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Original Article

Thiadiazolidinone-8, a GSK3β inhibitor, ameliorates aldosterone-induced cardiac inflammation and fibrosis by regulating autophagy

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Abstract: Aldosterone (Aldo)-salt-induced cardiovascular inflammation plays an important role in the pathogenesis of cardiac fibrosis. GSK-3β contributes to inflammatory cardiac diseases, and thiadiazolidinone-8 (TDZD-8) is able to repress the expression of inflammatory cytokines by acting as a specific GSK-3β inhibitor. However, the role of TDZD-8 in Aldo-salt-induced cardiac inflammation and fibrosis has not been clearly documented. In the present study, rats were treated with Aldo-salt in the absence or presence of TDZD-8 for 4 weeks, and then hemodynamic and cardiac parameters were assayed at various time points. We found that the expression levels of pro-inflammatory cytokines (IL-1β and TNF-a) and fibrosis (TGF-β and collagen I) were increased in cardiac tissues by Aldo-salt infusion, whereas TDZD-8 treatment reversed these alterations. TDZD-8 also suppressed Aldo-salt-induced endothelial-to-mesenchymal transition (EndoMT), as indicated by increased expression of VE-cadherin and decreased expression of a-SMA. Furthermore, TDZD-8 upregulated the protein levels of LC3-II in cardiac tissues, and p62 degradation, indicating that autophagy was activated by TDZD-8 in cardiac tissues. More importantly, autophagy inhibition by specific inhibitors attenuated the function of TDZD-8 in inhibiting EndoMT and perivascular fibrosis. Taken together, these results demonstrate that TDZD-8 plays a protective role in Aldo-salt-induced cardiac fibrosis by activating autophagy.

Keywords: TDZD-8, aldosterone, GSK-3β, cardiac inflammation, cardiac fibrosis, autophagy

Introduction

Cardiovascular disease (CVD) continues to be the first cause of mortality and morbidity worldwide [1, 2]. Although advancements have been made in the diagnosis and therapy of CVD, there is still a critical need for novel diagnostic biomarkers to decrease the morbidity, and novel therapeutic interventions to decrease the mortality.

Aldosterone (Aldo), which is secreted from the adrenal cortex, is a mineralocorticoid hormone that acts classically by the activation of intracellular mineralocorticoid receptor (MR) [3]. Studies have shown that Aldo-salt plays a crucial role in regulating blood pressure and electrolytic balance [4, 5]. Clinical and preclinical evidence showed that Aldo-salt plays an important pathophysiological role in cardiac remodeling by promoting cardiac fibrosis [6]. Aldo-salt is involved in cardiac remodeling, specifically it induces inflammation, oxidative stress and fibrosis [7-9]. Emerging data reported that chronic inflammation plays an important role in the pathogenesis of cardiac fibrosis and hypertension [10, 11].

Glycogen synthase kinase 3β (GSK3β), an evolutionarily conserved serine/threonine protein kinase, was initially identified as a key regulator of insulin-dependent glycogen synthesis [12]. Recent studies have shown that GSK3β is involved in cardiac growth during development and in response to stress [13]. GSK3β is also an important positive regulator of inflammatory and fibrotic processes [14, 15]. Jope *et al* first identified the role of GSK3β in the regulation of inflammation [16], demonstrating that GSK3β activity is indispensable for full stimulation of the production of pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis fac-

tor α (TNF-α) and IL-1β [16, 17]. Additionally, GSK3β inhibition by specific inhibitors greatly decreases pro-inflammatory cytokine production and increases anti-inflammatory cytokine production [18]. Previous studies have demonstrated that the activity of GSK-3β is negatively regulated by S9 phosphorylation [19, 20].

Thiadiazolidinone-8 (TDZD-8) is a specific inhibitor of GSK3β activity [21]. Xie *et al* demonstrated that GSK3β inhibition using TDZD-8 has a potent therapeutic effect by ameliorating L-dopa-induced dyskinesia in 6-OHDA parkinsonian rats [22]. However, its role in regulating cardiac fibrosis and injury is still unclear.

In the present study, the role of TDZD-8 in inhibiting Aldo-salt-induced cardiac inflammation and fibrosis was investigated. We found that TDZD-8 treatment suppressed Aldo-salt-induced cardiac inflammation and injury. Moreover, TDZD-8 promoted autophagy activation in cardiac tissues. Notably, autophagy inhibition attenuated the role of TDZD-8 in suppressing perivascular fibrosis. Therefore, all results suggest that TDZD-8 plays a protective role in Aldo-salt-induced cardiac inflammation and fibrosis, at least in part by activating autophagy.

Materials and methods

Animal model

All animal care and experimental procedures complied with requirements of the Ethics Committee of Experimental Animals at Anhui Medical University. Six-week old male Wistar rats (220-250 g) were obtained from Shanghai Model Organisms Center, Inc. (Shanghai, China). All rats were housed in traditional open cages in a pathogen-free facility under normal feeding and lighting conditions. Rats were randomly divided into three groups (11 rats in each group): 1) Vehicle control: Rats were subcutaneously treated with vehicle (sunflower oil) only for 3 weeks; 2) Aldo-salt: Aldo-salt was dissolved in sunflower oil, and rats were subcutaneously treated with Aldo-salt (1 mg/kg each day) and 1% NaCl as drinking water for 3 weeks; and 3) Aldo-salt combined with TDZD-8: The rats were subcutaneously treated with Aldo-salt (1 mg/kg/day) and TDZD-8 (1.5 mg/kg/day) and 1% NaCl as drinking water for 3 weeks.

Reagents

Aldo-salt, TDZD-8 (the GSK-3β inhibitor) and 3-MA (the autophagy inhibitor) were obtained from Target Mol (Boston, USA). The 4'6-diamidino-2-phenylindole (DAPI; Life Technologies) was used for nuclear staining.

Western blotting

SDS-PAGE under denaturing conditions was performed to separate the extracted heart proteins, which were then transferred to a PVDF membrane. Blocking with 5% BSA in TBST, the PVDF membranes were incubated with specific primary antibodies in a refrigerator at 4°C overnight (GSK3β:ZG004, 1:250, Invitrogen, CA, USA; p-GSK3β:44-604G, 1:200, Invitrogen; LC3B:MAB85582, 0.1 μg/mL, R&D Systems, MN, USA; p62:ab56416, 1 μg/mL, Abcam, MA, USA; VE-cadherin:ab166715, 1:1,000, Abcam; a-SMA:ab5694, 1 μg/mL, Abcam). The membrane was then washed 3 times with TBST, and then incubated with ECL HRP-conjugated secondary antibodies for 1 h and imaged with an Odyssey Imaging System (LI-COR Biosciences). Each assay was carried out in triplicate and statistically quantified by ImageJ software.

Total RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was isolated from cardiac tissues with TRIzol (Invitrogen), and then 1 μg RNA was converted into cDNA using a Revert Aid First Strand cDNA Synthesis Kit (Shnghai Haoran Biological Technology CO., Ltd), as the manufacturer's instructions. The mRNA levels of IL-1β, TNF-α, TGF-β, Col I, LC3B and p62 were measured with qPCR, and β-actin was used as the internal control. The primers are listed in Supporting Table S1.

Histological analysis

Rats were treated with the assigned reagents, and then the left ventricles were fixed with 4% paraformaldehyde, embedded in paraffin and cut into 4 μm-thick sections as previously described [18]. Masson's trichrome staining was carried out with serial sections. The percentage of perivascular collagen deposition by trichromatic staining in total perivascular area was calculated, and the color cube function of

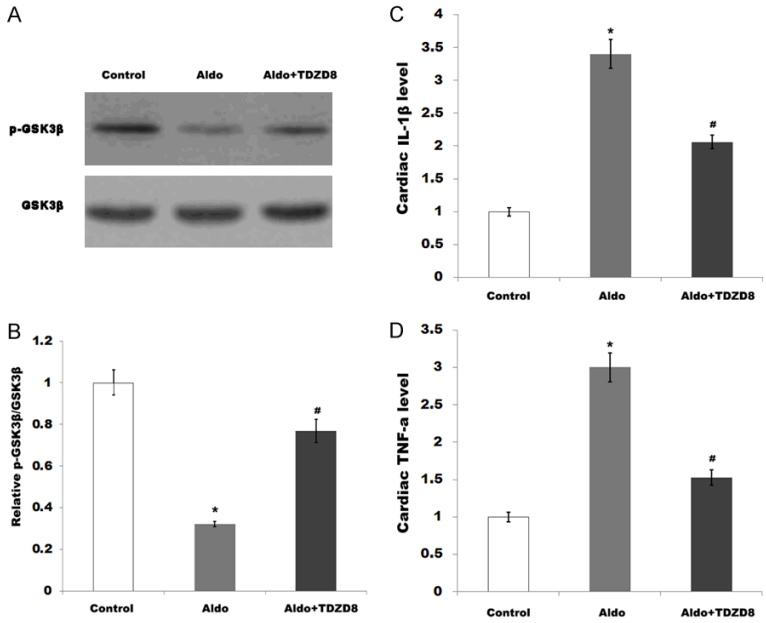

Figure 1. TDZD-8 inhibited GSK3β activation and decreased Aldo-salt-induced cardiac inflammation. (A) Western blot analysis of p-GSK3β level in the heart tissues after treatment with Aldo-salt, or Aldo-salt and TDZD8. This assay was performed in triplicate. (B) Quantitative analysis of western blot data showed in (A). (C) The mRNA levels of IL-1β (C) and TNF-α (D) were analyzed by quantitative real-time PCR. β-actin was set as the internal control. This assay was repeated at least three times. *$P < 0.05$ vs control, #$P < 0.05$ vs Aldo.

the software was used to evaluate perivascular fibrosis.

Statistical analysis

Each experiment was carried out in triplicate, and all results are presented as the mean ± S.D. Averaged data were compared with an unpaired Student's t-test (**Figure 4**) or one-way ANOVA (**Figures 1**-**3** and **5**), followed by the Scheffé test. The level of significance was set at $P < 0.05$.

Results

TDZD-8 inhibited GSK3β activation and decreased Aldo-salt-induced cardiac inflammation

To investigate the role of GSK3β in regulating Aldo-salt-induced cardiovascular inflammation and fibrosis, rats were treated with Aldo-salt combined with TDZD-8 (a specific GSK3β inhibitor), and hemodynamic and cardiac parameters were assessed. Aldo-salt treatment led to a significant increase in diastolic blood pressure (DBP) and systolic blood pressure (SBP) (**Table 1**). Aldo-salt also increased the ratio of heart weight (HW) to body weight (BW), and decreased heart rate (**Table 1**).

GSK3β expression and activation was assessed because TDZD-8 has been described as a specific inhibitor of GSK3β. **Figure 1A** and **1B** showed that TDZD-8 treatment could not change the total GSK3β protein level, but that TDZD-8 upregulated phosphor-GSK3βS9 levels at PSer9 in the heart tissues, indicating that TDZD-8 inhibited GSK3β activation. Functionally, TDZD-8 treatment significantly inhibited Aldo-salt-induced upregulation of cardiac IL-1β and TNF-α mRNA levels (**Figure 1C** and **1D**).

TDZD-8 inhibited Aldo-salt-induced cardiac fibrosis

Given that EndoMT contributes to the development of various cardiovascular diseases, we next investigated the effects of Aldo-salt and TDZD-8 on EndoMT. As shown in **Figure 2A** and **2B**, Aldo-salt treatment resulted in decreased mRNA and protein levels of VE-cadherin and an increased expression level of a-SMA, indicating that Aldo-salt induced EndoMT. As expected, TDZD-8 significantly suppressed Aldo-salt-induced EndoMT (**Figure 2A** and **2B**).

We then investigated the role of TDZD-8 in regulating cardiac fibrosis as chronic inflammation and EndoMT playing critical roles in the pathogenesis of cardiac fibrosis and hypertension [10, 11]. To verify this, we assessed the expression levels of transforming growth factor-β (TGF-β) and collagen type I (Col I), which are a profibrotic marker and extracellular matrix protein, respectively [23]. As shown in **Figure 3A** and **3B**, Aldo-salt upregulated the mRNA and protein levels of TGF-β and Col I in cardiac tissues, whereas TDZD-8 treatment significantly inhibited the Aldo-salt-induced upregulation of TGF-β and Col I. Perivascular fibrosis in the left

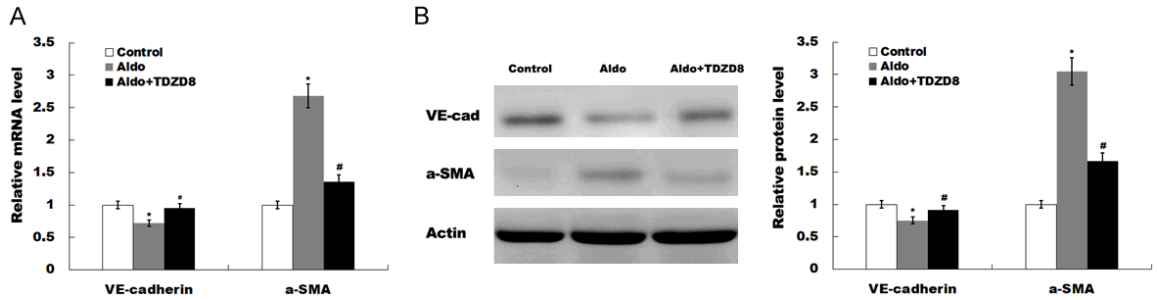

Figure 2. TDZD-8 inhibited Aldo-induced EndoMT. A. The mRNA levels of VE-cadherin and a-SMA in cardiac tissues were analyzed by quantitative real-time PCR after Aldo-salt treatment with or without TDZD-8. β-actin was set as the internal control. B. The protein levels of VE-cadherin and a-SMA were analyzed by western blot after Aldo-salt treatment with or without TDZD-8. Quantitative and statistical analysis was conducted based on data from least three repeats. *$P < 0.05$.

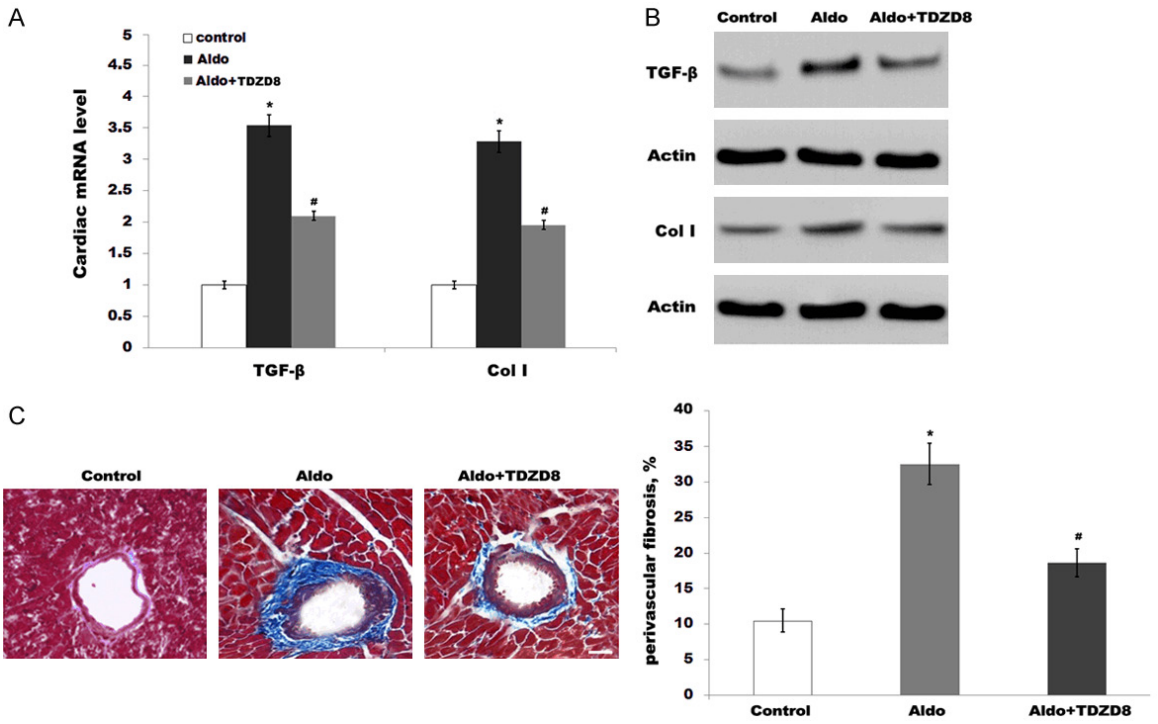

Figure 3. TDZD-8 inhibited Aldo-induced cardiac fibrosis. Cardiac mRNA (A) and protein (B) levels of TGF-β and Col I were assessed by quantitative real-time PCR. (C) IHC analysis for indicating the collagen deposition and perivascular fibrosis after Aldo-salt treatment, or Aldo and TDZD8 treatment. Bar, 50 μM. Quantitative analysis was conducted after the assay was performed in triplicate. *$P < 0.05$ vs control, #$P < 0.05$ vs Aldo-salt.

ventricle was then assayed via the deposition of collagen around the vasculature, as previously described [24]. **Figure 3C** presented the representative results of collagen deposition and the quantification of fibrosis. The results showed that Aldo-salt treatment caused perivascular fibrosis, whereas TDZD-8 significantly suppressed Aldo-salt-induced perivascular fibrosis. These data demonstrated that TDZD-8 could effectively suppress Aldo-salt-induced cardiac inflammation and fibrosis.

TDZD-8 activated cell autophagy in cardiac tissues

We then investigated whether TDZD-8 regulated autophagy activation and TDZD-8 inhibited cardiac fibrosis by regulating autophagy. As

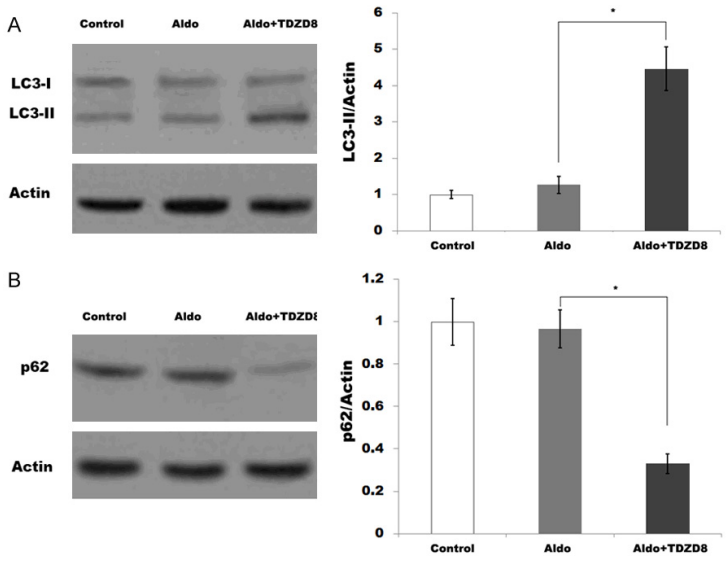

Figure 4. TDZD-8 activates activated autophagy in cardiac tissues. A. LC3-I and LC3-II protein levels were analyzed by western blotting after Aldo treatment, in the presence or absence of TDZD8. Quantitative and statistical analysis was conducted based on at least three repeats. B. p62 protein level was analyzed by western blot after Aldo treatment in the presence or absence of, or Aldo and TDZD8. Quantitative and statistical analysis was conducted based on at least three repeats. *$P < 0.05$.

shown in **Figure 4A**, additional treatment of TDZD-8 markedly upregulated the protein levels of LC3-II (the marker of autophagy activation) compared with Aldo-salt alone in the cardiac tissues, indicating that autophagy was activated after TDZD-8 treatment. The multifunctional ubiquitin-binding protein p62/SQSTM1 is an autophagy substrate [25]. The autophagy flux was also verified by assessing the reduction of the p62/SQSTM1 protein level following TDZD-8 treatment (**Figure 4B**).

TDZD-8 inhibited Aldo-salt-induced cardiac fibrosis by activating autophagy

TDZD-8 *inhibited* Aldo-salt-induced cardiac inflammation and fibrosis, and contributed to autophagy activation. Therefore, we next investigated whether TDZD-8 inhibited Aldo-salt-induced cardiac inflammation and fibrosis by activating autophagy. Here, 3-methyladenine (3-MA) was used to specifically attenuate LC3-II upregulation and destroy the formation of autophagosomes, as previously described [26]. **Figure 5A** and **5B** showed that the level of cardiac inflammation and perivascular fibrosis was upregulated after Aldo-salt treatment, whereas TDZD-8 could reverse this alteration. Notably, autophagy inhibition by 3-MA significantly suppressed the function of TDZD-8 in inhibiting cardiac inflammation and perivascular fibrosis. Collectively, current data confirmed that TDZD-8 protects against Aldo-salt-induced cardiac injury, at least in part by activating autophagy.

Discussion

In the present study, we investigated the potential role of a GSK3β inhibitor (TDZD-8) in alleviating Aldo-salt-induced cardiac inflammation and fibrosis. The results suggest that: (I) TDZD-8 inhibited GSK3β activation in cardiac tissues treated with Aldo-salt; (II) TDZD-8 inhibited Aldo-salt-induced cardiac inflammation, EndoMT and fibrosis; (III) TDZD-8 contributed to the activation of autophagy in cardiac tissues; and (IV) TDZD-8 inhibited cardiac injury by activating autophagy. These data demonstrate the important role of TDZD-8 and autophagy in regulating Aldo-salt-induced inflammation and fibrosis and suggested that TDZD-8 possesses a potential therapeutic effect for alleviating salt-sensitive cardiac injury.

Aldosterone promotes inflammatory response by inducing the production of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide, which stimulate the activation of the pro-inflammatory transcription factor activator protein (AP)-1 and nuclear factor kappa B (NF-κB) [27, 28]. In the heart, Aldo-salt-induced generation of ROS could activate Ca^{2+} calmodulin (CaM)-dependent protein kinase II (CaMKII). The activation of CaMKII promotes left ventricular remodeling following myocardial infarction [27, 29]. Recent studies have reported that GSK3β is centrally involved in Aldo-induced podocyte death [30]. Aldosterone-induced GSK3β activation leads to hyperphosphorylation and the over-activation of GSK3β substrates, and results in subsequent cell injury and death [30]. Ischemia/reperfusion [I/R] injury results in an increased S9 phosphorylation of GSK3β and thus inhibits GSK3β activity [20].

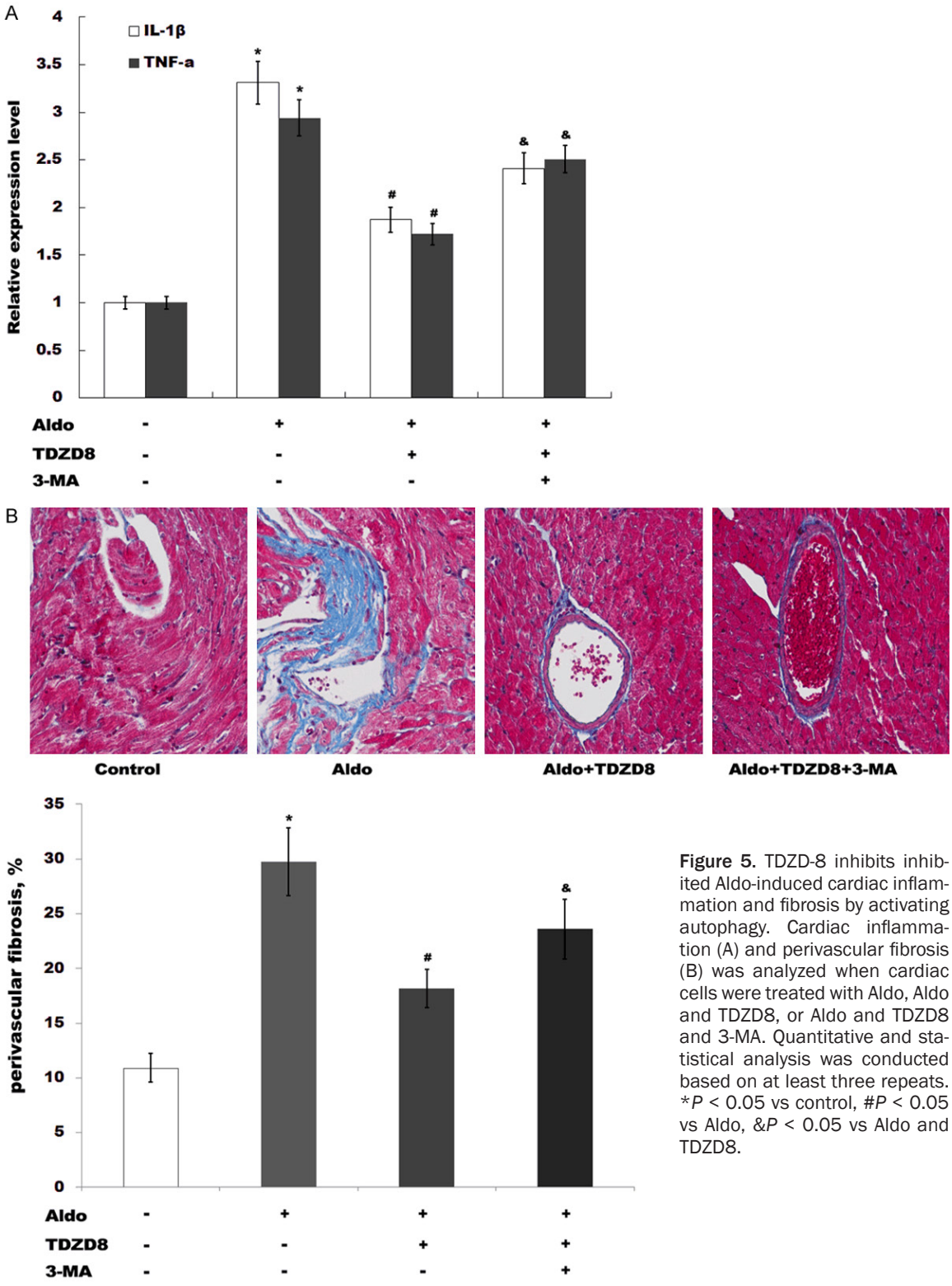

Figure 5. TDZD-8 inhibits inhibited Aldo-induced cardiac inflammation and fibrosis by activating autophagy. Cardiac inflammation (A) and perivascular fibrosis (B) was analyzed when cardiac cells were treated with Aldo, Aldo and TDZD8, or Aldo and TDZD8 and 3-MA. Quantitative and statistical analysis was conducted based on at least three repeats. *$P < 0.05$ vs control, #$P < 0.05$ vs Aldo, &$P < 0.05$ vs Aldo and TDZD8.

Recent studies have demonstrated that the therapeutic effect of GSK3β inhibitors are associated with the suppression of the inflammatory response. Inhibition of GSK3β results in decreased activation of the pro-inflammatory transcription factor NF-κB. Additionally, GSK3β inhibition contributes to the production of the anti-inflammatory cytokine IL-10 [31]. In this

Table 1. Physiological and hematological parameters in Aldo-treated rats

	Control	Aldo	Aldo + TDZD8
SBP, mm Hg	138 ± 0.6	153 ± 0.4*	144 ± 0.3#
DBP, mm Hg	92 ± 0.9	122 ± 1.3*	107 ± 0.8#
HR, beats/min	331 ± 6.8	269 ± 9.2*	317 ± 5.8#
HW/BW, mg/g	2.61 ± 0.02	2.90 ± 0.03*	2.72 ± 0.01#

Note: Aldo = aldosterone; SBP = systolic blood pressure; DBP = diastolic blood pressure; HW = heart weight; BW = body weight. Values are presented as mean ± SEM. *$P < 0.05$ vs control. #$P < 0.05$ vs Aldo group.

study, we investigated the role of TDZD-8, a specific GSK3β inhibitor, in the regulation of Aldo-induced cardiac inflammation and fibrosis. Our results demonstrated that Aldo induces a significant increase in DBP and SBP, but additional treatment with TDZD-8 inhibits Aldo-salt-induced cardiac dysfunction and hypertrophy. Functionally, TDZD-8 suppresses the Aldo-induced upregulation of cardiac IL-1β and TNF-α levels. We also investigated the role of TDZD-8 in regulating Aldo-salt-induced cardiac fibrosis. Aldo-salt-increases TGF-β and Col I expression in cardiac tissues, whereas TDZD-8 treatment also markedly suppresses the Aldo-induced upregulation of TGF-β and Col I. Furthermore, TDZD-8 suppresses Aldo-induced perivascular fibrosis.

Autophagy is a lysosome-mediated intracellular catabolic process by which cells remove their damaged organelles; thus, it plays a significant role in the regulation of intracellular homeostasis and cell survival. Emerging studies demonstrated that inactivation of autophagy promotes the progression of cardiovascular and renal disease [32, 33]. In an addition, GSK3β inhibition triggers a profound autophagic response in salt-sensitive hypertension and renal fibrosis, under serum-free condition and ischemic mouse models [18, 20, 34, 35]. Based on these facts, we further investigated the role of autophagy in TDZD-8-regulated cardiac fibrosis. We found that TDZD-8 treatment contributes to the activation of autophagy. More important, autophagy inhibition by its specific inhibitors significantly decreases the effect of TDZD-8 in preventing perivascular fibrosis. The detailed mechanisms by which TDZD-8 activates autophagy deserve further study. Taken together, these data demonstrated that the TDZD-8/autophagy pathway possesses a potential therapeutic effect for alleviating the salt-sensitive cardiac injury.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

CVD, Cardiovascular disease; Aldo, Aldosterone; TDZD-8, Thiadiazolidinone-8; GSK-3β, Glycogen synthase kinase-3β; TGF-β, Transforming growth factor-β; Col I, collagen type I.

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Table S1. The primer used in the study

Gene name	Primer forward	Primer reverse
IL-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
TGF-β	GGCCAGATCCTGTCCAAGC	GTGGGTTTCCACCATTAGCAC
Col I	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC
LC3b	AACATGAGCGAGTTGGTCAAG	GCTCGTAGATGTCCGCGAT
p62	GCACCCCAATGTGATCTGC	CGCTACACAAGTCGTAGTCTGG