Silibinin Protects Against Isoproterenol-Induced Rat Cardiac Myocyte Injury Through Mitochondrial Pathway After Up-regulation of SIRT1

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Abstract. Terminally differentiated adult injured cardiac myocytes have been used for various animal models of heart failure. It has recently been shown that isoproterenol induces injury in rat neonatal cardiac myocytes via a β-adrenergic pathway, suggesting that it might be one of the factors involved in myocardial injury in heart failure in vivo. In the study, silibinin, a plant flavanoid from milk thistle was first evaluated for its protective effect against β-adrenergic agonist isoproterenol-induced injury in cultured rat neonatal cardiac myocytes. The viability, activation of lactate dehydrogenase (LDH), and content of maleic dialdehyde (MDA) were chosen for measuring the degree of cardiac myocytes injury. As a result, silibinin protected isoproterenol-treated rat cardiac myocytes from death and significantly decreased LDH release and MDA production. Silibinin increased superoxide dismutase activity, decreased [Ca²⁺], and increased mitochondrial membrane potential (ΔΨᵢ). Furthermore, the release of pro-apoptotic cytochrome c from mitochondria was reduced by silibinin. Silibinin increased the expression of anti-apoptotic Bcl-2 family protein Bcl-2, and up-regulation of SIRT1 inhibited the translocation of Bax from cytoplasm to mitochondria, which caused mitochondrial dysfunction and cell injury. These results demonstrate that silibinin protects against isoproterenol-induced cardiac myocytes injury through resuming mitochondrial function and regulating the expression of SIRT1 and Bcl-2 family members.

Keywords: silibinin, cardiac myocyte (rat), injury, mitochondrial membrane potential, SIRT1

Introduction

Accumulating evidence suggests that terminally differentiated adult cardiac myocytes undergo injury in various animal models of heart failure (1, 2). Because adult muscle cells have lost their proliferative capacity, the losses of viable cardiac myocytes due to injury result in a further decrease in cardiac function (3). Thus, identification of the signaling pathways that mediate cardiac myocyte cell death and survival is crucial to the elucidation of the molecular basis of cardiac muscle failure.

Norepinephrine is one of the injury inducing factors and the elevation of norepinephrine in plasma closely correlates with the severity and poor prognosis of heart failure (4). It has been recently shown that norepinephrine can induce apoptosis in cultured neonatal cardiac myocytes via a β-adrenergic pathway (5, 6), suggesting that it might be one of the factors involved in myocardial cell injury in heart failure in vivo.

Silibinin (Fig. 1A) is isolated from the seeds of Silybum marianum, a tall herb with prickly leaves and a milky sap. It has been clinically used as an antihepatotoxic agent for many liver diseases (7, 8). Recent reports...
B Zhou et al have shown that silibinin also has therapeutic aspects to reduce biliary cholesterol levels, intervene in hormone refractory human prostate cancer, and decrease the nephritic effects of chemical injury (9). In our preliminary study, we found that silibinin protected human malignant melanoma cells (A375-S2 cells) and human keratinocyte cell line (HaCaT cells) against UV irradiation, resulting in a reduced apoptotic ratio.

The starting point of our study was to search for compounds in Chinese herbal medicines that could protect against cardiac myocyte injury. It has been reported that isoproterenol induces apoptosis of cardiac myocytes in vivo and in vitro through β-adrenergic receptors (10–12). Thus, this study was conducted to determine the effect of silibinin on the β-adrenergic agonist isoproterenol stimulation-induced injury of cultured rat neonatal cardiac myocytes.

The heart is an organ that requires a constant high energy supply. The heart pump function quickly fails when ATP is not efficiently synthesized through mitochondrial oxidative phosphorylation. These deficiencies in the mitochondria are associated with the development of heart failure (13, 14). It has been implicated that mitochondrial function is regulated by Bcl-2 family proteins (15). This regulation is mainly in two important aspects: mitochondrial permeability transition pore (PTP) opening and the release of apoptogenic proteins including cytochrome c from mitochondria into cytosol (16, 17). Here we tried to identify the mitochondrial function and to examine quantitative alterations among Bcl-2, Bcl-XL, and Bax proteins and cytochrome c expression in isoproterenol stimulation-induced injury of cultured rat neonatal cardiac myocytes after silibinin treatment.

**Materials and Methods**

**Chemicals**

Silibinin (Lot: 0856-9902) was obtained from the Beijing Institute of Biologic Products (Beijing, China). The purity of silibinin was confirmed by HPLC to be higher than 99% as shown in Fig. 1B. Silibinin was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution. The DMSO concentration was kept below 0.1% in all the cell cultures and did not exert any detectable effect on cell growth or cell death.

Proteinase K, RNase A, dithiothreitol (DTT), 3-(4,5-dimetrylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3,3-diaminobenzidine tetrahydrochloride (DAB), isoproterenol, collagenase II, and rhodamine 123 (Rh-123) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Tianjin TBD (Tianjin, China). Dulbecco’s modified eagle medium (DMEM) was from Hyclone (Logan, UT, USA). Murine polyclonal antibodies against Bcl-XL and cytochrome c; rabbit polyclonal antibodies against Bax, Bcl-2, and SIRT1; and horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Cell culture**

Primary ventricular cardiac myocytes were prepared as previously described (18). Briefly, hearts from one- to two-day-old Sprague-Dawley rats were removed, the ventricles were pooled, and the ventricular cells were dispersed by digestion with collagenase II. The cells were preplated for 1 h to enrich the culture with myocytes (90% to 95% of cells after this step). Then the cells were cultured in media consisting of high glucose DMEM, 2 mM L-glutamine (GIBCO, Grand Island, NY,
USA), penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% FBS at 37°C, 5% CO₂ in a humidified atmosphere.

**Observation of morphologic changes**

Rat cardiac myocytes (5 × 10³) in high glucose DMEM containing 10% FBS were seeded in 6-well culture plates and cultured for 48 h. Silibinin was added 1 h before 10 µM isoproterenol treatment. The cellular morphologic changes were observed using phase contrast microscopy (Leica, Nussloch, Germany) at 48 h.

**Cell growth assay**

The cytotoxic effect of isoproterenol on cardiac myocytes was measured using the MTT assay as described (19). The cells were dispensed in 96-well flat-bottomed microtiter plates (NUNC, Roskilde, Denmark) at the density of 5 × 10⁴ cells/well. After 48 h incubation, they were treated with isoproterenol (10 µM) and/or various concentrations of silibinin for 48 h. The viability was calculated as follows:

\[
\text{Viability (§)} = \left( \frac{A_{490,\text{sample}} - A_{490,\text{blank}}}{A_{490,\text{control}} - A_{490,\text{blank}}} \right) \times 100
\]

**Maleic dialdehyde (MDA) assay**

A 0.2-ml aliquot of the supernatant and 1 ml of 20% trichloroacetic acid were transferred into the test tubes. The solutions were mixed well and then allowed to stand for 20 min. After addition of 2 ml 0.1 M HCl and 0.67% thiobarbituric acid, the solutions were allowed to stand in a water bath for 1 h at 95°C and then centrifuged at 250 × g at 4°C for 10 min. The absorbance of the supernatant was measured by using a spectrophotometer (UV-2201; Shimadzu, Kyoto) at a wavelength of 532 nm against the reagent blank.

**Lactate dehydrogenase (LDH) activity-based cytotoxicity assay**

LDH activity was measured as the LDH content released in the culture medium. The cells were incubated with various concentrations of silibinin for 1 h prior to isoproterenol (10 µM) treatment followed by incubation for 48 h. The substrate reaction buffer of LDH (0.5 mM l(+)-lactic acid, 0.66 mM INT, 0.28 mM PMS, and 1.3 mM NAD⁺ in pH 8.2 Tris-HCl) was added to collected culture medium. The absorbance value (A) at 490 nm of reaction for 1 and 5 min were assayed.

**Superoxide dismutase (SOD) assay**

WST working solution [2-(4-Iodophenyl)-3-(4-nitroph enyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] and enzyme working solution (the xanthine oxidase concentration was controlled so that the rate of O.D. change was 0.025 dA/min) were added to each sample and then mixed thoroughly. Cells were incubated at 37°C for 20 min. The absorbance was determined at 540 nm wavelength.

**[Ca²⁺]ᵢ assay**

At 30 min after addition of 0.5 ml of the Flura 2-AM (Molecular, St. Louis, MO, USA) working solution to the culture cells, the cells were harvested and washed twice in cold HBSS. Fluorescent calcium ions were detected after cells were incubated in the HEPES (pH 7.4) for 1 h. The fluorescence intensity of the supernatant was measured in a fluorescence spectrophotometer (model 650-60; Hitachi, Tokyo) at excitation wavelength of 360 nm.

**Determination of mitochondrial membrane potential (ΔΨ)**

To determine the change of ΔΨ in cardiac myocytes in various designated treatments, flow cytometry was applied using Rh-123 staining (20). Briefly, after treatment with or without silibinin, the cells were washed twice with cold PBS and resuspended in 1 × binding buffer at a concentration of 1 × 10⁶ cells/ml. Rh-123 at 5 µM was added to 100 µl of cell suspension. The tubes were vortexed gently and incubated at 37°C in the dark for 15 min. Flow cytometric analysis was carried out by FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). The results were analyzed by using Cell Quest software (Becton Dickinson).

**Western blot analysis**

After incubation for 48 h, both adherent and floating cardiac myocytes were collected. Western blot analysis was carried out as previously described (21) with some modification. The cells were lysed on ice in lysis buffer [50 mM HEPES (pH 7.4), 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF)], supplemented with protease inhibitors (100 µg/ml aprotinin, 10 µg/ml leupeptin, 100 µg/ml pepstatin) for 1 h. Protein concentration was determined by the Folin assay. The lysate was centrifuged at 16,000 × g at 4°C for 10 min, equal amounts of total proteins were mixed in 2 × loading buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% 2-mercaptoethanol, 10% glycerol, and 0.002% bromphenol blue], boiled 5 min, and run on a 12% or 15% SDS-polyacrylamide gel electrophoresis. Proteins were electrotransferred onto nitrocellulose membranes and detected with antibodies against Bcl-2, Bax, Bcl-XL, cytochrome c, SIRT1, and β-actin, followed by addition
of horseradish peroxidase (HRP)-conjugated secondary antibody and DAB as the HRP substrate.

**Preparation of mitochondria and cytosolic extracts**
After treatment with or without silibinin, the cells were collected by centrifugation at 200 × g at 4°C for 5 min and then washed twice with ice-cold PBS. The cell pellets were resuspended in ice-cold homogenizing buffer, including 250 nM sucrose, 20 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 1 µg/ml aprotinin, and 1 µg/ml leupeptin. After homogenization (40 strokes), the homogenates were centrifuged at 4,200 × g at 4°C for 30 min. The supernatants and the pellets were stored at −70°C until analysis by gel electrophoresis. The supernatant was used as the cytosol fraction, and the pellet was resolved in lysis buffer as the membrane fraction (22).

**Statistical analysis of the data**
All data represent at least three independent experiments and are expressed as the mean ± S.D., unless otherwise indicated. Statistical comparisons were made by Student’s t-test, P-values of less than 0.05 were considered to represent a statistically significant difference.

**Results**

**Morphologic changes of cardiac myocytes treated with silibinin and isoproterenol**
When rat cardiac myocytes were treated with 10 µM isoproterenol for 48 h with or without silibinin pre-culture, remarkable morphological changes were observed compared with the control group (Fig. 2). By 48 h, the majority of cardiac myocytes treated with isoproterenol had become round (Fig. 2B). The cells in the control group, silibinin (0.5 mM)-pre-treated and silibinin (0.5 mM)-treated alone groups did not show these injury features (Fig. 2: A, C and D).

**Inhibitory effect of silibinin on β-adrenergic agonist-induced death in cardiac myocytes**
Cardiac myocytes were treated with silibinin (0 – 0.7 mM) alone or isoproterenol (10 µM) for 48 h. The inhibition ratio against isoproterenol-treated cells ranged from 41.2 ± 1.62% to 91.8 ± 3.33% (Fig. 3A). It was found that silibinin protected isoproterenol-treated cardiac myocytes from cell death and it had no cytotoxic effects on the cells.

**Decrease the content of MDA in isoproterenol-treated by silibinin**
In unstimulated cardiac myocytes, only small amounts of MDA were detected. Treatment of unstimulated cells with silibinin (0 – 0.7 mM) for 48 h did not induce any change in MDA contents. Treatment with isoproterenol increased MDA production, whereas this increase was attenuated by silibinin with the increased concentrations (Fig. 3B).

**Inhibition of LDH release in isoproterenol-treated by silibinin**
LDH was released to the medium slightly in unstimulated or silibinin (0 – 0.7 mM) alone cases for 48 h. Isoproterenol significantly induced LDH release in cardiac myocytes. Pretreatment with silibinin markedly reduced isoproterenol-induced LDH release (Fig. 3C).

**Enhancement of SOD activities in silibinin**
Treatment with silibinin (0 – 0.7 mM) alone for 48 h did not cause any change in SOD activities. Treatment with isoproterenol induced attenuation of SOD activity, whereas the activity was recovered by silibinin (Fig. 4).

**Content of [Ca²⁺] in silibinin-treated cardiac myocytes**
As shown in Fig. 5, exposure of cardiac myocytes to
isoproterenol caused a marked increase in \([\text{Ca}^{2+}]_i\), compared with that of untreated cells, whereas the

increase of \([\text{Ca}^{2+}]_i\) was partially reversed by silibinin treatment.

Protective effect of silibinin on mitochondrial function

Mitochondrial \(\Delta \Psi\) was detected with Rho-123 staining by flow cytometric analysis. As shown in Fig. 6, the cells were incubated with 0.2 or 0.5 mM silibinin for 48 h, and mitochondrial \(\Delta \Psi\) did not change compared with untreated cells (Fig. 6: A – C). Exposure of cardiac myocytes to isoproterenol caused a marked dissipation of \(\Delta \Psi\) (Fig. 6D) compared with that of untreated cells,
whereas the loss of $\Delta\Psi$ was partially reversed by silibinin treatment (Fig. 6: E and F).

**The expression of cytochrome c and Bcl-2 family proteins in silibinin-treated cardiac myocytes**

As the central conduit of the intrinsic injury pathway, mitochondria regulate the release of apoptogenic proteins including cytochrome c. To investigate the effect of silibinin on the release of cytochrome c from the mitochondria into the cytosol, the expression of cytochrome c in the cytosol and mitochondria were examined by Western blotting analysis. The cytosolic cytochrome c was markedly augmented after isoproterenol-treatment, but this increase was obviously reversed by silibinin treatment (Fig. 7).

Isoproterenol-induced injury has been determined to significantly affect mitochondria (23), so expressions of mitochondrial proteins (Bcl-2 family members) were detected by Western blot analysis. Cardiac myocytes were incubated with isoproterenol for 48 h with or without silibinin and then Bcl-2, Bcl-X<sub>L</sub>, and Bax

![Fig. 6. The recovery of the lost mitochondrial $\Delta\Psi$ in isoproterenol-injured cardiomyocytes by silibinin. A: The cells were incubated in medium alone and detected at 48 h. B and C: The cells were incubated with 0.2 mM (B) and 0.5 mM (C) silibinin for 48 h, harvested, and stained with 5 $\mu$M Rh-123 and then detected using a flowcytometry. D: The cells were incubated in 10 $\mu$M isoproterenol alone and detected at 48 h. E and F: The cells were incubated with 0.2 mM (E) and 0.5 mM (F) silibinin for 1 h prior to the treatment with isoproterenol (10 $\mu$M) and then continued to be incubated for 48 h.](image)

![Fig. 7. Effects of silibinin on the expression of cytochrome c in isoproterenol-treated cardiomyocytes. The cells were incubated with silibinin for 1 h prior to the treatment with isoproterenol (10 $\mu$M) and then continued to be incubated for 48 h. Cytochrome c proteins both in the cytosol and the mitochondria were detected by 15% SDS-PAGE.](image)

![Fig. 8. Effects of silibinin on the expression in Bcl-2 family proteins in isoproterenol-treated cardiomyocytes. The cells were incubated with silibinin for 1 h prior to the treatment with isoproterenol (10 $\mu$M) and then continued to be incubated for 48 h. Cell lysates were separated by 12% SDS-PAGE, and the expression of Bcl-2, Bcl-X<sub>L</sub>, and Bax were detected by Western blot analysis.](image)
expression of whole cell lysate were analyzed. As shown in Fig. 8, after silibinin treatment, Bcl-XL and Bax protein expressions were not affected, and Bcl-2 expression was markedly increased. Therefore, the expression of the apoptotic antagonist (Bcl-2) was related to the mechanism of silibinin’s anti-apoptotic effect.

Enhanced expression of SIRT1 participated in the inhibitory effect of silibinin on injury of cardiac myocytes

To further investigate the effect of silibinin in response to isoproterenol-treatment, Bax protein expression in the cytosol and mitochondria were compared by Western blotting analysis. As shown in Fig. 9, the increased level of Bax in the mitochondria by isoproterenol-treated was attenuated by silibinin treatment, and Bax was relocated in the cytosol. SIRT1, the deacetylase for ku70, could block translocation of Bax to mitochondria. After incubation with 10 µM isoproterenol, the level of SIRT1 significantly decreased, whereas silibinin up-regulated its expression (Fig. 10).

Discussion

Since LDH and MDA are released when cell injury occurs, the viability of cardiac myocytes, the activation of LDH, and the content of MDA were chosen for measurement of the degree of cardiac myocyte injury in this study. The results demonstrated that silibinin could decrease the content of LDH and MDA and thus could protected cardiac myocytes from injury.

SOD is a metalloenzyme whose active center is occupied by copper and zinc and sometimes, by manganese or iron. SOD plays an extremely important role in the protection of all aerobic life-systems, including humans, against oxygen toxicity and the free radicals derived from oxygen. MDA and SOD are usually detected as the test parameters (24). In this study, it was found that treatment with silibinin could markedly increase SOD activity, which could scavenge free radicals and attenuate the production of lipoperoxide.

In our study, the beating rhythmically of silibinin-treated group was observed as the control group. Beating rate of the cells was greatly increased at the early stage of isoproterenol (10 µM) treatment, and it was weakened in a time-dependent manner. At 48 h, cell beating was not observed. However, by adding silibinin and isoproterenol simultaneously, we could observe the steady cell beating during the 48-h period. It has been reported that the beating rate greatly influences oxygen demand and thus affects cell injury (25). It was possible that isoproterenol induced an increase in beating rate at the early stage, thus it could induce lipoperoxide, such as MDA, assembled. At the later stage, excessive lipoperoxide induced cell injury, and thus the cell beating was suspended. This was consistent with our results on the content of MDA and activity of SOD. Silibinin could prevent shrinking of the isoproterenol-induced cells and
thus resume rhythmic beating, and thereby reverse the changes of MDA and SOD simultaneously. These suggested that the cardiac myocyte protective effects of silibinin might be related to preservation of the cell contraction by blocking isoproterenol-induced oxidative stresses.

The numerous ion transport pathways involved in the regulation of [Ca$^{2+}$], is critical in mediating both contractile and electrical activity of cardiac myocytes (26). In normal cardiac function, mitochondrial Ca$^{2+}$ transport pathways are thought to play a significant role in the coordination of ATP supply and demand. Elevation of [Ca$^{2+}$], has been reported to induce injury in some cell types through activation of a Ca$^{2+}$-dependent phosphatase calcineurin (27). Isoproterenol induced cardiomyocyte apoptosis through the Ca$^{2+}$-calcineurin dependent pathway (28); and in this study, silibinin reversed the increase in [Ca$^{2+}$], and thus rescued the cardiomyocytes from injury.

Recently, Ca$^{2+}$-mobilizing agents have been reported to dephosphorylate Bad by activating calcineurin and to enhance Bad heterodimerization with Bcl-Xl, leading to apoptosis through mitochondrial instability (29). Mitochondria comprise about 30% of the total intracellular volume within a mammalian cardiomyocyte (30, 31). The mitochondrion is an organelle that synthesizes ATP by oxidative phosphorylation through electron transport and contains a double membrane (32). Finally, ATP is synthesized by the transport of protons into the mitochondrial matrix through mitochondrial H+ATPase. Injury stimuli often perturb mitochondrial function by decreasing the membrane potential as well as oxygen consumption (33). The mitochondrial ∆Ψ assay showed that silibinin could reverse the dissipation of membrane potential, so it could protect mitochondrial function.

Collapse of the mitochondrial membrane potential indicates the opening of a large conductance channel known as the PTP. Several studies have suggested that PTP opening is regulated by bcl-2 family proteins (34). Increased expression of the mitochondrial stabilizing Bcl-2 proteins can be transcriptionally regulated through a stress-responsive signaling pathway, which subsequently antagonizes mitochondrial dysfunction and cytochrome c release (35). Our result demonstrated that silibinin induced cardiac myocyte expression of bcl-2 protein, which prevents permeability transition pore opening, and therefore cytochrome c release decreased. These events might be one of the mechanisms of silibinin-mediated stabilization of the mitochondrial membrane. However, the activity of bcl-2-related proteins is regulated not only by the quantity of these proteins, but also by their subcellular localization and post-translational modification. The precise mechanisms remain to be further elucidated.

SIRT1, a NAD$^+$-dependent histone deacetylase, is known to inhibit stress-induced apoptosis by deacetylating Bax and p53 (36). Under normal conditions, Bax is rendered inactive in the cytosol by its tight association with a DNA repair factor Ku70. In response to the cellular damage or stress, two lysine sites K539 and K542 of Ku70 are acetylated by the acetyltransferases and the Ku70-Bax interaction is disrupted, allowing Bax to localize to mitochondria and initiate apoptosis (37). It has been proved that SIRT1 efficiently deacetylates the two lysines of Ku70 that are critical for blocking translocation of Bax to mitochondria in calorie restriction (38). In the present study, the total Bax expression was not changed in the whole process, whereas the location of Bax was changed in the silibinin treated cells. After isoproterenol treatment, cytoplasmic Bax translocated to mitochondria, and subsequently it might be bound to Bcl-2 or Bcl-Xl, abrogating their antiapoptotic effects. Silibinin blocked the translocation of Bax by up regulation of SIRT1 and protected myocardial cells from injury. In this process, the increased level of SIRT1 might deacetylate Ku70 factor and subsequently prevents Bax from moving to the mitochondria. Therefore, SIRT1 and its upstream pathways play a key role in the inhibition of myocardial cell injury and the recovery of mitochondrial function during silibinin treatment.

Our results collectively suggest that silibinin protected against β-adrenergic injury through resuming the function of mitochondria and Bcl-2 family members and SIRT1.

In this study, we did not determine DNA fragmentation and laddering, so it is not clear that the cause of isoproterenol-induced cardiac cell death is apoptosis or not. Furthermore, it has been clarified that silibinin could affect the down-stream mitochondrial pathway and related proteins, but whether silibinin directly exerts an effect on the β-adrenergic receptor and the target of silibinin are still not clear. Consequently, further investigation is necessary to clarify the effect of silibinin and its protective mechanism.

References

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