Puerarin attenuates the daunorubicin-induced apoptosis of H9c2 cells by activating the PI3K/Akt signaling pathway via the inhibition of Ca^{2+} influx

WEIHUA LI[1], MIN LU[2], YANHONG ZHANG[3], DANQIN XIA[1], ZEBIN CHEN[4], LINHUA WANG[5], NINA YIN[3] and ZHIGANG WANG[6]

[1]Department of Cardiology, Affiliated Liyuan Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430077; [2]Department of Human Anatomy and Embryology, Medical College of Henan University of Science and Technology, Luoyang, Henan 471003; [3]Department of Anatomy, College of Basic Medicine, Hubei University of Chinese Medicine; [4]Acupuncture and Moxibustion College, Hubei University of Chinese Medicine/Hubei Provincial Collaborative Innovation Center of Preventive Treatment by Acupuncture and Moxibustion, Wuhan, Hubei 430065; [5]Department of Traditional Chinese Medicine, Hubei Rongjun Hospital, Wuhan, Hubei 430079; [6]Department of Pathogen Biology, College of Basic Medicine, Hubei University of Chinese Medicine, Wuhan, Hubei 430065, P.R. China

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Abstract. Puerarin extracted from *Radix Puerariae* is well known for its pharmacological effects, including antioxidant, anti-inflammatory, neuroprotective and cardioprotective properties. In this study, we aimed to investigate the effects of puerarin on the daunorubicin (DNR)-induced apoptosis of H9c2 cells and to elucidate the potential mechanisms involved. MTT assay and flow cytometry were performed to evaluate cell cytotoxicity and apoptosis, respectively. Western blot analysis was used to assess changes in the expression levels of proteins, including caspase-3, Akt and phosphorylated Akt (p-Akt). Ratiometric imaging of intracellular calcium (Ca^{2+}) using cells loaded with Fura-2 was also carried out. Our results revealed that puerarin pre-treatment protected the H9c2 cells against DNR-induced cytotoxicity by inhibiting cell apoptosis, which was also confirmed by the decrease in the expression of cleaved caspase-3. Additionally, p-Akt activation was associated with the suppressive effects of puerarin. Following pre-treatment with puerarin, the extracellular Ca^{2+} influx was restrained and this resulted in a reduction in the intracellular Ca^{2+} levels; these effects were abrogated by LY294002 [an inhibitor of phosphatidylinositol 3-kinase (PI3K)]. The inhibition of Ca^{2+} influx suggested that the PI3K/Akt signaling pathway participated in the suppressive effects of puerarin against H9c2 cell apoptosis. Taken togher, our findings demonstrate that puerarin attenuates the DNR-induced apoptosis of H9c2 cells by activating the PI3K/Akt signaling pathway via the inhibition of Ca^{2+} influx, suggesting that puerarin may be a potential cardioprotective agent for use in the clinical treatment of cardiomyopathy triggered by DNR.

Correspondence to: Dr Zhigang Wang, Department of Pathogen Biology, College of Basic Medicine, Hubei University of Chinese Medicine, 1 Huangjiahu West Road, Hongshan, Wuhan, Hubei 430065, P.R. China
E-mail: wangzhigang1979@hotmail.com

Dr Nina Yin, Department of Anatomy, College of Basic Medicine, Hubei University of Chinese Medicine, 1 Huangjiahu West Road, Hongshan, Wuhan, Hubei 430065, P.R. China
E-mail: yinnina-hbtcm@hotmail.com

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Introduction

Daunorubicin (DNR), a anthracycline chemotherapeutic drug, is widely used in the treatment of various types of cancer, including acute leukemia, breast cancer, soft tissue sarcoma and aggressive lymphoma (1). However, the clinical application of DNR is always restricted due to its cumulative and dose-dependent cardiotoxicity (2). Increasing evidence indicates that cardiomyocyte apoptosis may contribute to the progression of anthracycline-based cardiotoxicity (3). The mechanisms involved in DNR-induced cardiotoxicity include reactive oxygen species (ROS) production, caspase activation and cell cycle arrest, which ultimately result in cardiomyocyte apoptosis (4).

Cell apoptosis is an essential process which maintains the dynamic equilibrium under normal conditions (5). Calcium (Ca^{2+}) is a pivotal regulator of cell survival, and the sustained elevation of intracellular Ca^{2+} concentrations can activate various Ca^{2+} signals connected to apoptosis (6). A recent study reported that BN52021, a platelet activating factor antagonist, protected H9c2 cells against DNR-induced death by decreasing Ca^{2+} influx and attenuating the activation of p38 mitogen-activated protein kinase (MAPK) signaling (7). In addition, as previously demonstrated, the overexpression of calreticulin suppresses phosphatidylinositol 3-kinase (PI3K)/Akt

signaling and causes the increased apoptosis of H9c2 cells via altered Ca^{2+} homeostasis (8). Thus, it is suggested that the disruption of Ca^{2+} homeostasis can lead to caspase activation and subsequent cellular apoptosis (9).

The traditional Chinese medicine, puerarin, known as Ge-gen in Chinese, is isolated from *Radix puerariae* as an active component and possesses a series of pharmacological activities, including antioxidant, anti-osteoporotic, anti-inflammatory, anti-apoptotic, neuroprotective and cardioprotective properties (10). Puerarin can protect against apoptosis in a variety of cell types, including osteoblasts, microglia, neurons and cardiomyocytes (11-14). However, its role in the DNR-induced apoptosis of H9c2 cells and the underlying mechanisms remain unclear.

In this study, we demonstrate that puerarin attenuates H9c2 cell apoptosis induced by DNR by promoting the activation of the PI3K/Akt signaling pathway via the inhibition of Ca^{2+} influx. These findings provide new insight into the the detailed mechanisms underlying the cardioprotective effects of puerarin. Puerarin may thus have potential for use as an alternative drug in the treatment of cardiomyopathy indcued by DNR.

Materials and methods

Materials. Rat H9c2 cardiomyocytes were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). DNR was obtained from Pfizer, Inc. (New York, NY, USA). Specific inhibitors, including PD98059 (MAPK inhibitor), LY294002 (PI3K inhibitor), SB203580 (p38 MAPK inhibitor) and SP600125 (JNK inhibitor) were purchased from Sigma-Aldrich, (St. Louis, MO, USA).

H9c2 cell culture. H9c2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml streptomycin and 100 U/ml penicillin) (all from Gibco Life Technologies, Carlsbad, CA, USA) at 37˚C in a humified incubator with 5% CO_2.

Cell treatments. The H9c2 cells were treated with various concentrations of puerarin (1-100 μg/ml; Sigma-Aldrich) for 24 h. The cells were then cultured for an additional 24 h in the presence of 1 μM DNR to induce cell apoptosis. The cells were then collected for use in the follow-up experiments.

Treatment of cells with signaling pathway inhibitors. The H9c2 cells were treated with various signaling pathway inhibitors, including ERK1/2 inhibitor (PD98059, 10 μM), PI3K inhibitor (LY294002, 10 μM), p38 MAPK inhibitor (SB203580, 10 μM) and JNK inhibitor (SP600125, 10 μM), for 30 min prior to the addition of puerarin. Following 24 h of incubation, the cells were treated with 1 μM DNR for 24 h. The suppressive effects of puerarin on DNR-induced apoptosis were then evaluated.

MTT assay. The H9c2 cell suspensions ($5x10^4$ cells/ml) were seeded in 96-well plates at approximately 200 μl per well, and $1x10^4$ cells per well for MTT assay. At the end of the drug incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) working solution (0.5 mg/ml) was added, and the plates were incubated for an additional 4 h at 37˚C. Following centrifugation, the medium was replaced with dimethyl sulfoxide (DMSO) (Sigma-Aldrich). The absorbance of each well at 570 nm was measured using a plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell viability was calculated as follows: inhibitory rate of H9c2 cell viability (%) = (OD value of control group - OD value of test group) x100%/OD value of control group.

Flow cytometric detection of apoptosis. The apoptotic cells were detected using a FITC Apoptosis Detection kit (BD Pharmingen, San Diego, CA, USA). The H9c2 cells were collected, washed and resuspended in buffer containing 5 μl of Annexin V-FITC and 5 μl of propidium iodide (PI). Following incubation for 15 min in the dark at room temperature, the fluorescence of the cells was analyzed using BD FACSCanto II with Diva software (BD Biosciences, San Diego, CA, USA). The apoptotic cells contained the early apoptotic cells (Annexin V^+ and PI^-) and the late apoptotic or necrotic cells (both Annexin V^+ and PI^+).

Enzymatic assay for caspase-3. Caspase-3 activity was detected using a Caspase-3 Activity Assay kit (BestBio, Shanghai, China). The cells were harvested and extracted on ice in lysis buffer for 15 min. The lysates were centrifuged and the supernatants were assessed for protein content. Subsequently, supernatant samples containing 50 μg proteins were incubated with 90 μl reaction buffer and 10 μl caspase-3 substrate (N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide) at 37˚C for 2 h in the dark. The optical density (OD) values were measured at 405 nm using a microtiter plate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Western blot analysis. The H9c2 cells were collected, washed with ice-cold sterile phosphate-buffered saline (PBS), and lysed with RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.2% deoxycholic acid and 1 mM PMSF, 1:100 protease inhibitor cocktail). The lysates were centrifuged and supernatants were analyzed for protein concentration using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc). An equal amount of the proteins (30 μg) was separated by 10% SDS-PAGE and transferred onto a PVDF membrane. The blotted membrane was then blocked with 5% non-fat dry milk for 1 h at room temperature and incubated overnight at 4˚C with primary antibodies [1:1,000, rabbit antibodies to cleaved caspase-3 (9661), caspase-3 (9665), phosphorylated Akt (p-Akt; 4058), Akt (4685) and mouse antibodies to β-actin (3700)] (Cell Signaling Technology, Inc., Danvers, MA, USA). The immunoreactive bands were further incubated with peroxidase-conjugated anti-mouse (7076)/rabbit (7074) secondary antibodies for 1 h at room temperature, were then detected by the enhanced chemiluminescence (ECL) method and captured using a scanner (HP Scanjet 7400C; Hewlett-Packard, Palo Alto, CA, USA). The protein intensities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cytosolic Ca^{2+} measurement. Ratiometric imaging of intracellular Ca^{2+} using cells loaded with Fura-2 was carried out as previously described (15). The H9c2 cells were grown in round coverslips (30 mm) under normal culture conditions, unless otherwise indicated. Coverslips with cells were placed in a cation-safe solution composed of 107 mM NaCl, 7.2 mM KCl,

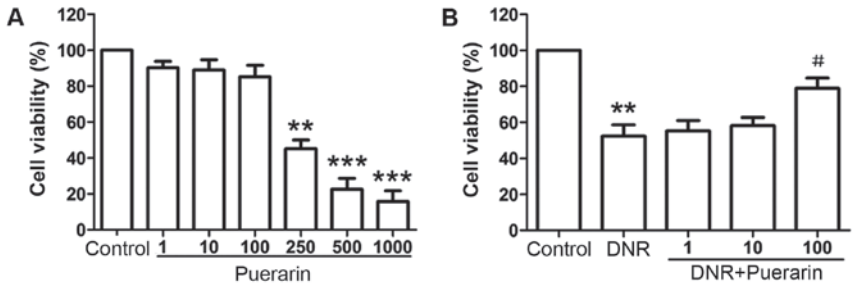

Figure 1. Protective effects of puerarin on daunorubicin (DNR)-induced cytotoxicity. H9c2 cells were seeded in 96-well plates and cultured for 2 h for stabilization. (A) H9c2 cells were treated with various concentrations of puerarin for 24 h. Cell viability was then determined by MTT assay. (B) The viability of H9c2 cells was determined by MTT assay following pre-treatment with various concentrations of puerarin (1, 10 and 100 μg/ml) for 24 h, followed by exposure to 1 μM DNR for 24 h. **P<0.01 and ***P<0.001 vs. control; $^{\#}$P<0.05 vs. DNR, n=3.

1.2 mM $MgCl_2$, 11.5 g lucose, 20 mM HEPES/NaOH (pH 7.3) and loaded with Fura-2/AM (2 μM final concentration) for 30 min at 37˚C. The cells were washed, and de-esterification was allowed for a minimum of 15 min. Ca^{2+} measurements were made using a Leica DMI 6000B fluorescence microscope controlled by the slidebook software (Intelligent Imaging Innovations, Inc., Denver, CO, USA). Fluorescence emission at 505 nm was monitored, while alternating excitation wavelengths between 340 and 380 nm at a frequency of 0.5 Hz; intracellular Ca^{2+} measurements are shown as 340/380 nm ratio obtained from groups (15) of single cells. External solutions contained: 135 mM NaCl, 5.4 mM KCl, 10 mM HEPES, 0.02 mM NaH_2PO_4, 2 mM Mg^{2+}, 10 g glucose (pH 7.4).

Statistical analysis. The results were analyzed with statistical analysis software SPSS 16.0 (SPSS, Inc., Chicago, IL, USA), and the values are represented as the means ± SD. Differences between 2 groups were evaluated using the Student's t-test. One-way analysis of variance (ANOVA) was used for multi-group comparisons with Tukey's post hoc test. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Puerarin suppresses DNR-induced cytotoxicity in H9c2 cells. When the H9c2 cells were treated with low concentrations of puerarin (1, 10 and 100 μg/ml), no significant changes in cell viability were observed apart from a slight decrease compared with the controls. However, the viability of the H9c2 cells was inhibited in a dose-dependent manner following treatment with higher concentrations of puerarin (250-1,000 μg/ml) (Fig. 1A), suggesting strong cytotoxicity to H9c2 cells treated with high concentrations of puerarin.

According to the inhibitory effects of puerarin at various concentrations on H9c2 cell viability (IC_{50}=168.5 μg/ml) (Table I), a concentration range of puerarin (1, 10 and 100 μg/ml) was selected for use in this study. As shown in Fig. 1B, treatment with DNR significantly suppressed the viability of the H9c2 cells compared with the controls (P<0.01). Importantly, puerarin at 100 μg/ml markedly suppressed DNR-induced cytotoxicity (DNR vs. puerarin, 52.38±6.22% vs. 78.98±5.65%; P<0.05).

Puerarin inhibits the DNR-induced apoptosis of H9c2 cells. By flow cytometric analysis, we observed that DNR markedly induced the apoptosis of H9c2 cells (control vs. DNR, 6.21±1.30% vs. 21.25±2.05%, P<0.001). Pre-treatment with puerarin at 100 μg/ml significantly inhibited DNR-induced apoptosis (DNR vs. puerarin, 21.25±2.05% vs. 12.28±1.52%; P<0.05) (Fig. 2A and B). In line with the Annexin V/PI staining results, the enzymatic activity of caspase-3 was also markedly decreased following treatment with puerarin prior to exposure to DNR (P<0.05) (Fig. 2C).

Table I. Effects of puerarin on the viability of H9c2 cells.

Puerarin (μg/ml)	OD 570 (mean ± SD)	Inhibition rate (%)
0	0.992±0.006	0
1	0.895±0.014	9.716±0.563
10	0.883±0.018	10.038±0.862
100	0.845±0.021	13.718±1.056
250	0.447±0.016	53.582±1.256
500	0.224±0.013	76.202±1.124
1,000	0.156±0.011	84.535±0.682

OD, optical density.

Puerarin promotes p-Akt (Ser473) activation which is suppressed by DNR. As shown in Fig. 3, puerarin markedly decreased the DNR-induced protein expression of cleaved caspase-3 (P<0.01). In addition, treatment with DNR markedly suppressed the phosphorylation of Akt (Ser473) compared with the control (P<0.01). Conversely, treatment with puerarin prior to exposure to DNR evidently promoted p-Akt (Ser473) activation (P<0.001) (Fig. 4).

Puerarin restricts Ca^{2+} influx in H9c2 cells treated with DNR. As shown in Fig. 5, DNR induced an increment in Ca^{2+} influx in a time-dependent manner. Of note, puerarin clearly restricted the DNR-induced Ca^{2+} influx (P<0.05), suggesting that the inhibition of Ca^{2+} influx was involved in the cardioprotective effects of puerarin.

Puerarin attenuates DNR-induced apoptosis via the PI3K/Akt signaling pathway. We treated the H9c2 cells with various inhibitors, including PD98059, LY294002, SB203580 and SP600125

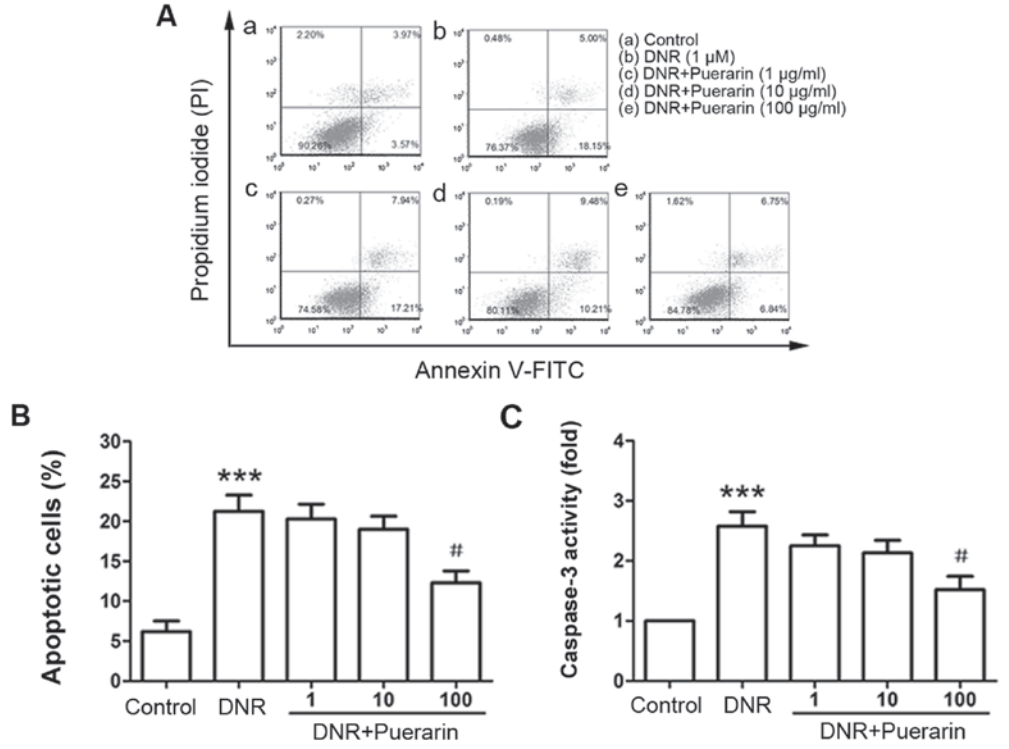

Figure 2. Puerarin attenuates daunorubicin (DNR)-induced apoptosis. H9c2 cells were treated with 1 μM DNR and cultured for 24 h following exposure to various concentrations of puerarin (1-100 μg/ml) for 24 h. (A) Cell apoptosis was detected by flow cytometric analysis following staining with Annexin V and propidium iodide (PI). A representative result is shown. (B) Combined results of 3 separate FACS analyses depicting the mean levels of apoptosis cells (Annexin V^+PI^- and Annexin V^+PI^+). (C) Enzymatic assay for caspase-3 activity in H9c2 cells. ***P<0.001 vs. control; $^{\#}$P<0.05 vs. DNR, n=3.

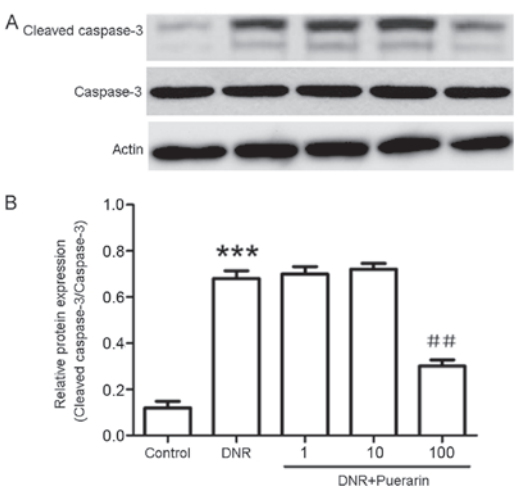

Figure 3. Puerarin suppresses caspase-3 protein expression in daunorubicin (DNR)-treated H9c2 cells. Cells were treated with 1 μM DNR and cultured for 24 h following exposure to various concentrations of puerarin (1-100 μg/ml) for 24 h. (A) Protein expression of caspase-3 was detected by western blot analysis. A representative result is shown. (B) Activated fragment of caspase-3 was assessed by semi-quantitative analysis. ***P<0.001 vs. control; $^{\#\#}$P<0.01 vs. DNR, n=3.

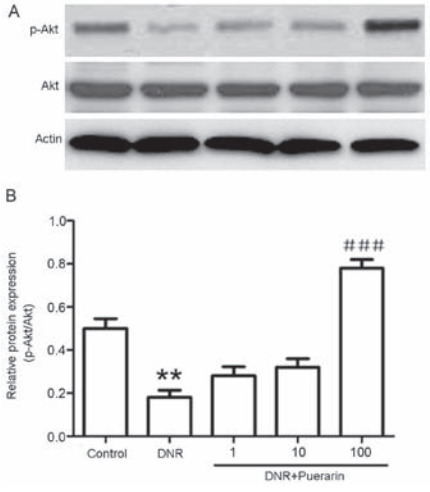

Figure 4. Puerarin increases the expression of phosphorylated-Akt (Ser473) in H9c2 cells treated with daunorubicin (DNR). H9c2 cells were treated with 1 μM DNR and cultured for 24 h following exposure to various concentrations of puerarin (1-100 μg/ml) for 24 h. (A) Protein expression levels of total Akt and p-Akt (Ser473) were detected by western blot analysis. A representative result is shown. (B) Relative protein expression (p-Akt/AKT) was assessed by semi-quantitative analysis. **P<0.01 vs. control; $^{\#\#\#}$P<0.001 vs. DNR, n=3.

prior to treatment with puerarin. As shown in Fig. 6D, pre-treatment with the PI3K inhibitor, LY294002, markedly reversed the inhibition of Ca^{2+} influx by puerarin (P<0.05); however, the ERK inhibitor (PD98059), p38 MAPK inhibitor (SB203580) and JNK inhibitor (SP600125) (Fig. 6) did not exert any significant effects.

Discussion

At present, the avoidance of the cardiotoxic side-effects of DNR without compromising its antitumor efficacy in clinical treatment is a major challenge. In this study, we found that puerarin exerted protective effects against DNR-induced cytotoxicity in H9c2 cells by suppressing apoptosis, promoting the activation

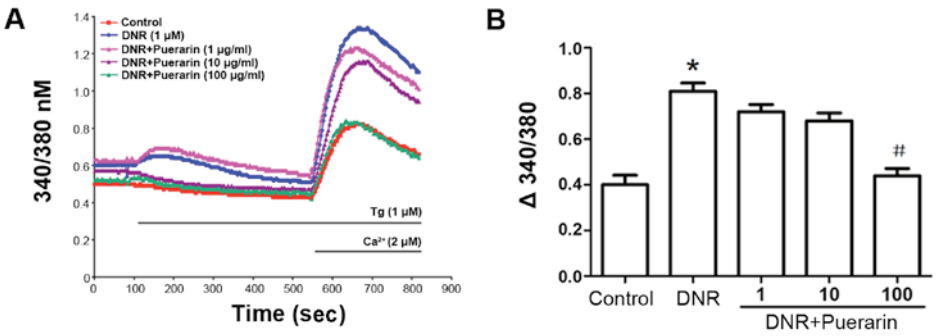

Figure 5. Inhibition of Ca^{2+} influx is involved in the cardioprotective effects of puerarin. H9c2 cells were treated with 1 μM daunorubicin (DNR) and cultured for 24 h following exposure to various concentrations of puerarin (1-100 μg/ml) for 24 h. (A) The intracellular Ca^{2+} measurements were determined as a 340/380 nm ratio obtained from different groups. A representative trace of intracellular Ca^{2+} changes is shown. (B) Involvement of inhibiting Ca^{2+} influx in the suppressive effects of puerarin on the DNR-induced apoptosis of H9c2 cells. *P<0.05 vs. control; #P<0.05 vs. DNR, n=3.

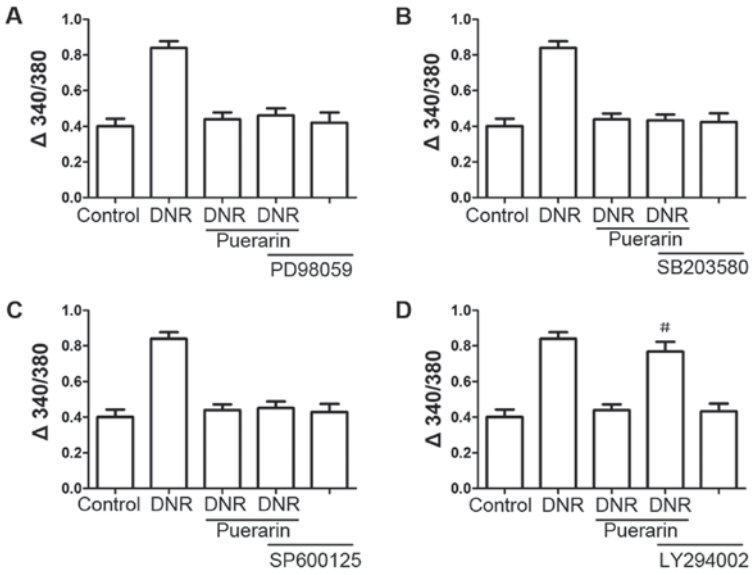

Figure 6. (A-D) The phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002, attenuates the cardioprotective effects of puerarin. H9c2 cells were treated with inhibitors, including PD98059, LY294002, SB203580 and SP600125 for 30 min prior to the addition of 100 μg/ml puerarin. Following 24 h of incubation, the cells were treated with 1 μM daunorubicin (DNR) for 24 h. The suppressive effects of puerarin on DNR-induced apoptosis were antagonized by the PI3K inhibitor (LY294002, 10 μM), but not by the ERK1/2 inhibitor (PD98059, 10 μM), p38 MAPK inhibitor (SB203580, 10 μM), or JNK inhibitor (SP600125, 10 μM). #P<0.05 vs. DNR with puerarin, n=3.

of the PI3K/Akt signaling pathway and the inhibition of caspase-3 expression. Simultaneously, the decreased Ca^{2+} influx was involved in the cardioprotective effects of puerarin; these effects were abolished by the PI3K inhibitor, LY294002. To our knowledge, this study is the first to report the direct effects of puerarin on DNR-mediated cardiotoxicity.

Accumulating evidence indicates that puerarin plays differential roles in cell apoptosis related to distinct cell types. As shown in a previous study, in human mantle Z138 cells, puerarin induced dose-dependent cytotoxicity, and exerted anti-proliferative and pro-apoptotic effects by inhibiting the PI3K/Akt/nuclear factor-κB (NF-κB) pathway (16). A recent study indicated that puerarin inhibited the proliferation and induced the apoptosis of U251 and U87 human glioblastoma cells. Treatment with puerarin suppressed the expression of p-Akt and Bcl-2 and promoted the expression of Bax and cleaved caspase-3 in cells (17). In line with the above-mentioned studies, treatment with puerarin has also been shown to result in the dose-dependent inhibition of the growth of various cancer cell lines, including HT-29 colon cancer, SMMC-7721 hepatocellular carcinoma, and HS578T, MDA-MB-231 and MCF-7 cells (18-20). Based on these findings, we speculated that puerarin exerted its antitumor effects by inhibiting proliferation and promoting apoptosis, although its pro-apoptotic characteristics are mainly observed in cancer cells. In the present study, low concentrations of puerarin (1, 10 and 100 μg/ml) exerted little cytotoxicity; however, higher concentrations of puerarin (250-1,000 μg/ml) decreased the viability of the H9c2 cells (Fig. 1A). In contrast to previous findings (18-20), puerarin at 100 μg/ml significantly attenuated DNR-induced cell apoptosis and cleaved caspase-3 expression (Figs. 2 and 3). Thus, various concentrations of puerarin thus contribute to distinct cell responses as regards apoptosis. Therefore, further investigations are required for more in depth clarifications.

It is possible that different molecular mechanisms are involved in the anti-apoptotic effects of puerarin. A previous study reported that puerarin attenuated amyloid-β (Aβ)-induced microglial apoptosis by activating the PI3K-dependent pathway in association with Akt phosphorylation, which inhibited the

activation of caspase-3 apoptotic cascade (21). The PI3K/Akt signaling pathway was proposed as an essential pathway in the prevention of cell apoptosis and plays a critical role in cell survival. Puerarin may have an ability to attenuate the progression of cardiac hypertrophy induced by pressure overload by targeting PI3K/Akt signaling (14). Puerarin pre-treatment may attenuate myocardial hypoxia/reoxygenation injury by inhibiting autophagy via the Akt signaling pathway (22). A recent study also indicated that puerarin prevented the TNF-α-induced apoptosis of PC12 cells by activating the PI3K/Akt signaling pathway (23). These studies suggested that the PI3K/Akt pathway may be involved in the biological effects of puerarin. Our results also revealed that puerarin on inhibited the DNR-induced apoptosis of H9c2 cells and promoted p-Akt activation (Fig. 4), which was associated with its cardioprotective effects.

It has been demonstrated that puerarin exerts protective effects on the cardiovascular system and nervous system, and against osteoporosis, liver injury, diabetes and inflammation *in vitro* and *in vivo* and (24 and refs therein). The cardioprotective effects of puerarin are partly attributed to its effects on Ca^{2+} in cardiomyocytes (25). Puerarin has been shown to block L-type Ca^{2+} channels in ventricular myocytes from Langendorff rat heart preparations, and to inhibit the amplitude of peak Ca^{2+} and decrease the level of Ca^{2+} flux (26). Accompanying mechanical strain, intracellular Ca^{2+} plays a role in regulating signaling through the PI3K/Akt pathway in osteoblasts (27). Cytoplasmic Ca^{2+} overload results in cytotoxicity, and an increased cytosolic Ca^{2+} concentration induces cellular apoptosis (28). In this study, we illustrated that the inhibition of Ca^{2+} influx participated in the suppressive effects of puerarin on the DNR-induced apoptosis of H9c2 cells (Fig. 5), which was abolished by the PI3K inhibitor, LY294002, suggesting that the inhibition of the Ca^{2+} influx was related to the activation of PI3K/Akt signaling (Fig. 6). Further studies are required in order to elucidate the precise mechanisms underlying the cardioprotective effects of puerarin.

In conclusion, the present study demonstrated that puerarin attenuated the DNR-induced apoptosis of H9c2 cells by activating the PI3K/Akt signaling pathway via the inhibition of Ca^{2+} influx, which contributed to decreased caspase-3 activation. These findings partly clarify the detailed mechanisms underlying the cardioprotective effects of puerarin, suggesting that puerarin may have potential for use as a cardioprotective drug in the treatment of cardiotoxicity triggered by DNR.

Acknowledgements

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