Puerarin inhibits NHE1 activity by interfering with the p38 pathway and attenuates mitochondrial damage induced by myocardial calcium overload in heart failure rats

<table>
<thead>
<tr>
<th><strong>Journal:</strong></th>
<th>Acta Biochimica et Biophysica Sinica</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Manuscript ID:</strong></td>
<td>ABBS-2023-314.R5</td>
</tr>
<tr>
<td><strong>Manuscript Type:</strong></td>
<td>Original Article</td>
</tr>
<tr>
<td><strong>Date Submitted by the Author:</strong></td>
<td>05-Nov-2023</td>
</tr>
</tbody>
</table>
| **Complete List of Authors:** | Pan, Guo-pin; Xinxiang Medical University  
Cui, Bao-yue; Xinxiang Medical University; Nanyang Second General Hospital  
Han, Ming-ming; Xinxiang Medical University  
Lin, Lai-biao; Xinxiang Medical University  
Li, Yin-lan; Heilongjiang University of Chinese Medicine  
Wang, Ling; Xinxiang Medical University  
Guo, Shuang; Hubei University of Science and Technology College of Computer Science and Technology  
Yin, Ya-ling; Xinxiang Medical University  
Zhan, He-qin; Xinxiang Medical University  
Li, Peng; Xinxiang Medical University; Hubei University of Science and Technology |
| **Keywords:** | Puerarin, p38 pathway, NHE1, heart failure, TGF-β, pro-inflammatory cytokines |
Original Article

Puerarin inhibits NHE1 activity by interfering with the p38 pathway and attenuates mitochondrial damage induced by myocardial calcium overload in heart failure rats

Guopin Pan\textsuperscript{1}, Baoyue Cui\textsuperscript{1,3}, Mingming Han\textsuperscript{1}, Laibiao Lin\textsuperscript{1}, Yinlan Li\textsuperscript{4}, Ling Wang\textsuperscript{1}, Shuang Guo\textsuperscript{2}, Yaling Yin\textsuperscript{1,*}, Heqin Zhan\textsuperscript{1,*}, and Peng Li\textsuperscript{1,2,*}

\textsuperscript{1} Sino-UK Joint Laboratory of Brain Function and Injury and Department of Physiology and Neurobiology, Henan International Joint Laboratory of Cardiovascular Remodeling and Drug Intervention, School of Basic Medical Sciences, College of Pharmacy, Xinxiang Medical University, Xinxiang 453003, China, \textsuperscript{2} Hubei Key Laboratory of Diabetes and Angiopathy, Hubei University of Science and Technology, Xianning 437100, China, \textsuperscript{3} Nanyang Second General Hospital, Nanyang473001, China, and \textsuperscript{4} College of Pharmacy, Heilongjiang University of Chinese Medicine, Heilongjiang 150040, China

*Correspondence address. Tel: +86-15837341636; E-mail: pengli@xxmu.edu.cn (P.L.) / Tel: +86-13781966576; E-mail: \texttt{041130@xxmu.edu.cn} (H.Z.) / Tel: +86-13663737650; E-mail: yalingyin@xxmu.edu.cn (Y.Y.)

Received: 01-Jul-2023

Accepted: 11-Sep-2023

Running title: Puerarin inhibits NHE1 activity

The Author(s) 2023. This is an open access article distributed under the terms of the Creative Commons Attribution License (\url{https://creativecommons.org/licenses/by/4.0/}).
Abstract

Previous studies have shown that puerarin plays a key role in protecting humans and animals from cardiovascular diseases. The exact mechanism of the therapeutic effect of puerarin on various cardiovascular diseases (protective effect on cardiomyocytes) is still unclear. In the present study, we identified the role of puerarin in an animal model of experimental heart failure (HF) and explored its underlying mechanisms. The HF rat model was induced by intraperitoneal injection of adriamycin (ADR), and puerarin was administered intragastrically at low, medium, and high concentrations. We demonstrate that puerarin significantly improves myocardial fibrosis and inflammatory infiltration and, as a result, improves cardiac function in ADR-induced HF rats. Mechanistically, we find for the first time that puerarin inhibits overactivated \( \text{Na}^+\text{H}^+ \) exchange isoform 1 (NHE1) in HF, which may improve HF by decreasing \( \text{Na}^+ \) and \( \text{Ca}^{2+} \) ion concentrations and attenuating mitochondrial damage caused by calcium overload; on the other hand, puerarin inhibits the activation of the p38 pathway in HF, reduces the expression of TGF-\( \beta \) and proinflammatory cytokines, and suppresses myocardial fibrosis. In conclusion, our results suggest that Puerarin is an effective drug against HF and may play a protective role in the myocardium by inhibiting the activation of p38 and its downstream NHE1.

Keywords: puerarin; p38 pathway; NHE1; heart failure; TGF-\( \beta \); proinflammatory cytokines

1. Introduction

HF, with high morbidity and mortality, is the outcome of many end-stage heart diseases that result in a heavy health burden [1, 2]. Current HF therapy focuses on symptomatic treatment or delays the progression of the disease by reducing heart rate and cardiac preload and afterload [3]. Despite significant innovations in the medical treatment of HF in recent decades, its incidence continues to increase [4], making it worthwhile to understand the underlying pathological changes in HF and to find
valuable medicines.

The prevention and cure of HF with natural products are applicable and valuable research fields because plant sources are considered to be less toxic, with fewer side effects than synthetic medicines. Puerarin (Pue) is the main bioactive ingredient isolated from the root of the wild legume *Pueraria lobata* (wild) [5] and has been widely studied due to its multiple pharmacological effects. Emerging studies over the past few decades have demonstrated the efficacy of Pue in the prevention and treatment of cardiovascular diseases (including atherosclerosis, hypertension, cardiac hypertrophy, cardiovascular complications of diabetes, myocardial infarction, and HF) [6]. The therapeutic effects of Pue on a variety of cardiovascular diseases work through multiple targets and pathways. There is increasing evidence that Pue can modulate Na\(^+\) [7], K\(^+\) [8, 9], and Ca\(^{2+}\) [10] channels and regulate the concentration of these ions in cardiomyocytes to exert its anti-cardiac injury effects. However, studies of the effects of Pue on specific ion channels have been performed by using various channel inhibitors, not by directly recording ion currents, and other regulatory mechanisms for changes in the concentrations of these ions cannot be excluded.

Na\(^+\)/H\(^+\) exchange isoform 1 (NHE1) is a ubiquitously expressed housekeeping glycoprotein that plays a key role in maintaining intracellular pH and regulating intracellular sodium and calcium concentrations through the exchange of intracellular H\(^+\) and extracellular Na\(^+\) [11-13]. Previous reports have suggested that enhanced expression and activity of NHE1 are associated with the development of different cardiac pathologies[14], including cardiomyocyte hypertrophy (CH) [15, 16] and ischemia/reperfusion (I/R) injury [17], in various experimental models. The elevated Na\(^+\) level in HF [18] may be related to the increase in Na\(^+\)/H\(^+\) exchangers. However, there are no studies on the effect of Pue on NHE1 expression in the HF myocardium. Previously, the ADR-induced rats were used for HF studies. ADR is a potent anti-neoplastic agent employed in a variety of carcinomas. However, the use of ADR has been limited due to its severe cardiotoxic side effect, which causes fatal congestive heart failure [19, 20].

In this study, we determine the pharmacological effects of Pue in an ADR-induced rat HF model and to further explore the potential cardioprotective mechanisms of Pue by studying its effect on NHE1 in the myocardium.
2. Materials and Methods

2.1 Key reagents

ADR was purchased from Duly Biotechnology (Nanjing, China); Pue was purchased from Selleck Chemicals (Houston, TX, USA). p38 MAPK antibody (mouse, Cat No. 66234-1-lg) and NHE1 antibody (mouse, Cat No. 67363-1-lg) were from Proteintech (Wuhan, China); phospho-p38 MAPK (Thr180+Tyr182) (rabbit, Cat No. ab4822), GAPDH (rabbit, Cat No. ab9485), and β-Actin (rabbit, Cat No. ab8227) were from Abcam (Cambridge, USA).

2.2 Study design

Male Sprague–Dawley rats (8 weeks old, 180–220 g) were provided by the Experimental Animal Center of Zhengzhou University (Henan Experimental Animal Center). Before the beginning of the experiment, the rats were adaptively fed for one week. All rats were kept individually in cages at a room temperature of 18–22°C with a 12 hr light/dark cycle and allowed free access to food and water. The animal model of HF was induced by ADR [21]. Briefly, rats were injected intraperitoneally with ADR (ADR group) (3 mg/kg) once a week for 6 consecutive weeks with a cumulative dose of 18 mg/kg. A 0.9% sodium chloride solution was given as a vehicle control (control group). For the in vivo study, we predetermined the group size and considered sample loss. Animals that died during the experiment were excluded from the study. The predetermined group size was retained by adding replacement animals to the affected group. Rats that received ADR injections and survived were randomly divided into four groups: ADR group, Pue low-dose treatment group (50 mg/kg, Pue-L), Pue middle-dose treatment group (100 mg/kg, Pue-M), and Pue high-dose treatment group (150 mg/kg, Pue-H) (n=10 each group). Pue was dispersed in ddH₂O to a final concentration of 37.5 mg/mL and administered via gavage per day for 4 weeks. The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal protocol was reviewed and approved by the Ethics Committee of Xinxiang Medical University (Xinxiang, China).

2.3 Echocardiography
Echocardiography was performed as described previously [22]. On the final day of the experiment, all rats were anaesthetized with pentobarbital sodium (35 mg/kg intraperitoneally) plus carprofen (5 mg/kg subcutaneously). The echocardiography of all rats was performed with a GE Vivid 7 (GE Health Medical, Milwaukee, USA). Ultrasonography with standard parasternal and apical views was performed in rats in the left lateral recumbent position. Then, high-quality 2-dimensional and M-mode images of the left ventricle from the parasternal short-axis views [23] were obtained. Images were digitized in cine-loop format and stored. Left ventricular (LV) ejection fraction (EF%) and stroke volume (SV) were taken as measurements of LV systolic function. All echocardiography was performed by the same investigator, who was blinded to the treatments.

2.4 Myocyte preparation

Adult rat ventricular myocyte isolation was performed by a modification of a previously reported method [24]. Briefly, hearts were removed from rats anaesthetized with sodium pentobarbital (60 mg/kg intraperitoneal) and immediately attached to an aortic cannula. The heart was first perfused with CaCl$_2$-free modified Tyrode’s solution for 5 min and digested with 0.3 mg/mL collagenase (Type 2, Worth-ington Biochemical, Freehold, USA), 0.4 mg/mL hyaluronidase (Type-S; Sigma, St Louis, USA) and 25 μM CaCl$_2$-containing modified Tyrode’s solution for 7 to 12 min. Then, the left ventricle was minced into small pieces with the same solution containing 0.25 mg/mL collagenase, 2 mg/dL trypsin and 50 μmol CaCl$_2$ and incubated for 10 min for further digestion. Isolated cells were maintained at 37°C in a 5% CO$_2$, 95% atmosphere and used for experiments within 6 hr after dissociation.

2.5 Loading of Fluo-3-AM or sodium Green-AM for fluorescence microscopy

Intracellular free Ca$^{2+}$ ([Ca$^{2+}$]i) and Na$^+$ ([Na$^+$]i) concentrations in single ventricular heart cells were measured with the calcium and sodium-sensitive fluorescent probes Fluo-3 and Sodium Green-AM (Molecular Probes, Oregon, USA)[25, 26]. Cells were loaded according to the method described elsewhere [27]. Frozen stocks of Fluo 3-AM or Sodium Green-AM were reconstituted in DMSO and diluted to a final concentration of 13.5 μM in Tyrode’s-BSA. Pluronic acid was added to the initial Sodium Green preparation to facilitate cell loading. Myocytes were
attached to laminin-coated glass coverslips and then incubated for 45 min at room
temperature, washed for 40 min, and further incubated for 15 min at room temperature
to complete the hydrolysis of acetoxymethyl ester groups. The intensity of the
fluorescence increases with an increase in \([\text{Ca}^{2+}]_i\) and \([\text{Na}^+]_i\). Fluorescence intensity in
the entire cell volume was detected, and average calcium or sodium fluorescence
intensity values were calculated for each group. Free \([\text{Ca}^{2+}]_i\) and \([\text{Na}^+]_i\) are represented
as the mean fluorescence intensity values relative to normal levels.

2.6 Histopathologic Analysis

Coronal sections of heart samples were fixed in 4% paraformaldehyde for 24-48 hr, dehydrated, and embedded in paraffin. Sections 4 μm thick were cut and stained
with hematoxylin and eosin (H&E) as described previously [28]. Masson’s Trichrome
staining was performed using a reagent kit from Solarbio (Beijing, China) as described
previously [29]. The area of myocardial fibrosis was quantified using a colored high-
definition pathological image-text analysis system (HPIAS-1000, Wuhan, China). The
ratio of fibrosis area to total myocardial area represents the degree of myocardial
fibrosis.

2.7 Gomori silver staining

Reticulum fibre staining was performed according to the Gomori method (Gomori
1947) using a Gomori silver impregnation staining kit (G1800; Solarbio Science &
Technology, Beijing, China). Briefly, the stationary process, paraffin embedding and
dehydration procedure were the same as described previously. Subsequently, the
oxidizing agent Gomori was added to the samples for 5 min at room temperature, and
the samples were washed with running water for 30 s. Then, the sections were rinsed in
the following solutions: 2.5% oxalic acid (2 min), 5% iron alum (5 min), and silver
ammonia solution (3 min). The reducing agent Gomori was added to the samples for 1
min and washed with running water for 10 min at room temperature. Then, the sections
were dehydrated through a graded series of ethanol, cleared in xylene, and mounted
with neutral gum.

2.8 Electron microscope quantitative analysis

The myocardial mitochondrial morphology was observed by electron microscopy.
In brief, hearts from all groups were cut into small 1 mm thick pieces and immediately fixed in 2.5% glutaraldehyde. Then, ultrathin sections were examined and imaged with a JEM-1400Plus transmission electron microscope (Electron Optics Laboratory, Tokyo, Japan). The mitochondrial area and aspect ratio (the ratio of length/width) were quantified by ImageJ. According to its volume ratio in three-dimensional space, the relevant secondary parameters volume density (Vv), shape factor (PE), average area (S) and average perimeter (L) are calculated.

2.9 Construction and analysis of the Pue-HF-common target protein network

Network pharmacological analysis of the core target of Pue therapy for HF was performed using the method we reported previously [30]. Briefly, used of TCM (http://bionet.ncpsb.org/batman-tcp/), SwissTarget BATMAN - Prediction (http://www.swisstargetprediction.ch/) and GeneCards database (https://www.genecards.org/) to obtain the target of puerarin and treatment of heart failure respectively, and then obtained the targets of Puerarin consociation sibeline targets for the treatment of heart failure in the VENNY platform (https://bioinfogp.cnb.csic.es/tools/venny/). The processed Pue was then molecularly docked to NHE1 using AutoDock Tools (https://autodock.scripps.edu/).

2.10 qPCR

Tissues were homogenized in TRIzol Reagent (Thermo Fisher Scientific), and total RNA was extracted according to the manufacturer’s instructions. The RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher). RNA was reverse-transcribed using the PrimeScript RT-PCR Kit from TaKaRa (RR037A) (Otsu, Shiga, Japan) according to the manufacturer’s protocol. PCR was performed using SYBR green qPCR mix (#CW0957M, from CoWin Biosciences, Taizhou, Jiangsu Province, China) on a StepOne Real-Time PCR System (Thermo Fisher). Ct values were obtained and normalized to the levels of mRNA of GAPDH, and then the relative level of mRNA expression was calculated using the 2^−ΔΔCt method.

2.11 Western blot

Western blotting was performed as described previously [31]. Briefly, the total proteins were boiled in SDS sample buffer for 10 min. Proteins were separated by
SDS–PAGE and were electrically transferred to polyvinylidene difluoride membranes. Membranes were blocked for 2 h at room temperature in TBS containing 5% nonfat milk and incubated with specific primary antibodies at 4°C overnight on an orbital shaker. Blots were then washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hr at room temperature. Immunoreactive proteins were visualized using the ECL Substrate and captured using a gel imaging instrument (Bio-Rad, Hercules, USA). Band densitometry analysis was performed with ImageJ.

2.12 Immunohistochemistry (IHC)

IHC was performed using conventional methods as previously described [32]. Briefly, antigen retrieval was conducted using 10 mM sodium citrate buffer (pH 6.0) in a microwave oven for 15 min at 98°C, and endogenous peroxidase activity was quenched using 3% hydrogen peroxide (37°C for 10 min). After blocking with normal horse serum, the sections were incubated with primary antibody at 4°C overnight, followed by subsequent incubation with horseradish peroxidase-conjugated secondary antibody at room temperature for 60 min. The slides were then processed with a 3,3′-diaminobenzidine (DAB) substrate kit (#3400; Thermo Fisher, Waltham, USA). Morphometric analyses were performed using ImageJ software in an operator-blind manner. For each sample, at least 5 randomly selected fields were analysed, and the data were averaged.

2.13 Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). n represents the number of independent samples but not technical replicates. Multiple comparisons were analysed using one-way analysis of variance (ANOVA) followed by post hoc Tukey’s test. Data without a Gaussian distribution were log transformed before statistical analysis. Statistical analysis was performed using Prism software (GraphPad, San Diego, USA). All tests were run as two-tailed tests. A value of $P < 0.05$ was considered significant.

3. Results

3.1 Pue attenuates myocardial injury and myocardial fibrosis in ADR-induced HF
The major cardiac structural remodelling of HF includes cardiac hypertrophy, fibrosis, inflammatory cell infiltration and edema, and even myocardial necrosis [33]. Myocardial fibre disruption and increased collagen with interstitial edema were observed in rat cardiomyocytes after 6 weeks of ADR induction. Pue treatment attenuated myocardial fibre breaks and edema, which was particularly significant in the high-dose group (Figure 1A,B).

The content of reticular fibres (Figure 1C-D) and collagen fibres (Figure 1E-F) in ADR-induced rat myocardium was significantly increased, while middle- and high-dose Pue treatment reduced the expression of collagen fibres and reticular fibres in myocardium and significantly attenuated the degree of myocardial fibrosis.

3.2 Pue improves LV systolic function in rats with HF

Next, we measured the EF and SV (both reliable indicators of LV systolic function) by ultrasonography to evaluate the LV systolic function in each group of rats. Both myocardial EF (Figure 1G,) and SV (Figure 1I,J) were significantly decreased in ADR-induced rats, indicating that ADR induction not only damages the myocardium structure but also causes ventricular systolic dysfunction. In contrast, high-dose Pue treatment greatly increased the EF, resulting in an increase in SV, suggesting that Pue improves the contractile function of the heart (at least the LV).

3.3 Pue regulates sodium and calcium concentration homeostasis

Whether ADR induction causes an imbalance in intracellular sodium and calcium ion concentrations and whether Pue can regulate intracellular sodium and calcium concentrations are our next concerns. The results showed that ADR induced an increase in intracellular free sodium (Figure 2A,B) and calcium (Figure 2C,D) ion concentrations in rat myocardium with significant differences, while high-dose Pue treatment significantly reduced intracellular free sodium and calcium ion concentrations and maintained intracellular ion concentration homeostasis.
3.4 Pue alleviates mitochondrial damage in ADR-induced HF rats

By mitochondrial electron microscopy (Figure 2E), mitochondrial swelling and striatum rupture were observed in the ADR group, which could be reversed by Pue treatment. Quantitative analysis of mitochondrial structure showed that Vv, PE, S and L were significantly increased in the middle- and high-dose Pue groups compared with the ADR group (Figure 2F).

3.5 Pue reduces NHE1 protein expression

In the heart, hyperactivation of NHE1 has been linked to the development of different pathologies, such as myocardial fibrosis and HF [34]. However, the effect of Pue on NHE1 in the myocardium has not been reported. The molecular docking results showed that the binding energy of Puerarin-NHE1 was -8.0 kcal/mol, indicating good binding between Puerarin and NHE1 (Figure 3A). To explore whether the protective effect of Pue against HF was related to NHE1, we examined the expression of NHE1 in the myocardium of each group by immunohistochemistry and Western blotting. In ADR-induced rat myocardium, both immunohistochemical (Figure 3B) and Western blot (Figure 3D) results showed that ADR activated NHE1 in the myocardium, while medium and high doses of Pue reduced the activation of NHE1 ($P < 0.05$). It is suggested that Pue may exert myocardial protective effects by inhibiting the excessive activation of NHE1 and affecting the intracellular sodium ion concentration in the myocardium.

3.6 Pue decreases the expression levels of relevant cytokines in a rat HF model.

TGF-β is an important fibrogenic cytokine [35], and IL-1β, IL-6, and TNF-α [36-38] are the major proinflammatory markers in the fibrotic response of the heart in mammalian cells. The qPCR results showed that TGF-β, TNF-α and IL-6 mRNA levels were significantly upregulated in the ADR-induced myocardium of rats, while high-dose Pue treatment decreased the mRNA expression of these cytokines (Figure 4A). These results indicate that Pue treatment can inhibit the progression of ADR-induced myocardial fibrosis and has significant anti-inflammatory effects, which may be one of
the possible mechanisms by which Pue could attenuate myocardial fibrosis in rats with HF.

3.7 Pue inhibits the p38 pathway in ADR-induced HF rats

Total p38 protein expression and the phosphorylation level of p38 in the myocardium of each group were measured separately. As shown in Figure 4B,D, the phosphorylation level of p38 was elevated significantly in ADR-induced rat myocardium, and high-dose Pue treatment significantly attenuated the phosphorylation of p38 and inhibited the activation of p38.

4. Discussion

The major findings

In this study, we found that Pue significantly improved myocardial fibrosis and inflammatory infiltration and improved cardiac function in HF rats by establishing an ADR-induced HF model. Mechanistically, we found for the first time that Pue inhibited the overactivated NHE1 in HF, which may alleviate mitochondrial damage caused by calcium overload and improve HF by reducing the concentration of Na⁺ and Ca²⁺ ions; on the other hand, Pue inhibited the activation of the p38 pathway in HF, reduced the expression of TGF-β, and inhibited myocardial fibrosis.

Reduced inflammatory response

Inflammation plays an important role in the pathophysiological process of HF. These proinflammatory cytokines can stimulate the release of many other inflammatory cytokines and transcription factors, activating immune cell transdifferentiation to proinflammatory and profibrotic subpopulations, which may promote myocardial hypertrophy and fibrosis [39-41]. The mRNA level of proinflammatory cytokines in the myocardium was significantly reduced after 4 weeks of continuous treatment with Pue (150 mg/kg) (Figure 4A), and myocardial pathological examination also showed reduced myocardial fibre disruption and alleviated interstitial edema. These results confirmed that Pue significantly attenuated the inflammatory response in the myocardium of ADR-induced HF rats.

TGF-β signaling pathway
In this experimental study, we specifically labelled collagen fibres and reticular fibres in rat myocardium. We found that Pue treatment attenuated ADR-induced collagen (Figure 1E) and reticular fibre content in rat myocardium (Figure 1C), inhibited the formation and development of fibrosis, increased myocardial LV EF and SV (Figure 1G and 1I), and greatly improved myocardial function. In cardiac tissue, activation of the TGF-β signaling pathway is associated with the development of cardiomyocyte hypertrophy and HF [42, 43]. Additionally, microvascular inflammation stimulates the secretion of TGF-β by monocyte-derived macrophages, which induces cardiac fibroblast proliferation and stimulates the phenotypic transformation of cardiac fibroblasts to myofibroblasts as well as extracellular matrix production. Myofibroblasts deposit collagen, and the increase in collagen may lead to fibrosis [44]. Moreover, TGF-β may simultaneously block matrix degradation by decreasing protease synthesis and increasing the levels of protease inhibitors [45]. We examined the expression of TGF-β in the myocardium of each group and found that Pue decreased the expression of TGF-β in ADR-induced myocardium, suggesting that the effect of Pue in reducing myocardial fibrosis may be related to the inhibition of TGF-β expression in myocardial tissue.

Decreased Na⁺ and Ca²⁺ ion concentrations

Some studies have reported that sodium (Na⁺) and calcium (Ca²⁺) ions are closely associated with HF [46]. Different studies have also confirmed that Pue can exert myocardial protective effects by regulating Na⁺ and Ca²⁺ ion concentrations in cardiomyocytes [6]. We confirmed that ADR induced an increase in intracellular free Na⁺ and Ca²⁺ ion concentrations in rat myocardium and that high-dose Pue treatment indeed significantly reduced intracellular free Na⁺ and Ca²⁺ ion concentrations (Figure 2A-D), but the mechanism is not fully understood.

Reduction in NHE1 activity

NHE1 plays an important role in maintaining Na⁺ and Ca²⁺ ion concentrations in the myocardium [15]. More importantly, exacerbated NHE1 activity has been associated with pathological cardiac processes [47]. Activation of NHE1 allows intracellular Na⁺ to accumulate and a consequent increase in Ca²⁺ transient amplitude through reverse Na⁺/Ca²⁺ exchange with subsequent activation of deleterious pathways, including the calcium/calmodulin-dependent protein kinase-histone deacetylase signaling pathway and Ca²⁺-dependent prohypertrophic signaling molecules [48], inducing increased cross-sectional area of cardiomyocytes, interstitial fibrosis, and
For Peer Review
decreased cardiac function. Dysregulation of Ca^{2+} homeostasis is a hallmark of HF, and the resultant Ca^{2+} overload contributes to mitochondrial dysfunction [49]. Our results revealed for the first time that Pue could affect NHE1 expression and inhibit ADR-induced NHE1 activation in an experimental HF model (Figure 3B and 3D). We hypothesized that inhibition of NHE1 overactivation, reduction of [Na^{+}]_i accumulation, and prevention of Ca^{2+} overload and the resulting mitochondrial dysfunction may be potential cardioprotective mechanisms of Pue. In fact, NHE exchanger inhibitors have been proven to protect the heart against I/R injury [50, 51] and inhibit the development of myocardial hypertrophy and HF.

p38 MAPK and NHE1 activity

Different kinases are associated with the regulation of NHE1. It has been found that p38MAPK can regulate NHE1 activity [52] and plays an important role in the regulation of cardiac remodelling and cardiac contractility [53-55]. Consistently, our results suggest that p38 is activated in ADR-induced rat myocardium and that Pue dose-dependently inhibits p38 activation. Most studies have shown that p38 activation promotes the development of HF due to extracellular matrix remodelling and cardiac fibrosis by activating the TGF-β signaling pathway [56-58]. This is consistent with our results that Pue inhibits p38 activation and reduces cardiac fibrosis and proinflammatory cytokine production, suggesting that p38 blockade and thus inhibition of the TGF-β signaling pathway may be a possible mechanism by which Pue exerts its cardioprotective effects.

p38 MAPK and cardiac fibrosis

p38 also controls cardiomyocyte contractility, and p38 activation has been shown to have an anti-inotropic effect [59-61]. Two main possibilities are proposed: altering intracellular pH and phosphorylating contractile proteins, thereby desensitizing cardiomyocytes to calcium, but the exact mechanism of inhibition of myofilament responsiveness to Ca^{2+} is not clear. Our experimental results offer the possibility that p38 activation may cause intracellular calcium overload via sodium-calcium exchange by causing enhanced NHE1 activity and thus decreased calcium sensitivity of myofilaments. Pue inhibits the activation of p38, which inhibits p38-mediated cardiac fibrosis and proinflammatory cytokine production on the one hand, further improving the sensitivity of myocardium to calcium and enhancing myocardial contractility by inhibiting the activation of NHE1. This needs further validation.

Side effects of Pue
Pue, as a vasodilator, dilates blood vessels and reduces myocardial oxygen consumption, which is the basic principle of its therapeutic effect on cardiovascular diseases. However, it is necessary to pay attention to the choice of dosage to avoid causing adverse reactions such as hypotension.

Conclusion and Limitations

In conclusion, Pue is an effective drug for the treatment of HF in the ADR-induced HF rat model. Our experimental results suggest that Pue may exert myocardial protective effects by inhibiting the activation of p38MAPK and its downstream NHE1 activation, inhibiting myocardial fibrosis and the production of proinflammatory cytokines, attenuating mitochondrial damage caused by calcium overload and improving myocardial contractile function. This study investigated the effects of Pue on HF and the potential mechanisms mainly at the animal level, lacking validation of in vitro experiments and did not further characterize the interaction of p38 with NHE1 and the effect of NHE1 knockdown on the efficacy of Pue. We suppose that it would be interesting and important to characterize the interaction of p38 with NHE1 in vitro as well as to observe the efficacy of Pue using NHE1 knockout animals in a separate study.

Acknowledgement

The authors thank Mrs. Qian Xu for technical assistance.

Conflict of Interest

The authors declare that they have no conflict of interest.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (Nos. 82271460 and U1804197), the Research Foundation of Henan Province (Nos. 202300410308 and HNGD2022067), the National High-End Foreign Expert Recruitment Plan of China (No. G2022026006L), and the Research Foundation of Xinxiang Medical University (Nos. XYBSKYZZ505319 and XYBSKYZZ201626).

References


9 Ravens U, Cerbai E. Role of potassium currents in cardiac arrhythmias. Europace 2008,
10: 1133-1137


11 Mraiche F, Oka T, Gan XT, Karmazyn M, Fliegel L. Activated NHE1 is needed to induce early cardiac hypertrophy in mice. Basic Res Cardiol 2011, 106: 603-616


14 Fliegel L. Regulation of the Na(+)/H(+) exchanger in the healthy and diseased myocardium. Expert Opin Ther Targets 2009, 13: 55-68


19 Yu, W., Qin, X., Zhang, Y., Qiu, P., Wang, L., Zha, W., & Ren, J. Curcumin suppresses doxorubicin-induced cardiomyocyte pyroptosis via a PI3K/Akt/mTOR-dependent manner. Cardiovascular diagnosis and therapy 2020, 10(4), 752–769.

20 Yu, W., Sun, H., Zha, W., Cui, W., Xu, L., Min, Q., & Wu, J. Apigenin Attenuates Adriamycin-Induced Cardiomyocyte Apoptosis via the PI3K/AKT/mTOR Pathway. Evidence-based complementary and alternative medicine : eCAM, 2017, 2590676.


25 Satoh H, Sperelakis N. Identification of the hyperpolarization-activated inwards current in

Protection by oral pretreatment with taurine against the negative inotropic effects of low-calcium medium on isolated perfused chick heart. Cardiovasc Res 1983, 17: 620-626


Res 2022, 177: 106120

33 Li Z, Zhao H, Wang J. Metabolism and Chronic Inflammation: The Links Between Chronic Heart Failure and Comorbidities. Front Cardiovasc Med 2021, 8: 650278


experimental validation. Pulmonary pharmacology & therapeutics 2022, 73-74, 102121.


50 Karmazyn M. Mechanisms of protection of the ischemic and reperfused myocardium by


Signaling. Circulation 2017, 135: 2041-2057


Figure legends

Figure 1. Pue prevented the progression of myocardial fibrosis and improved left ventricular function in ADR-induced HF rats (A) HE staining. (B) Normal cardiomyocyte ratio of HE-stained cells. (C) Gomori silver staining. (D) Quantitative analysis of the reticular fibre area in the myocardial tissue of each group. (E) Masson
staining. (F) Quantitative analysis of collagen fibre area in each group. (G and I)
Representative two-dimensional and M-mode short-axis echocardiographic images of
left ventricular function. (H and J) Quantitative analysis of echocardiography
parameters (EF% and SV) in each group. Data were analysed by using one-way
ANOVA. All data are expressed as the mean ± SEM (n = 5 for B/D/F; n = 6 for
panels H/J). *p < 0.05 vs the Con group; #p < 0.05 vs the ADR group.

Figure 2. Pue reduced intracellular free sodium and calcium ion concentrations
and attenuated mitochondrial damage caused by calcium overload

Representative immunofluorescence images of [Na⁺]i (A) and [Ca²⁺]i (C) in single
ventricular heart cells of each group. (B) Quantitation of cytoplasmic Na⁺
fluorescence. (D) Quantitation of cytoplasmic Ca²⁺ fluorescence. (E) Mitochondrial
transmission electron microscopy. (F) Quantitative analysis of mitochondrial
structure. Data are the mean ± SEM. *P < 0.05 vs the Con group; #P<0.05 vs the
ADR group, one-way ANOVA (n = 4 for panels A/C; n = 6 for E).

Figure 3. Effects of Pue on NHE1 expression in myocardial tissue in ADR-
induced HF rats (A) Overall effect diagram of Pue and NHE 1 docking. (B)
Immunohistochemistry images of NHE1 in myocardial tissues of each group. (C)
Quantitative data of the positive expression of NHE1. (D) Representative western
blots of NHE1 expression. (E) Statistical analysis of the protein expression of NHE1.
Data are the mean ± SEM. *P < 0.05 vs the Con group; #P < 0.05 vs the ADR group,
one-way ANOVA (n = 5 for panels B; n = 4 for D).

Figure 4. Pue inhibited p38 activation and reduced cardiac fibrosis and
proinflammatory cytokine production in the myocardial tissue of ADR-induced
HF rats (A) The mRNA levels of IL-Iβ, IL-6, TNF-α and TGF-β in the myocardial
tissue of each group. (B) Representative western blots of p-p38 expression. (C) Statistical analysis of the protein expression of p-p38. (D) Immunofluorescence staining of p-p38 in myocardial tissues of each group. (E) Quantitative analysis of p-p38. Data are the mean ± SEM. *P < 0.05 vs the Con group; #P < 0.05 vs the ADR group, one-way ANOVA (n = 4).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence (5′→3′)</th>
<th>Reverse sequence (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>AATCTCACAGCAGCATCTCGACAAG</td>
<td>TCCACGGGCAAGACATAGGTAGC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>ATGGGCTCCCTCTCATCAGTTCC</td>
<td>CCTCCGCTTGGTGTTTGCTAC</td>
</tr>
<tr>
<td>IL-6</td>
<td>ACTTCCAGCCAGTGCTTCTTG</td>
<td>TGGTCTGTGTGTGGTTATCCTC</td>
</tr>
<tr>
<td>TGF-β</td>
<td>GACCGCAACAACGCAATCTATGAC</td>
<td>CTGGAHTGCTTCCGAATGTC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GACATGCCGCCTGGAGAAGC</td>
<td>AGCCAGGATGCCCTTGTAG</td>
</tr>
</tbody>
</table>

Table 1. qPCR primer sequences
Highlights

In this study, we characterize the potential cardioprotective effects of Puerarin and explore the underlying mechanisms. Our results suggest that Puerarin is an effective drug against heart failure and may play a protective role in the myocardium by inhibiting the activation of p38 and its downstream NHE1.

(1) Puerarin inhibits the over-activated Na\(^+\)/H\(^+\) exchange isoform 1 (NHE1) in HF which may improve HF by regulating Na\(^+\) and Ca\(^{2+}\) ion concentrations.

(2) Pueraria alleviates myocardial fibrosis and improves left ventricular systolic function in ADR-induced heart failure rats.

(3) Puerarin inhibits the activation of the p38 pathway in HF, and p38 blockade and thus inhibition of the TGF-β signaling pathway may be a possible mechanism by which Puerarin exerts its cardioprotective effects.