Original Article

Uric acid preconditioning alleviated doxorubicin induced JNK activation and Cx43 phosphorylation associated cardiotoxicity via activation of AMPK-SHP2 signaling pathway

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Background: Doxorubicin is an anthracycline antibiotic, which is effective for treating various malignancies such as leukemias and lymphomas. However, its serious cumulative dose-dependent cardiotoxicity limits its clinical application. Previous studies have shown that doxorubicin-associated cardiotoxicity is closely related to adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK). Uric acid is known to exert a strong antioxidant function and moderate protection on the nerves. However, its cardioprotective properties have not been established. This study aimed to investigate the potential effect of uric acid preconditioning on doxorubicin-induced cardiotoxicity and the involvement of AMPK signaling in this process.

Methods: An acute cardiotoxicity model of doxorubicin was established by intraperitoneal injection of a single dose of doxorubicin (20 mg/kg) in mice. Uric acid (62.5, 125, and 250 mg/kg) was intragastrically administered to mice one day before doxorubicin treatment and then continuously administered every 24 h for 8 consecutive days. The mortality rate and weight of the mice were recorded every day. Electrocardiograms (ECG) and serum biochemicals were detected with an ECG instrument and enzyme-linked immunosorbent assay kit (Elisa) respectively. A real-time cell analyzer (RTCA) was used to investigate the cytotoxicity of doxorubicin *in vitro*. Cell signaling was assayed by western blotting.

Results: Uric acid (125 mg/kg) preconditioning increased the survival rate and body weight of doxorubicin-treated mice. Uric acid also effectively alleviated prolongation of the doxorubicin-induced QT interval, slowed heart rate, and reduced the plasma levels of aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatine kinase (CK) in plasma in mice. Moreover, uric acid strongly activated AMPK and Src homology 2 domain-containing protein tyrosine phosphatase (SHP2), inhibiting doxorubicin-induced expression phosphorylated-c-Jun N-terminal kinases (JNK) and phosphorylated-connexin 43 (Cx43) *in vitro* and *in vivo* and effectively reversing the doxorubicin-induced decreased viability of H9C2 myocardial cells *in vitro*.

Conclusions: We demonstrated that uric acid preconditioning alleviated doxorubicin-induced cardiotoxicity through the AMPK-SHP2-JNK-Cx43 signaling pathway.

Keywords: Uric acid; preconditioning; doxorubicin; cardiotoxicity; connexins 43 (Cx43)

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Introduction

Cancer is a life-threatening, significant health burden that is rapidly emerging as the foremost cause of death in the 21st century. Although several molecular targeting drugs are widely used, chemotherapy continues to remain the most effective form of tumor treatment. Doxorubicin, a representative anthracycline, is a prominent chemotherapy drug found to be effective in the treatment of several cancers, including hematological malignancies, soft tissue sarcomas, and solid tumors. However, the long-term use of this potent antineoplastic agent is limited by its severe side effects, with cardiotoxicity identified as a particularly common side effect (1). While reducing the dose or dexrazoxane administration can relieve doxorubicin-induced cardiotoxicity (2), the dosage of dexrazoxane is strictly controlled. Therefore, identifying new strategies to improve doxorubicin-induced cardiotoxicity is critical.

Doxorubicin has been proposed to induce cardiotoxicity via redox cycling and reactive oxygen species (ROS) generation, which leads to the destruction of myocardial mitochondria, production of substantial ROS levels and cell death by apoptosis or necrosis (3). Doxorubicin can also cause cardiac conduction disorder, leading to arrhythmia. In doxorubicin-treated beagle dogs and rats, doxorubicin-induced acute cardiotoxicity manifested as abnormal conduction, including bradycardia, ST-segment elevation, prolongation of QT intervals, and QRS widening (4). However, the underlying mechanism of how doxorubicin affects cardiac conduction is not precise.

The two branches of arrhythmia, including abnormalities in cardiac pacing and conduction, may co-exist in doxorubicin-induced heart disease. Connexins 43 (Cx43) are integral membrane proteins that form gap junctions to enable the direct cytoplasmic exchange of information and substances between adjacent cells and contribute to cardiac conduction. Notably, the propagation of cardiac action potentials and the maintenance of a regular beating rhythm are mediated by the gap junctions (5). There is evidence that suggests that gap junctions regulate electrical coupling and are vital for normal impulse propagation through the heart. Additionally, the reduction of gap junctions (Cx43) leads to reduced intercellular coupling, thereby lowering conduction velocity (6). Studies have shown that chemotherapy may cause down-regulation of Cx43 total protein and upregulation of its phosphorylated form, p-Cx43 (7). However, the extensive knowledge in this field, the prevention of chemotherapy-induced upregulation of p-Cx43 and the resulting conduction abnormalities has not been established.

Since doxorubicin-induced cardiotoxicity is associated with both ROS-induced myocardial injury and inflammation-induced conduction disorder (8), the identification of a safe and stable compound that can ameliorate both adverse effects of doxorubicin were critical. After extensive screening, uric acid was selected, which is a compound with strong antioxidant capacity. Uric acid is an important, naturally occurring, physiological antioxidant, iron stinger mixture, and free radical scavenger (9). Despite previous reports of the neuroprotective effects displayed by uric acid, the underlying mechanism remains unclear (10). Also, we observed that plasma levels of uric acid often increased in patients after chemotherapy. Although previously considered to be associated with chemotherapy-induced kidney damage, this phenomenon has rarely been linked with other adverse events.

Furthermore, the level of uric acid in the body was shown to increase during cellular stress (11). While our previous research has focused on AMPK, the cellular energy stress molecule, involved in inflammatory and repair processes (12). Evidence has also indicated that doxorubicin-induced cardiotoxicity is closely related to AMPK (13-16). This study explored the protective effect of elevated uric acid on doxorubicin-induced cardiotoxicity. Furthermore, we investigated if this protective effect was mediated through AMPK signaling.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/atm-20-3105).

Methods

Chemicals and reagents

All the reagents were purchased from Sigma-Aldrich (St.

Louis, MO, USA), except for doxorubicin and shp099, which were obtained from Med Chem Express (New Jersey, USA); Antibody for phosphorylated Cx43, phosphorylated SHP2, phosphorylated JNK (Thr183/Tyr185), and phosphorylated AMPK (Thr172) was obtained from Cell Signaling Technology (Beverly, MA, USA). Fetal bovine serum (FBS) was purchased from Gibco, and other cell culture media and supplements were purchased from KenGEN (KenGEN BioTECH, China).

Animals and treatment

Male ICR mice (18–22 g) at 8 weeks of age were provided by the Experimental Animal Center at Nanjing Medical University, Nanjing, China. The mice had free access to food and water and were housed in groups of five to six per cage under pathogen-free conditions with soft bedding under controlled temperature (22±2 ℃) and a 12-hour light/dark cycle (lights on at 8:00 am). All rodent experiments were approved by the Animal Research Committee of Nanjing Medical University and maintained following the guidelines of the China Council on Animal Management (Animal ethical code authorization number: IACUC1707012).

Sixty mice were randomly divided into six groups (n=10 in each group): control; doxorubicin; doxorubicin + uric acid (62.5, 125, and 250 mg/kg); doxorubicin + allopurinol. Doxorubicin was dissolved in sterile saline (0.9% NaCl); uric acid and allopurinol were suspended in 0.5% CMC-Na solution, and before given it to mice, it would be resuspended by slightly concussion. Acute cardiotoxicity was induced by a single dose of doxorubicin (20 mg/kg) via intraperitoneal (*i.p.*) injection in mice. Various concentrations of uric acid (62.5, 125, and 250 mg/kg) and allopurinol (40 mg/kg) were intragastrically (*i.g.*) administered one day before doxorubicin treatment, and then continuously administered for 8 days every 24 h. To prevent uric acid from being metabolized by uricase in the body, the mice were injected with Oteracil potassium (an inhibitor of uricase) 5 min before uric acid treatment. The control and doxorubicin groups were *i.g.* administered with 0.5% carboxymethylcellulose (CMC-Na) solution. The mortality rate and body weight of the mice were recorded every day.

Cell cultures

H9C2 cells were maintained in Dulbecco's modified Eagle's Medium (DMEM; KenGEN Bio TECH, China) supplemented with 10% (v/v) FBS(Gibco), penicillin (100 U/mL), and streptomycin (100 U/mL). All cells were kept in a humidified chamber with 5% CO_2 at 37 ℃. A total of 105 cells were plated in a six or 12-well plate overnight before treatment with uric acid (1.25, 2.5, 5 mg/dL) for 2 hours. Cells were then treated with doxorubicin 1 μM, AMPK activator 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) (10 μM), compound C (10 μM), and Shp099 (10 μM) for 12 hours. Cells were subsequently harvested and analyzed by immunoblot assay.

Cardiomyocyte damage assessment

Serum and tissue samples were extracted from each mouse and stored at −80 ℃ for further analysis. The following cardiac injury markers were identified using commercial kits (R&D Systems, USA) and detected according to the manufacturer's instructions: creatine kinase MB (CK-MB); lactate dehydrogenase (LDH) and Aspartate aminotransferase (AST). Concentrations were calculated by referring to a standard curve, according to the manufacturer's instructions.

ECG

Essentially, mice were anesthetized with chloral hydrate (400 mg/kg, *i.p.*) before undergoing ECG for heart rate and QT interval analysis. Electrodes were placed under the skin right hind limb, right front limb, and left hind limb. The results were recorded using the MP150 ECG module (BIOPAC, USA). The duration of each recording was at least 5 min at 50 mm/s with a voltage of 1 mV/cm.

Real-time cell analyzer (RTCA)

Doxorubicin-induced cardiotoxicity was determined using the xCELLigence platform (real-time cell analyzer, RTCA). Briefly, duplicate wells of E-plate 16 were seeded with H9C2 cells at 10^4/well and cultured for 24 hours in complete culture medium containing FBS. The xCELLigence RTCA system was used to monitor cellular kinetics across microelectronic sensors integrated into the bottom of the plate.

Western blotting

Samples (cells or heart tissue) underwent lysis in radioimmunoprecipitation assay (RIPA) lysis buffer. The protein concentrations were determined by BCA Protein

Assay (Thermo Fisher, Waltham, MA, USA). Protein (40–80 μg) was loaded and separated by SDS-PAGE before undergoing electrophoretic transfer onto polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA, USA). The membranes were blocked with 5% bovine serum albumin for two hours at room temperature, probed with antibodies overnight at 4 ℃ with the primary antibodies, and then incubated with HRP-coupled secondary antibodies. The primary antibodies used included p-AMPK (Thr172) (1:1,000), AMPK (1:1,000), p-JNK (Thr183/Tyr185) (1:1,000), JNK (1:1,000), p-SHP2 (1:1,000), SHP2 (1:1,000), p-Cx43 (1:1,000), total Cx43 (1:1,000). For loading control, the blots were probed with the antibody for β-actin (1:1,000). The filters were subsequently developed by enhanced chemiluminescence reagents (PerkinElmer, Waltham, MA, USA) with secondary antibodies (Chemicon, Billerica, MA, USA). Data were acquired with the Molecular Imager (Gel DocTM XR, 170-8170) and analyzed with Quantity One-4.6.5 (Bio-Rad Laboratories, Berkeley, CA, USA).

Statistical analyses

GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA) was used to conduct all the statistical analyses. Kaplan Meier Survival analysis was completed using the Log-rank (Mantel-Cox) test. The student's *t*-test evaluated the differences between the two groups. The data from more than two groups were evaluated by one-way ANOVA, followed by Tukey's multiple comparisons test or two-way ANOVA followed by Bonferroni post hoc tests. Results were expressed as mean ± SEM of three independent experiments. P<0.05 was deemed to be statistically significant.

Results

Uric acid preconditioning alleviated doxorubicin-induced cardiotoxicity

As shown in *Figure 1A*, compared to the control group 10/10 (100%), the survival rate of doxorubicin-treatment mice was only 1/10 (10%) on day 8, whereas uric acid (125 mg/kg) preconditioning increased the survival rate to 4/10 (40%). Interestingly, allopurinol, a drug used to lower uric acid, also increased the mortality rate of doxorubicin-treated mice (*Figure 1A*). Additionally, preconditioning with uric acid (125 mg/kg) significantly alleviated the loss of body weight compared to that in the doxorubicin-treated group (P<0.05; *Figure 1B*). Based on the results shown in *Figure 1*, uric acid (125 mg/kg) was selected for subsequent analysis, including ECG, myocardial enzyme spectrum testing, and western blot analysis in mice. As shown in *Figure 2A,B,C,D*, doxorubicin reduced cardiac function, characterized by bradycardia, QT prolongation, and slowed heart rate in mice. However, preconditioning with uric acid effectively reversed the doxorubicin-induced slowed heart rate and partially hampered the increase in QT interval and QT(c), induced by doxorubicin (P<0.05). Uric acid preconditioning significantly reverted doxorubicin-induced elevated AST, LDH, and CK levels in the plasma *in vivo*. The results suggest that uric acid preconditioning may play a cardioprotective role (P<0.05; *Figure 2E,F,G,H*). ECG analysis was subsequently performed to assess whether uric acid preconditioning alleviated doxorubicin-induced conduction abnormalities. Moreover, to prevent uric acid being metabolized by uricase in the body, mice were injected with Oteracil potassium 5 min before uric acid treatment. On day 5 after modeling, we detected the level of uric acid in the plasma of the mice. The results showed that the uric acid level in uric acid-treated mice was higher than that in the control group of mice (P<0.001; *Figure 2I*). Therefore, uric acid protected against doxorubicin-induced cardiotoxicity in mice.

Uric acid significantly increased AMPK phosphorylation in vitro and in vivo

To investigate the effect of uric acid on AMPK, *in vitro* analysis was performed in H9C2 cells, which were co-cultured with various concentrations of uric acid for two hours. Western blot results showed that 5 mg/dL uric acid sufficiently induced the activation of AMPK *in vitro* (P<0.001; *Figure 3A*), where phosphorylated AMPK (p-AMPK) levels increased in a time-dependent manner *in vitro* (P<0.001; *Figure 3B*), but did not affect the level of total AMPK expression *in vitro* (*Figure 3A,B*).The effect of uric acid on AMPK *in vivo* was subsequently assessed by *i.g.* administered uric acid at varying concentrations of for 2 days. The results indicated significant upregulated p-AMPK levels *in vivo* at doses of 125 and 250 mg/kg of uric acid, but did not affect the level of total AMPK expression *in vivo* (P<0.001; *Figure 3C*).

Uric acid alleviated doxorubicin-induced cardiotoxicity and abnormal phosphorylation of Cx43 in an AMPK-dependent manner

We found that doxorubicin time-dependently increased

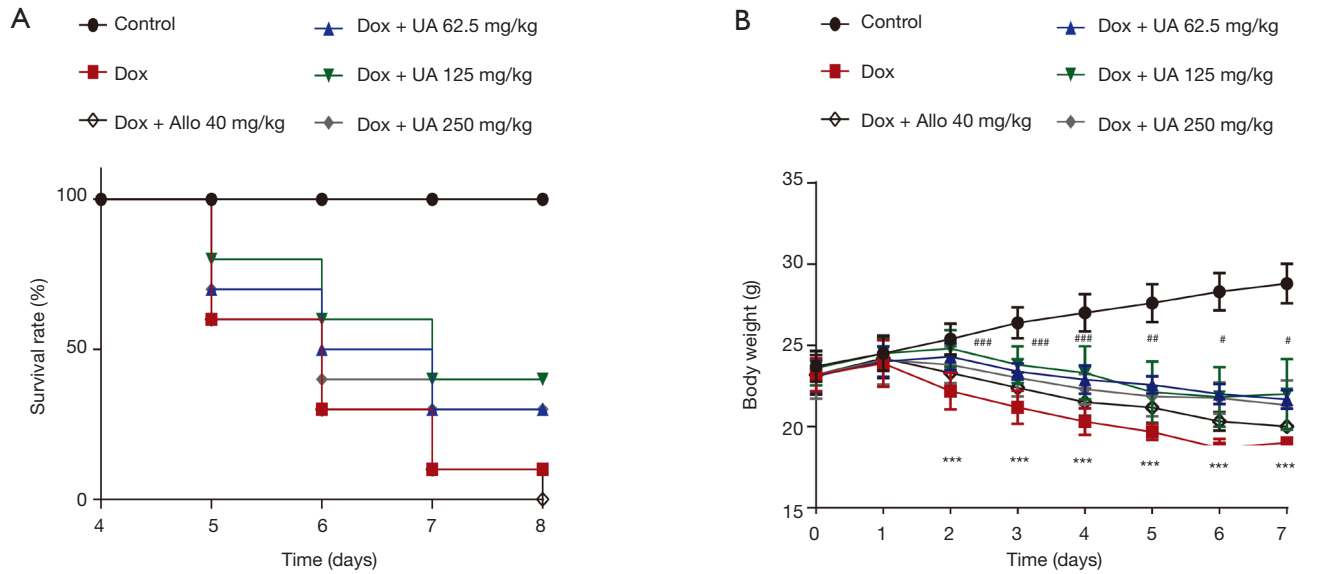

Figure 1 Uric acid preconditioning significantly increased the survival rate and body weight of doxorubicin-treated mice. (A) Kaplan Meier survival analysis showed that uric acid preconditioning increased the survival rate of mice compared to doxorubicin treatment. The Log rank (Mantel-Cox) test was used to determine significance (n=10 of each group). (B) Body weight was increased in the uric acid-treatment group. Data are presented as the mean ± SEM. Significant difference was detected by one-way or two-way analysis of variance (***, P<0.001 *vs.* control group; #, P<0.05, ##, P<0.01, and ###, P<0.001 *vs.* doxorubicin-treatment alone group, Bonferroni post hoc tests). Allo, allopurinol; Dox, doxorubicin; UA, uric acid.

the phosphorylation levels of Cx43 and JNK *in vivo* and reduced the expression of total Cx43 but did not obviously affect the levels of total JNK in mice (P<0.001; *Figure 3D*). In contrast, uric acid preconditioning increased the expression of total Cx43 *in vivo* (P<0.001; *Figure 4A*) and reversed the increase of p-Cx43 and p-JNK caused by doxorubicin *in vitro* and *in vivo* (P<0.001; *Figure 4A,B,C*). Moreover, preconditioning with AMPK inhibitor (compound C) abolished the inhibition of uric acid in the doxorubicin treatment group *in vitro* (P<0.001; *Figure 4C*). More importantly, western blot analysis indicated that AMPK agonist AICAR also reduced doxorubicin-induced phosphorylation of Cx43 *in vitro* (P<0.001; *Figure 4C*). Results obtained by RTCA demonstrated that uric acid (5 mg/dL) preconditioning for 2 hours significantly remitted doxorubicin-induced cardiotoxicity. Pretreatment with AICAR mimicked the protective effect of uric acid *in vitro*, whereas pretreatment with the AMPK inhibitor (compound C) reversed the protective effect of uric acid on doxorubicin-induced cardiotoxicity *in vitro* (P<0.05; *Figure 4D*). Taken together, uric acid preconditioning inhibited abnormal phosphorylation of Cx43 and alleviated doxorubicin-induced cardiotoxicity, possibly in an AMPK dependent manner.

Uric acid reduced doxorubicin-induced cardiotoxicity through the AMPK-SHP2-JNK-Cx43 signaling pathway axis

The above data showed that activated AMPK effectively reduced doxorubicin-induced cardiotoxicity, and previous studies indicated that resveratrol (an AMPK activator) activates SHP2 (17). Therefore, we further investigated whether uric acid preconditioning activates SHP2 in an AMPK-dependent manner to result in JNK-Cx43 signaling inhibition. Our results showed that uric acid significantly induced the expression of p-SHP2 *in vitro* and *in vivo* (P<0.001; *Figure 3A,B,C*). Furthermore, compound C (10 μM) reversed uric acid-induced upregulation of p-SHP2 *in vitro* (P<0.001; *Figure 4C*). Uric acid also significantly inhibited doxorubicin-induced upregulation of p-JNK and p-Cx43 *in vitro* and *in vivo* (P<0.001; *Figure 4A,B*), as expected, and compound C reversed these effects *in vitro* (P<0.001; *Figure 4C*). Moreover, we investigated whether the cardioprotective effect of uric acid was SHP2-dependent. As shown in *Figure 4C*, shp099 (an

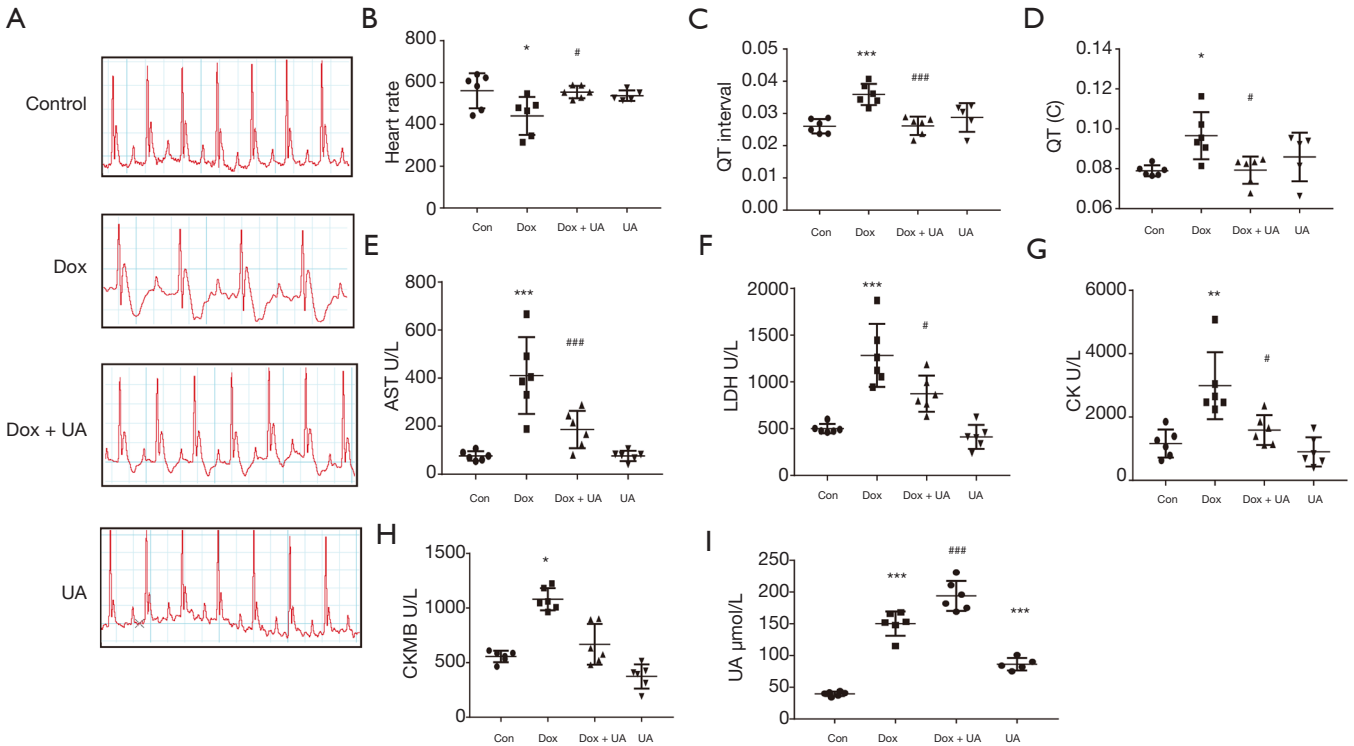

Figure 2 Uric acid preconditioning alleviated doxorubicin-induced cardiac abnormal conduction in mice. (A) Representative images of ECG indicated that doxorubicin induced conduction abnormalities, including slowed heart rate (B) and prolongation of QT intervals (C) and QT(c) (D), whereas preconditioning with uric acid (125 mg/kg) significantly alleviated doxorubicin-induced abnormal conduction in mice. ELISA kits were used to detect the levels of cardiomyocyte injury markers, including AST (E), LDH (F), CK (G), CK-MB (H), and UA (I). Data are presented as the mean ± SEM. n=6 in each group. Significant difference was observed by one-way or two-way analysis of variance (*, P<0.05, **, P<0.01, and ***, P<0.001 *vs.* control group; $^{\#}$, P<0.05, and $^{\#\#\#}$, P<0.001 *vs.* doxorubicin-treatment alone group, Bonferroni post hoc tests). Dox, doxorubicin; UA, uric acid.

SHP2 inhibitor) abolished the reduction in doxorubicin-induced phosphorylated JNK by uric acid (P<0.001) and cardioprotective effect of uric acid *in vitro*. Overall, these results demonstrate that uric acid preconditioning reduced doxorubicin-induced cardiotoxicity, possibly via the AMPK-SHP2-JNK-Cx43 signaling pathway.

Discussion

We demonstrated that doxorubicin-induced cardiotoxicity was inhibited by uric acid, which occurred through upregulation of SHP2 via AMPK both *in vivo* and *in vitro*. Furthermore, preconditioning with uric acid inhibited doxorubicin-induced upregulation of phosphorylated JNK and Cx43. In contrast, inhibition of SHP2 abolished the reductions in doxorubicin-induced phosphorylated JNK and Cx43 by uric acid and the protective effect of uric acid on H9C2 myocardial cells *in vitro*. These results reveal that uric acid inhibited doxorubicin-induced cardiotoxicity by modulating the AMPK-SHP2-JNK-Cx43 signaling pathway (*Figure 5*).

Uric acid is a product of the catabolism of purine nucleotides, and previous studies have shown that excessively high levels of uric acid increase the risk of heart disease and gout (18-20). Evidence also indicated that uric acid has a powerful antioxidant effect (21,22). Yasutake *et al.* found that uric acid ameliorates indomethacin-induced enteropathy in mice through its antioxidant activity (23). Additionally, systemic administration of uric acid over a range of doses is safe and can increase the body's antioxidant capacity (24). Studies have also demonstrated that febuxostat, a drug which lowers uric acid, which

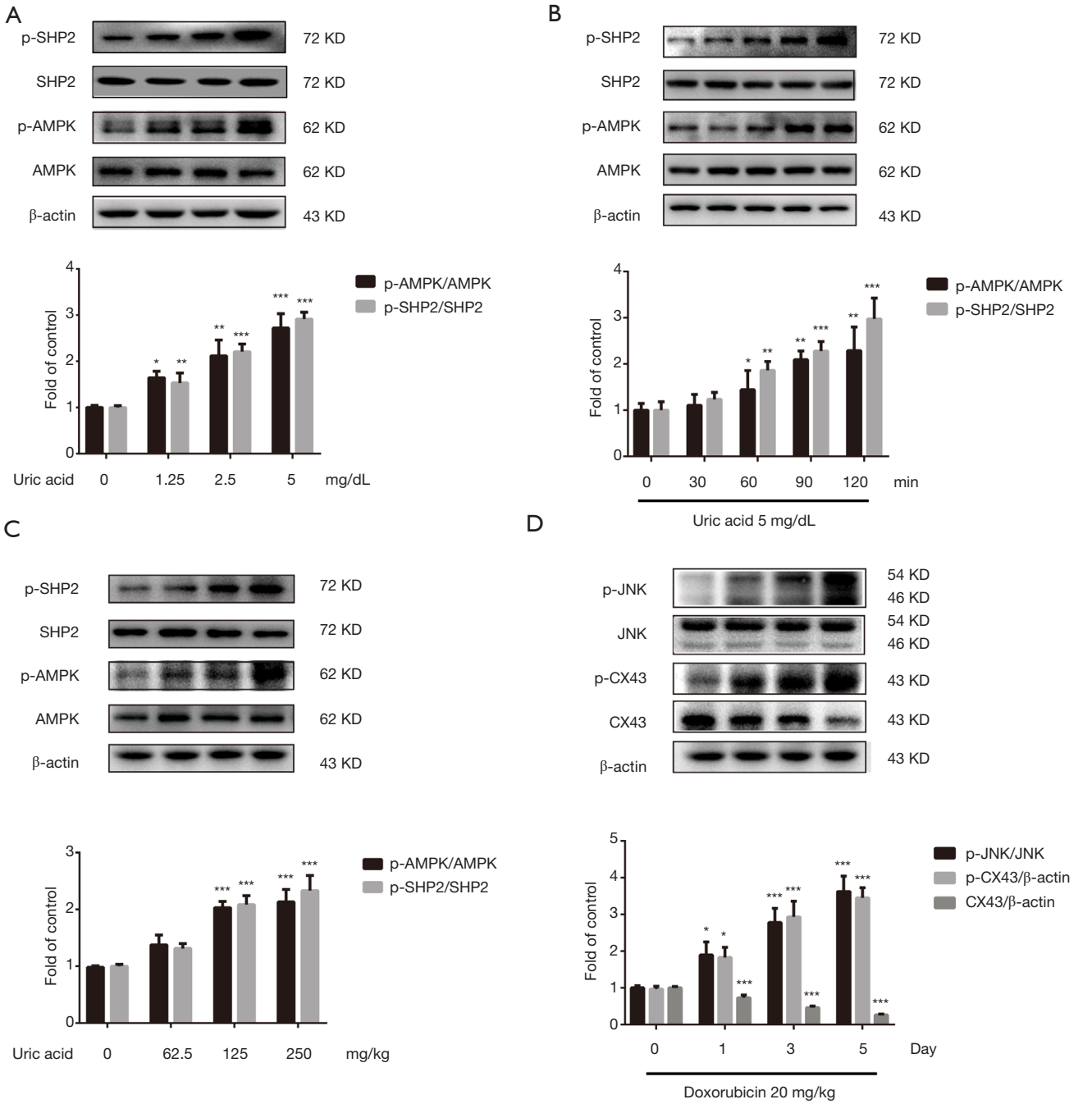

Figure 3 Uric acid preconditioning significantly induced activation of AMPK and SHP2 both *in vivo* and *in vitro*. (A) Uric acid treatment for 2 h significantly increased the levels of p-AMPK/AMPK and p-SHP2/SHP2 in H9C2 cells *in vitro*. (B) Uric acid treatment (5 mg/dL) time-dependently increased he levels of p-AMPK/AMPK and p-SHP2/SHP2 in H9C2 cells *in vitro*. (C) Consecutive administration of uric acid (125 and 250 mg/kg, *i.g.*) for 2 days significantly up-regulated the level of p-AMPK/AMPK and p-SHP2/SHP2 in heart tissue *in vivo*. (D) Intraperitoneal injection of a single dose of doxorubicin (20 mg/kg) markedly elevated the expression levels of p-JNK/JNK and p-Cx43 but reduced the total Cx43 level on day 5 *in vivo*. Data are presented as the mean ± SEM of three individual experiments. Significant difference by one-way or two-way analysis of variance (*, P<0.05, **, P<0.01, and ***, P<0.001 *vs.* control group; Bonferroni post hoc tests).

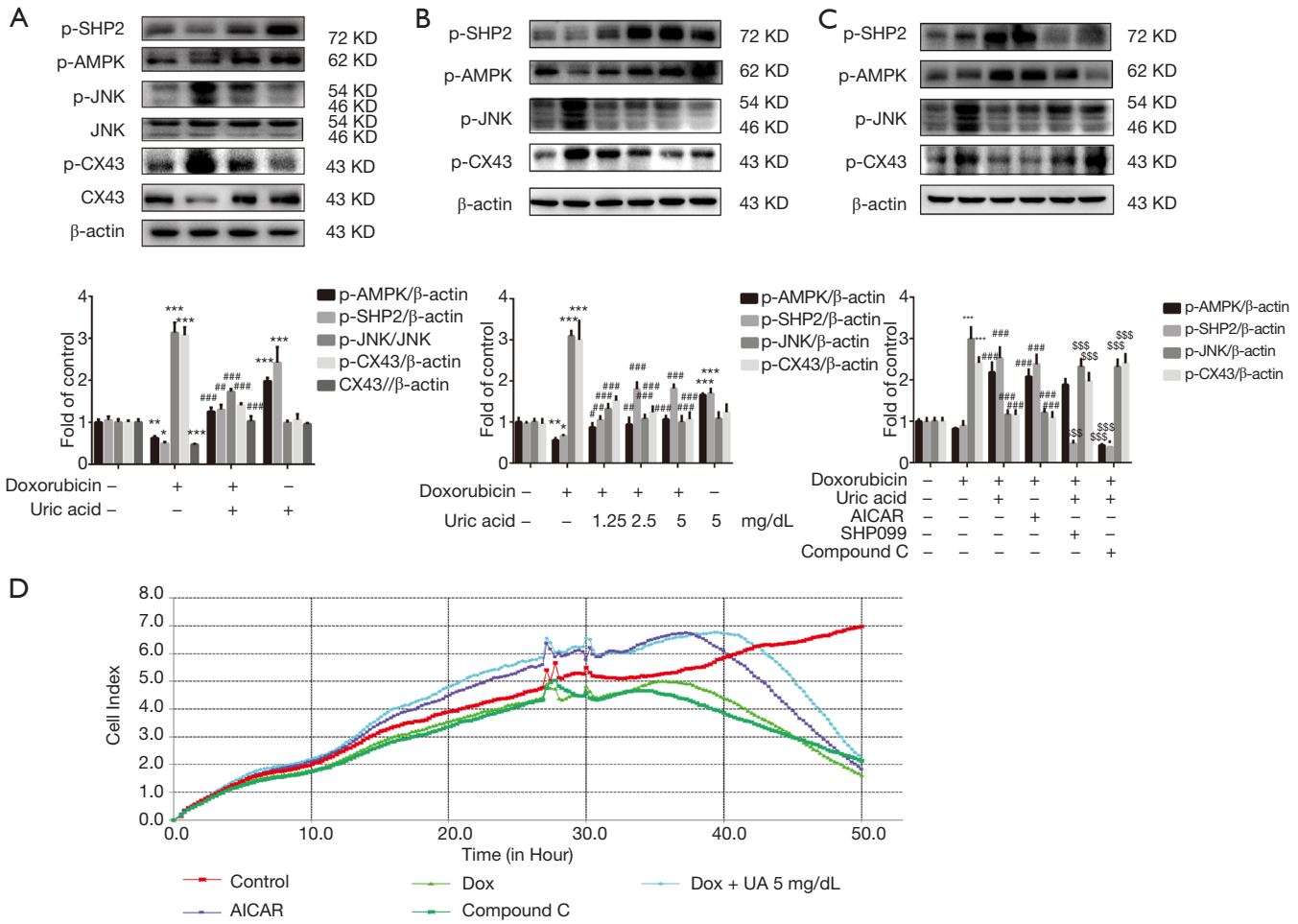

Figure 4 Uric acid preconditioning alleviated doxorubicin-induced cardiotoxicity via the AMPK-SHP2-JNK-Cx43 signaling axis. (A) Representative western blot images showing the levels of p-AMPK, p-SHP2, p-JNK/JNK, p-Cx43, and total Cx43 in heart tissue *in vivo*. (B) H9C2 cells were pretreated with uric acid (1.25, 2.5, 5 mg/dL) for 2 h, and then treated with doxorubicin (1 μM) for 12 h. Western blot images showing the levels p-AMPK, p-SHP2, p-JNK, and p-Cx43 in H9C2 cells *in vitro*. (C) H9C2 cells were treated with compound C (10 μM) and Shp099 (10 μM) 30 min before uric acid (5 mg/dL) administration, and then after 12 h of doxorubicin (1 μM) treatment. Western blot samples (n=3 of each group) were collected, and the images show the levels p-AMPK, p-SHP2, p-JNK, and p-Cx43 in H9C2 cells *in vitro*. (D) Real-time cell analyzer results showed that preconditioning with uric acid (5 mg/dL) or AICAR (10 μM) for 2 h significantly alleviated doxorubicin-induced cardiotoxicity *in vitro*, whereas these effects were abolished by compound C (10 μM). Data are presented as the mean ± SEM of three individual experiments. Significant difference by one-way or two-way analysis of variance (*, P<0.05, **, P<0.01 *vs.* *, P<0.001 control group; $^{\#}$, P<0.05, $^{\#\#}$, P<0.01 *vs.* $^{\#\#\#}$, P<0.001 the doxorubicin-treatment alone group; $^{\$\$\$}$, P<0.001 *vs.* doxorubicin and uric acid treatment group; Bonferroni post hoc tests).

can increase the incidence of cardiovascular disease in patients, suggesting that lowering of uric acid is not always beneficial (25). However, persistent hyperuricemia may induce the apoptosis of cardiomyocytes by activating calpain-1 and endoplasmic reticulum stress (26), and repeated administration of uric acid may be unfavorable in patients with joint and renal disease, it is also difficult to determine the absolute serum concentration beyond which the risk is significantly increased. Additionally, in patients with arthritis and tumor lysis syndrome, even uric acid concentrations beyond 1,200 μM do not necessarily cause gout (27). Moreover, renal impairment in the presence of chronic hyperuricemia is more often attributable to other factors, such as hypertension or diabetes, rather than the

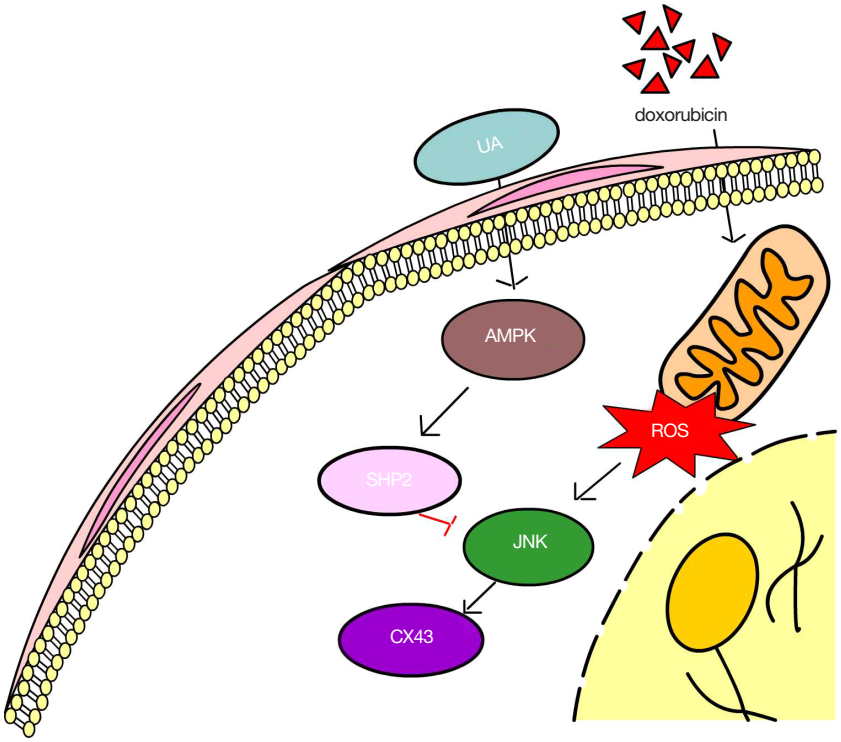

Figure 5 Schematic indicated that uric acid preconditioning could inhibit doxorubicin-induced the changes of JNK-Cx43 gap junctions and conduction abnormalities via AMPK-SHP2 signaling pathway. Doxorubicin could significantly induce the activation of JNK-Cx43 signaling pathway, and lead to gap junctions changes and conduction abnormalities, and then mediate the production of cardiac toxicity. However, uric acid preconditioning could significantly activate AMPK and induce the upregulation of SHP2, which could in turn suppress the change of Cx43 gap junctions and conduction abnormalities, and alleviate doxorubicin-mediated cardiotoxicity.

serum uric acid concentration itself. Thus, increased uric acid levels do not always have negative effects. In this study, we found that the uric acid-lowering medicine allopurinol increased the mortality rate of doxorubicin-treatment mice. In addition, uric acid preconditioning significantly increased the survival rate and body weight of mice and reduced the levels of AST, CK, and LDH relative to doxorubicin-treated mice. Additionally, studies have shown that uric acid does not cause tumor drug resistance to doxorubicin (28). Based on these results, increased endogenous uric acid levels may have a certain protective effect on the heart.

Previous studies have linked gap junctions (Cx43) to cardiac conduction. The role of gap junctions is dependent on the phosphorylation status of Cx43. Connexin phosphorylation is an interaction between protein kinase and phosphatase, but the exact pathway is unknown (29). The stimulation of Cx43 phosphorylation was shown to decrease the permeability of cardiomyocyte gap junctions. Changes in Cx43 phosphorylation and localization may also cause gap junction dysfunction in heart failure, slow conduction velocities, and arrhythmias (30). Therefore, regulating Cx43 phosphorylation levels may be a potential strategy in improving conduction in heart failure. However, the mechanism underlying doxorubicin-induced changes in gap junctions is still unclear.

Moreover, JNK reportedly plays an essential role in the regulation of Cx43 and p-CX43. JNK activation led to a loss of total Cx43 protein and increased p-CX43 levels, which significantly reduced conduction velocity (31). Furthermore, it also can reduce Cx43-mediated cell-to-cell communication (32). Additionally, knockdown of the carboxyl terminal domain of Cx43 lead to an increase in the infarct size and increased susceptibility to arrhythmias following acute coronary occlusion in a previous study (33). Consistent with previous studies, we found that doxorubicin could significantly upregulated p-JNK and p-Cx43 *in vitro* and *in vivo* and induced abnormal conduction in the heart, such as bradycardia, QT prolongation, and arrhythmia,

whereas preconditioning with uric acid effectively reversed these effects.

Next, we further investigated how uric acid modulated the JNK-Cx43 signaling pathway to alleviate doxorubicin-induced cardiotoxicity. Recent studies have shown that metformin can resist doxorubicin-induced cardiomyocyte injury by activating AMPK (13,14). Doxorubicin has been demonstrated to reduce AMPK activity in rat heart tissue (15,34). These observations ultimately led us to reasonably believe that doxorubicin-induced cardiotoxicity might be AMPK-related. Additionally, previous studies postulated the existence of a particular relationship between AMPK and SHP2 (35). However, these findings were not conclusive. Additionally, some researchers revealed that an SHP-2 deletion in post-migratory neural crest cells results in impaired cardiac sympathetic innervation (36). Another study showed that the deletion of Ptpn11 (SHP2) in cardiomyocytes causes dilated cardiomyopathy, thus indicating the protective role of SHP2 in adult cardiac function (37). In this study, uric acid induced significant upregulation of p-AMPK and p-SHP2 but inhibited abnormal phosphorylation of JNK and Cx43 mediated by doxorubicin *in vitro* and *in vivo*. Moreover, preconditioning with AICAR mimicked the protective effect of uric acid on cardiomyocytes *in vitro*, whereas preconditioning with compound C reversed these effects and abolished the protective effect of uric acid on cardiomyocytes. Although there is some controversy regarding the effect of uric acid on AMPK (15,38,39), we found that uric acid preconditioning activated AMPK-SHP2 signaling and alleviated doxorubicin-induced alterations in the JNK-Cx43 gap junction and abnormal conduction.

Based on our results, increased of endogenous uric acid levels may help to protect against doxorubicin-induced cardiotoxicity by activating the AMPK-SHP2 signaling pathway. However, how uric acid activates AMPK-induced SHP2 activation, whether uric acid has a cardioprotective effect in other animals such as rats and beagles, and the potential value of this novel therapeutic strategy in the clinic require further analyses.

Conclusions

In conclusion, we provided experimental evidence to demonstrated that uric acid preconditioning alleviated doxorubicin-induced cardiotoxicity via the AMPK-SHP2-JNK-Cx43 signaling axis. Our results demonstrated that uric acid preconditioning significantly upregulated SHP2 expression in an AMPK-dependent manner, and inhibited doxorubicin-induced phosphorylation of gap junction protein, Cx43, to alleviate doxorubicin-induced cardiotoxicity.

Acknowledgments

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Footnote

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References

1. Rochette L, Guenancia C, Gudjoncik A, et al. Anthracyclines/trastuzumab: new aspects of cardiotoxicity and molecular mechanisms. Trends Pharmacol Sci 2015;36:326-48.
2. Saad SY, Najjar TA, Al-Rikabi AC. The preventive role of deferoxamine against acute doxorubicin-induced cardiac, renal and hepatic toxicity in rats. Pharmacol Res 2001;43:211-8.
3. Zhang S, Liu X, Bawa-Khalfe T, et al. Identification of the molecular basis of doxorubicin-induced cardiotoxicity. Nat Med 2012;18:1639-42.
4. Xin Y, Zhang S, Gu L, et al. Electrocardiographic and biochemical evidence for the cardioprotective effect of antioxidants in acute doxorubicin-induced cardiotoxicity in the beagle dogs. Biol Pharm Bull 2011;34:1523-6.
5. Kizana E, Ginn SL, Allen DG, et al. Fibroblasts can be genetically modified to produce excitable cells capable of electrical coupling. Circulation 2005;111:394-8.
6. Gutstein DE, Morley GE, Vaidya D, et al. Heterogeneous expression of Gap junction channels in the heart leads to conduction defects and ventricular dysfunction. Circulation 2001;104:1194-9.
7. Pecoraro M, Ciccarelli M, Fiordelisi A, et al. Diazoxide Improves Mitochondrial Connexin 43 Expression in a Mouse Model of Doxorubicin-Induced Cardiotoxicity. Int J Mol Sci 2018;19:757.
8. Ichikawa Y, Ghanefar M, Bayeva M, et al. Cardiotoxicity of doxorubicin is mediated through mitochondrial iron accumulation. J Clin Invest 2014;124:617-30.
9. Itahana Y, Han R, Barbier S, et al. The uric acid transporter SLC2A9 is a direct target gene of the tumor suppressor p53 contributing to antioxidant defense. Oncogene 2015;34:1799-810.
10. Amaro S, Laredo C, Renu A, et al. Uric Acid Therapy Prevents Early Ischemic Stroke Progression: A Tertiary Analysis of the URICO-ICTUS Trial (Efficacy Study of Combined Treatment With Uric Acid and r-tPA in Acute Ischemic Stroke). Stroke 2016;47:2874-6.
11. Du Y, Chen CP, Tseng CY, et al. Astroglia-mediated effects of uric acid to protect spinal cord neurons from glutamate toxicity. Glia 2007;55:463-72.
12. Xiang HC, Lin LX, Hu XF, et al. AMPK activation attenuates inflammatory pain through inhibiting NF-kappaB activation and IL-1beta expression. J Neuroinflammation 2019;16:34.
13. Zilinyi R, Czompa A, Czegledi A, et al. The Cardioprotective Effect of Metformin in Doxorubicin-Induced Cardiotoxicity: The Role of Autophagy. Molecules 2018;23:1184.
14. Chen J, Zhang S, Pan G, et al. Modulatory effect of metformin on cardiotoxicity induced by doxorubicin via the MAPK and AMPK pathways. Life Sci 2020;249:117498.
15. Gratia S, Kay L, Potenza L, et al. Inhibition of AMPK signalling by doxorubicin: at the crossroads of the cardiac responses to energetic, oxidative, and genotoxic stress. Cardiovasc Res 2012;95:290-9.
16. Timm KN, Tyler DJ. The Role of AMPK Activation for Cardioprotection in Doxorubicin-Induced Cardiotoxicity. Cardiovasc Drugs Ther 2020;34:255-69.
17. Haider UG, Roos TU, Kontaridis MI, et al. Resveratrol inhibits angiotensin II- and epidermal growth factor-mediated Akt activation: role of Gab1 and Shp2. Mol Pharmacol 2005;68:41-8.
18. Wu AH, Gladden JD, Ahmed M, et al. Relation of serum uric acid to cardiovascular disease. Int J Cardiol 2016;213:4-7.
19. Grossman C, Grossman E, Goldbourt U. Uric acid variability at midlife as an independent predictor of coronary heart disease and all-cause mortality. PLoS One 2019;14:e0220532.
20. Shiozawa A, Szabo SM, Bolzani A, et al. Serum Uric Acid and the Risk of Incident and Recurrent Gout: A Systematic Review. J Rheumatol 2017;44:388-96.
21. Glantzounis GK, Tsimoyiannis EC, Kappas AM, et al. Uric acid and oxidative stress. Curr Pharm Des 2005;11:4145-51.
22. Tasaki E, Sakurai H, Nitao M, et al. Uric acid, an important antioxidant contributing to survival in termites. PLoS One 2017;12:e0179426.
23. Yasutake Y, Tomita K, Higashiyama M, et al. Uric acid ameliorates indomethacin-induced enteropathy in mice through its antioxidant activity. J Gastroenterol Hepatol 2017;32:1839-45.
24. Waring WS, Webb DJ, Maxwell SR. Systemic uric acid administration increases serum antioxidant capacity in healthy volunteers. J Cardiovasc Pharmacol 2001;38:365-71.
25. Messerli FH, Burnier M. Cardiovascular disease and uric acid: is the not-so-innocent bystander becoming a true culprit and does the US black box warning for febuxostat indicate that not all uric acid lowering is beneficial? Eur Heart J 2019;40:1787-9.
26. Yan M, Chen K, He L, et al. Uric Acid Induces

Cardiomyocyte Apoptosis via Activation of Calpain-1 and Endoplasmic Reticulum Stress. Cell Physiol Biochem 2018;45:2122-35.
27. Leach M, Parsons RM, Reilly JT, et al. Efficacy of urate oxidase (uricozyme) in tumour lysis induced urate nephropathy. Clin Lab Haematol 1998;20:169-72.
28. Cantor JR, Abu-Remaileh M, Kanarek N, et al. Physiologic Medium Rewires Cellular Metabolism and Reveals Uric Acid as an Endogenous Inhibitor of UMP Synthase. Cell 2017;169:258-72.e17.
29. Jabr RI, Hatch FS, Salvage SC, et al. Regulation of gap junction conductance by calcineurin through Cx43 phosphorylation: implications for action potential conduction. Pflugers Arch 2016;468:1945-55.
30. Akar FG, Spragg DD, Tunin RS, et al. Mechanisms underlying conduction slowing and arrhythmogenesis in nonischemic dilated cardiomyopathy. Circ Res 2004;95:717-25.
31. Petrich BG, Gong X, Lerner DL, et al. c-Jun N-terminal kinase activation mediates downregulation of connexin43 in cardiomyocytes. Circ Res 2002;91:640-7.
32. Yan J, Kong W, Zhang Q, et al. c-Jun N-terminal kinase activation contributes to reduced connexin43 and development of atrial arrhythmias. Cardiovasc Res 2013;97:589-97.
33. Maass K, Chase SE, Lin X, et al. Cx43 CT domain influences infarct size and susceptibility to ventricular tachyarrhythmias in acute myocardial infarction. Cardiovasc Res 2009;84:361-7.
34. Tokarska-Schlattner M, Zaugg M, da Silva R, et al. Acute toxicity of doxorubicin on isolated perfused heart: response of kinases regulating energy supply. Am J Physiol Heart Circ Physiol 2005;289:H37-47.
35. Nerstedt A, Cansby E, Amrutkar M, et al. Pharmacological activation of AMPK suppresses inflammatory response evoked by IL-6 signalling in mouse liver and in human hepatocytes. Mol Cell Endocrinol 2013;375:68-78.
36. Lajiness JD, Snider P, Wang J, et al. SHP-2 deletion in postmigratory neural crest cells results in impaired cardiac sympathetic innervation. Proc Natl Acad Sci U S A 2014;111:E1374-82.
37. Kontaridis MI, Yang W, Bence KK, et al. Deletion of Ptpn11 (Shp2) in cardiomyocytes causes dilated cardiomyopathy via effects on the extracellular signal-regulated kinase/mitogen-activated protein kinase and RhoA signaling pathways. Circulation 2008;117:1423-35.
38. Kimura Y, Yanagida T, Onda A, et al. Soluble Uric Acid Promotes Atherosclerosis via AMPK (AMP-Activated Protein Kinase)-Mediated Inflammation. Arterioscler Thromb Vasc Biol 2020;40:570-82.
39. Luo C, Lian X, Hong L, et al. High Uric Acid Activates the ROS-AMPK Pathway, Impairs CD68 Expression and Inhibits OxLDL-Induced Foam-Cell Formation in a Human Monocytic Cell Line, THP-1. Cell Physiol Biochem 2016;40:538-48.

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