Original Paper

Puerarin Enhances Ca^{2+} Reuptake and Ca^{2+} Content of Sarcoplasmic Reticulum in Murine Embryonic Stem Cell-Derived Cardiomyocytes via Upregulation of SERCA2a

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Key Words

Puerarin • Murine embryonic stem cells • Cardiomyocytes • Sarcoplasmic Reticulum • SERCA2a

Abstract

Background/Aims: The embryonic stem cell-derived cardiomyocytes (ES-CMs) serve as potential sources for cardiac regenerative therapy. However, the immature sarcoplasmic reticulum (SR) function of ES-CMs prevents its application. In this report, we examined the effect of puerarin, an isoflavone compound, on SR function of murine ES-CMs. **Methods:** Murine ES-CMs were harvested by embryoid body-based differentiation method. Confocal calcium imaging and whole-cell patch clamps were performed to assess the function of SR. The mRNA expression levels of SR-related genes were examined by quantitative PCR. The protein expression of sarcoplasmic reticulum calcium-ATPase 2a (SERCA2a) was evaluated by immunofluorescent and western blot. **Results:** Long-term application of puerarin promotes basic properties of spontaneous calcium transient with increased amplitude, decay velocity, and decreased duration. Puerarin fails to alter $I_{\text{Ca,L}}$ but increases the Ca^{2+} content of SR. Puerarin-treated ES-CMs have intact SR Ca^{2+} cycling with more SR Ca^{2+} reuptake. Long-term application of puerarin asynchronously upregulates the mRNA and protein expression of SERCA2a, as well as the transcripts of calsequestrin and triadin in developing ES-CMs. Application of puerarin during the stage of post-cardiac differentiation upregulates dose-dependently the transcripts of SERCA2a, phospholamban and triadin which can be reversed by the inhibitors of the PI3K/Akt and MAPK/ERK signaling pathways, but shows no effect on the protein expression of SERCA2a. **Conclusion:** This study demonstrates that long-term puerarin treatment enhances Ca^{2+} reuptake and Ca^{2+} content via upregulation of SERCA2a.

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Introduction

The pluripotent stem cells, including embryonic stem (ES) cells, can differentiate into beating cardiomyocytes (CMs). ES cell-derived CMs (ES-CMs) are widely accepted as a promising resource for cell replacement therapy of cardiac disease and a powerful model for investigating the development, maturation as well as pathological remodeling of heart [1-2]. However, accumulating data suggest that current culture system produces primitive ES-CMs with undeveloped structure and contractile function [1, 3-4]. *In vivo* studies of transplantation of such primitive ES-CMs [5] have shown only partial or transient restoration of heart pumping function. Moreover, there will be a risk of non-synchronized contraction and arrhythmias from transplanting such immature CMs to host heart [6].

In adult CMs, the sarcoplasmic reticulum (SR) is the main source and depot of cytosolic Ca^{2+} . Extracellular Ca^{2+} enters into the cytosol *via* sarcolemmal L-type Ca^{2+} channels (LTCC) distributed in t-tubules during action potential, which then trigger Ca^{2+} release from the SR *via* ryanodine receptor (RyR) resulting in sliding of aligned myofilaments and ultimately the muscle contraction. In diastolic period, Ca^{2+} is pumped into the SR *via* SR calcium-ATPase (SERCA) and removed out of cytosol by sarcolemmal sodium-calcium exchanger (NCX). Such an increase and decrease cycle in cytosol $[\text{Ca}^{2+}]_i$ is known as Ca^{2+} transient [7], which determines the contractile force of CMs. In adult heart, the contraction force increases in response to an increase of the rate of stimulation during exercise or stress conditions. This phenomenon is termed the positive force-frequency relationship (FFR). During positive FFR, an increased SR Ca^{2+} content and Ca^{2+} transient amplitude result from augmented SR Ca^{2+} release and reuptake at higher rates [8]. Another contractile feature of adult CMs related to SR function is the potentiation of the first contraction after a resting period, which is mainly dependent on the Ca^{2+} remaining in the SR and termed the post-rest potentiation [9].

Early studies indicate that both sarcolemmal Ca^{2+} entry and SR Ca^{2+} release through RyR are essential for producing force of contraction in murine ES-CMs (mES-CMs) [10-11]. RyR knockout mES-CMs showed slow, weak and irregular Ca^{2+} transient, suggesting that the RyR-released Ca^{2+} would directly contribute to the spontaneous Ca^{2+} transient and following contraction in mES-CMs even at early immature developmental stage [12]. The Ca^{2+} influx *via* LTCC is more dominating in the earlier stage, although its contribution to the Ca^{2+} transient decreases with the development of RyR [13]. Nevertheless, we observed recently that mES-CMs at the late developmental stage show a negative FFR and only a minor post-rest potentiation, demonstrating an underdeveloped functional SR compared to that of both embryonic ventricular CMs at the same developmental stage and adult CMs [1]. In many cases, the contraction of human ES-CMs (hES-CMs) is dependent on trans-sarcolemmal Ca^{2+} influx, but not on the SR Ca^{2+} releases [14]. These cells represent immature Ca^{2+} handling properties, reflected by smaller peak amplitude, slower rise and decay kinetics [6, 14]. The nonfunctional SR in hES-CMs is further evidenced by the absence of post-rest potentiation and the negative FFR [14]. Some crucial Ca^{2+} handling genes and proteins, such as calsequestrin (CSQ) and phospholamban (PLB), are also absent in hES-CMs [14].

Nevertheless, it is still possible to optimize ES-CMs with enhanced capability of Ca^{2+} handling, to ensure a robust and long-term improvement in heart function after successful cell transplantation [15]. Concerning the maturation of ES-CMs with enhanced contractile functions before their clinic application, it is essential to optimize the current culture system with a safe, economic and efficient substance or approach. Up to now, several studies have examined the effects of transgenic technique [16], electrical stimulation [13], cytokines application [5] and prolonged *in vitro* culture [15] on the contractile and structural maturation of ES-CMs. The improvement of Ca^{2+} handling properties in ES-CMs has been reported by above mentioned studies. Still safer and more economical method to drive maturation of ES-CMs *in vitro* are demanded.

Puerarin, a main isoflavone glycoside in the traditional Chinese medicine radix of *Pueraria lobata* (Willd.) Ohwi, has been widely utilized in the treatment of cardiovascular diseases [17-18]. So far, anti-inflammatory [19], anti-oxidative [20] and anti-arrhythmia

[21] effects of puerarin have been proven. Puerarin also shows acute inhibitory effects on the functional ion channels, such as Na^+ channels [12], inward rectified K^+ channels [21] and LTCC [22], in isolated CMs. Accumulated studies have shown that the PI3K/Akt, JAK/STAT, JNK, ERK1/2 and MAPK signaling pathways mediate the bio-effects of puerarin on various types of cells, i.e. CMs [2, 17, 20], astrocytes [23], neurons [24], and retinal ganglion cells [10]. Our previous research found that puerarin could enhance the specialization of mES cells into ventricular-like CMs [25], inhibits the proliferation of mES-CMs [2], while improves the t-tubule development of mES-CMs [4], suggesting an ameliorated excitation-contraction coupling of these cells in the presence of puerarin. However, it is still uncertain whether puerarin has effect on the Ca^{2+} handling maturation of ES-CMs.

In the present study, we examined the hypothesis that the treatment of puerarin on mES-CMs during *in vitro* cardiac differentiation would enhance calcium handling properties. To accomplish this hypothesis, mES-CMs were harvested by embryoid body-based differentiation method with or without puerarin treatment. Confocal calcium imaging, whole-cell patch clamp, immunofluorescent, western blot and quantitative PCR were employed to compare the functional properties and the molecular expression of key proteins between the cells. We found that puerarin-treated mES-CMs have intact SR Ca^{2+} cycling with more powerful SR Ca^{2+} reuptake and greater SR Ca^{2+} load. Our results suggest that these cells might generate greater force and perhaps restore impaired cardiac function upon transplantation more efficiently.

Materials and Methods

Ethics Statement

Animal work was conducted according to relevant national and international guidelines and was approved by Hubei Science and Technology Agency (2005-50).

Culture of mES-CMs

The undifferentiated mES cell line D3 (ATCC, Manassas, VA, USA) were cultured and differentiated into spontaneously beating CMs as previously described [26]. The hanging drop method was used to induce cardiac differentiation. The differentiation medium consisted of Iscove's Modified Dulbecco's Medium, 20% fetal bovine serum (FBS), 1% non-essential amino acids, 100 units/mL penicillin-streptomycin, 2 mM L-glutamine, and 0.1 mM β -mercaptoethanol, as previously described [25]. Puerarin (100 μM , National Institutes for Food and Drug Control, Beijing, China) was applied throughout cardiac differentiation (from day 0 to day 20 of differentiation, long-term puerarin treatment) or during the stage of post-cardiac differentiation (from day 10 to day 16 of differentiation). Cells cultured with differentiation medium containing 0.05% dimethyl sulfoxide (DMSO, Sigma) were served as controls. Medium was changed every two days. All cultivation medium and reagents were purchased from Gibco if not otherwise indicated.

Isolation of mES-CMs

Spontaneous beating areas with or without long-term puerarin treatment were mechanically microdissected at day 16 of differentiation, and were dissociated into single cells by incubation in collagenase II (1 mg/mL, Roche, Mannheim, Germany) at 37°C for 30 min. Cells were then plated onto gelatin-coated glass coverslips, and cultured with culture medium without puerarin and DMSO for at least 24h for further functional experiments.

Confocal imaging

The cytosolic $[\text{Ca}^{2+}]_i$ was measured using fluo-3/AM dye, dissolved in DMSO, and confocal microscope as described previously [27]. Briefly, single mES-CMs at day 16 of differentiation were incubated with 10 μM fluo-3/AM for 30 min at 37°C and then perfused with Tyrode solution containing (mM): 140 NaCl, 5.4 KCl, 1.8 CaCl_2 , 1.0 MgCl_2 , 10.0 glucose, and 10 HEPES (pH 7.4). Spontaneous calcium transients were recorded with the line scan using an LSM 700 confocal microscopy (Carl Zeiss) and analyzed with Interactive Data Language software (IDL, Research Systems). Amplitude, maximal rise velocity ($V_{\text{max rise}}$) and maximal decay velocity ($V_{\text{max decay}}$) of calcium transients were analyzed by use Origin software (Originlab). For the effects

of ryanodine and thapsigargin, the results were expressed as percent change to baseline.

Electrophysiological recordings

The electrophysiological recordings of single mES-CMs were performed at day 16 of differentiation with or without long-term puerarin treatment. LTCC current ($I_{Ca,L}$) was recorded from a holding potential of -80 mV to test potentials using the whole-cell patch-clamp technique with an Axopatch 1D amplifier and the pClamp9.0 software (Axon Instruments). Bath solution contained (in mM) 120 NaCl, 5 KCl, 2 CaCl₂, 20 tetraethylammonium-Cl, 1 MgCl₂ and 10 HEPES (pH adjusted to 7.4 with TEA-OH). Pipettes solution contained (in mM): 120 CsCl, 3 MgCl₂, 5 MgATP, 10 EGTA and 5 HEPES (pH adjusted to 7.4 with CsOH). Pipette resistances were in the range of 2–4 MΩ. To avoid contamination by Na⁺ channels, TTX (Tetrodotoxin citrate, 15 μM) was added. A prepulse of -40 mV for 50 ms was executed to inactivate T-type Ca²⁺ current ($I_{Ca,T}$). $I_{Ca,L}$ was elicited by 300 ms pulses to test potentials (-40 to +50 mV) and then a single pulse to 0 mV with a duration of 300 ms. Experiments were performed at 37°C.

The inactivation velocity of the current can be delineated by the inactivation time constant τ , which was derived from a single exponential fitting. The fitting equation is: $y = y_0 + A(1 - \exp[-t/\tau])$, where y is the inactivation part of current trace (from peak to steady), t is time, τ is the inactivation time constant, and y_0 and A are the offset and amplitude coefficients, respectively. In this study, the time constant from the current trace with maximum activation (at -10 mV) was used for comparison. Data were analyzed by pClamp software (version 10.5).

RT-PCR and quantitative real-time PCR analysis

Total RNA was extracted using Trizol (Invitrogen, USA). RT-PCR Kit (TRT-101) was used for cDNA synthesis and DNA amplification, according to the manufacturer's instructions. The PCR program was under the following conditions: 94 °C for 2 min, 35 cycles of 30 s at 95 °C, 30 s at 52–60 °C (based on the target) and 45 s at 72 °C, 5 min at 72°C for final PCR product extension. Real-time PCR was performed in triplicate using MxPRO3000 detector (Strata gene Technology Company) with SYBR Green Real-time PCR Master Mix plus (Toyobo). The expression of GAPDH was used for internal normalization at both RT-PCR and real-time PCR. All primers were listed in Table 1. The 2^{-ΔΔCt} method was used to perform the relative quantification of PCR products.

Immunofluorescent analysis

The areas with beating cells were sectioned into small pieces and dissected into single cells. Cells were plated onto coverslips for 48 hours, washed with PBS and fixed with 4% paraformaldehyde. After permeabilization with 0.5% TritonX- 100, coverslips were blocked with 1% BSA, followed by incubation with anti-SERCA2 antibody (1:100 dilution; Abcam, UK) overnight at 4°C and with FITC Goat anti-mouse antibody (1:200 dilution; Proteintech, USA) for 1h. After two further washes in PBS, cells were counterstained with 1 μg/ml of the DNA dye 4', 6-dianidino-2-phenylindole (DAPI) for 10 min. To avoid the batch-to-batch variation, we performed immunofluorescent staining and took all images by the confocal microscope (Olympus FV1000 Viewer; Japan) at the same day. Our setup and software allow us to apply the same setting parameters to all samples we examined. The analysis was performed independently with image-pro-plus software by three operators. The expression of SERCA2a of each mES-CMs were evaluated with integrated optical density (IOD) values. IOD = area × density.

Table 1. Primers for RT-PCR and quantitative real-time PCR analysis. F: forward and R: reverse. ✱ for quantitative real-time PCR

Gene	Primer sequence (forward/reverse)	Ta (°C)	No. of cycles	Product size (bp)
NCX	F: CAGCTTCCAAAAGTGAATCGA R: GTCCCTCTCATCGACTTCCAAAA	58	35	104
PLB	F: CAGTGGCTAAGTGTGGCATAAGTGT R: TGTGAGGACCCAGTGAGCTATTTC	55	35	306
✱PLB	F: TCTCCCTACTTTTGCTTCCTG R: TATGTCTGCTGCTGATCTGCATC	55	35	167
RyR2	F: GACGGCAGAAGCCACTCACCTCGG R: CCTGCAGAGAACTGACAACCTGG	60	35	333
SERCA2a	F: TGTGTGATGTGGAGGAAATGTGTA R: TACAACCTGAAGGCATGCATTACAA	52	35	247
✱SERCA2a	F: TGGAAACAACCCGGTAAAGAGT R: CACCAAGGGGCATAATGAGCAG	55	40	113
✱CSQ	F: TGAACCTTCCCACGTACGATG R: AAACCTAATCGTGGCGGTCAAC	55	40	189
✱junctin	F: AGCATCGCAGAACCATCCAAA R: GCATCTGTCACTGCTTCACTG	55	40	84
✱Triadin	F: CGACAACCAACAGGGTGATAG R: ACCATGTGATAATCAGAGCGATG	55	40	150
✱GAPDH	F: TGACCACAGTCCATGCCATC R: GACGGACACATTGGGGGTAG	55	40	204

Western blot

The proteins were extracted from mES-CMs at day 10 and/or day 16 of differentiation after long-term or short-term puerarin treatments. Their concentrations were determined using a BCA Protein Assay Kit (BOSTER). Adult murine heart served as positive control. 25 µg of protein samples were subjected to 12% SDS-PAGE and analyzed by blotting with anti-SERCA2 antibody (1:1000 dilution; Abcam, UK).

Cell Signaling Inhibitors

The wortmannin (W, 1 µM, Sigma-Aldrich) and PD0325901 (PD, 1 µM, Selleck), specific inhibitors for the PI3K/Akt and MAPK/ERK respectively, were used to investigate PI3K/Akt and MERK/ERK signaling pathways mediated the effect of puerarin. Cells at day 16 of differentiation were pretreated with Opti-MEM for 2 h, and then treated with above-mentioned differentiation medium containing W or PD for 2 h, finally cultured in differentiation medium containing puerarin or DMSO.

Statistics

Data are expressed as mean ± SEM. The differences were tested using the two-tailed unpaired or paired *t* test if not otherwise indicated. Statistical significance was accepted when *p* < 0.05. The *r*² and *p*-values for linear correlation were determined using unweighted linear regression.

Results

Long-term application of puerarin affects basic properties of spontaneous calcium transient

Spontaneous calcium transient were recorded from single beating mES-CMs treated with or without puerarin as shown in Fig. 1a. Compared with control cells (*n* = 45), the puerarin-treated mES-CMs (*n* = 54) presented significantly higher peak amplitude (*p* < 0.05) and faster *V*_{max decay} (*p* < 0.01), while shorter decay time (*p* < 0.05) and duration (*p* < 0.05) (Table 2). There was no significant difference between the frequency and *V*_{max rise} of control cells and that of puerarin-treated cells (*p* > 0.05). Taken together, these data indicate that puerarin facilitates the occurrence of mature properties of spontaneous calcium transients in mES-CMs.

*Long-term application of puerarin fails to alter *I*_{Ca,L} but increases the *Ca*²⁺ content of SR*

In mature CMs, during calcium-induced calcium release (CICR), cytosolic *Ca*²⁺ is rapidly elevated by *Ca*²⁺ influx via LTCC and *Ca*²⁺ release from the SR via RyR. Thus, we next investigated whether a greater *Ca*²⁺ influx via LTCC accounted for the increased amplitude of spontaneous calcium transient in the puerarin-treated cells using patch clamp technique. We found that the *I*_{Ca,L} density in cells treated with (*n* = 11) and without puerarin (*n* = 7) were no significant difference (Fig. 1b and c). The inactivation time constant *τ* of *I*_{Ca,L} was 50.80 ± 8.55 in puerarin-treated cells (*n* = 11), which was comparable with that of control cells (59.16 ± 11.43, *n* = 7, *p* > 0.05). This indicates that the entry of *Ca*²⁺ via LTCC obviously is not affected by puerarin.

We next studied whether the increased amplitude of spontaneous calcium transients could be caused by a greater *Ca*²⁺ release from the SR via RyR. The effect of caffeine, an opener of RyR, on cytosolic *Ca*²⁺ was assessed. The peak amplitude of *Ca*²⁺ transient induced by caffeine (10 mM) was significantly larger in the puerarin-treated cells (*n* = 14) compared to the control cells (*n* = 19, *p* < 0.05, Fig. 1f). The *V*_{decay} in the puerarin-treated cells was significantly larger (*p* < 0.05) than that in the control cells (Fig. 1g). These results imply that puerarin enhances the *Ca*²⁺ content of SR.

Puerarin-treated mES-CMs show similar response to ryanodine, but a greater sensitivity to thapsigargin

To further assess whether puerarin affects *Ca*²⁺ release from SR and reuptake to the SR, we next compared the effects of ryanodine and thapsigargin on *Ca*²⁺ transient in mES-CMs

Table 2. The effects of puerarin on the spontaneous Ca^{2+} transients. Values are expressed as mean $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$. $V_{\text{max rise}}$, the maximum velocity of rise; T_{rise} , the rise time; $V_{\text{max decay}}$, the maximum velocity of decay; T_{decay} , the decay time

	n	Amplitude ($\Delta F/F_0$)	$V_{\text{max rise}}$ ($\Delta F/F_0/\text{s}$)	T_{rise} (ms)	$V_{\text{max decay}}$ ($\Delta F/F_0/\text{s}$)	T_{decay} (ms)	Duration (ms)	Frequency (Transients/s)
Control	45	0.83 $\pm .05$	43.95 $\pm$ 9.55	52.53 $\pm$ 3.32	9.60 $\pm$ 1.40	160.06 $\pm$ 10.50	212.57 $\pm$ 12.79	0.89 $\pm$ 0.31
Puerarin	54	1.01 $\pm 0.06^*$	57.70 $\pm$ 7.86	53.39 $\pm$ 5.56	15.00 $\pm$ 1.42*	111.63 $\pm$ 5.58**	165.03 $\pm$ 7.57**	1.01 $\pm$ 0.34

Fig. 1. Long-term application of puerarin fails to alter $I_{\text{Ca,L}}$ but increases the Ca^{2+} content of SR. (a) The representative traces of spontaneous Ca^{2+} transient in mES-CMs treated without (left) or with (right) puerarin. (b) The voltage-clamp protocol (upper) and the representative $I_{\text{Ca,L}}$ traces recorded from mES-CMs treated with (lower, right) or without (lower, left) puerarin. (c) The current-voltage curves of $I_{\text{Ca,L}}$ in mES-CMs treated with (filled circles) or without puerarin (open circles). (d and e) The representative traces of caffeine-induced Ca^{2+} transient. (f and g) Comparison of amplitude (f) and $V_{\text{max decay}}$ (g) of caffeine-induced Ca^{2+} transient. Values are expressed as mean $\pm$ SEM. $n = 7$ and 11 for control and puerarin treatment group, respectively (c). $n = 19$ and 14 for control and puerarin treatment group, respectively (f and g). *, $p < 0.05$ vs. control.

treated with or without puerarin. Ryanodine is a specific inhibitor of RyR. After application of ryanodine ($10 \mu\text{M}$) for 30 min, we found a comparable percentage of mES-CMs with or without puerarin treatment were irresponsive to ryanodine (3 out of 16 puerarin-treated cells and 4 out of 19 untreated cells, Fig. 2a). These irresponsive cells were excluded for the further analysis. We found that the ryanodine-responsive cells with or without puerarin treatment showed the comparable decreases in amplitude (41.26 ± 5.80 % in puerarin-treated cells, $n = 13$, $p < 0.05$ vs. baseline; 37.68 ± 5.39 % in untreated cells, $n = 15$, $p < 0.05$ vs. baseline) and $V_{\text{max rise}}$ (44.01 ± 12.21 % in puerarin-treated cells, $n = 13$, $p < 0.05$ vs. baseline; 37.66 ± 9.72 % in untreated cells, $n = 15$, $p < 0.05$ vs. baseline) of spontaneous Ca^{2+} transients ($p > 0.05$, puerarin-treated group vs. untreated group) (Fig. 2b and c).

Thapsigargin is the SERCA inhibitor. Interestingly, after application of thapsigargin ($0.5 \mu\text{M}$) for 15 min, we found a greater percentage of untreated cells (42 %, 8 out of 19 cells) were irresponsive to thapsigargin compared with that of puerarin-treated cells (26 %, 5 out

Fig. 2. Effects of ryanodine and thapsigargin on spontaneous Ca^{2+} transient of mES-CMs. (a) The percentages of ryanodine responsive and insensitive cells treated with ($n = 16$) or without ($n = 19$) puerarin. (b) The representative traces of spontaneous Ca^{2+} transient before and after application of ryanodine. (c) Comparison of normalized amplitude and $V_{\text{max rise}}$ of Ca^{2+} transient of ryanodine responsive cells. Dashed line presents 100 %, $n = 15$ and 13 in control cells and puerarin-treated cells, respectively. (d) The percentages of thapsigargin responsive and insensitive cells treated with ($n = 19$) or without ($n = 19$) puerarin. (e) The representative traces of spontaneous Ca^{2+} transient before and after application of thapsigargin. (f) Comparison of normalized amplitude and $V_{\text{max decay}}$ of Ca^{2+} transient of thapsigargin responsive cells. Dashed line presents 100 %, $n = 11$ and 14 in control cells and puerarin-treated cells, respectively. *, $p < 0.05$. **, $p < 0.01$ vs. dashed line.

of 19 puerarin-treated mES-CMs, Fig. 2d). Moreover, as we excluded irresponsive cells from both two groups, the rest of puerarin-treated and untreated cells showed the comparable decreases in amplitude and $V_{\text{max decay}}$ of spontaneous Ca^{2+} transient ($n = 14$ in puerarin-treated group vs. $n = 11$ in untreated group, $p > 0.05$). The peak amplitude and $V_{\text{max decay}}$ were significantly decreased by 43.39 ± 5.38 % and 61.71 ± 4.29 % ($n = 14$, $p < 0.05$) in puerarin-treated cells, and 41.39 ± 5.38 % and 55.60 ± 7.29 % ($n = 11$, $p < 0.05$) in untreated cells, respectively (Fig. 2e and f).

Collectively, the puerarin-treated mES-CMs showed the similar response to ryanodine, but were more sensitive to thapsigargin compared with control cells. The above observations, together with results of caffeine, demonstrate that puerarin enhances Ca^{2+} content of the SR and Ca^{2+} reuptake to SR, but does not affect RyR function.

Long-term application of puerarin upregulates the mRNA and protein expression of SERCA2a as well as the transcripts of CSQ and triadin (Trd) in developing mES-CMs

We next deciphered the putative molecular mechanism of the Ca^{2+} handling properties in puerarin-treated and -untreated cells by RT-PCR and quantitative real-time PCR analysis. The mRNA level of RyR2, SERCA2a, NCX, PLB, CSQ, Trd and junctin (Jn) were measured at day 10, day 16 and day 20 of differentiation. All of the mRNAs were detectable in mES-CMs (Fig. 3a). Among them, the transcript level of SERCA2a at day 10 was significantly higher in the puerarin-treated cells than that in the -untreated cells ($p < 0.05$, $n = 5$, Fig. 3b), but this difference was not observed in day 16 and day 20. However, the transcript levels of CSQ and Trd were significantly elevated in the puerarin-treated cells at day 16 and day 20 ($n = 8$ for CSQ, $n = 3$ for Trd, $p < 0.05$) (Fig. 3c and d). The protein level of SERCA2a in mES-CMs at day 16 of differentiation was detected by immunofluorescence with semi-quantitative analysis. We found that the protein level of SERCA2a was significantly elevated by applying puerarin throughout differentiation ($n = 74$ at the puerarin-treated group, $n = 69$ at the

Fig. 3. Long-term application of puerarin upregulates the transcripts of SERCA2a, PLB, Trd and the protein level of SERCA2a in developing mES-CMs. (a) Representative RT-PCR image of SERCA2a, PLB, NCX, RyR2 and CSQ. (b-e) Real-time PCR analysis of the mRNA levels of SERCA2a (b, n = 5), CSQ (c, n = 8), Trd (d, n = 5) and Jn (e, n = 5) in mES-CMs treated with or without puerarin. (f) Representative images of SERCA2a immunostaining in mES-CMs treated with (upper) or without (lower) puerarin at day 16 of differentiation. (g) Semi-quantitative analysis of fluorescent integrated optical density (IOD) values (IOD = area × density) showed SERCA2a expression in mES-CMs treated with puerarin (n = 74) and -untreated group (n = 69). (h) Images of western blot of SERCA2a at day 10 and day 16 of differentiation with or without long-term puerarin treatment. Adult mouse whole heart served as positive controls. Results are expressed as means ± SEM. *, p < 0.05. **, p < 0.01 vs. control. P, puerarin. C, control. PLB, phospholamban.

SERCA2a, sarcoplasmic reticulum Ca^{2+} -ATPase. CSQ, calsequestrin. Jn, Junction. RyR, ryanodine receptor. NCX, Na- Ca^{2+} exchanger. Trd, triadin.

untreated group, $p < 0.01$, Fig. 3f, g). Western blot experiments further confirmed that long-term puerarin treatment increased the expression of SERCA2a protein at both day 10 and day 16 of differentiation (Fig. 3h), resulting in the greater SR Ca^{2+} reuptake and greater Ca^{2+} content of SR.

Puerarin applied during the stage of post-cardiac differentiation dose-dependently affects the transcripts of SERCA2a, PLB, Trd and Jn, but has no effect on the protein expression of SERCA2a

To explore the effective time point of puerarin treatment, we next applied different dose of puerarin (50 μM , 100 μM , 200 μM) from day 10 to day 16 of differentiation (the stage of post-cardiac differentiation), and total RNA was collected at day 16 of differentiation. The transcript level of SERCA2a was up-regulated in cells treated with 100 μM and 200 μM puerarin than control group. Puerarin treatment increased dose-dependently transcript levels of PLB and Trd while decreased that of NCX. The gene expressions of Jn was down-regulated in the puerarin-treated cells than that in the -untreated cells. Besides, the expressions of RyR2 and CSQ were no remarkable difference between two groups ($p < 0.05$, n = 5, Fig. 4a).

We also detected the SERCA2a protein expression of mES-CMs at day 16 of differentiation after treatment with 100 μM puerarin during day10-16. No significant difference was observed between two groups at day16 ($p > 0.05$, n=127 at the puerarin-treated group, n=136 at the untreated group) (Fig. 4c and d).

Fig. 4. Application of puerarin during post-cardiac differentiation upregulates the transcripts of SERCA2a, PLB, Trd but not the protein level of SERCA2a in mES-CMs. The puerarin applied at post-cardiac differentiation (from day 10 to day 16 of differentiation). (a) Real-time PCR analysis of mRNA levels of SERCA2a, PLB, NCX, RyR2, CSQ, Trd and Jn (n = 5) in mES-CMs treated with or without puerarin at day 16 of differentiation. (b) Representative images of SERCA2a immunostaining in mES-CMs treated with (upper) or without (lower) puerarin at day 16 of differentiation. (c) Semi-quantitative analysis of fluorescent IOD values showed SERCA2a expression in mES-CMs treated with puerarin (n = 127) and -untreated group (n = 136). (d) Images of western blot of SERCA2a at day 16 of differentiation with or without short-term puerarin treatment. (e) Effect of wortmannin and PD0325901, the inhibitor of PI3K/Akt and MAPK/ERK pathways, on puerarin-induced upregulation of transcription level of SERCA2a, PLB and triadin at day 16 of differentiation (n = 4). Results are expressed as means $\pm$ SEM. *, p < 0.05. **, p < 0.01 vs. control. #, p < 0.05. ##, p < 0.01 vs puerarin. \$, p < 0.05. \$\$, p < 0.01 vs DMSO + W. &, p < 0.05. &&, p < 0.01 vs DMSO+PD. P, puerarin. C, control. PLB, phospholamban. SERCA2a, sarcoplasmic reticulum Ca^{2+} -ATPase. CSQ, calsequestrin. Jn, Junction. RyR, ryanodine receptor. NCX, Na- Ca^{2+} exchanger. Trd, triadin. W: wortmannin, PD: PD0325901.

Inhibitions of the PI3K/Akt and MAPK/ERK signaling pathways reverse the puerarin-induced upregulations of SERCA2a, PLB and Trd

To further elucidate the mechanism of effect of puerarin on the mES-CMs, we examined the effect of specific inhibitors for the PI3K/Akt and MAPK/ERK, W (1 μM) and PD (1 μM) respectively, on SERCA2a, PLB and Trd expressions. As shown in Fig. 4e, both W and PD effectively reversed the puerarin-induced upregulation of transcription level of SERCA2a, PLB and Trd (n = 4 for SERCA2a, p < 0.01; n = 4 for PLB, p < 0.05; n = 4 for Trd, p < 0.05). These results suggest that the PI3K/Akt and MAPK/ERK pathways involve in the effect of puerarin mentioned above.

Discussion

In this study, our major findings are: (1) Long-term application of puerarin affects basic properties of spontaneous calcium transient with increased amplitude, decay velocity, and decreased duration; (2) puerarin fails to alter $I_{\text{Ca,L}}$ but increases the Ca^{2+} content of SR; (3) puerarin-treated mES-CMs have intact SR Ca^{2+} cycling with more powerful SR Ca^{2+} reuptake; (4) long-term application of puerarin asynchronously upregulates the mRNA and protein expression of SERCA2a in developing mES-CMs; (5) puerarin applied during the stage of

post-cardiac differentiation dose-dependently upregulates the transcripts of SERCA2a, PLB and Trd which can be relieved by the inhibitors of the PI3K/Akt and MAPK/ERK signaling pathways, but has no effect on the protein expression of SERCA2a.

The Ca^{2+} transient is a key event to couple the excitation and the following contraction of CMs. The Ca^{2+} transient amplitude depends steeply on SR Ca^{2+} content, and the latter is controlled mainly by the activity of four Ca^{2+} handling proteins: LTCC, RyR, SEACA and NCX [28]. The amplitude and kinetic properties of Ca^{2+} transient are crucial for the force producing, and widely used as major indexes for the contractile maturation [13, 15].

The Ca^{2+} influx via LTCC is the first step to cause CICR in CMs. Guo et al. investigated rapid effect of puerarin on $I_{\text{Ca-L}}$ in isolated rat ventricular myocytes by the whole-cell recording and pipettes solution containing 10 mM EGTA. They reported that puerarin (2.4 mM) exerts a transient inhibitory action on $I_{\text{Ca-L}}$ in a time-dependent manner, and elevates the I-V curve of $I_{\text{Ca-L}}$. The maximum inhibitory effect of puerarin was observed within 6 min after application of puerarin, and vanished after 7 min in their study [22]. Lin et al. employed the whole-cell recording of patch-clamp technology and pipette solution with 10 mM EGTA to examine rapid effect of puerarin on $I_{\text{Ca-L}}$ in rat hippocampal neurons. However, they found that application of puerarin (30 μM) fails to affect the amplitude as well as the I-V curve of $I_{\text{Ca-L}}$ [29]. In the present study, puerarin (100 μM) was applied throughout cardiac differentiation (from day 0 to day 20 of differentiation). The electrophysiological recordings (Fig. 1) were performed in puerarin-free bath solution and pipettes solution with 10 mM EGTA. In consistence with data from Lin et al., there were no significant differences of the current density, the inactivation time constant τ , and the I-V curve of $I_{\text{Ca-L}}$ between the puerarin-treated cells and control cells, suggesting that long-term treatment of puerarin fails to alter $I_{\text{Ca-L}}$ [29].

Lundy et al. reported an enhancement of functional maturation of hES-CMs by prolonged *in vitro* culture. They found that prolonged culture results in an increase in Ca^{2+} release and reuptake rates with no change in the maximum amplitude [15]. However, data linked to the changes of Ca^{2+} handling related proteins with the functional changes were missing in their study. Liu et al. have reported a facilitated maturation of Ca^{2+} handling properties of transgenic hES-CMs with CSQ overexpression. CSQ is a high-capacity but low affinity Ca^{2+} binding protein in the SR, playing an important role in regulating excitation-contraction coupling and CICR in the heart [16, 30-31]. It serves as a major Ca^{2+} buffer that modulate RyR activity through Jn and Trd, and also as a reservoir for Ca^{2+} readily accessible for CICR [31]. In these studies, overexpression of CSQ increased Ca^{2+} transient amplitude and SR Ca^{2+} content of hES-CMs without altering LTCC current [16].

In the present study, long-term puerarin application increased the transcript levels of CSQ, Trd and Jn at day 16 and day 20 of differentiation. However, short-term application of puerarin enhanced the gene expression of Trd but not the CSQ. Moreover, we found no detectable protein expression of CSQ in both puerarin-treated group and control group (data not shown). Therefore, inconsistent with the above mentioned report, the changes of Ca^{2+} transients in our study cannot be explained by enhancing CSQ expression. One possibility is that the elevated protein expressions of Trd or Jn might account for the changes of Ca^{2+} handling maturation of these cells, because the transcript of Trd is upregulated by both long-term and short-term treatment of puerarin. However, we did not examine the protein level of either Trd or Jn in this study, it is yet unclear whether Trd and Jn protein play roles in the puerarin-induced changes of Ca^{2+} transients, and therefore further investigation is still needed.

SERCA2a, a major Ca^{2+} transporter responsible for $[\text{Ca}^{2+}]_i$ decline even at an early differentiation stage *in vivo* and *in vitro*, is one of four key Ca^{2+} handling proteins determining the SR Ca^{2+} content and the amplitude of the Ca^{2+} transient [28]. Theoretically, in the steady state, the Ca^{2+} influx on each beat must equal to the Ca^{2+} efflux. If only activity of SERCA2a is altered, both Ca^{2+} influx and efflux will be unchanged [28]. Enhancement of SERCA2a activity will accelerate SR Ca^{2+} reuptake, increase the SR Ca^{2+} content, and thence elevate the Ca^{2+} transient amplitude. In our study, long-term puerarin treatment resulted in a faster decay velocity and shorter duration of Ca^{2+} transient of mES-CMs than those of control cells,

indicating a more powerful SR Ca^{2+} reuptake. Consistently, these cells have more abundant SERCA2a expression with regular alignment at day 16 of differentiation. In patients with heart failure, the expression level and activity of SERCA2a is attenuated, contributing directly to deficient SR Ca^{2+} uptake of CMs and impaired cardiac contractile function [32]. Data have shown that a reduced SERCA2a expression decreases the amplitude and the decay rate of Ca^{2+} transients, therefore reduces SR Ca^{2+} content in mice [28, 33]. Contrarily, results from heart failure model have convincingly shown that overexpression of SERCA2a improves contractile function of the heart by increasing SR Ca^{2+} transport [34]. Recently, phase 1 and phase 2 trials have shown that an AAV1/SERCA2a gene therapy improves effectively cardiac function in advanced heart failure [32, 35].

We found that the protein level of SERCA2a at day 16 of differentiation was increased by long-term puerarin treatment with no changes of mRNA level at the same time point. Accumulated data suggested that the relation between mRNA and protein is not strictly linear, but has a complex dependence [36]. We think that different regulation mechanisms (such as synthesis and degradation rates), acting on both the synthesized mRNA and the synthesized protein of SERCA2a, affected the amount of the two molecules differentially.

PLB is a small protein, comprising 52 amino acid residues, working as a reversible inhibitor of the cardiac SERCA activity [37]. It is a critical mediator of the β -adrenergic stimulatory effects, and its phosphorylation/dephosphorylation balance determines the extent of SERCA inhibition, which in turn is associated with functional alterations of the cardiac SR and contractility [14, 37-38]. In the present study, however, we found a puerarin-induced upregulation of both SERCA2a and PLB mRNA level, which might attenuate the activity of SERCA and in turn slow down the decay velocity of Ca^{2+} transient. Nevertheless, Dolinkov et al. firstly reported that immature SR function of hES-CMs might result from the nonfunctional expression of SERCA in the absence of PLB, suggesting the importance of expression of PLB protein in developing ES-CMs [14]. Secondly, as mentioned above, the phosphorylation/dephosphorylation balance of PLB determines the activity of SERCA. In failing hearts, it has been reported that the depressed calcium cycling is associated with reduced SERCA2a expression and increased inhibition by dephosphorylated PLB [37]. Cardiac-specific overexpression of PLB inhibited SR Ca^{2+} uptake, Ca^{2+} load and contractile parameters. However, these inhibitory effects relieved by isoproterenol stimulation and phosphorylation of PLB [39]. Hence, our finding might be explained by the puerarin-induced changes in the phosphorylation/dephosphorylation balance of PLB, which in turn further enhance the SERCA activity. Deciding whether this is valid will await studies directly investigating the expression and function of PLB protein.

Additionally, recent studies identified that the inhibitor-1 (I-1) of protein phosphatase 1 (PP1), the small heat shock protein 20 (Hsp20), the HS-1 associated protein X-1 (HAX1) and the intra-luminal histidine-rich calcium binding protein (HRC) are associated with PLB and take part in controlling SR Ca^{2+} -transport [40]. These protein players interact with each other physically and directly, mediating a fine-cross talk between SR Ca^{2+} uptake, storage and release [40]. Whether puerarin also affects the expression and function of these regulatory proteins is not clear. More detailed studies are still needed to fully understand the underlying mechanism of the effect of puerarin on the functional development of SR.

Researches demonstrated that signal pathways such as PI3K/Akt and MAPK/ERK were involved in biological activities of puerarin [2, 17, 20, 23]. Consistently, we found that the upregulation of SERCA2a, PLB and Trd induced by puerarin are abolished by inhibitors of both PI3K/Akt and MAPK/ERK signaling, suggesting their roles in puerarin-mediated functional maturation of mES-CMs through SERCA2a.

Limitation of the current study is the lack of real force measurement, which could provide further evidence for the effect of puerarin on the contractile maturation of ES-CMs. Future studies would benefit from the force measurements of ES-CMs on rigid substrates, i.e. avital heart slice or tissue engineering based scaffold. The precise mechanism and the detailed downstream signaling events, which mediated the effect of puerarin in this study, are still not fully clear, and need to be further investigated.

Conclusion

Our results suggest that long-term puerarin treatment enhances Ca^{2+} reuptake and Ca^{2+} content via upregulation of SERCA2a. Our findings provide a new insight into the biological effects of puerarin on the functional maturation of ES-CMs *in vitro*, and contribute to a better understanding of the cardioprotection mechanism of puerarin in the cardiovascular diseases.

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Disclosure Statement

The authors declare no competing financial interests.

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