Article

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AZA Protects against Cardiomyocyte Injury by Ameliorating Mitochondrial Dysfunction Attenuating Oxidative Stress, Inflammation and Apoptosis via VDAC1

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Background: Azathioprine (AZA) is a purine-derived drug used for immunosuppression. The molecular mechanisms by which AZA protects cardiomyocytes remain unclear. This experiment will further elaborate, on the basis of the previous study, the mechanism of AZA's protection against hypoxia-induced cardiomyocyte injury in high glucose conditions.
Methods: We utilized a high glucose (HG) and hypoxia/reoxygenation (H/R) cell model and a diabetic Sprague-Dawley (SD) rat ischaemia/reperfusion (I/R) model to detect cellular calcium ions, mitochondrial membrane potential, and reactive oxygen species (ROS) levels using Fluo-4 AM, MitoTracker Red, and ROS Assay Kit. Malondialdehyde (MDA), superoxide dismutase (SOD) and pro-inflammatory cytokines (interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α) levels were measured using the appropriate kits. Cellular energy metabolism was analyzed by oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). The expression of Voltage-dependent anion channel 1 (VDAC1), Nucleotide-binding oligomerization domain-like receptor protein 3 (NALP3), Nuclear factor-kappa B p65 (NF-κB p65), ATP synthase subunit alpha (ATP5A1), ATP synthase subunit beta (ATP5B), B-cell lymphoma 2 (Bcl-2), Caspase-3 and Bcl-2 associated X protein (Bax) was detected.
Results: It was shown in the HG+H/R cell model that AZA up-regulated the levels of calcium ions ($p < 0.05$) and mitochondrial membrane potential ($p < 0.05$). AZA reduced ROS accumulation ($p < 0.01$) and oxidative stress marker MDA levels ($p < 0.01$), improved cellular energy metabolism, and increased expression of the antioxidant defense enzyme SOD ($p < 0.05$). AZA treatment inhibited VDAC1, NALP3, and NF-κB activation, upregulated ATP synthase (ATP5A1, ATP5B), and Bcl-2 expression, as well as inhibited apoptosis by downregulating Bax and Caspase3 expression in HG+H/R cells and I/R rat cardiomyocytes ($p < 0.001$). We constructed a VDAC1 siRNA cell model, and knockdown of VDAC1 significantly promoted the expression of ATP synthases (ATP5A1, ATP5B) under HG+H/R conditions ($p < 0.001$).
Conclusion: Our data reveal the molecular mechanisms by which AZA protects cardiomyocytes from injury induced by HG+H/R and I/R in diabetic rats. We demonstrate that AZA ameliorates mitochondrial dysfunction, oxidative stress, inflammation, and apoptosis by modulating VDAC1 and ATP synthase expression. Our findings suggest that AZA may be a potential therapeutic agent for mitigating myocardial ischemic injury in diabetes.

Keywords: azathioprine; diabetic cardiomyopathy; oxidative stress; inflammation; mitophagy

Introduction

There are currently 463 million cases of diabetes mellitus globally, and this number could increase to 700 million by 2045 [1]. This disease is the eighth leading cause of death worldwide, and accounts for 11.3% of deaths [2]. Diabetic cardiomyopathy is a clinical condition in which the myocardium of patients with diabetes mellitus has abnormal structure and performance [3]. Cardiovascular diseases, such as myocardial infarction caused by myocardial ischemia and hypoxia, are important life-threatening factors for patients with diabetes; in contrast to patients without diabetes, patients with diabetes are more sensitive to ischemic injury [4]. Myocardial ischemia is more likely to occur in patients with diabetes than in those without diabetes [5]. Medications for the treatment of diabetes not only control blood glucose but also blood pressure, lipids, uric acid and many other risk factors and, most importantly, have cardiovascular protective effects [6]. Therefore, reducing and preventing myocardial injury in patients with diabetes is an urgent challenge.

Myocardial ischemia refers to the insufficient supply of oxygen and nutrients to myocardial cells, resulting in functional damage to myocardial cells, which in turn results in increased myocardial infarction size, cardiac insufficiency, and even death [7]. Cardiomyocyte

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apoptosis is the result of increased production of reactive oxygen species (ROS) in a low oxygen environment [8]. Voltage-dependent anion channel 1 (VDAC1) is a major transporter protein that mediates bidirectional solute movement between the cytoplasm and mitochondria [9]. An increase in the affinity of phosphorylated VDAC1 for B-cell lymphoma 2 (Bcl-2) associated X protein (Bax) leads to increased ROS production, collapse of the mitochondrial membrane potential, and increased opening of the mPTP, resulting in increased apoptosis. This is in contrast to the actions of dephosphorylated VDAC1, which ameliorates cellular myocardial injury [10]. VDAC1 is a downstream factor of Hes1, and overexpression of the Hes1 protein can downregulate the expression of the VDAC1 protein, reduce the production of ROS, stabilize the mitochondrial membrane potential, and inhibit apoptosis in H9C2 cardiomyocytes [11]. It has also been demonstrated that miR-7a-5p targets VDAC1 and that reducing VDAC1 enhances the protection of miR-7a-5p against hypoxia/reoxygenation (H/R)-induced cellular damage [12]. In summary, VDAC1 is a crucial component of the process of cardiomyocyte injury. However, in astrocytes, mitochondrial dysfunction is accompanied by reduced levels of both ATP synthase ATP5A and ATPB and VDAC1 [13]. In renal studies, the presence of VDAC1 stimulates renal mitochondrial respiration and ATP production, reduces mitochondrial fission, and promotes the recovery of mitochondrial function, kinetics, renal morphology, and renal function [14]. VDAC1 plays different roles in mitochondrial ATP production.

Azathioprine (AZA) is a purine antimetabolite immunosuppressant that is frequently used for immunosuppressive therapy in recipients who have undergone transplantation or who suffer from autoimmune diseases [15]. In our previous research, we showed that AZA decreased the size and severity of myocardial infarction and injury in ischaemia/reperfusion (I/R) diabetic rats and downregulated the serum creatine kinase isoenzyme (CK-MB) and myeloperoxidase (MPO) levels. AZA alleviated the damage caused to the myocardium in diabetic rats after myocardial infarction by decreasing oxidative stress, cardiomyocyte apoptosis, and inflammatory responses [16]. In our study, we utilized a high glucose (HG) and H/R cell model and an I/R model in diabetic Sprague-Dawley (SD) rats to investigate further whether AZA exerts a cardiomyocyte-protective effect through the improvement of mitochondrial function and ATP synthase production via VDAC1 on the basis of previous work.

Materials and Methods

Cell Culture and Administration and Viability Assay

H9C2 cardiomyocytes (SCSP-5211, National Collection of Authenticated Cell Cultures, https://www.cellbank.org.cn/) were maintained in Dulbecco's Modified Essential Medium (DMEM, Gibco, 11995500, Shanghai, China) plus 10% fetal bovine serum (FBS, Genial Biological, G24-70500, Brighton, CO, USA) and 1% penicillin-streptomycin (biosharp, BL505A, Beijing, China) at 37 °C, in an incubator equipped with humidity, 95% air and 5% CO_2. Mycoplasma detection showed no mycoplasma contamination in the H9C2 cell samples. When the cellular confluence was about 85%, they were detached with 0.25 g/L trypsin (Gibco, 25200-072, Shanghai, China). 1.0×10^6 of cardiomyocytes were inoculated in a 25 cm^2 culture flask for passage. Every 2 days, the medium was replaced and the cardiomyocytes were passaged every 3–4 days. The cardiomyocytes in logarithmic growth phase were utilized for subsequent assays.

The cardiomyocytes were seeded onto a culture plate that contained 96 wells (1×10^5/mL) and treated with 10, 20, 40, 60, 80 and 100 μM AZA (MedChemexpress, HY-B0256, Shanghai, China) for 24 h. Dimethyl sulfoxide (DMSO) (Solarbio, D8371, Beijing, China) was utilized for dissolving AZA. As a control, equal amounts of DMSO were added. Cell viability was assayed utilizing cell counting kit-8 (CCK-8) assay kit (Jiancheng, Nanjing, China) following the manufacturer's instructions. 10 μL of CCK-8 reagent was added to the culture medium at each well, followed by continuous incubation lasting 4 h in darkness at 37 °C. The absorbance values were detected at 450 nm with a microplate reader (Rayto, RT-6000, Shenzhen, China). The Relative cell viability was obtained by calculating the ratio of the absorbance value treated with different AZA concentrations to that treated without AZA.

Transfection

VDAC1 siRNA was purchased from company (SangonBiotech, China, https://www.sangon.com/) and the sequences are as follows: *VDAC1* siRNA1 (5′-3′): Sense: GCCUCCCACAUAUGCUGAUTT, Antisense: AUCAGCAUAUGUGGGAGGCTT. Lipofectamine 8000 (Beyotime, C0533FT, Shanghai, China) were used for all transfection in accordance with the manufacturer's instructions. Silencing efficiency after 48 h transfection were detected by western blotting.

Cell Experiment Grouping and Administration

To create a high glucose (HG) environment, 50% glucose injection was added into the medium, the final concentration of glucose was 30 mM, and the cell culture was continued for 24 h. To induce hypoxia/reoxygenation (H/R) injury, the cells were pretreated with low serum (2% FBS) for 6 hours, followed by hypoxia (95% N_2+5% CO_2) for 21 hours. Then the culture conditions were changed to normal conditions (95% air+5% CO_2) for 6 h. The experimental cells were divided into Control group: cells cultured under normal oxygen conditions, DMSO group: cells cultured under DMSO treatment, HG+H/R+DMSO group: cells with H/R induced damage under HG environment

and added DMSO concentration the same as DMSO group, HG+H/R+AZA group: cells with H/R induced damage under HG environment and treated with 20 μM AZA.

Animals

24 male Sprague-Dawley rats that were 6–8 weeks old, and each weighed 200–220 grams were purchased from Charles River Company (https://www.vitalriver.com/) and reared in 22 ± 2 °C, 55 ± 5% humidity and 12 h day/night cycle with free drink and food. This study strictly followed the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and was approved by the Ethics Committee of Sichuan Vocational College of Health and Rehabilitation (2020-881). The experiment was conducted after 7 days feeding.

Animals Experimental Protocol and Drug Administration

Four groups were created by randomly assigning 24 male Sprague-Dawley rats (6 rats in each group, n = 6): (1) sham operation (2) Control+ischaemia/reperfusion (I/R); (3) diabetes mellitus (DM)+I/R; (4) DM+I/R+AZA. The DM rat model and I/R rat model were generated as previously described [16]. For I/R rat model, the rats were first administered pentobarbital sodium for anesthesia, a 2/0-T thread was employed to secure the ligation of the left anterior descending (LAD) branch following a left thoracotomy. The sham operation group did not ligate the rats. During the 120 minutes following 30 minutes of ischemia, the reperfusion was maintained. The DM rat model was constructed by intraperitoneally administering 60 mg/kg STZ, the rats with fasting glucose concentration higher than 16.8 mmol/L were successful models of DM rats. For DM+I/R+AZA group, diabetic rats were given intragastric administration of 3 mg/kg AZA for 5 d [15,17,18]. After the establishment of the DM rat model, I/R model and 5 days of AZA administration, the rats were then anaesthetized with sodium thiopental via intraperitoneal injection, blood was collected through cardiac puncture and centrifuged to obtain serum for further CK-MB, MPO, MDA and SOD detection. Myocardial tissues were harvested and rinsed with physiological saline solution for Western blot and immunohistochemical staining. At the end of experiment, rats were first administered with 50 mg/kg pentobarbital sodium for anesthesia then euthanized by cervical dislocation.

Endoplasmic Reticulum (ER) Activity Measurement

ER-Tracker Red staining was conducted in accordance with the instructions of ER-Tracker Red kit (C1041, Beyotime, Shanghai, China). H9C2 cardiomyocytes were washed twice with PBS. Afterwards, incubation with1 mM pre-warmed ER-tracker dye reagent was carried out lasting 30 min at 37 °C. ER activity was investigated utilizing a fluorescence microscope (Olympus, IX73, Tokyo, Japan). The fluorescence intensity was measured by using Image-Pro Plus 6.0 software (MEDIA CYBERNETICS, Rockville, MD, USA). The stronger the fluorescence intensity, the higher the ER activity.

Detection of Free Calcium Concentration [Ca^{2+}]i

H9C2 cardiomyocytes were recalcified by gradient with Tyrode's solution containing 0.5% bovine serum albumin (BSA) and $CaCl_2$, with a final $CaCl_2$ concentration of 1 mmol/L. The cardiomyocyte calcium (Ca^{2+}) fluorescent indicator Fluo-4/AM (F14201, Invitrogen™, Wilmington, MA, USA) was used to indicate [Ca^{2+}]i. The cardiomyocytes were incubated at 37 °C in darkness with 5 μmol/L Fluo-4/AM for 0.5 h. Afterwards, they were washed for twice with PBS to remove unbound indicator. The fluorescence density of [Ca^{2+}]i in the cardiomyocytes was detected under a fluorescence microscope. The fluorescence intensity was measured by using Image-Pro Plus 6.0 software. The stronger the fluorescence intensity, the higher the concentration of [Ca^{2+}]i in the cell.

Mitochondrial Function Measurement

MitoTracker Red probe (mitochondrial red fluorescent probe) is capable of specifically labeling the biologically active mitochondria. H9C2 cardiomyocytes were incubated with 0.2 μM MitoTracker Red CMXRos staining reagent (C1049B, Beyotime, Shanghai, China) at 37 °C lasting 30 min. After replacing with fresh culture medium, mitochondria were investigated utilizing a fluorescence microscope. The fluorescence intensity was measured by using Image-Pro Plus 6.0 software.

Intracellular ROS Measurement

The use of a fluorescent probe called dichlorodihydro-fluorescein diacetate (DCFH-DA; D6883, Sigma, Saint Louis, MS, USA) allowed for the examination of intracellular ROS levels. In the absence of light, 50 μM DCFH-DA was incubated in the cardiomyocytes at 37 °C for 30 min. Afterwards, the cardiomyocytes were washed for three times with pre-cold PBS buffer. Utilizing a fluorescence microscope with 488 nm excitation and 525 nm emission wavelengths, intracellular ROS fluorescence images were obtained. The fluorescence intensity can be evaluated by Image-Pro Plus 6.0 software to detect the level of intracellular ROS.

Measurement of Malondialdehyde (MDA) and Superoxide Dismutase (SOD)

According to the manufacturer's protocols, the levels of MDA in cardiomyocyte lysate were detected through Lipid Peroxidation Malondialdehyde Assay Kit (Jiancheng, A003-4-1, Nanjing, China). The activity of SOD in cardiomyocyte lysate was assayed with SOD activity assay kit (Jiancheng, A001-1-2, Nanjing, China). Utilizing a microplate reader, absorbance values at the 450 nm wave-

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length were measured. According to the formula in the kit instructions, calculate the optical density (OD) value obtained by measurement.

Measurement of Proinflammatory Cytokines

Cell culture medium of H9C2 cardiomyocytes were harvested. Corresponding enzyme-linked immunosorbent assay kits (Jiancheng, H002-1-2, H007-1-2, H052-1-2, Nanjing, China) were applied for quantifying the amount of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in accordance with the standard instructions. OD values were measured by enzymograph and IL-1β, IL-6 and TNF-α levels were calculated through standard curve.

Western Blot

Both total proteins of H9C2 cardiomyocytes and myocardial tissues were extracted through RIPA lysing buffer. Protein concentrations were evaluated with BCA Bradford protein assay kit. Afterwards, proteins were combined with loading buffer and brought to a boil for 10 min. 40 μg extracts were separated via electrophoresis utilizing SDS-PAGE (8%), followed by transference to PVDF membranes. Following blockage with 5% non-fat dry milk lasting 2 h, the membranes were incubated for a whole night at 4 °C with primary antibody against Bax (1:5000, 50599-2-Ig, Proteintech, Wuhan, China), ATP5A1 (1:2500, 66037-1-Ig, Proteintech, Wuhan, China), B-cell lymphoma 2 (Bcl-2) (1:1000, BA0412, BOSTER, Pleasanton, CA, USA), ATP synthase subunit beta (ATP5B) (1:3000, 17247-1-AP, Proteintech, Wuhan, China), Caspase-3 (1:2000, DF6020, Affinity, Melbourne, FL, USA), VDAC1 (1:2000, 55259-1-AP, Proteintech, Wuhan, China), Nucleotide-binding oligomerization domain-like receptor protein 3 (NALP3) (1:500, DF7438, Affinity, Melbourne, FL, USA), NK-κB p65 (1:1000, 10745-1-AP, Proteintech, Wuhan, China), and β-actin (1:1000, 20536-1-AP, Proteintech, Wuhan, China) as well as horseradish peroxidase-conjugated secondary antibody (1:5000, ZB-2301 and ZB-2305, ZSGB-BIO, Beijing, China) for 2 h at room temperature. Using an upgraded chemiluminescence kit, protein bands were created. Protein expressions were normalized on the basis of β-actin examined on the same blot to a control for the relative signal intensity. Band intensity was quantified using ImageJ2X v2.1.4.7 software (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemical and Immunofluorescent Staining

For cell experiment, H9C2 cardiomyocytes were seeded onto culture slides, followed by immobilization with 4% paraformaldehyde (LEAGENE, DF0135, Beijing, China) lasting 30 min. For animal experiment, myocardial tissue embedded in paraffin was cut to a thickness of 5 μm. Both cells and tissue slices were then permeated with 0.1% Triton X-100 (Solarbio, 9002-93-1, Beijing, China) lasting 30 min. After blockage with 2% BSA (Biotopped, A6020A, Beijing, China) lasting 60 min at room temperature, incubations with primary antibody of NALP3 (1:100, DF7438, Affinity, Melbourne, FL, USA), VDAC1 (1:200, 55259-1-AP, Proteintech, Wuhan, China), ATP5A1 (1:50, 66037-1-Ig, Proteintech, Wuhan, China), ATP5B (1:200, 17247-1-AP, Proteintech, Wuhan, China), were implemented at 4 °C overnight. For immunohistochemical staining, the slices were incubated with HRP-labeled secondary antibody (PV-6000, ZSGB-BIO, Beijing, China) lasting 2 h at room temperature. For immunofluorescent staining, the slices were incubated with Alexa Fluor® 488 Conjugate (ZF-0316, ZSGB-BIO, Beijing, China) or Alexa Fluor® 594 Conjugate (ZF-0313, ZSGB-BIO, Beijing, China) antibody lasting 2 h at room temperature. DAPI was utilized for counterstaining nuclei. Images were observed with a light or fluorescence microscope.

Analysis of Cell Energy Phenotype and Cellextracellular Flux

We used a Seahorse XFp analyzer (Agilent) to measures oxygen consumption rate (OCR) and extracellular acidification rate (ECAR)/PER of living cells in 96-well plates. Briefly, H9C2 was seeded at 6×10^3 per well onto Seahorse XF cell culture microplates. H9C2 cells were pretreated, ECAR and OCR was measured according to the instructions after 24 h of routine incubation. The cell growth medium in the cell culture microplate was replaced with preheated assay solution using a multichannel pipette, and the cell culture microplate was placed in a CO_2 free incubator set at 37 °C for 45 minutes to 1 hour. OCR was measured at steady state, after which oligomycin (1 μM), FCCP (1.5 μM) and rotenone/antamicin A (0.5 pM) mixture were added to the Wells, and the oxygen consumption rate of the cells was obtained according to the method recommended by the manufacturer. For ECAR, first, cells were incubated in glycolytic stress test detection solution without glucose and sodium pyruvate, and ECAR was measured. Glycolysis was measured by injecting glucose, Oligomycin and 2-deoxy-glucose (2-DG) at saturated concentrations. Seahorse XF test report analysis method was used to analyze the data.

Serum CK-MB, MPO, MDA and SOD Level

Creatine kinase isoenzyme (CK-MB; E006-1-1), myeloperoxidase (MPO; A044-1-1), malonaldehyde (MDA; A003-1-2) and superoxide dismutase (SOD; A001-3-2) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Serum CK-MB, MPO, MDA and SOD levels were then detected via enzyme-linked immunosorbent assay (ELISA) methods.

Statistical Analysis

Data are expressed as mean ± SEM. One-way ANOVA was applied for significance between means. Post hoc comparison between individual means was conducted by Tukey multiple comparison tests. $p < 0.05$ was considered statistical significance.

Results

AZA Affects ER Activity, Intracellular [Ca^{2+}]i Accumulation, Mitochondrial Abundance, ROS Accumulation, Oxidative Stress and Secretion of Proinflammatory Cytokines in HG and H/R-Induced H9C2 Cardiomyocytes

As shown in results of CCK-8 assay, 10 and 20 μM AZA did not significantly affect the viability of cardiomyocytes (Fig. 1A). On the basis of above data, 20 μM AZA was utilized for this work. ER activity was examined through ER-Tracker Red staining. HG and H/R dramatically weakened ER activity of cardiomyocytes ($p < 0.001$) (Fig. 1D,E). Moreover, the ER activity was heightened by AZA exposure ($p < 0.05$). This indicated that AZA administration was capable of heightening ER activity of H9C2 cardiomyocytes in the context of HG and H/R. Similarly, HG and H/R significantly reduced the fluorescence intensity of [Ca^{2+}]i in the cardiomyocytes ($p < 0.001$) (Fig. 1F,G) and AZA treatment was capable of enhancing the fluorescence intensity of Ca^{2+} in cardiomyocytes in the context of HG and H/R ($p < 0.05$). Improving mitochondrial quality control may attenuate diabetic cardiomyopathy [19]. Chronic hyperglycemia results in the persistent production of ROS that enables to damage macromolecules within the cells [20]. Consistently, HG and H/R-exposed cardiomyocytes had the reduced abundance of mitochondria ($p < 0.001$) (Fig. 1H,I), and AZA administration elevated the mitochondrial abundance of H9C2 cardiomyocytes in the context of HG and H/R ($p < 0.05$). The generation of ROS was enhanced in HG and H/R-exposed H9C2 cardiomyocytes ($p < 0.001$) (Fig. 1J,K). Following AZA administration, HG and H/R-induced ROS accumulation was dramatically mitigated ($p < 0.01$). Oxidative stress is linked to diabetic cardiac dysfunction and cardiomyopathy [21]. As shown in Fig. 1B,C, in comparison to normal conditions, MDA exhibited the increased levels and SOD levels displayed the significant reduction ($p < 0.001$) in HG and H/R-exposed cardiomyocytes which were reversed by AZA administration ($p < 0.05$). Myocardial inflammation leads to the secretion of cytokines. In this study, increased levels of IL-1β, IL-6 and TNF-α were observed in the cardiomyocytes exposed to HG and H/R compared to DMSO group ($p < 0.001$) (Fig. 1L–N). Under AZA administration, the levels of above proinflammatory cytokines exhibited the remarkable reduction. These findings demonstrated that AZA had an anti-inflammatory property in HG and H/R -induced cardiomyocytes.

AZA Alleviates NALP3 Inflammasome, Apoptosis and ATP Synthesis in HG and H/R-Exposed H9C2 Cardiomyocytes

For in-depth investigation of the mechanisms by which AZA exerted an anti-inflammatory, alleviates apoptosis and promoting ATP synthesis property in the cardiomyocytes with the HG and H/R conditions, expression of NALP3, ATP5A1, ATP5B, Bax, Bcl-2, Caspase-3, Nuclear factor-kappa B p65 (NF-κB p65) and VDAC1 were detected through western blotting. As a result, the expression of NALP3, NF-κB p65, Bax, Caspase-3 and VDAC1 were decreased under AZA administration in HG and H/R-exposed cardiomyocytes ($p < 0.001$). On the contrary, the expression of ATP5A1, ATP5B and Bcl-2 were increased ($p < 0.001$) (Fig. 2). Further immunohistochemical and immunofluorescent (Fig. 3) staining also demonstrated AZA's inhibitory effects on HG and H/R-induced H9C2 cardiomyocytes's NALP3 inflammasome activation and ATP synthesis.

AZA Improves Energy Metabolism of H9C2 Cells Induced by HG and H/R

Considering the key role of high glucose in energy metabolism, we used OCR and ECAR assays to find that compared with the control group, the HG+H/R+DMSO group significantly reduced cell energy metabolism ($p < 0.001$). However, the combined use of AZA in the HG+H/R group increased significantly to maintain cell energy metabolism ($p < 0.05$) (Fig. 4A–F). Under HG and H/R conditions, the expression of VDAC1 was up-regulated ($p < 0.001$), and the expression of ATP5A1 and ATP5B was down-regulated ($p < 0.001$). Furthermore, the knockdown of VDAC1 significantly enhanced the expression of ATP5A1 and ATP5B ($p < 0.001$) (Fig. 4G–L). Therefore, it can be inferred that VDAC1 modulates cellular energy metabolism by modulating ATP synthesis.

AZA Plays a Protective Role in the Myocardium of Diabetic Rats by Regulating Energy Metabolism, Apoptosis and Inflammation

After DM and I/R treatment, the expression of VDAC1 was up-regulated and the expression of ATP5A1 and ATP5B was down-regulated in diabetic rats ($p < 0.001$). However, the intervention of AZA significantly decreased the expression of VDAC1 and promoted the expression of ATP5A1 and ATP5B ($p < 0.001$) (Fig. 5). In diabetic rats, the regulation of AZA on apoptosis-related proteins (Bax, Caspase-3, Bcl-2), NALP3 inflammatome and ATP synthesis (VDAC1, ATP5A1, ATP5B) (Fig. 6) reflects the myocardial protective effect of AZA on diabetic rats.

AZA Improves the Oxidative Stress Brought on by Myocardial Injury in Diabetic Rats

By quantifying factors associated with oxidative stress and serum markers of myocardial injury, we found that the

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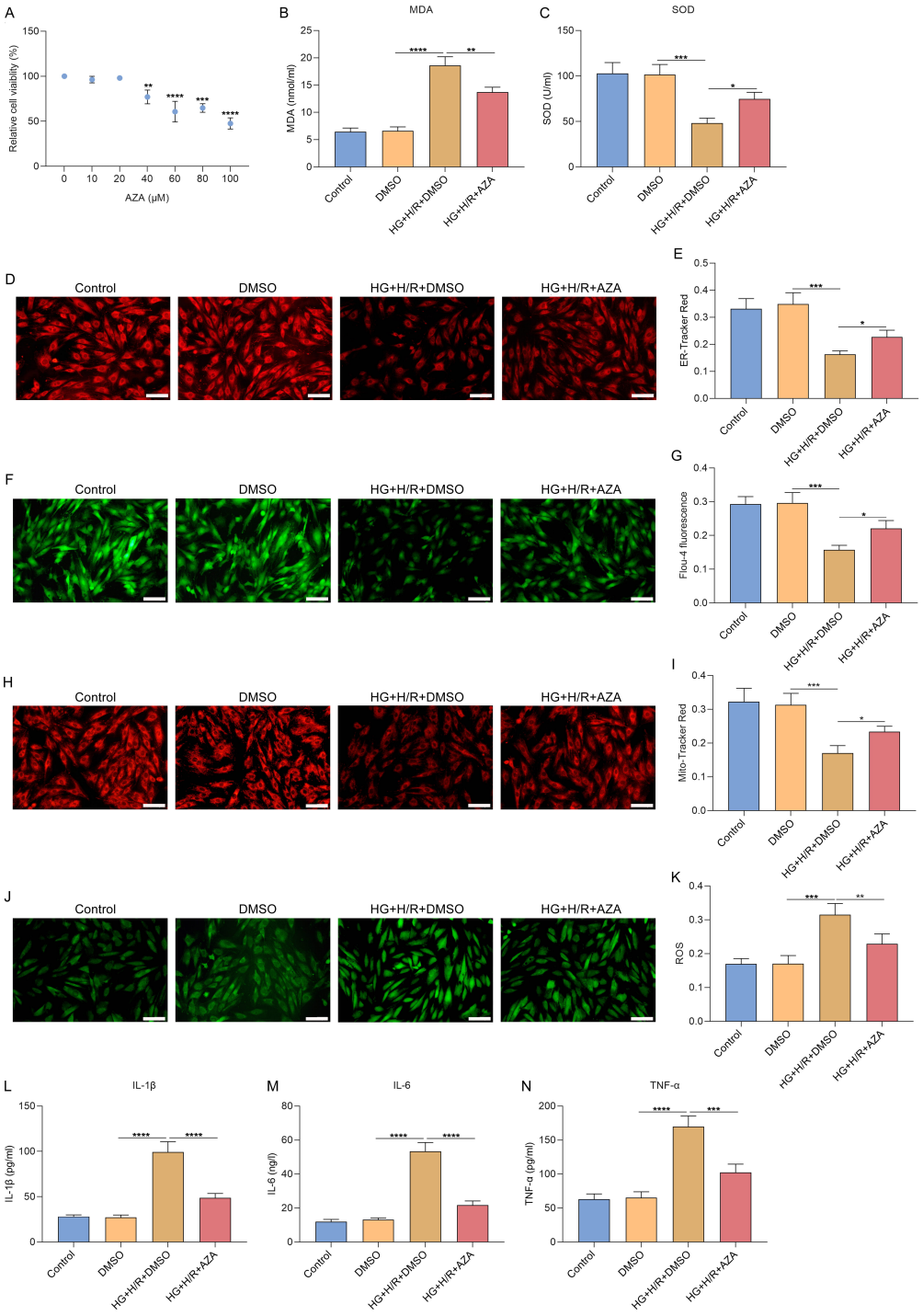

Fig. 1. Effects of azathioprine (AZA) on cell viability related indexes of high glucose (HG) and hypoxia/reoxygenation (H/R)-induced H9C2 cardiomyocytes. (A) Cell viability of H9C2 cardiomyocytes exposed to 0, 10, 20, 40, 60, 80 and 100 μM AZA. (B,C) The levels of malondialdehyde (MDA) and superoxide dismutase (SOD) in the lysate of the cardiomyocytes exposed to HG and H/R or AZA. (D,E) ER-Tracker Red staining for ER activity in H9C2 cardiomyocytes with HG and H/R or AZA administration. (F,G) Measurement of the fluorescence intensity of $[Ca^{2+}]i$ in H9C2 cardiomyocytes with HG and H/R or AZA treatment utilizing Ca^{2+} fluorescent indicator Fluo-4/AM. (H,I) Mito-Tracker Red staining for the abundance of mitochondria in H9C2 cardiomyocytes with HG and H/R or AZA administration. (J,K) Measurement of intracellular reactive oxygen species (ROS) levels in the cardiomyocytes with HG and H/R or AZA administration utilizing DCFH-DA. (L–N) Measurement of the levels of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in the cardiomyocytes that were exposed to HG and H/R or AZA. Bar, 100 μm. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. n = 3. DMSO, Dimethyl sulfoxide; ER, Endoplasmic Reticulum; Ca^{2+}, cardiomyocyte calcium.

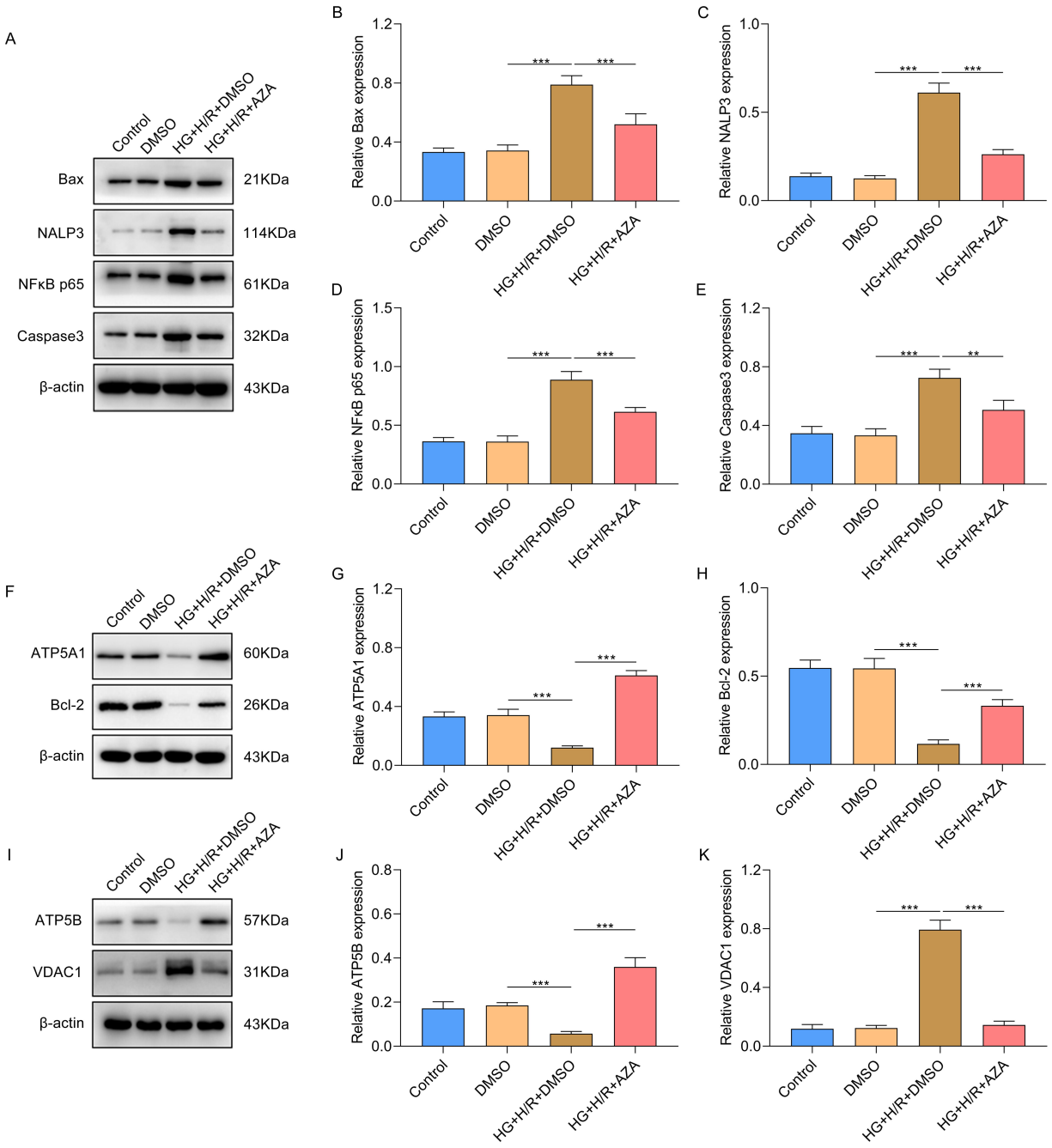

Fig. 2. AZA alleviates NALP3 inflammasome, apoptosis and ATP synthesis in HG and H/R-exposed H9C2 cardiomyocytes. (A–K) Western blot of the protein expressions of Bcl-2 associated X protein (Bax), Nucleotide-binding oligomerization domain-like receptor protein 3 (NALP3), Nuclear factor-kappa B p65 (NF-κB p65), ATP synthase subunit alpha (ATP5A1), B-cell lymphoma 2 (Bcl-2), ATP synthase subunit beta (ATP5B), Caspase-3 and Voltage-dependent anion channel 1 (VDAC1) in H9C2 cardiomyocytes with HG and H/R or AZA administration. $^{**}p < 0.01$; $^{***}p < 0.001$. n = 3.

I/R-induced serum CK-MB, MPO, MDA and SOD levels of diabetic rats were higher ($p < 0.001$) than in the Control+I/R group, but that the AZA treatment caused a decrease in these levels ($p < 0.001$) (Fig. 7). These results indicated that AZA treatment could improve the oxidative stress brought on by myocardial injury in diabetic rats.

Discussion

Preventing and reducing diabetic cardiomyopathy remains a major challenge in the treatment and management of diabetes mellitus [22]. Oxidative damage, inflammation and cell death are the main drivers of diabetes-induced cardiomyocyte damage and inevitably lead to cardiac dysfunc-

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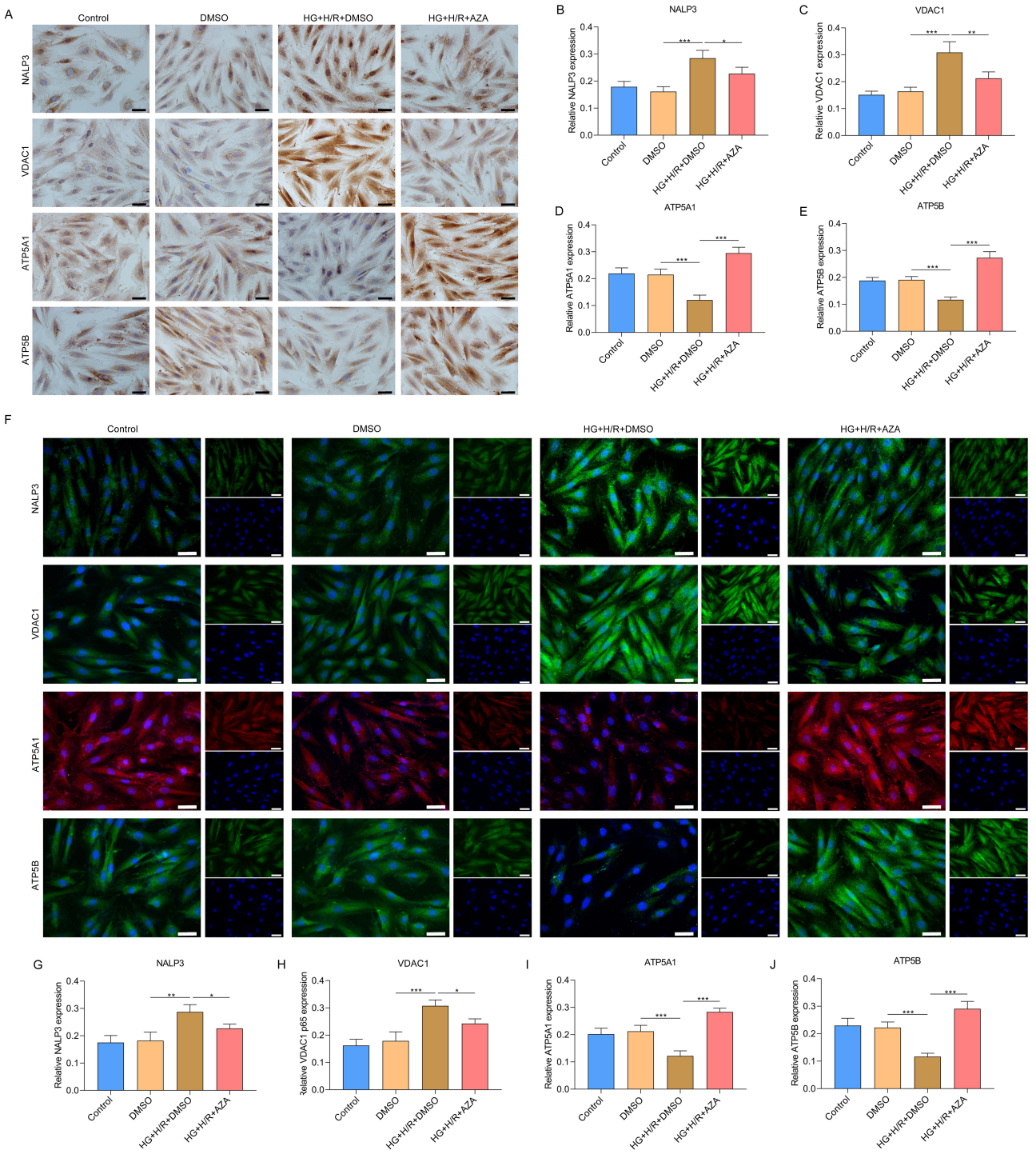

Fig. 3. AZA suppresses NALP3 inflammasome and VDAC1/ATP5A1/ATP5B signaling in HG and H/R-exposed H9C2 cardiomyocytes. (A–E) Immunohistochemical staining of the expressions of NALP3, VDAC1, ATP5A1 and ATP5B in H9C2 cardiomyocytes exposed to HG and H/R or AZA. Bar, 40 μm. (F–J) Immunofluorescent staining of the expressions of NALP3, VDAC1, ATP5A1 and ATP5B in H9C2 cardiomyocytes exposed to HG and H/R or AZA. Bar, 40 μm. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. n = 3.

tion [23]. Our study investigated the mechanisms and cardioprotective effects of AZA in cardiomyocyte injury induced by HG and H/R. AZA reduces the vulnerability of cardiomyocytes by improving mitochondrial function and mitochondrial ATP synthase production, as well as reducing oxidative stress and inflammation. These findings indicate its use as a potentially effective preventative and therapeutic measure for diabetic cardiomyopathy.

Under normal conditions, cardiomyocyte calcium (Ca^{2+}) regulates mitochondrial function [24]. Excess Ca^{2+}

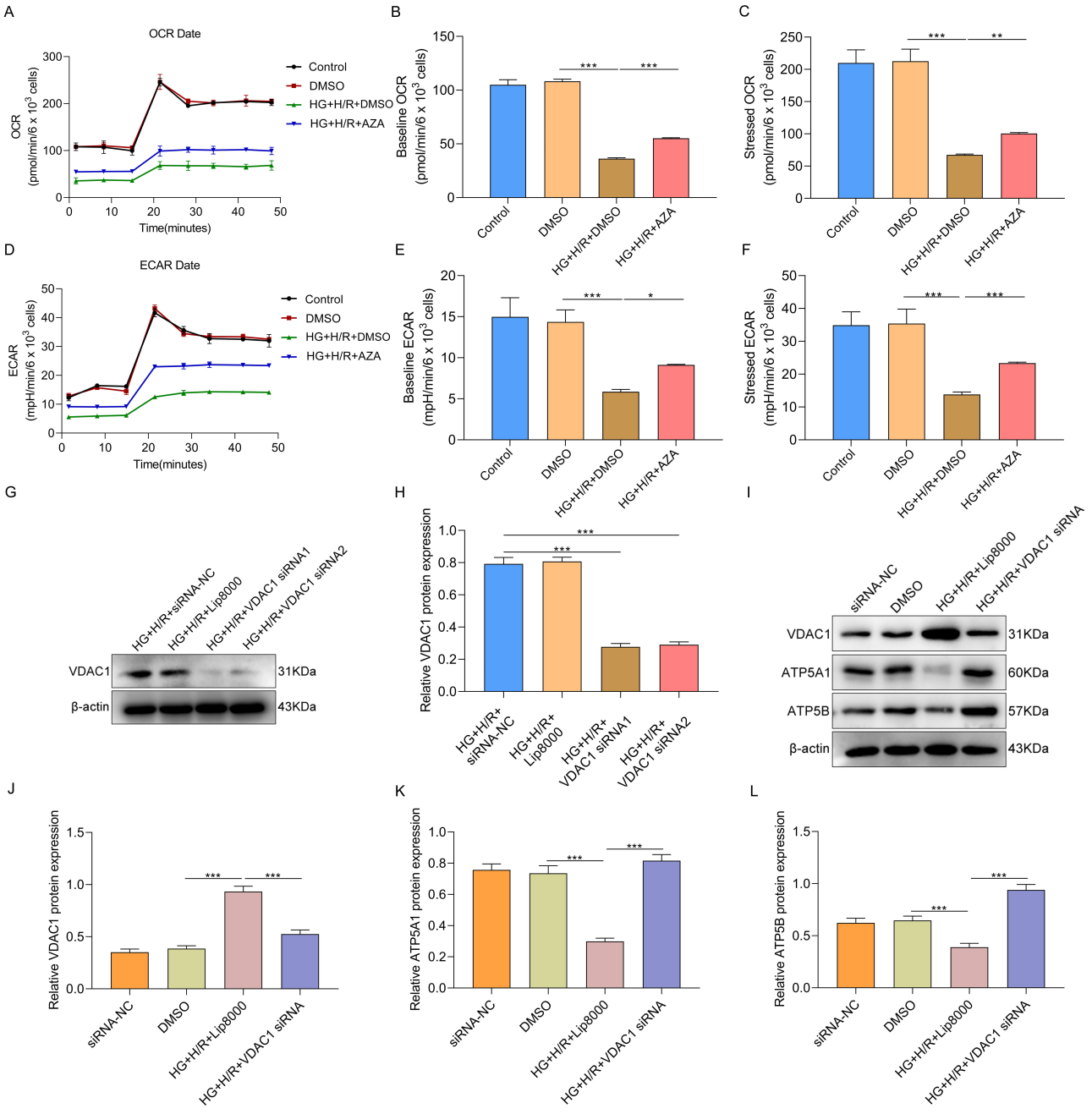

Fig. 4. AZA improves low energy metabolism and VDAC1 regulates the expression of ATP5A1 and ATP5B in HG and H/R-treated H9C2 cardiomyocytes. (A–F) Results of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in H9C2 cardiomyocytes exposed to HG and H/R or AZA. (G–L) Detection of VDAC1 downstream signal ATP5A1, ATP5B expression in each group, we conducted the following experiments using VDAC1 siRNA. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. n = 3.

in the mitochondria leads to an increase in reactive oxygen species (ROS). This causes a change in mitochondrial permeability and disruption of mitochondrial function, leading to cardiomyocyte death and heart failure [25]. During myocardial ischemia/reperfusion injury (MI/RI), reactive oxygen species (ROS) readily oxidize NO to peroxynitrite, leading to secondary cardiomyocyte injury [26]. Hypoxia in cardiomyocytes reduces mitochondrial membrane potential, limits ATP synthase synthesis, and ultimately leads to mitochondrial dysfunction. The use of drugs to improve mitochondrial dysfunction effectively improves the degree of cardiomyocyte injury, inhibits myocardial apoptosis and fibrosis, and attenuates myocardial infarction by maintaining mitochondrial function and regulating the NO pathway [27]. In the present study, using the HG+H/R cell model, we observed that HG+H/R mediated a decrease in calcium ion levels and mitochondrial membrane potential in cardiomyocytes, induced ROS production, and decreased cel-

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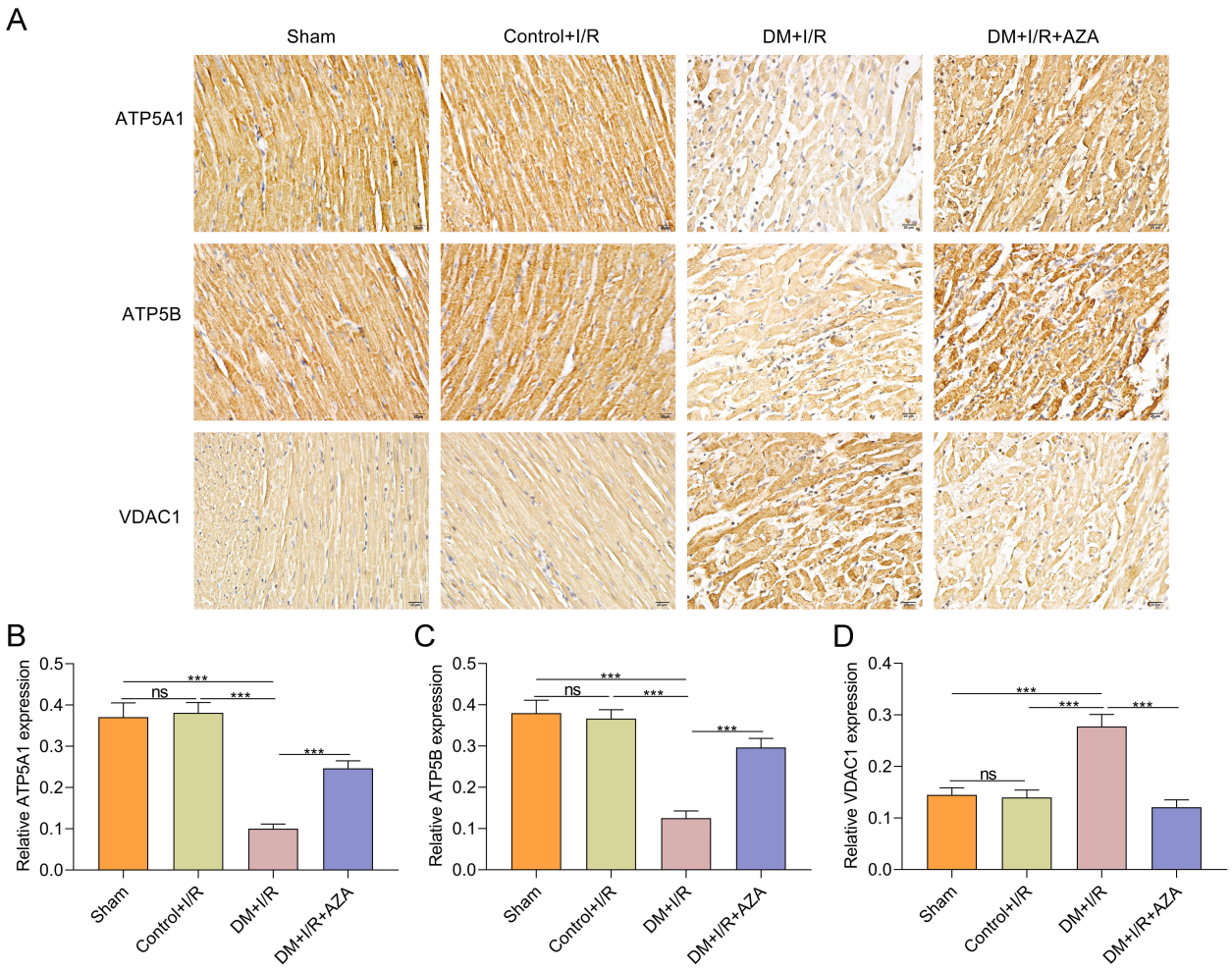

Fig. 5. The impact of AZA on energy metabolism-related protein expression in diabetic rats after ischaemia/reperfusion (I/R). (A–D) Immunohistochemical staining of the expressions of VDAC1, ATP5A1 and ATP5B in diabetic rats after I/R or AZA treatment. Bar, 20 μm. ns, no significance, ***$p < 0.001$. n = 6. DM, diabetes mellitus.

lular energy metabolism. AZA treatment significantly improved mitochondrial dysfunction and energy metabolism and reduced oxidative stress in HG/H-induced cardiomyocytes. It has been suggested that AZA prevents ROS production in vascular endothelial cell injury, attenuates endothelial cell dysfunction, and reduces vasospasm [28,29]. These findings are similar to our results because of the protective effect against cellular damage. It has also been suggested that AZA may cause adverse effects in atrial arrhythmias, but there are no reports of direct effects of AZA on cardiomyocytes or mitochondrial function in heart tissue [30,31]. The present study showed that AZA improved mitochondrial function and cellular energy metabolism. However, we did not find any arrhythmias in our preliminary animal model studies, either because they were incidental or because the low dose we used did not cause arrhythmia. It is undeniable that such reports are helpful in studying the role of AZA, and we will pay particular attention to them in our future studies.

According to recent research, myocardial ischemia can cause an imbalance between the oxidative and antioxidative processes of the body, which can result in a high production of ROS and oxidative stress damage [32]. MDA is a byproduct of membrane lipid peroxidation, and the degree of oxidative stress experienced by cardiomyocytes is directly reflected in the expression of MDA. SOD functions as a crucial barrier to inhibit oxygen radical damage, reducing oxygen radicals and protecting cells from damage [33]. According to reports, ROS production is known to spike within minutes of myocardial HG+H/R injure, and in diabetic states, elevated levels of oxidative stress, and mitochondrial dysfunction may heighten ROS production and therefore exacerbate myocardial HG+H/R injury [34]. We found that AZA treatment reduced ROS accumulation in cardiomyocytes, decreased the expression of the oxidative stress enzyme MDA, and enhanced the expression of SOD, an enzyme involved in antioxidant stress. And as shown in the results of the previous experiments [16], in I/R-treated diabetic rats, the expression of the main indi-

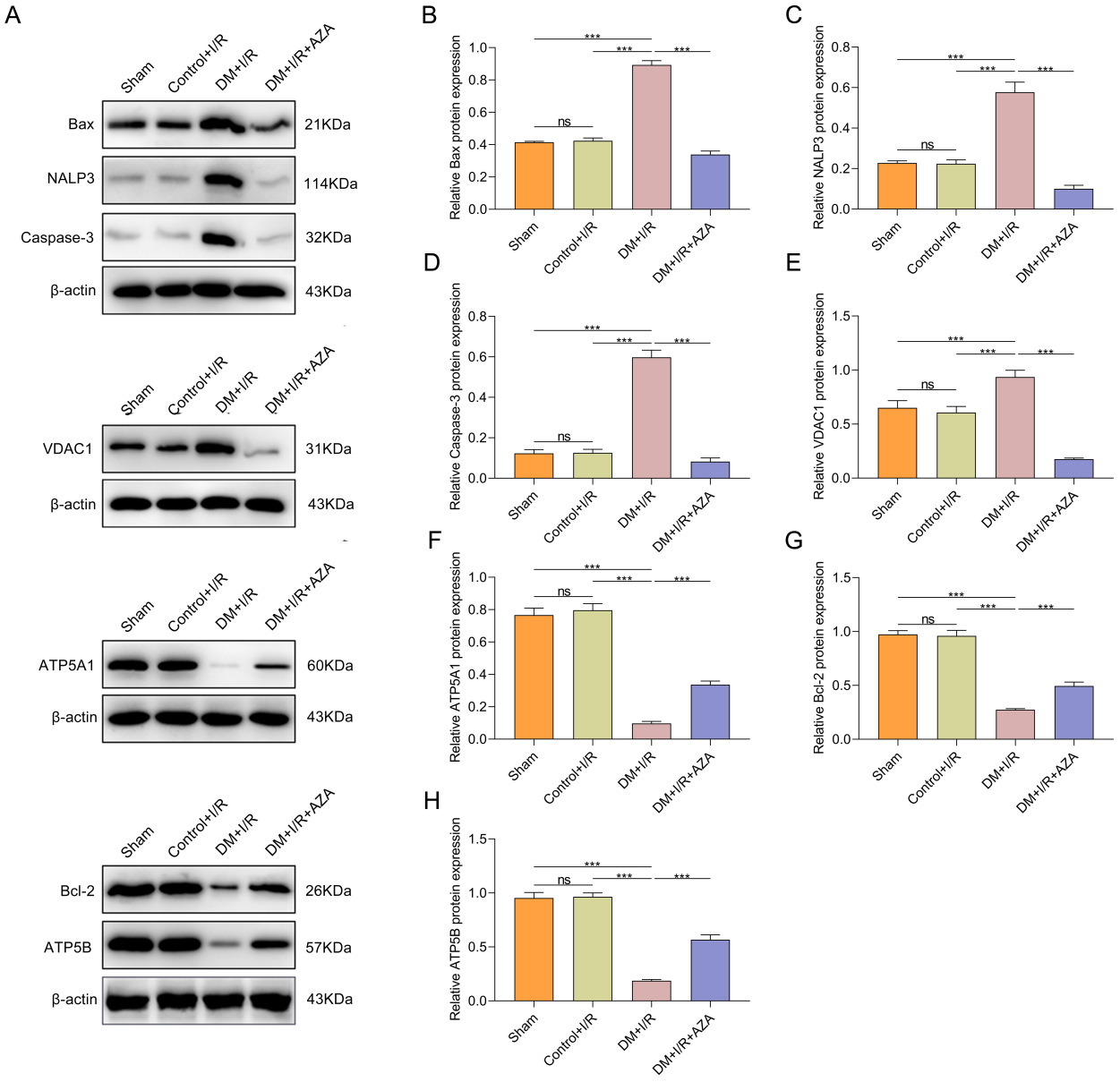

Fig. 6. The impact of AZA on apoptosis and ATP synthesis, and protein expression linked to the NALP3 inflammasome in diabetic rats following I/R. (A–H) Western blot of the expressions of Bax, NALP3, NF-κB p65, ATP5A1, Bcl-2, ATP5B, Caspase-3 and VDAC1 in diabetic rats after I/R or AZA treatment. ns, no significance, ***$p < 0.001$. n = 3.

cators of myocardial injury (CK-MB and MPO) was significantly increased, and then decreased by the addition of AZA treatment. When combined, these findings supported the cardioprotective effects of AZA in cellular and animal models, and further clarified that AZA exerts its protective effects by improving mitochondrial function, reducing oxidative stress, and regulating cellular metabolism.

VDAC1 acts as a "gatekeeper" for the mitochondria, controlling energy metabolism between the mitochondria and the rest of the cell [9]. In mitochondrial calcium homeostasis regulation, VDAC1 is responsible for the uptake of calcium ions at the outer mitochondrial membrane, which regulates mitochondrial calcium homeostasis and prevents cell death due to too much or too little calcium ions [35]. However, mitochondrial calcium homeostasis regulatory mechanisms are also critical for mitochondrial membrane potential and ATP synthase production [26]. Upregulation of ATP5A1 restores ATP synthase activity, reduces diabetic cardiomyopathy in a mouse model of type 1 diabetes [36]. Coxsackievirus B3 (CVB3) infection induces Calpain1 accumulation in cardiomyocyte mitochondria, leading to ATP5A1 segregation, overproduction of mitochondrial ROS, promotion of NLRP3 inflammasome activation, and induced cellular pyrokinesis [37]. In the present experiments, VDAC1 expression was significantly increased and ATP5A1 and ATP5B expression significantly decreased in

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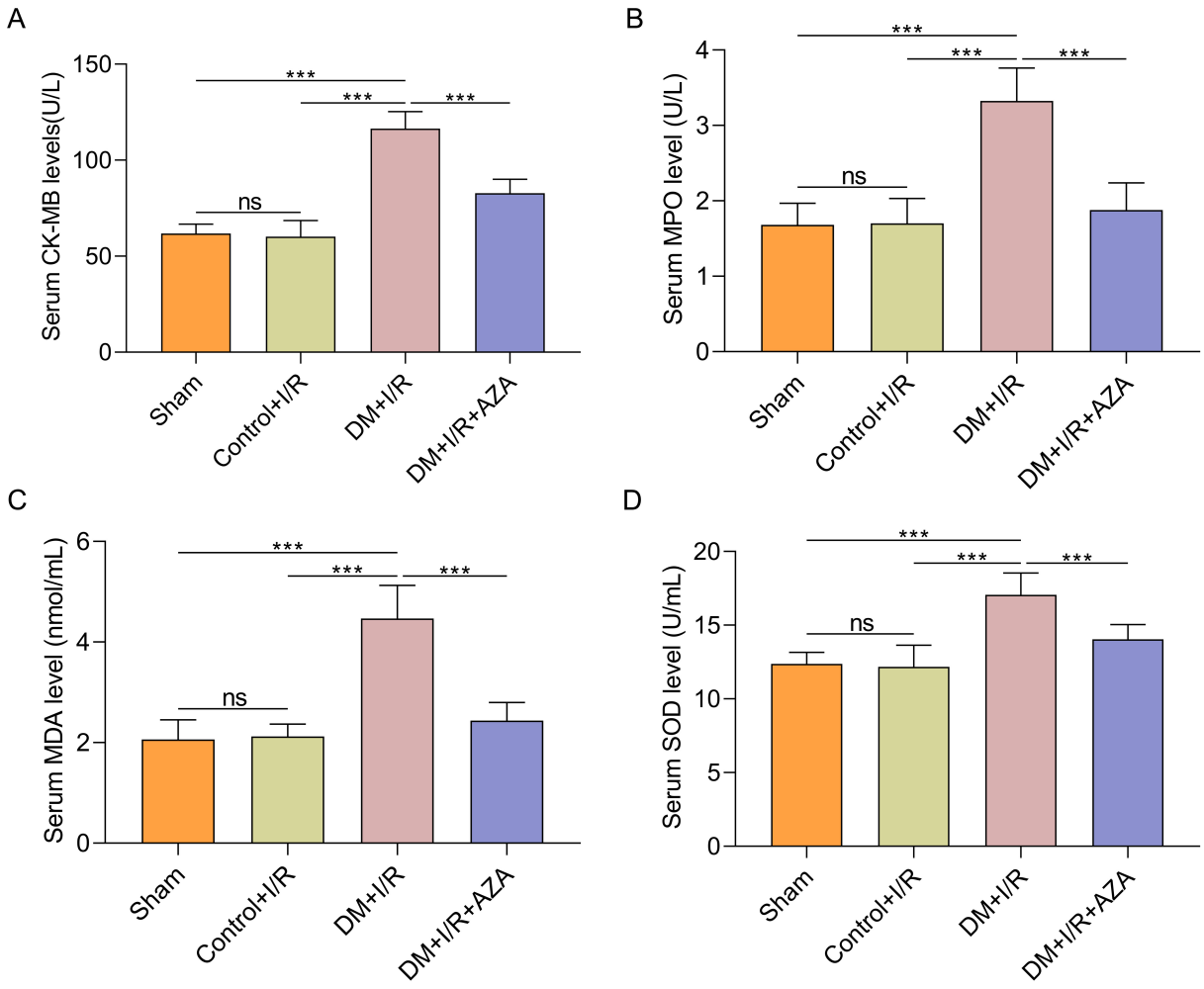

Fig. 7. AZA improves the oxidative stress induced by myocardial injury in diabetic rats. (A–D) Serum creatine kinase isoenzyme (CK-MB), myeloperoxidase (MPO), MDA and SOD levels in diabetic rats following I/R or AZA were detected by enzyme-linked immunosorbent assay (ELISA). ns, no significance, ***$p < 0.001$. n = 6.

both the cardiomyocyte model induced by HG+H/R as well as the diabetic I/R rats. Interestingly, AZA treatment subsequently decreased VDAC1 expression and upregulated ATP5A1 and ATP5B expression. To confirm the relationship between the role of VDAC1 and ATP5A1 and ATP5B expression, we found that VDAC1 siRNA was able to downregulate ATP5A1 and ATP5B expression, further suggesting that AZA may regulate the formation of ATP synthase (ATP5A1 and ATP5B) by decreasing VDAC1. We hypothesise that AZA is able to regulate intracellular calcium ion levels most likely by modulating VDAC1 signaling. These data provide a reference for elucidating the action of AZA in cardiomyocyte protection.

Myocardial ischaemia and hypoxia affect not only energy supply but also cell proliferation and apoptosis [38]. Targeting VDAC1, miR-7a-5p contributes to H/R-induced cardiomyocyte apoptosis by downregulating pro-apoptotic proteins cleaved caspase-3 and Bax and upregulating anti-apoptotic protein Bcl-2 [12]. We observed that AZA also has an anti-apoptotic effect, but this result contradicts the results of Nam HJ [39]. This is most likely related to the abnormal mitochondrial function of the tumor cells as well as the dose concentration. We also found in our CCK-8 experiments that cell proliferative activity decreased with increasing concentrations of AZA. This reinforces the fact that AZA should be used at an appropriate dose to be protective.

Inflammatory factors are important mediators of cell necrosis during myocardial ischaemia and hypoxia [40]. Pro-inflammatory and pro-fibrotic genes are expressed more frequently when NF-κB is activated [41]. The NLRP3 inflammasome, a central part of the inflammatory mechanism, is activated to release a number of inflammatory factors that cause myocardial injury [42,43]. Monocytes secrete TNF-α, which exacerbates the inflammatory response and promotes neutrophils and other pro-inflammatory cytokines [44]. IL-1β is thought to initiate neutrophil adhesion to endothelial cells [45]. IL-6 is another important pro-

inflammatory cytokine strongly associated with myocardial injury [46]. In previous studies in animal models, NLRP3 was significantly upregulated in cardiomyocytes after H/R, and there was a notable increase in the expression of IL-1β, IL-18, and TNF-α, whereas the opposite effect occurred after application of AZA [47]. Comparable outcomes were noted in the cardiomyocyte model induced by HG+H/R, and we have demonstrated that AZA has a multifaceted role in anti-inflammatory and anti-oxidative stress.

In conclusion, AZA can improve mitochondrial function, reduce oxidative stress, inhibit inflammation and apoptosis. Studies have shown that AZA is safe and effective in the long-term treatment of immune-mediated diseases [48]. VDAC1 not only participates in energy production, but also plays an important role in the regulation of inflammation-induced apoptosis and oxidative stress pathways [49]. Our experimental results show that AZA can significantly reduce the expression of VDAC1 in HG+H/R cell models and diabetic rat ischemia/reperfusion (I/R) models. We hypothesized that AZA plays the important role in mitochondrial function, reducing oxidative stress, inhibiting inflammation and apoptosis by regulating the expression of VDAC1.

In this study, H9C2 cardiomyocytes were used as the cell model, but they are not real cardiomyocytes, but derived from rat embryonic heart myoblasts, and their biological characteristics were significantly different from those of mature cardiomyocytes. Although they cannot completely simulate the process of myocardial ischemia/reperfusion injury, this study provides a reference for further research. It seems to be insufficient to evaluate the effect of constructing a rat cardiomyopathy model only from the three aspects of blood glucose level, myocardial injury related indexes and myocardial function. Therefore, in future studies, we will increase the detection of myocardial infarction size, myocardial tissue structure and myocardial function.

Conclusion

Our current study demonstrates that AZA has a protective effect on cardiomyocytes when HG+H/R and I/R are present. The mechanisms by which AZA exerts its cardioprotective effects appear to be multifaceted. The cardioprotective effects were associated with improved mitochondrial function; increased intracellular calcium ion accumulation; decreased ROS accumulation and oxidative stress; decreased secretion of proinflammatory cytokines; inhibition of NALP3 inflammatory vesicles; decreased activation of VDAC1, NF-κB signaling; increased activation of ATP5A1, ATP5B signaling; and improved cellular energy metabolism to avoid apoptosis. The above data suggest that AZA may be a new therapeutic candidate for the prevention of diabetic cardiomyocyte dysfunction.

Availability of Data and Materials

All data included in this study are available upon request by contact with the corresponding author. Available at Cuijie Lu.

Author Contributions

CL, LL and JN contributed to the concept and designed the research study. JN, JQ and DL performed the experiments. SC and ZC analyzed and interpretated the data. CL, SC and ZC wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

This study strictly followed the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and was approved by the Ethics Committee of Sichuan Vocational College of Health and Rehabilitation (2020-881).

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

References

[1] García-Díez E, López-Oliva ME, Caro-Vadillo A, Pérez-Vizcaíno F, Pérez-Jiménez J, Ramos S, *et al*. Supplementation with a Cocoa-Carob Blend, Alone or in Combination with Metformin, Attenuates Diabetic Cardiomyopathy, Cardiac Oxidative Stress and Inflammation in Zucker Diabetic Rats. Antioxidants. 2022; 11: 432.

[2] Cherney DZI, Charbonnel B, Cosentino F, Dagogo-Jack S, McGuire DK, Pratley R, *et al*. Effects of ertugliflozin on kidney composite outcomes, renal function and albuminuria in patients with type 2 diabetes mellitus: an analysis from the randomised VERTIS CV trial. Diabetologia. 2021; 64: 1256–1267.

[3] Komuro I, Kadowaki T, Bodegård J, Thuresson M, Okami S, Yajima T. Lower heart failure and chronic kidney disease risks associated with sodium-glucose cotransporter-2 inhibitor use in

Japanese type 2 diabetes patients without established cardiovascular and renal diseases. Diabetes, Obesity & Metabolism. 2021; 23: 19–27.

[4] Mfeukeu-Kuate L, Meyanui VA, Jingi AM, Ndobo-Koe V, Mballa F, Ntep-Gweth M, *et al*. Prevalence and determinants of silent myocardial ischemia in patients with type 2 diabetes in Cameroon: a cross-sectional study. The Pan African Medical Journal. 2022; 42: 41.

[5] Aniskevich S, Renew JR, Chadha RM, Irizarry-Alvarado JM. Pharmacology and Perioperative Considerations for Diabetes Mellitus Medications. Current Clinical Pharmacology. 2017; 12: 157–163.

[6] Lu Q, Liu J, Li X, Sun X, Zhang J, Ren D, *et al*. Empagliflozin attenuates ischemia and reperfusion injury through LKB1/AMPK signaling pathway. Molecular and Cellular Endocrinology. 2020; 501: 110642.

[7] Han D, Wang Y, Chen J, Zhang J, Yu P, Zhang R, *et al*. Activation of melatonin receptor 2 but not melatonin receptor 1 mediates melatonin-conferred cardioprotection against myocardial ischemia/reperfusion injury. Journal of Pineal Research. 2019; 67: e12571.

[8] Chang X, Zhang T, Meng Q, ShiyuanWang, Yan P, Wang X, *et al*. Quercetin Improves Cardiomyocyte Vulnerability to Hypoxia by Regulating SIRT1/TMBIM6-Related Mitophagy and Endoplasmic Reticulum Stress. Oxidative Medicine and Cellular Longevity. 2021; 2021: 5529913.

[9] Gatliff J, East D, Crosby J, Abeti R, Harvey R, Craigen W, *et al*. TSPO interacts with VDAC1 and triggers a ROS-mediated inhibition of mitochondrial quality control. Autophagy. 2014; 10: 2279–2296.

[10] Tian M, Xie Y, Meng Y, Ma W, Tong Z, Yang X, *et al*. Resveratrol protects cardiomyocytes against anoxia/reoxygenation via dephosphorylation of VDAC1 by Akt-GSK3 β pathway. European Journal of Pharmacology. 2019; 843: 80–87.

[11] Wang L, Lai S, Zou H, Zhou X, Wan Q, Luo Y, *et al*. Ischemic preconditioning/ischemic postconditioning alleviates anoxia/reoxygenation injury via the Notch1/Hes1/VDAC1 axis. Journal of Biochemical and Molecular Toxicology. 2022; 36: e23199.

[12] Lu H, Zhang J, Xuan F. MiR-7a-5p Attenuates Hypoxia/Reoxygenation-Induced Cardiomyocyte Apoptosis by Targeting VDAC1. Cardiovascular Toxicology. 2022; 22: 108–117.

[13] Ha BG, Heo JY, Jang YJ, Park TS, Choi JY, Jang WY, *et al*. Depletion of Mitochondrial Components from Extracellular Vesicles Secreted from Astrocytes in a Mouse Model of Fragile X Syndrome. International Journal of Molecular Sciences. 2021; 22: 410.

[14] Nowak G, Megyesi J, Craigen WJ. Deletion of VDAC1 Hinders Recovery of Mitochondrial and Renal Functions After Acute Kidney Injury. Biomolecules. 2020; 10: 585.

[15] Zhang C, Zhang M, Qiu W, Ma H, Zhang X, Zhu Z, *et al*. Safety and efficacy of tocilizumab versus azathioprine in highly relapsing neuromyelitis optica spectrum disorder (TANGO): an open-label, multicentre, randomised, phase 2 trial. The Lancet. Neurology. 2020; 19: 391–401.

[16] Lu C, Liu L, Chen S, Niu J, Li S, Xie W, *et al*. Azathioprine pretreatment ameliorates myocardial ischaemia reperfusion injury in diabetic rats by reducing oxidative stress, apoptosis, and inflammation. Clinical and Experimental Pharmacology & Physiology. 2021; 48: 1621–1632.

[17] Igarashi Y, Nakatsu N, Yamashita T, Ono A, Ohno Y, Urushidani T, *et al*. Open TG-GATEs: a large-scale toxicogenomics database. Nucleic Acids Research. 2015; 43: D921–D927.

[18] Tanaka Y, Takahashi K, Hattori N, Yokoyama H, Yamaguchi K, Shibui Y, *et al*. The influence of serial 50 µL microsampling on rats administered azathioprine, the immunosuppressive drug. Toxicology Reports. 2023; 10: 334–340.

[19] Yu LM, Dong X, Xue XD, Xu S, Zhang X, Xu YL, *et al*. Melatonin attenuates diabetic cardiomyopathy and reduces myocardial vulnerability to ischemia-reperfusion injury by improving mitochondrial quality control: Role of SIRT6. Journal of Pineal Research. 2021; 70: e12698.

[20] Wang Y, Zheng X, Li L, Wang H, Chen K, Xu M, *et al*. *Cyclocarya paliurus* ethanol leaf extracts protect against diabetic cardiomyopathy in db/db mice via regulating PI3K/Akt/NF-κB signaling. Food & Nutrition Research. 2020; 64.

[21] Raish M, Ahmad A, Bin Jardan YA, Shahid M, Alkharfy KM, Ahad A, *et al*. Sinapic acid ameliorates cardiac dysfunction and cardiomyopathy by modulating NF-κB and Nrf2/HO-1 signaling pathways in streptozocin induced diabetic rats. Biomedicine & Pharmacotherapy. 2022; 145: 112412.

[22] Chiasson JL, Le Lorier J. Glycaemic control, cardiovascular disease, and mortality in type 2 diabetes. Lancet. 2014; 384: 1906–1907.

[23] Yan M, Li L, Wang Q, Shao X, Luo Q, Liu S, *et al*. The Chinese herbal medicine Fufang Zhenzhu Tiaozhi protects against diabetic cardiomyopathy by alleviating cardiac lipotoxicity-induced oxidative stress and NLRP3-dependent inflammasome activation. Biomedicine & Pharmacotherapy. 2022; 148: 112709.

[24] Werley CA, Boccardo S, Rigamonti A, Hansson EM, Cohen AE. Multiplexed Optical Sensors in Arrayed Islands of Cells for multimodal recordings of cellular physiology. Nature Communications. 2020; 11: 3881.

[25] Zhou B, Tian R. Mitochondrial dysfunction in pathophysiology of heart failure. The Journal of Clinical Investigation. 2018; 128: 3716–3726.

[26] Wang Z, Yang N, Hou Y, Li Y, Yin C, Yang E, *et al*. L-Arginine-Loaded Gold Nanocages Ameliorate Myocardial Ischemia/Reperfusion Injury by Promoting Nitric Oxide Production and Maintaining Mitochondrial Function. Advanced Science. 2023; 10: e2302123.

[27] Zou H, Liu G. Inhibition of endoplasmic reticulum stress through activation of MAPK/ERK signaling pathway attenuates hypoxia-mediated cardiomyocyte damage. Journal of Receptor and Signal Transduction Research. 2021; 41: 532–537.

[28] Chang CZ, Wu SC, Kwan AL, Lin CL, Hwang SL. 6-Mercaptopurine reverses experimental vasospasm and alleviates the production of endothelins in NO-independent mechanism-a laboratory study. Acta Neurochirurgica. 2011; 153: 939–949.

[29] Krötz F, Keller M, Derflinger S, Schmid H, Gloe T, Bassermann F, *et al*. Mycophenolate acid inhibits endothelial NAD(P)H oxidase activity and superoxide formation by a Rac1-dependent mechanism. Hypertension. 2007; 49: 201–208.

[30] Cassinotti A, Massari A, Ferrara E, Greco S, Bosani M, Ardizzone S, *et al*. New onset of atrial fibrillation after introduction of azathioprine in ulcerative colitis: case report and review of the literature. European Journal of Clinical Pharmacology. 2007; 63: 875–878.

[31] Eisen HJ, Kobashigawa J, Keogh A, Bourge R, Renlund D, Mentzer R, *et al*. Three-year results of a randomized, double-blind, controlled trial of mycophenolate mofetil versus azathioprine in cardiac transplant recipients. The Journal of Heart and Lung Transplantation. 2005; 24: 517–525.

[32] De Keulenaer GW, Ushio-Fukai M, Yin Q, Chung AB, Lyons PR, Ishizaka N, *et al*. Convergence of redox-sensitive and mitogen-activated protein kinase signaling pathways in tumor necrosis factor-alpha-mediated monocyte chemoattractant protein-1 induction in vascular smooth muscle cells. Arteriosclerosis, Thrombosis, and Vascular Biology. 2000; 20: 385–391.

[33] Yin Y, Han W, Cao Y. Association between activities of SOD,

MDA and Na^+-K^+-ATPase in peripheral blood of patients with acute myocardial infarction and the complication of varying degrees of arrhythmia. Hellenic Journal of Cardiology. 2019; 60: 366–371.

[34] Kurian GA, Rajagopal R, Vedantham S, Rajesh M. The Role of Oxidative Stress in Myocardial Ischemia and Reperfusion Injury and Remodeling: Revisited. Oxidative Medicine and Cellular Longevity. 2016; 2016: 1656450.

[35] Belosludtsev KN, Serov DA, Ilzorkina AI, Starinets VS, Dubinin MV, Talanov EY, *et al*. Pharmacological and Genetic Suppression of VDAC1 Alleviates the Development of Mitochondrial Dysfunction in Endothelial and Fibroblast Cell Cultures upon Hyperglycemic Conditions. Antioxidants. 2023; 12: 1459.

[36] Ni R, Zheng D, Xiong S, Hill DJ, Sun T, Gardiner RB, *et al*. Mitochondrial Calpain-1 Disrupts ATP Synthase and Induces Superoxide Generation in Type 1 Diabetic Hearts: A Novel Mechanism Contributing to Diabetic Cardiomyopathy. Diabetes. 2016; 65: 255–268.

[37] Liu X, Li M, Chen Z, Yu Y, Shi H, Yu Y, *et al*. Mitochondrial calpain-1 activates NLRP3 inflammasome by cleaving ATP5A1 and inducing mitochondrial ROS in CVB3-induced myocarditis. Basic Research in Cardiology. 2022; 117: 40.

[38] Hermann DM, Xin W, Bähr M, Giebel B, Doeppner TR. Emerging roles of extracellular vesicle-associated non-coding RNAs in hypoxia: Insights from cancer, myocardial infarction and ischemic stroke. Theranostics. 2022; 12: 5776–5802.

[39] Nam HJ, Kim YE, Moon BS, Kim HY, Jung D, Choi S, *et al*. Azathioprine antagonizes aberrantly elevated lipid metabolism and induces apoptosis in glioblastoma. iScience. 2021; 24: 102238.

[40] Krijnen PAJ, Nijmeijer R, Meijer CJLM, Visser CA, Hack CE, Niessen HWM. Apoptosis in myocardial ischaemia and infarction. Journal of Clinical Pathology. 2002; 55: 801–811.

[41] Ren X, Wang L, Chen Z, Hou D, Xue Y, Diao X, *et al*. Foxtail Millet Improves Blood Glucose Metabolism in Diabetic Rats through PI3K/AKT and NF-κB Signaling Pathways Mediated by Gut Microbiota. Nutrients. 2021; 13: 1837.

[42] Dong W, Yang R, Yang J, Yang J, Ding J, Wu H, *et al*. Resveratrol pretreatment protects rat hearts from ischemia/reperfusion injury partly via a NALP3 inflammasome pathway. International Journal of Clinical and Experimental Pathology. 2015; 8: 8731–8741.

[43] Tschopp J, Martinon F, Burns K. NALPs: a novel protein family involved in inflammation. Nature Reviews. Molecular Cell Biology. 2003; 4: 95–104.

[44] Devkar ST, Kandhare AD, Zanwar AA, Jagtap SD, Katyare SS, Bodhankar SL, *et al*. Hepatoprotective effect of withanolide-rich fraction in acetaminophen-intoxicated rat: decisive role of TNF-α, IL-1β, COX-II and iNOS. Pharmaceutical Biology. 2016; 54: 2394–2403.

[45] Kandhare AD, Alam J, Patil MVK, Sinha A, Bodhankar SL. Wound healing potential of naringin ointment formulation via regulating the expression of inflammatory, apoptotic and growth mediators in experimental rats. Pharmaceutical Biology. 2016; 54: 419–432.

[46] Tambewagh UU, Kandhare AD, Honmore VS, Kadam PP, Khedkar VM, Bodhankar SL, *et al*. Anti-inflammatory and antioxidant potential of Guaianolide isolated from Cyathocline purpurea: Role of COX-2 inhibition. International Immunopharmacology. 2017; 52: 110–118.

[47] Huang Y, Sun X, Juan Z, Zhang R, Wang R, Meng S, *et al*. Dexmedetomidine attenuates myocardial ischemia-reperfusion injury in vitro by inhibiting NLRP3 Inflammasome activation. BMC Anesthesiology. 2021; 21: 104.

[48] Yewale RV, Ramakrishna BS, Doraisamy BV, Basumani P, Venkataraman J, Jayaraman K, *et al*. Long-term safety and effectiveness of azathioprine in the management of inflammatory bowel disease: A real-world experience. JGH Open. 2023; 7: 599–609.

[49] Hu H, Guo L, Overholser J, Wang X. Mitochondrial VDAC1: A Potential Therapeutic Target of Inflammation-Related Diseases and Clinical Opportunities. Cells. 2022; 11: 3174.