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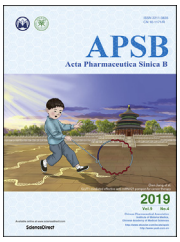

ORIGINAL ARTICLE

Matrine attenuates oxidative stress and cardiomyocyte apoptosis in doxorubicin-induced cardiotoxicity *via* maintaining AMPKα/UCP2 pathway

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KEY WORDS

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Abstract Oxidative stress and cardiomyocyte apoptosis are involved in the pathogenesis of doxorubicin (DOX)-induced cardiotoxicity. Matrine is well-known for its powerful anti-oxidant and anti-apoptotic capacities. Our present study aimed to investigate the effect of matrine on DOX-induced cardiotoxicity and try to unearth the underlying mechanisms. Mice were exposed with DOX to generate DOX-induced cardiotoxicity or normal saline as control. H9C2 cells were used to verify the effect of matrine *in vitro*. DOX injection triggered increased generation of reactive oxygen species (ROS) and excessive cardiomyocyte apoptosis, which were significantly mitigated by matrine. Mechanistically, we found that matrine ameliorated DOX-induced uncoupling protein 2 (UCP2) downregulation, and UCP2 inhibition by

Abbreviations: 4-HNE, 4-hydroxynonenal; ACC, acetyl-CoA carboxylase; AMPKα, 5′-AMP-activated protein kinase α; ANOVA, analysis of variance; BAX, BCL-2-associated X protein; BCL-2, B-cell lymphoma 2; C-caspase 3, cleaved-caspase3; CCK-8, cell counting kit 8; CK-MB, creatine kinase isoenzymes; cTnT, cardiac isoform of Tropnin T; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; DMEM, Dulbecco's modified Eagle's medium; DOX, doxorubicin; FBS, fetal bovine serum; FS, fractional shortening; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HW, heart weight; LDH, lactate dehydrogenase; MDA, malondialdehyde; BCA, bicinchoninic acid; PPARs, peroxisomal proliferators-activated receptors; ROS, reactive oxygen species; SOD2, superoxide dismutase 2; T-caspase3, total-caspase3; TL, tibia length; Top2, topoisomerase-II; TUNEL, TdT-mediated dUTP nick end-labelling; UCP2, uncoupling protein 2

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genipin could blunt the protective effect of matrine on DOX-induced oxidative stress and cardiomyocyte apoptosis. Besides, 5′-AMP-activated protein kinase α2 (*Ampkα2*) deficiency inhibited matrine-mediated UCP2 preservation and abolished the beneficial effect of matrine in mice. Besides, we observed that matrine incubation alleviated DOX-induced H9C2 cells apoptosis and oxidative stress level *via* activating AMPKα/UCP2, which were blunted by either AMPKα or UCP2 inhibition with genetic or pharmacological methods. Matrine attenuated oxidative stress and cardiomyocyte apoptosis in DOX-induced cardiotoxicity *via* maintaining AMPKα/UCP2 pathway, and it might be a promising therapeutic agent for the treatment of DOX-induced cardiotoxicity.

1. Introduction

Doxorubicin (DOX) is a widely used and highly potent chemotherapeutic agent to treat various types of cancers[1]. However, the clinical usage of DOX is greatly limited by its dose-dependent cardiotoxicity, which eventually leads to irreversible degenerative cardiomyopathy and congestive heart failure[2]. Despite the limited elucidation of pathogenesis of DOX-induced cardiotoxicity, accumulating evidence suggested that excessive production of ROS is the main factor involved in DOX-induced cardiotoxicity, which leads to oxidative damage and cardiomyocyte apoptosis, and eventually the occurrence of heart failure[3,4].

Mitochondria are the major source of intracellular ROS. The excessive production of ROS induces oxidative damage to biological macromolecules, including lipids, proteins and DNA, and disrupts cellular membrane structure and functions[5,6]. Mitochondrial uncoupling proteins (UCPs) are now well accepted as mitochondrial metabolite transporters, which mainly located in the inner membrane of mitochondria[7,8]. It has been reported that UCPs family could partially dissipate the proton concentration gradient of the inner mitochondria membrane, and play critical roles in controlling intracellular ROS homeostasis and preventing oxidative stress[9]. UCP2, a novel member of the UCPs family, sharing 60% sequence identity with the well-known thermogenic uncoupling protein 1 (UCP1) from brown adipose tissue, is the predominant type of UCPs in the heart[10]. Previous studies indicated that *Ucp2* deficiency exacerbated oxidative stress and delayed liver regeneration in mice, whereas adenovirus-mediated UCP2 overexpression inhibited ROS production and protected endothelial cells from oxidative damage[11,12]. Besides, activation of UCP2 allowed for the prevention of ischemia-reperfusion induced oxidative stress and preservation of mitochondrial function[13]. Previously, Dhamrait et al.[14] reported that gene mutation of *UCP2* in man that led to a decreased UCP2 expression could reduce total antioxidant status. More importantly, a recent study demonstrated that knockdown of UCP2 could exacerbate DOX-induced cardiomyocyte apoptosis and myocardial oxidative stress[15]. 5′-AMP-activated protein kinase α (AMPKα) is a highly conserved eukaryotic serine/threonine protein kinase, and exerts beneficial effects in many physiological and pathological process, including energy homeostasis, endoplasmic reticulum stress, cardiac hypertrophy and fibrosis, etc.[16,17]. Current available studies showed that AMPKα also played essential roles in regulating oxidative stress and cell survival[18,19]. Kim et al.[20] found that AMPKα activation decreased iron-induced ROS production and inhibited cell apoptosis. Ceolotto et al.[21] further confirmed that activation of AMPKα prevented hyperactivity of NADPH oxidase and protected endothelial cells against glucose-induced oxidative damage. All these data defined indispensable roles of UCP2 and AMPKα in the regulation of oxidative stress and cell apoptosis. Therefore, targeting AMPKα and UCP2 may be of great therapeutic interest for the treatment of DOX-induced cardiotoxicity.

Matrine, a natural compound extracted from the root of *Sophora flavescens* Ait, has been shown several pharmacological activities, including anti-inflammatory, anti-fibrotic capacities, and served as a therapeutic agent clinically for the treatment of viral hepatitis and dementia[22–24]. Previous studies showed that matrine and its derivatives reduced inflammatory response and attenuated sepsis-induced organ injury[24]. In addition, Liu et al.[25] implied that matrine treatment increased the expression of NF-E2-related factor 2 and heme oxygenase-1, and suppressed ROS production in experimental autoimmune encephalomyelitis. Moreover, matrine administration resulted in an apparent decrease of ROS formation and prevented cardiomyocyte apoptosis in diabetic hearts[26]. Based on these data, we suppose that matrine might be a promising therapeutic agent against DOX-induced cardiotoxicity. Therefore, the present study aimed to evaluate the protective effect of matrine on DOX-induced cardiotoxicity and tried to elucidate the underlying mechanisms.

2. Materials and methods

2.1. Reagents and antibodies

Matrine (M5319), DOX (D1515) and genipin (G4796) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Primary antibodies for the following proteins were purchased from Cell Signaling Technology (Danvers, MA, USA): UCP2 (1:1000), total-caspase3 (T-caspase3, 1:1000), cleaved-caspase3 (C-caspase3, 1:1000), BCL-2-associated X protein (BAX, 1:1000), T-AMPKα (1:1000), phosphorylated-AMPKα (P-AMPKα, 1:1000), T-acetyl-CoA carboxylase (T-ACC, 1:1000), p-ACC (1:1000) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:1000). Anti-B-cell lymphoma 2 (BCL-2, 1:1000), superoxide dismutase 2 (SOD2, 1:1000) and 4-hydroxynonenal (4-HNE, 1:200 for staining) were obtained from Abcam (Cambridge, UK). Dihydroethidium (DHE) was obtained from Keygen Biotech (Nanjing, China), and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Beyotime Biotechnology (Shanghai, China). Malondialdehyde (MDA) assay kit, total SOD assay kit and NADPH oxidase assay kit were all purchased from Nanjing Jiancheng

Bioengineering Institute (Nanjing, China). The cell counting kit-8 (CCK-8) was obtained from Dōjindo Laboratories (Kumamoto, Japan). ApopTag® Plus *In Situ* Apoptosis Fluorescein Detection Kit was purchased from Millipore (Billerica, MA, USA). Small interfering RNA against UCP2 (si*Ucp2*) and its negative control (si*RNA*) were synthesized by RiboBio (RiboBio Co. Ltd, Guangzhou, China). Replication-defective adeno-vectors carrying small hairpin RNA against *Ampkα2* (sh*Ampkα2*) were obtained from Vigene Bioscience (Rockville, MD, USA), and the efficiency was confirmed by western blot as previously described[27,28]. Anti-rabbit/mouse EnVision^{TM+}/HRP reagent was purchased from Gene Technology (Shanghai, China). The bicinchoninic acid (BCA) protein assay kit was obtained from Pierce (Rockford, IL, USA).

2.2. Animals and treatment

All animal care and experimental procedures were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University (Wuhan, China) and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication, revised 2011). Adult male C57/B6 mice (weight: 23.5–27.5 g, age: 8–10 weeks) were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China). Mice were bred in the specific pathogen free conditions with a controlled temperature (20–25 °C) and suitable humidity (50 ± 5%). The animals were allowed free access to food or water, and were housed with regular 12/12 h light/dark cycles. After an adaptive feeding for 1 week, the mice were randomly exposed to intraperitoneal injections of DOX (4 mg/kg) weekly for consecutive 4 weeks to generate DOX-induced cardiotoxicity or equal volume of normal saline (NS) as a control. To investigate the effect of matrine, mice were intragastrically administered with matrine (200 mg/kg/day) simultaneously from the first injection of DOX referring to previous studies[26,29]. All the mice were monitored and weighed weekly, and were sacrificed after 4-week matrine treatment, with hearts collected for further experiment. For UCP2 inhibition, genipin (30 mg/kg) was administered biweekly by intraperitoneal injection for two times referring to previous data[30]. To verify the involvement of AMPKα, *Ampkα2* knockout (KO) mice were used, and the source has been described in our previous studies[27].

2.3. Echocardiography and hemodynamics

Echocardiography and invasive hemodynamic monitoring were performed referring to our previous studies[27]. Briefly, mice were lightly anaesthetized with isoflurane (1.5%) and subjected to a MyLab 30CV ultrasound (Esaote SpA, Genoa, Italy) to detect the morphological and functional parameters of the heart, averaged from three to five cardiac cycles. Hemodynamic variables were collected by the PowerLab system (AD Instruments Ltd., Oxford, UK) using a 1.4-French Millar pressure-volume catheter (SPR-839; Millar Instruments, Houston, TX) and the data were analyzed using the PVAN data analysis software.

2.4. Immunohistochemistry staining

The paraffin-embedded heart sections were dewaxed, rehydrated and incubated with citric acid buffer for antigen retrieval, and then they were treated with 3% hydrogen peroxide and 10% goat serum to block endogenous peroxidase and the nonspecific binding of the antibody. After incubated with primary antibodies against 4-HNE (1:100) at 4 °C overnight, sections were incubated with anti-rabbit/mouse EnVision^{TM+}/HRP reagent for 1 h at 37 °C. After being visualized with diaminobenzidin for 2 min at room temperature, these sections were observed and analyzed by two investigators in a blinded manner.

2.5. Western blot and quantitative real-time PCR

Western blot and quantitative real-time PCR were performed according to our previous studies[31]. Total proteins were extracted from murine hearts or cell lysates, and the protein concentration was determined by BCA protein assay kit. Then, the proteins were separated on 10% SDS-PAGE and transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA; No. IPFL00010). Membranes were blocked with 5% skim milk for 1 h at room temperature, and incubated with the primary antibodies at 4 °C overnight followed with the secondary antibodies for an additional 1 h. The images were detected and quantified by Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Total RNA was extracted using TRIzol reagent and reverse transcribed with Maxima First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland; 04896866001). The expression level of each individual transcript was normalized to *Gapdh*.

2.6. Cell culture and treatment

H9C2 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, USA) with 10% fetal bovine serum (FBS, GIBCO, USA). After being starved for 16 h to synchronize, H9C2 cells were assigned randomly to incubate with matrine (200 μmol/L) or equal volume of vehicle in the presence or absence of DOX (1 μmol/L) for 24 h[32]. To knock down the expression of UCP2 *in vitro*, cells were transfected with si*Ucp2* (50 nmol/L) for 24 h using Lipo6000^{TM} transfection reagent (Beyotime, Shanghai, China) according to the manufacturer's protocol as our previous described[33]. The efficiency was confirmed by western blot, and we chose the most effective sequence for further study. To evaluate the role of AMPKα in matrine-mediated protective effects, we knocked down AMPKα with sh*Ampkα2* as previously described[34]. The efficiency was determined with western blot. Cell viability was detected by CCK-8 according to the protocol.

2.7. Oxidative stress detection in vivo and in vitro

ROS generation was detected by DHE staining *in vivo* and DCFH-DA staining *in vitro*. Briefly, frozen heart sections or H9C2 cells were incubated with DHE (5 μmol/L) or DCFH-DA (5 μmol/L) at 37 °C for 30 min in a dark chamber. Fluorescent images were observed with a fluorescence microscope (Olympus IX53, Tokyo, Japan) in a blinded manner. To further assess oxidative stress level, the content of MDA, total SOD activity and NADPH oxidase activity in the myocardium or H9C2 cells were measured according to our previous study by commercially available kits[35].

2.8. TdT-mediated dUTP nick end-labelling (TUNEL) staining

A commercially available kit was used to detect apoptosis levels *in vivo* and *in vitro* as we previously described[36], and the images were captured *via* the OLYMPUS DX51 fluorescence microscope (Tokyo, Japan).

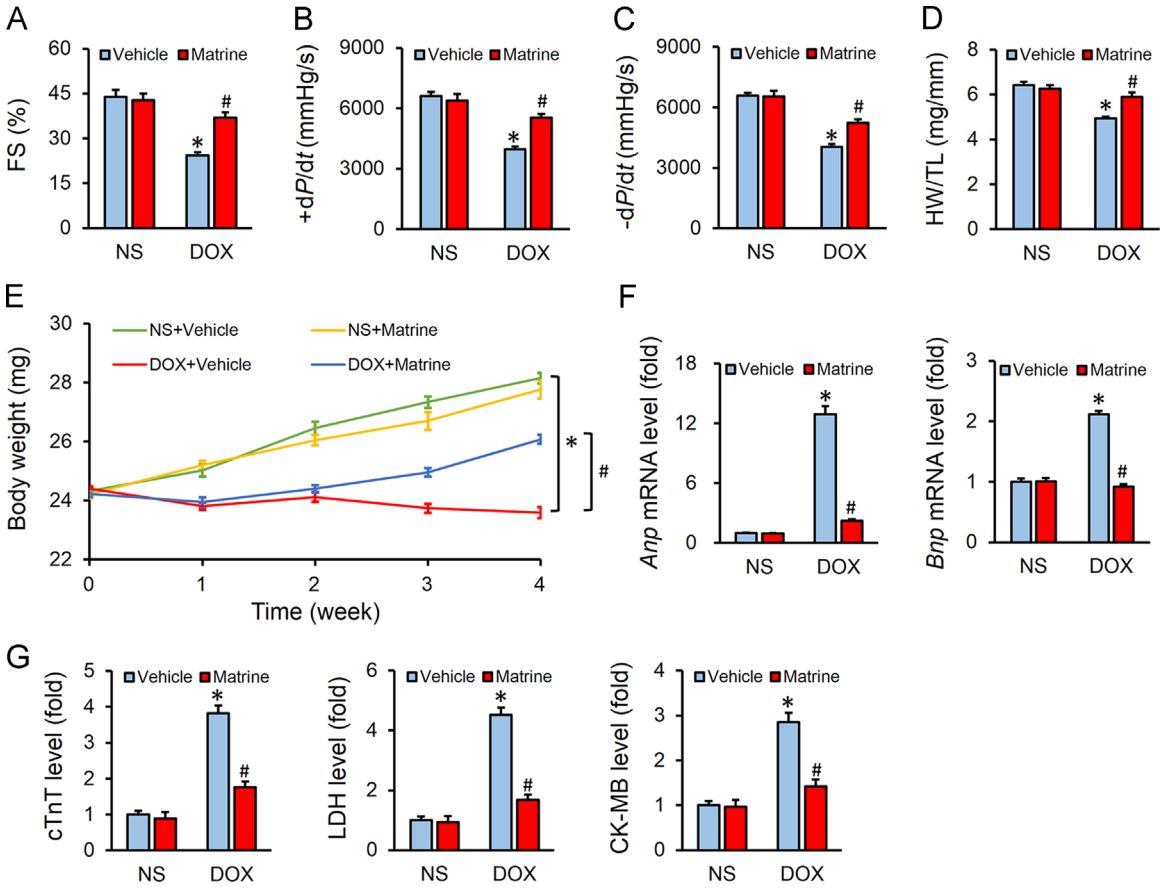

Figure 1 Matrine attenuated doxorubicin (DOX)-induced cardiotoxicity in mice. (A) Quantitative analysis of fraction shortening (FS) obtained from echocardiography ($n = 8$). (B) and (C) Hemodynamic analysis of mice with or without matrine treatment ($n = 8$). (D) Statistical results of the heart weight (HW)/tibia length (TL) ($n = 12$). (E) Body weight of four groups ($n = 12$). (F) Relative mRNA levels of *Anp*, *Bnp* ($n = 6$). (G) Plasma levels of cTnT, LDH and CK-MB in mice ($n = 6$). Values represent mean ± SEM. $^{*}P < 0.05$ *versus* NS+Vehicle; $^{\#}P < 0.05$ *versus* DOX+Vehicle.

2.9. *Biochemical analysis*

Serum levels of cardiac isoform of Tropnin T (cTnT), lactate dehydrogenase (LDH) and creatine kinase isoenzymes (CK-MB) were measured by an automatic biochemical analyzer (ADVIA® 2400, Siemens Ltd., China) as previously described[37].

2.10. *Statistical analysis*

All data in this research were presented as mean ± standard error of the mean (SEM) and analyzed using SPSS 22.0 software. One-way analysis of variance (ANOVA) followed by Tukey *post hoc* test was performed when comparing multiple groups, whereas differences in two groups were evaluated using unpaired Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. *Matrine attenuated DOX-induced cardiotoxicity in mice*

Mice were challenged with DOX (4 mg/kg, i.p.) weekly for consecutive 4 weeks to generate DOX-induced cardiotoxicity. As shown in Fig. 1A–C, DOX injection significantly impaired the left ventricular function as indicated by the decreased fractional shortening (FS) and ±d*P*/d*t*, which were significantly attenuated by matrine administration. However, matrine treatment alone showed no beneficial effects on heart function under basal conditions (Fig. 1A–C). Consistent with the fact that DOX injection results in massive loss of cardiac cells, we observed that heart weight (HW)/tibia length (TL) was markedly decreased in DOX-treated mice, which was mitigated with matrine protection (Fig. 1D). Previous studies indicated that chemotherapeutic agents-induced body weight loss predicted bad prognosis in cancer patients and DOX clinical usage is usually accompanied with decreased body weight[38,39]. Our present data suggested that systematic administration of matrine prevented DOX-induced body weight loss in mice (Fig. 1E). Matrine also overtly decreased the mRNA levels of *Anp* and *Bnp* in DOX-treated murine hearts (Fig. 1F). Myocardial injury was also assessed by the serum levels of cTnT, LDH and CK-MB, and we observed that matrine alleviated DOX-induced myocardial injury (Fig. 1G). Collectively, we concluded that matrine attenuated DOX-induced cardiotoxicity in mice.

3.2. *Matrine prevented oxidative damage in response to DOX insult*

Oxidative stress has been proved to be the primary mechanism in DOX-induced cardiotoxicity, which eventually resulted in massive cardiomyocyte apoptosis[4]. As shown in Fig. 2A and B, matrine administration decreased lipid peroxidation and ROS generation in

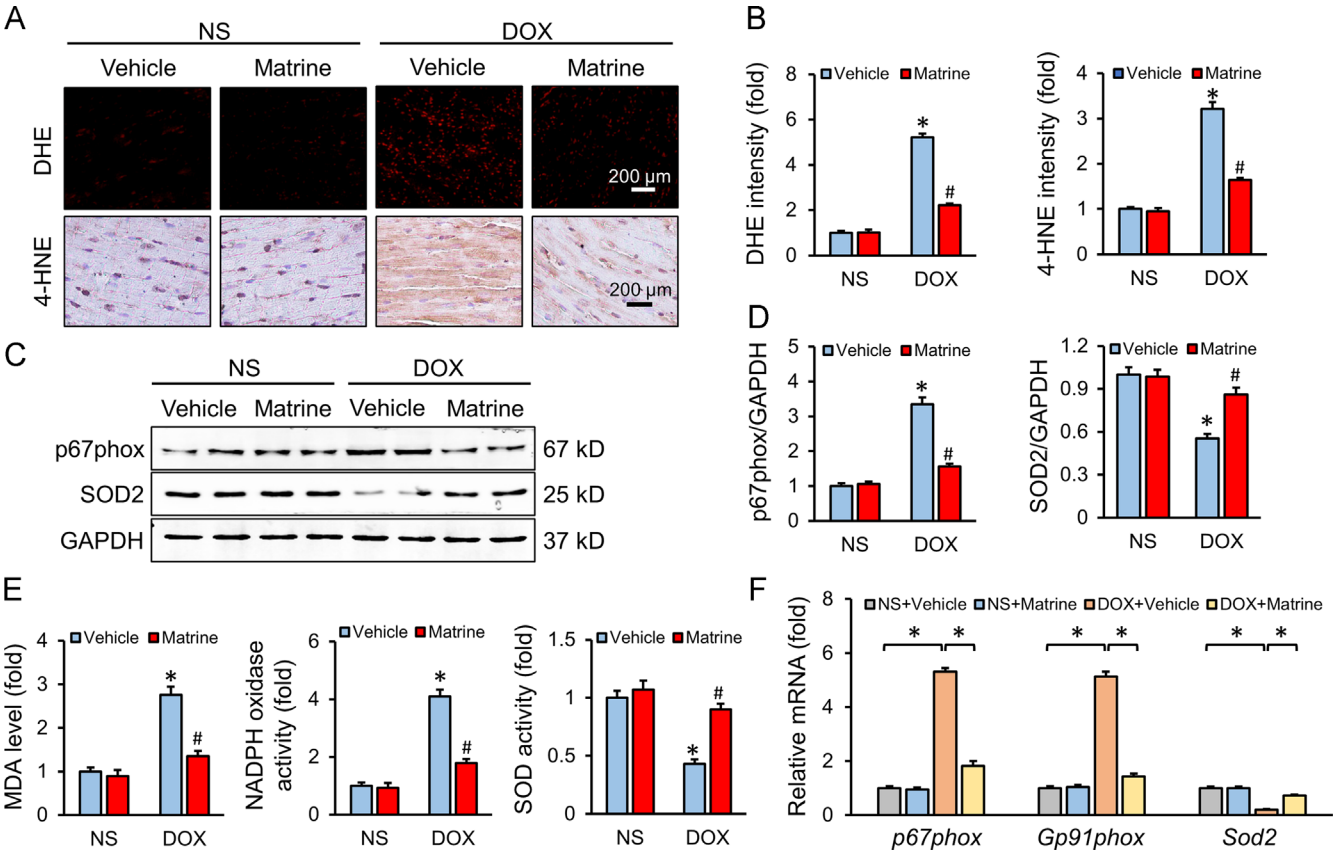

Figure 2 Matrine prevented oxidative damage in response to DOX insult. (A) and (B) Representative DHE and 4-HNE staining images and the statistical results ($n = 6$). (C) and (D) Representative Western blot and quantitative data ($n = 6$). (E) The content of MDA, NADPH oxidase activity and total SOD activity in the myocardium ($n = 6$). (F) Relative mRNA levels of *p67phox*, *Gp91phox* and *Sod2* ($n = 6$). Values represent the mean ± SEM. $^{*}P < 0.05$ *versus* NS+Vehicle; $^{\#}P < 0.05$ *versus* DOX+Vehicle.

DOX-treated murine hearts. Western blot results also verified the protective effect of matrine on DOX-induced oxidative stress, as confirmed by the decreased p67phox and increased SOD2 protein levels (Fig. 2C and D). We also observed that matrine administration reduced the abnormal MDA level and NADPH oxidase activity, and preserved total SOD activity (Fig. 2E). Such results were further confirmed by the mRNA levels of *p67phox*, *Gp91phox* and *Sod2* (Fig. 2F). All these data defined a beneficial effect of matrine on DOX-induced oxidative damage.

3.3. *Matrine attenuated DOX-induced cardiomyocyte apoptosis*

Cardiomyocyte apoptosis is an important event in the process of DOX-induced cardiotoxicity and contributes to the progression of cardiac dysfunction. Consistently, we found that DOX injection resulted in distinct cardiomyocyte apoptosis, which was significantly alleviated in mice with matrine protection (Fig. 3A and B). The protective effect on apoptosis was further confirmed by Western blot data showing that matrine decreased the expression of BAX, C-caspase3 and increased the BCL-2 level after DOX treatment (Fig. 3C and D).

3.4. *UCP2 was responsible for matrine-mediated beneficial effect on DOX-induced cardiotoxicity*

UCP2 is known to restrain ROS generation, and UCP2 knockdown exacerbated DOX-induced oxidative stress and cell death[40]. Consistent with previous studies, we observed that myocardial expression of UCP2 was downregulated by DOX, which was alleviated in mice with matrine protection (Fig. 4A). To further elucidate the role of UCP2 in matrine-mediated protective effects *in vivo*, we treated mice with genipin, an inhibitor of UCP2. As shown in Fig. 4B–G, UCP2 inhibition almost completely abolished matrine-induced downregulation of p67phox, BAX, C-caspase3 and upregulation of SOD2, BCL-2, which was further verified by alterations of *p67phox*, *Gp91phox* and *Sod2* in mRNA level (Fig. 4H). In addition, we found that the inhibitory effect of matrine on the abnormal MDA level, NADPH oxidase activity and total SOD activity was also abrogated in the presence of genipin (Fig. 4I). DOX-triggered myocardial injury, as assessed by serum levels of cTnT and LDH, was prevented in mice with matrine treatment, but not in mice treated with matrine together with genipin (Fig. 4J–K).

3.5. *Knockdown of UCP2 blunted the protective effects of matrine in vitro*

To investigate whether *Ucp2* deficiency could blunt the protective effects of matrine on oxidative stress and cardiomyocyte apoptosis *in vitro*, we knocked down the expression of UCP2 in H9C2 cells *via* si*Ucp2* and the efficiency was confirmed by

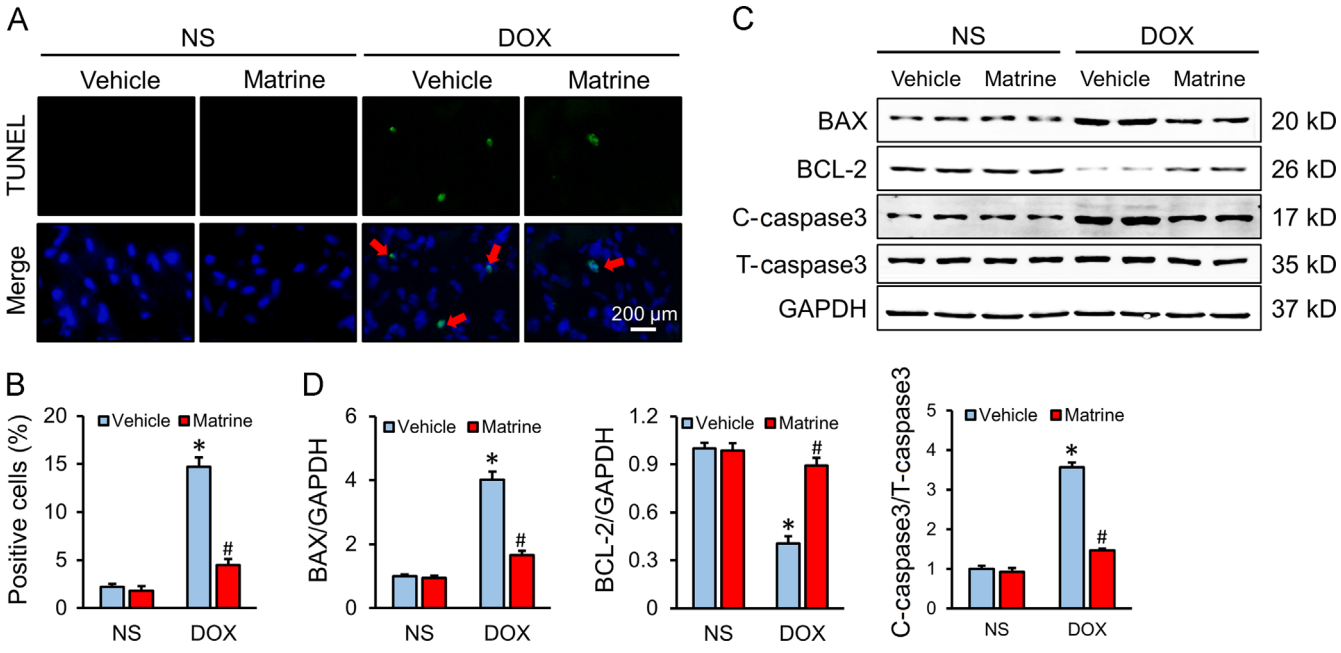

Figure 3 Matrine attenuated DOX-induced cardiomyocyte apoptosis. (A) and (B) Representative TUNEL staining images and the quantitative results for apoptosis in heart tissues ($n = 6$). (C) and (D) Western blot and statistical results in the indicated groups ($n = 6$). Red arrows indicate TUNEL positive staining. Values represent the mean ± SEM. $^{*}P < 0.05$ *versus* NS+Vehicle; $^{\#}P < 0.05$ *versus* DOX+Vehicle.

Western blot (Fig. 5A). Consistent with the data *in vivo*, we found that the inhibitory effect of matrine on ROS generation was blunted after the knockdown of UCP2 (Fig. 5B). Besides, matrine lost its effect on DOX-induced cardiomyocyte apoptosis in *Ucp2*-knockdown cells, as confirmed by the cell viability assay and TUNEL staining (Fig. 5C–D). Western blot results further confirmed that matrine-induced downregulation of p67phox, BAX, and C-caspase3, as well as upregulation of SOD2 and BCL-2, was counteracted in the absence of UCP2 (Fig. 5E–J). In line with the molecular alterations, we found that UCP2 knockdown negated matrine-mediated beneficial effects on lipid peroxidation and oxidative damage in DOX-treated H9C2 cells, as evidenced by the unaltered MDA, NADPH oxidase, SOD activity and LDH level (Fig. 5K–L). These results suggested that matrine alleviated DOX-induced oxidative stress and cardiomyocyte apoptosis *via* preserving the expression of UCP2.

3.6. *Matrine prevented DOX-induced downregulation of UCP2 via activating AMPKα*

We next explored the possible mechanism by which matrine prevented DOX-induced downregulation of UCP2. Previous studies implied that AMPK*α* was inactivated in response to DOX treatment[41]. More importantly, *Ampkα* silence reduced UCP2 level, whereas *Ampkα* activation increased the expression of UCP2[42]. Therefore, we speculated that matrine might prevent DOX-induced downregulation of UCP2 *via* activating AMPK*α*. As expected, we found that matrine treatment restored the decreased AMPK*α* activity induced by DOX incubation in H9C2 cells, which was further assessed by the increased phosphorylation of ACC (Fig. 6A and B). To gain evidence that AMPK*α* was responsible for the upregulation of UCP2, we knocked down *Ampkα* with adenoviruses in H9C2 cells, and the efficiency was evidenced by Western blot (Supporting Information Fig. S1). As shown in Fig. 6C and D, *Ampkα* knockdown abolished the restoration of UCP2 level by matrine *in vitro*. In line with the downregulation of UCP2, we found that *Ampkα* knockdown significantly abrogated matrine-mediated inhibitory effect on ROS generation and oxidative stress level (Fig. 6E–G), as well as the content of MDA, NADPH oxidase activity and total SOD activity (Fig. 6H). Besides, the protective effect of matrine on cardiomyocyte injury was also blunted, as evaluated by the cell viability, apoptosis-related proteins and serum LDH level (Fig. 6I–M). Therefore, we concluded that matrine prevented DOX-induced downregulation of UCP2 *via* activating AMPK*α*.

3.7. *Ampkα deficiency abolished the protective effect of matrine in vivo*

To further investigate whether matrine exerted its protective effects through AMPK*α*/UCP2 pathway axis *in vivo*, *Ampkα2* KO mice were used. Consistent with the data *in vitro*, we observed that *Ampkα* deletion suppressed matrine-induced upregulation of UCP2 in murine hearts (Fig. 7A). Besides, matrine-mediated downregulation of p67phox, BAX, and C-caspase3, as well as upregulation of SOD2 and BCL-2, was all reversed by *Ampkα* deletion (Fig. 7C and D). In line with the molecular alterations, matrine lost its protective effects on oxidative stress and cardiomyocyte apoptosis in *Ampkα*-deficient mice, as evidenced by the MDA level, SOD activity and serum myocardial injury markers (Fig. 7E–F). More importantly, we found that matrine could attenuate DOX-induced cardiac dysfunction in wild type mice, but not in *Ampkα*-deficient mice (Fig. 7G). The protective effect of matrine on HW/TL was also abolished in *Ampkα*-deficient mice (Fig. 7H). Collectively, these results suggested that the protective effects of matrine on oxidative stress and cardiomyocyte apoptosis were dependent on the activation of AMPK*α*/UCP2 axis.

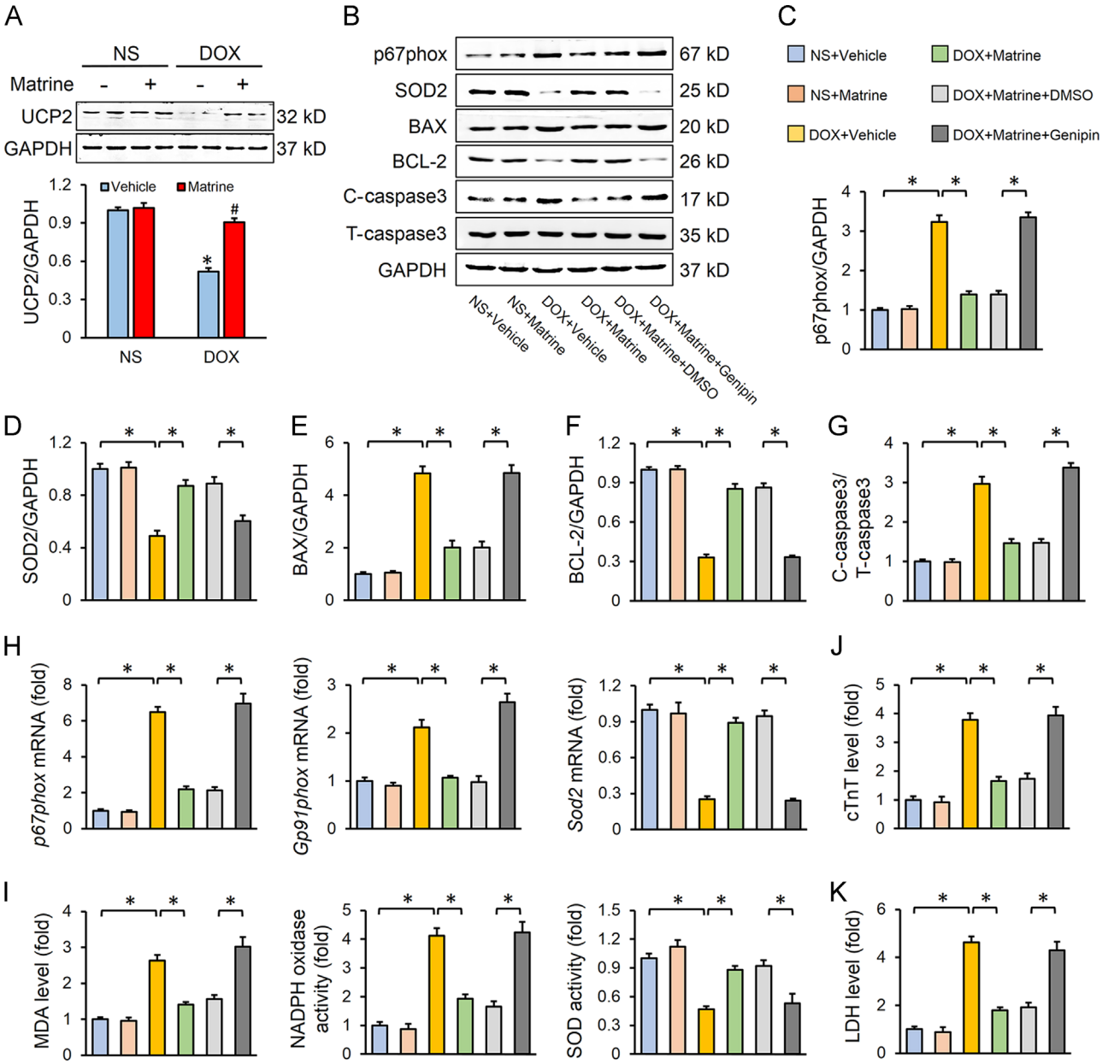

Figure 4 UCP2 was responsible for matrine-mediated beneficial effect on DOX-induced cardiotoxicity. (A) UCP2 expression level in murine hearts with or without DOX or matrine ($n = 6$; $^{*}P < 0.05$ *versus* NS+Vehicle, $^{\#}P < 0.05$ *versus* DOX+Vehicle). (B)–(G) Western blot and statistical results in the indicated groups ($n = 6$). (H) Relative mRNA levels of *p67phox*, *Gp91phox* and *Sod2* ($n = 6$). (I) The content of MDA, NADPH oxidase activity and total SOD activity in the myocardium ($n = 6$). (J)–(K) Plasma levels of cTnT and LDH ($n = 6$). Values represent the mean ± SEM. $^{*}P < 0.05$ *versus* the matched groups.

4. Discussion

DOX significantly increased the survival rate of cancer patient; nevertheless, many survivors suffer from DOX-induced cardiotoxicity with left ventricular dysfunction and heart failure, which markedly reduced the quality of life for survivors[1]. Thus, a promising pharmacological therapy for DOX-induced cardiotoxicity is urgently need to be addressed. In this study, we determined that matrine attenuated DOX-induced cardiotoxicity *via* inhibiting oxidative stress and cardiomyocyte apoptosis. Mechanistically, we found that matrine suppressed UCP2 downregulation in DOX-treated murine hearts, thus exerted beneficial effects on oxidative stress and cardiomyocyte apoptosis. And we also verified that AMPKα activation was responsible for restoring the expression of UCP2. Either AMPKα deletion or UCP2 inhibition with genetic or pharmacological methods abrogated matrine-mediated beneficial effects on oxidative stress and cardiomyocyte apoptosis both *in vivo* and *in vitro*. Therefore, our present study identified matrine as a promising therapeutic agent against DOX-induced cardiotoxicity.

Increased oxidative damage due to ROS overproduction has been actually implicated in the progression of DOX-induced cardiotoxicity[43]. Excessive ROS generation may cause DNA damage, mitochondrial dysfunction and protein synthesis inhibition, which were linked to the activation of apoptotic cascades and ultimately contribute to heart failure[44]. Zhu et al.[45] found that

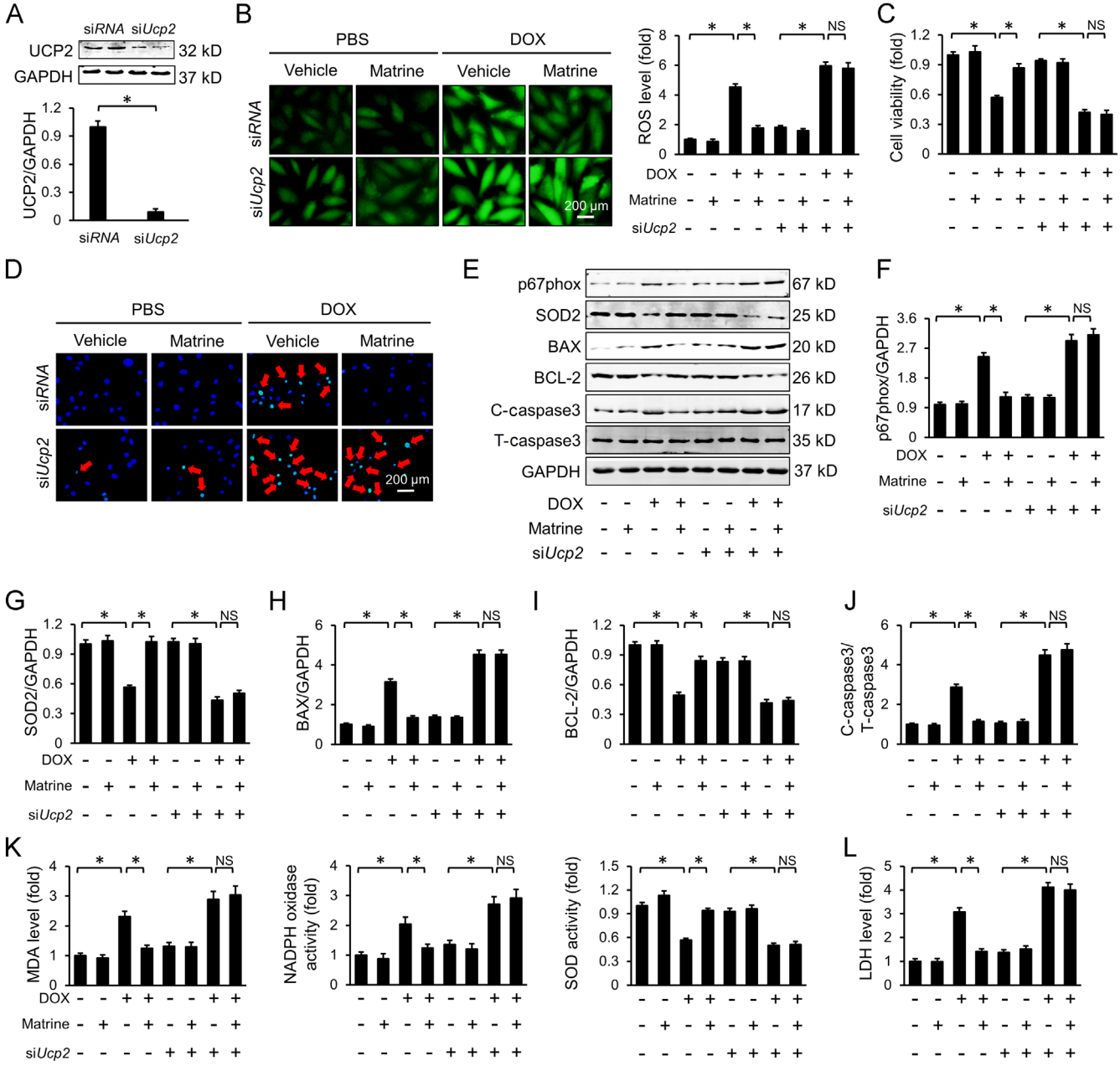

Figure 5 Knockdown of UCP2 blunted the protective effects of matrine *in vitro*. (A) Western blot for UCP2 and the quantitative data for H9C2 cells ($n = 6$). (B) Representative DCFH-DA images and the statistical results. (C) CCK-8 assay for cell viability ($n = 5$). (D) Representative images of TUNEL in H9C2 cells ($n = 5$). (E)–(J) Western blot and statistical results ($n = 6$). (K) The content of MDA, NADPH oxidase activity and total SOD activity in H9C2 cells ($n = 5$). (L) LDH levels in H9C2 cells ($n = 5$). Red arrows indicate TUNEL positive staining. Values represent the mean ± SEM. $^{*}P < 0.05$ *versus* the matched groups. NS indicates no statistical difference.

dietary nitrate supplementation decreased oxidative damage and protected against DOX-induced cardiomyopathy in mice. Moreover, Zhang et al.[43] previously defined topoisomerase-II β (Top2β) as an essential driver of DOX-induced oxidative stress and cardiomyocyte apoptosis. DOX application suppressed DNA replication and resulted in cell cycle arrest and apoptosis of cancer cells when binding to Top2α. However, when DOX bound to Top2β, it increased ROS generation, impaired mitochondrial function and triggered cardiomyocyte apoptosis for the absence of Top2α and the abundance of Top2β within the heart. These findings collectively suggested that oxidative damage was responsible for the occurrence of DOX-induced cardiac dysfunction. Accordingly, targeting oxidative stress, many cardioprotective agents including *N*-acetylcystiene, angiotensin converting enzyme inhibitors and statins have been tested in clinical trials with some efficacy for recovery of cardiac function but are not used prophylactically. Dexrazoxane is the only cardioprotective agent proven to be effective in the prevention and treatment of DOX-induced cardiotoxicity[46,47]. It is regarded as a derivative of EDTA and can decrease ROS generation through its iron chelating activity. However, long-term use of high-doses of dexrazoxane

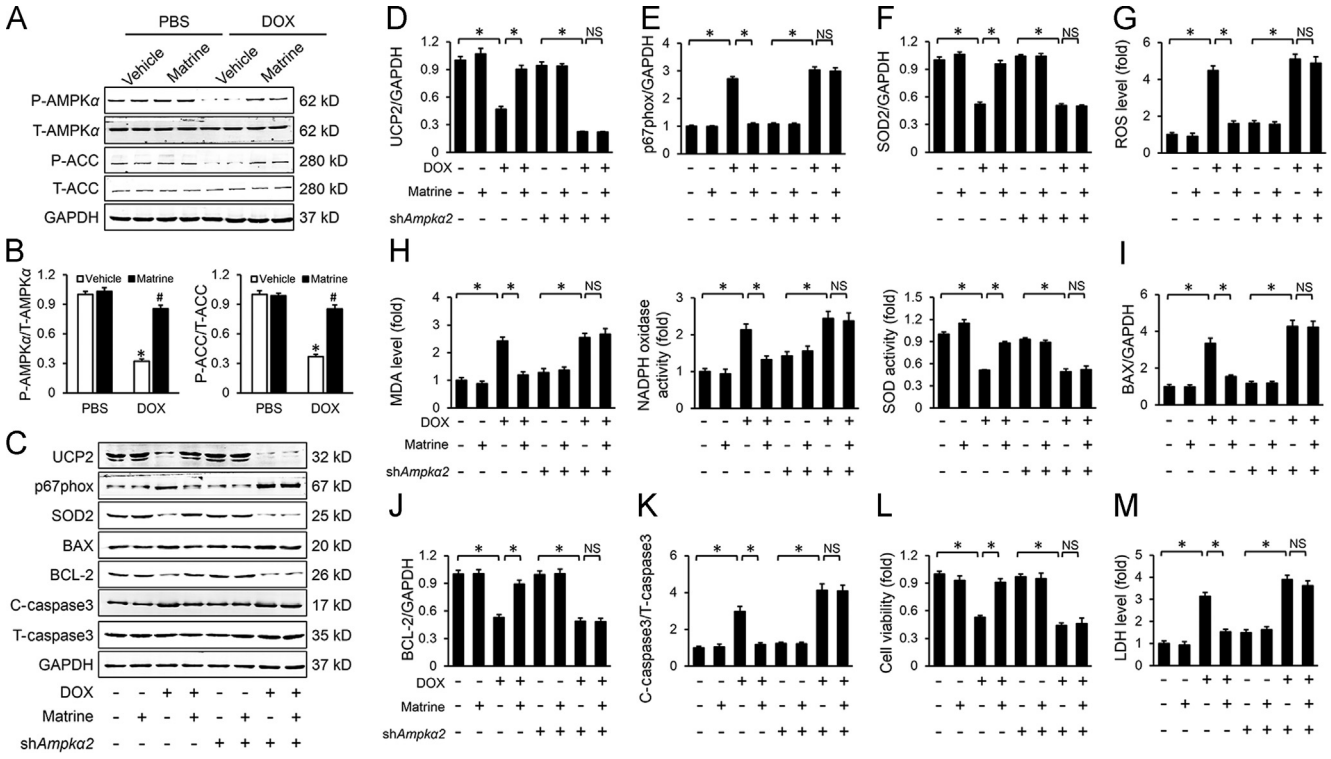

Figure 6 Matrine prevented DOX-induced downregulation of UCP2 *via* activating AMPKα. (A) and (B) Western blot and statistical results ($n = 6$). (C)–(F) Western blot and statistical results ($n = 6$). (G) Statistical results of intracellular ROS production detected by DCFH-DA in H9C2 cells ($n = 5$). (H) The content of MDA, NADPH oxidase activity and total SOD activity in H9C2 cells ($n = 5$). (I)–(K) Western blot and statistical results ($n = 6$). (L) Cell viability by CCK-8 ($n = 5$). (M) Relative LDH levels ($n = 5$). Values represent the mean ± SEM. $^{*}P < 0.05$ *versus* the matched groups. NS indicates no statistical difference.

resulted in severe side effects, such as hepatic toxicity, early death, etc. Furthermore, dexrazoxane also depletes Top2α and may reduce its chemotherapeutic capacities[48]. In the present study, we found that matrine prevented DOX-induced oxidative stress and cardiomyocyte apoptosis both *in vivo* and *in vitro*, and matrine administration attenuated cardiac dysfunction in response to DOX insult. More importantly, matrine has been clinically used for the treatment of hepatic tumors in the form of capsule or injection solution with little adverse effects (China Food and Drug Administration approvals H20010242 or H20044669; China Food and Drug Administration, 2014)[29,49]. In addition, numerous studies implied that matrine or its derivative WM130 showed beneficial effects on other cancer cells in basic experiment[50–52]. All these data proved that matrine could be an effective and safe therapeutic agent against DOX-induced cardiotoxicity.

UCP2 is increasingly recognized as an important target to restrain oxidative damage in the pathologic processes of various cardiovascular diseases, including atherosclerosis, hypertension and cardiac injury[53]. UCP2 exerted its protective effects on oxidative stress *via* uncoupling of respiration, that is, uncoupling oxygen consumption from ATP synthesis[54]. Previously, Teshima et al.[55] found that UCP2 overexpression reduced ROS production in mitochondria and protected cardiomyocytes from oxidative stress-induced cell death. In addition to its antioxidant effect, Turner et al.[10] also demonstrated that UCP2 overexpression markedly inhibited mitochondrial Ca^{2+} uptake and significantly prolonged the contractile effect. Recent studies indicated that myocardial UCP2 expression was downregulated in response to DOX and *Ucp2* deficiency further exacerbated DOX-induced oxidative stress and cardiomyocyte apoptosis[40]. In this study, we observed that matrine alleviated oxidative damage and cardiomyocyte apoptosis *via* preventing DOX-induced downregulation of UCP2, whereas pharmacological inhibition or genetic deletion of UCP2 abolished the beneficial effects both *in vivo* and *in vitro*. UCP2 protein expression has been proved to be regulated at multiple levels, and we found that AMPKα activation was responsible for matrine-mediated upregulation of UCP2, which is consistent with previous studies[56,57]. Long-chain fatty acid was the first described transcriptional activators of *Ucp2*. Reilly et al.[58] and Samec et al.[59] found that polyunsaturated fatty acids triggered UCP2 upregulation in 3T3-L1 preadipocytes. One possible candidate for transcriptional activation of *Ucp2* by long-chain fatty acid is peroxisomal proliferators-activated receptors (PPARs); however, no such PPAR response elements have been annotated within or in vicinity of the *Ucp2* gene. All this suggested that PPAR-mediated regulation of UCP2 appeared to be indirect[60]. AMPKα is a key controller of energy metabolism and survival during cellular stress, and plays a critical role in regulating lipid metabolism *via* PPAR-dependent or PPAR-independent manners[61]. Despite the exact mechanism through which AMPKα activation increases UCP2 expression remains unclear, previous studies also indicated that AMPKα activation increased the expression of UCP2, which is in line with our present study.

5. Conclusions

The present study found that matrine administration attenuated DOX-induced cardiotoxicity *via* suppressing oxidative stress and

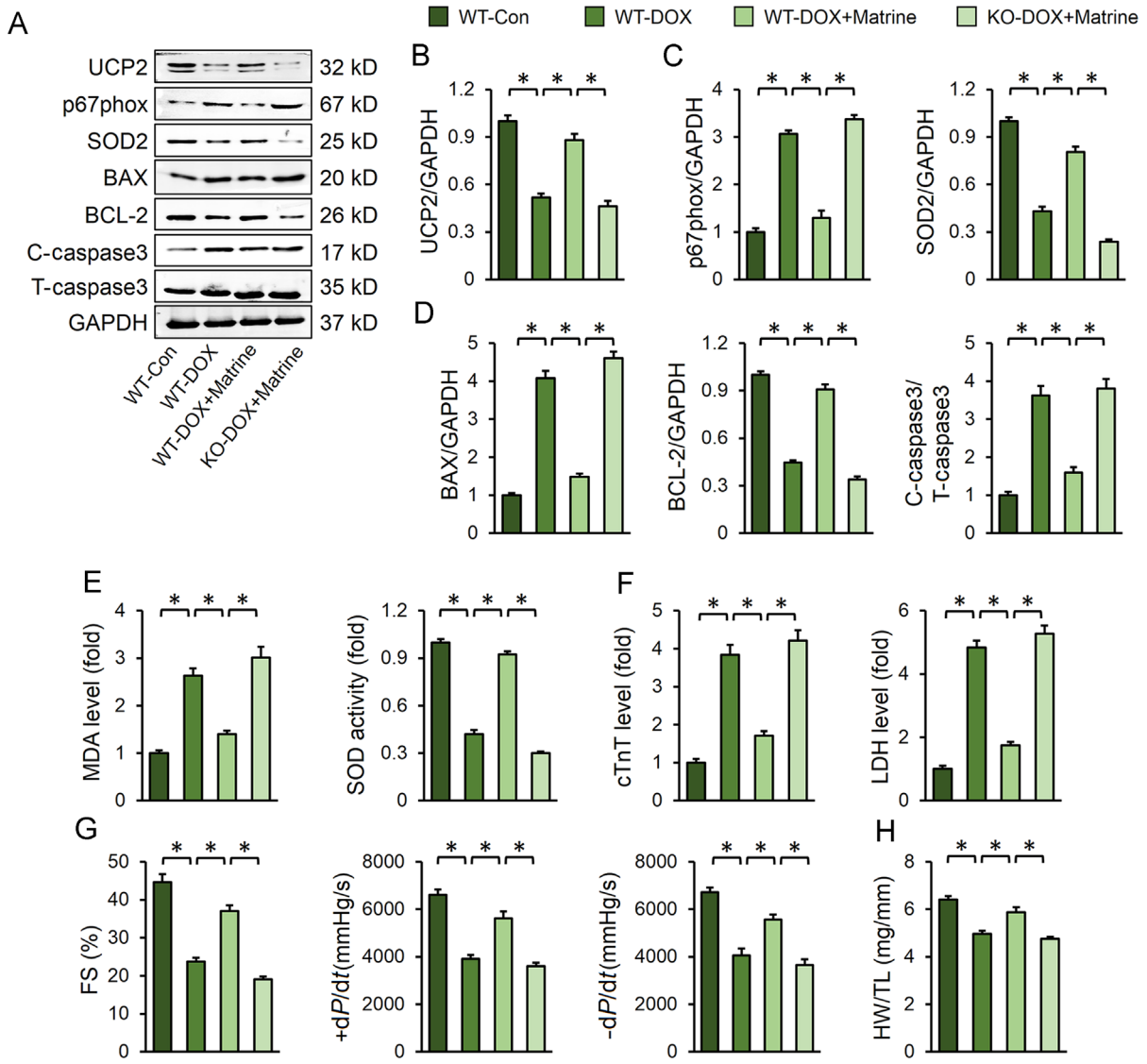

Figure 7 *Ampkα* deficiency abolished the protective effect of matrine *in vivo*. (A)–(D) Representative Western blot images and statistical results ($n = 6$). (E) The content of MDA and total SOD activity ($n = 6$). (F) Plasma levels cTnT and LDH ($n = 6$). (G) Heart function evaluated by echocardiography and hemodynamic analysis of mice ($n = 8$). (H) Statistical results of the HW/TL ($n = 12$). Values represent the mean ± SEM. $^{*}P < 0.05$ *versus* the matched groups.

cardiomyocyte apoptosis. Matrine treatment resulted in AMPKα activation and subsequently upregulated UCP2 expression to restrain ROS generation and oxidative damage. Our data identified matrine as a promising therapeutic agent for the treatment of DOX-induced cardiotoxicity.

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Appendix A. Supporting information

Supporting data associated with this article can be found in the online version at https://doi.org/10.1016/j.apsb.2019.03.003.

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