:** Representative immunoblot results for detecting the expression of cav-3 protein in the primary cultured neonatal rat cardiomyocytes treated with various extraneous free radicals. The cardiomyocytes were treated with exogenous \( \text{O}_2^- \) by incubating with xanthine/xanthine oxidase (X/XO), \( \text{H}_2\text{O}_2 \), NO donor intramolecular salt 1-[N, N-

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di-(2-aminoethyl) amino] diazen-1-ium-1, 2-diolate (DETA/NO) or peroxynitrite donor 3-morpholinosydnonimine (SIN-1) for 4h. A. Representative immunoblot results: a, control; b, DETA/NO 5 µmol/L (DETA/NO L); c, DETA/NO 10 µmol/L (DETA/NO H); d, X 50 µmol/L plus XO 0.025 U/ml (X/XO L); e, X 10 µmol/L plus XO 0.05 U/ml (X/XO H); f, SIN-1 5 µmol/L (SIN-1 L); g, SIN-1 10 µmol/L (SIN-1 H); h, H₂O₂ 0.005% (H₂O₂ L); i, H₂O₂ 0.01% (H₂O₂ H). B. Statistic analysis on the expression of cav-3 in the cardiomyocytes treated with DETA/NO, X/XO, SIN-1 and H₂O₂. Data are presented from 4 independent experiments (Mean±S.D.). Versus control group, * p<0.01, ** p<0.001.
Figure 3: Effects of cav-1, cav-2, cav-3 scaffolding domain peptides on the expression of CuZn-SOD protein in primary cultured neonatal rat cardiomyocytes under hypoxia-reoxygenation. The cardiomyocytes were exposed to normoxia or 4 h hypoxia plus 24 h reoxygenation. A, Representative immunoblot results for detecting the expression of CuZn-SOD protein: a, control; b, HR; c, HR+ control peptide (5 μmol/L); d, HR+cav-1 peptide (5 μmol/L); e, HR+cav-2 peptide (5 μmol/L); f, HR+cav-3 peptide (5 μmol/L). B, Statistic analysis on the expression of CuZn-SOD protein in the hypoxia-reoxygenated cardiomyocytes. Data are presented from 4 independent experiments (Mean±S.D.). Versus control group, **p<0.01; Versus HR group, ##p<0.01. C, Effects of cav-1 and cav-3 scaffolding domain peptides on SOD activities in primary cultured neonatal rat cardiomyocytes under hypoxia-reoxygenation. The cardiomyocytes were pretreated with control peptide (low dose, 5 μmol/L, middle dose, 10 μmol/L, high dose, 20 μmol/L), cav-1 peptide (low dose, 5 μmol/L, middle dose, 10 μmol/L, high dose, 20 μmol/L) or cav-3 peptide (low dose, 5 μmol/L, middle dose, 10 μmol/L, high dose, 20 μmol/L) at 30 min before subjected to 4 h hypoxia followed by 24 reoxygenation treatment. Data are presented from 4 independent experiments (Mean±S.D.). Versus control group, ** p<0.01; Versus HR group, ##p<0.01.

3.2 Down-regulation of cav-3 is associated with the aggravated oxidative injury in hypoxia-reoxygenated cardiomyocytes

We next addressed the relationship between the down-regulation of cav-3 and oxidative stress in the cardiomyocytes. We investigated the effects of cav-1, cav-2 and cav-3 scaffolding domain peptides on the expression of CuZn-SOD protein in the hypoxia and reoxygenated cardiomyocytes. As showed in Figure 3, the expression of CuZn-SOD protein and the activity of total SOD were down-regulated in the cardiomyocytes after exposed to 4 h hypoxia followed by 24 h reoxygenation. Interestingly, both the expression level of CuZn-SOD and activities of total SOD were preserved by pretreatment of cav-3 scaffolding domain peptide but not by cav-1 and cav-2 scaffolding domain peptides statistically. In addition, the SOD activity was restored by cav-3 scaffolding domain peptide in a dose-dependent manner (Figure 4). We then detected O$_2^-$ production in the cardiomyocytes quantitatively by using HEt-staining flow cytometry. As showed in Figure 4, both cav-1 and cav-3 scaffolding domain peptides significantly reduced the production of O$_2^-$. The cav-3 scaffolding domain peptide revealed better effect than the cav-1 scaffolding domain peptide. These results suggest that cav-3 peptide could protect the cardiomyocytes from oxidative injury via
preserving SOD activity and subsequently decreasing $O_2^-$ production in the hypoxia-reoxygenated cardiomyocytes.

**Figure 4:** Effects of cav-1, cav-2, cav-3 scaffolding domain peptides on $O_2^-$ production in primary cultured neonatal rat cardiomyocytes under hypoxia-reoxygenation. The cardiomyocytes were subjected to normoxia, 4 h hypoxia or 4 h hypoxia/24 h reoxygenation with and without caveolin peptides treatment. Prior to hypoxia, cav-1, cav-2, cav-3 peptides (5 µmol/L) were added into the cultured medium. HEt (10 µmol/L) was added into the medium for the detection of $O_2^-$. The fluorescence produced from the oxidation of HEt by $O_2^-$ was accounted by FACS analysis. A. Representative FACS results of $O_2^-$ production. B. Statistic analysis on the $O_2^-$ level. Data are presented as the HEt positive staining rates (%) from 3 independent experiments (Mean±S.D.). Versus control group, **p<0.01; Versus hypoxia group, @@p<0.01; Versus HR group, ##p<0.01.
3.3 Effects of cav-1 and cav-3 scaffolding domain peptides on the activity of caspase 3 and apoptotic cell death in hypoxia-reoxygenated cardiomyocytes

We next observed the effects of cav-1, cav-2 and cav-3 scaffolding domain peptides on regulating caspases pathway in the hypoxia-reoxygenated cardiomyocytes. Western blot analysis was used to detect the expression of caspase 3 protein. Ac-DEVD-AMC is a fluorogenic, tetrapeptide substrate that is cleaved by caspase-3 and was used for the determination of caspase-3 activity. As showed in Figure 5, the expression and activity of caspase-3 were up-regulated by the treatment of 4 h hypoxia and the increase of caspase-3 became pronounced after 4 h hypoxia plus 24 h reoxygenation. To observe the roles played by caveolins in regulating caspase-3 activation, the cav-1 and cav-3 scaffolding domain peptides were added into the cultured medium prior to the treatment of hypoxia-reoxygenation. Both cav-1 and cav-3 scaffolding domain peptides inhibited significantly the expressions and activity of caspase 3. The results indicate that both caveolin-1 and caveolin-3 could inhibit the caspase 3 apoptotic pathway.

A

![Caspase-3 and β-actin Western blots](image)

B

![Graph of relative level of caspase 3](image)
We finally detected apoptotic cell death with annexin V/PI dual staining flow cytometry. Annexin V can specifically stain with PS to detect the PS externalization, an early characteristics of apoptotic cell death. After loss of membrane integrity, PI can enter the cells and intercalate into DNA. Early apoptotic cells can be identified as the Annexin V<sup>+</sup>/PI<sup>+</sup> cells whereas the cells at late phase of apoptosis characteristically showed as Annexin V<sup>+</sup>/PI<sup>−</sup> cells. As showed in Figure 6, exposure to 4 h hypoxia induced both early and late apoptosis, showing Annexin V<sup>+</sup>/PI<sup>−</sup> cells and Annexin V<sup>−</sup>/PI<sup>+</sup> cells respectively, and the treatment of 4 h hypoxia plus 24 h reoxygenation remarkably increased the rates of early and late apoptosis. Both cav-1 and cav-3 scaffolding domain peptides significantly decreased the rates of early and late apoptotic cell death (Annexin V<sup>−</sup>/PI<sup>−</sup> cells and Annexin V<sup>−</sup>/PI<sup>+</sup> cells), but cav-3 scaffolding domain peptide showed better effects than cav-1 on the inhibition of late apoptotic cells. These results indicate that both cav-1 and cav-3 can protect the cardiomyocytes from hypoxia-reoxygenation injury.
Figure 6: Effects of cav-1, cav-2 cav-3 scaffolding domain peptides on apoptotic cell death in primary cultured neonatal rat cardiomyocytes under hypoxia-reoxygenation. The cardiomyocytes were subjected to normoxia, 4 h hypoxia or 4 h hypoxia/24 h reoxygenation with and without caveolin peptides treatment. Apoptotic cell death was quantified by FACS analysis after being stained with Annexin V and propidine iodide (PI). A. Representative FACS results of apoptotic cell death in the cardiomyocytes. B. Statistic analysis on early apoptosis (Annexin V<sup>+</sup>/PI<sup>-</sup>, phase 1) and late apoptosis (Annexin V<sup>+</sup>/PI<sup>+</sup>, phase 2). Data are presented as the positive staining rates (%) from 3 independent experiments (Mean±S.D.). Versus control group, **p<0.01; Versus hypoxia group, @@p<0.01; Versus HR group, ##p<0.01.
4. Discussion

This study provides an evidence for the first time to demonstrate that (1) cav-3 is a target protein of free radicals in cardiomyocyte; (2) cav-3 scaffolding domain peptide can ameliorate oxidative damage in the hypoxia-reoxygenated cardiomyocytes via the up-regulation of CuZn-SOD protein, the increase of SOD activity and inhibiting O$_2^-$ production and prevention of the activation of caspase-3; (3) cav-1 scaffolding domain peptide can also ameliorate oxidative damage in the hypoxia-reoxygenated cardiomyocytes without affecting SOD expression and activity.

In our results, the expression of cav-3 protein was down-regulated in the hypoxia-reoxygenated cardiomyocytes and the down-regulation of cav-3 was prevented by SOD/catalase but not L-NAME. X/XO is a classic chemical reaction system for generating O$_2^-$. Treatments of X/XO and H$_2$O$_2$ had similar effects to hypoxia-reoxygenation. High concentration of DETA/NO also revealed to down-regulate the expression of cav-3 protein of free radicals in cardiomyocyte apoptosis in myocardial ischemia-reperfusion injury. ONOO$^-$ exerts potent proapoptotic effects in cardiomyocytes in vitro and in the myocardium in vivo, characterized by the activation of caspase-3 and the cleavage of nuclear enzyme poly(ADP-ribose) polymerase (PARP). Peroxynitrite decomposition catalysts were reported to reduce myocardial infarct size and cardiomyocyte apoptosis in myocardial ischemia-reperfusion injury [42,43]. The peroxynitrite decomposition catalyst can isomerize ONOO$^-$ to nitrate and decrease its decomposition to highly reactive intermediates [44]. FeTMPyP treatment preserved the level of cav-3 protein in the hypoxia-reoxygenated cardiomyocytes, whereas ONOO$^-$ donor SIN-1 led to down-regulate the expression of cav-3 in the cardiomyocytes. The results indicate that ONOO$^-$ contributes to the down-regulation of the expression of caveolin-3 protein in the hypoxia-reoxygenated cardiomyocytes. Therefore, we conclude that caveolin-3 is the target protein of free radicals in the cardiomyocytes under hypoxia-reoxygenation injury.

There is an avenue to note that cav-1, cav-2 and cav-3 scaffolding domain peptides showed different antioxidant and cardioprotective effects. Although all subtypes of caveolins presented in the cardiomyocytes, the hypoxia-reoxygenation treatment didn’t affect the expression of cav-1 and cav-2. Treatment of cav-2 scaffolding domain peptide had no effects on the production of O$_2^-$ and the rates of apoptotic cell death. Thus, we focused on the studies of cav-1 and cav-3. Cav-3 scaffolding domain peptides significantly up-regulated the expression of CuZn-SOD, preserved SOD activity and decreased O$_2^-$ production in the hypoxia-reoxygenated cardiomyocytes. Although cav-1 scaffolding domain peptide had no effects on the expression and activity of SOD, the cav-1 peptide reduced the level of O$_2^-$ production and attenuated hypoxia-reoxygenation-induced apoptotic cell death. The results indicate that other mechanisms might contribute to the cav-1’s antioxidant and cardioprotective effects. Previous study suggests that cav-1 attenuated PMN-induced post-ischemia-reperfused cardiac contractile dysfunction via promoting release of NO [34]. Cav-1 and cav-3 interact with different apoptosis-related signals such as proapoptotic p38MAPKα and anti-apoptotic p38MAPKβ respectively, and such interaction functions as a molecular switch for the conversion of cell death signal into precondition-induced survival signal during myocardial ischemia-reperfusion injury [13]. Exposure of PS at the external leaflet of plasma membrane is probably the most prominent feature of the collapse of transbilayer asymmetry in mammalian cells. As an early apoptotic signal, the externalization of PS was found in ischemia-reperfused cardiomyocytes [45]. Apoptotic cells have the affinity for annexin V to PS [46-47]. Both cav-1 and cav-3 scaffolding domain peptides attenuated remarkably the rates of apoptotic cell death in the hypoxia-reoxygenated cardiomyocytes. Thus, it is necessary to further study the cardioprotective mechanisms of cav-1 and cav-3.

Caspase pathway is one of the critical apoptotic signal pathways in myocardial ischemia-reperfusion injury [31-32]. Active oxygen and nitrogen species like O$_2^-$, H$_2$O$_2$, NO, and ONOO$^-$.
are critical mediators in triggering apoptotic cell death via activating caspases pathways during myocardial ischemia-reperfusion injury [48-50]. Caspase-3 has been found to co-localize with caveolin-3 in the plasma membrane of the cardiac endothelial cells [33]. We found that the activation of caspase-3 was concordant with the down-regulation of cav-3 protein in the hypoxia-reoxygenated cardiomyocytes. Treatment of cav-3 scaffolding domain peptide inhibited the expression and the activity of caspase-3 in a dose-dependent way. Over-expression of CuZn-SOD was previously proved to inhibit the activations of caspase-3, caspase-8 and Fas expression and attenuate both apoptosis and the inflammatory response during ischemia-reperfusion injury [48]. Thus, the inhibitive effects of the cav-3 peptide on caspase-3 pro-apoptotic pathway could be, at lest in part, attributed to the enhanced CuZn-SOD activity and subsequently improving the scavenging effects on $O_2^-$. Interestingly, although cav-1 scaffolding domain peptide had no effects on the expression and activities of CuZn-SOD, the cav-1 peptide also reduced the activations of caspase-3 and inhibited apoptosis in the hypoxia-reoxygenated cardiomyocytes, indicating other unknown mechanisms might involve in the process. Further investigations are necessary to understand the cardioprotective mechanisms of cav-1 and cav-3.

Taken together, we can draw following conclusions: (1) Cav-3 plays an important role in preventing ischemia-reperfusion-induced cardiac apoptosis via enhancing antioxidant capacities and preventing caspase-3 activation; (2) Down-regulation of caveolin-3 induced by free radicals could be one of the pathways in oxidative damage in the cardiomyocytes exposed to hypoxia and reoxygenation.

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References


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