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Trimetazidine inhibits pressure overload-induced cardiac fibrosis through NADPH oxidase–ROS–CTGF pathway

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Aims Cardiac fibrosis contributes to the transition from compensated ventricular hypertrophy to heart failure, which can be promoted by connective tissue growth factor (CTGF). Trimetazidine (TMZ), an anti-angina drug, also has benefits in non-ischaemic heart disease. We wondered whether TMZ has an effect on cardiac fibrosis from pressure overload by downregulating CTGF.

Methods and results Male Sprague–Dawley rats underwent transverse aortic constriction (TAC) or sham operation and then after 20 weeks were assigned to receive TMZ or saline for another 5 weeks. TMZ significantly inhibited collagen accumulation, CTGF expression, and reactive oxygen species (ROS) production induced by TAC. Furthermore, the effects of TMZ on ROS, the upstream signal of CTGF synthesis signal transduction, were evaluated in cardiac fibroblasts. The result showed that the ROS level was reduced by TMZ on stimulation with angiotensin II. Additionally, the NADPH oxidase activity was ameliorated with TMZ by the regulation of translocation of its subunit Rac1.

Conclusion TMZ effectively inhibits myocardial fibrosis, perhaps through the NADPH oxidase–ROS–CTGF signalling pathway. Our findings may be used to provide new clues for the potential function of TMZ in pressure overload-induced myocardial fibrosis.

Keywords Trimetazidine • Cardiac fibrosis • Connective tissue growth factor • Reactive oxygen species • NADPH oxidase

1. Introduction

Cardiac fibrosis, defined as interstitial fibroblast proliferation and excessive extracellular matrix (ECM) deposition, leads to heart failure, arrhythmia, sudden cardiac death, and other serious complications.[1,2] Long-term pressure overload induces ventricular remodelling, including myocyte hypertrophy and interstitial fibrosis. Cardiac fibrosis is one of the most important factors contributing to the transition from compensated ventricular hypertrophy to heart failure.[3] Therefore, inhibiting the process of myocardial fibrosis is a crucial strategy in clinical treatment.

Trimetazidine, 1-(2,3,4-trimethoxybenzyl) piperazine dihydrocholoride (TMZ), is used for the treatment of ischaemic cardiomyopathy in the clinic. It decreases the utilization of free fatty acids for energy production by inhibiting mitochondrial 3-ketoacyl CoA thiolase (3-KAT) activity in beta-oxidation, thus resulting in increased consumption of non-lipid substrates, mainly glucose.[4,5] In addition to metabolic effects, TMZ was previously shown to have non-metabolic benefits at the ventricular myocyte level.[6,7] The mechanism is related to preservation of mitochondrial function, inhibition of inflammatory response, improvement of endothelial dysfunction, and reduction of oxidative stress, for example.[7–10] Although TMZ improves ejection fraction (EF) in patients of heart failure with or without ischaemic cardiomyopathy,[11–16] whether TMZ has an effect on myocardial fibrosis in compensated ventricular hypertrophy from pressure overload and its molecular basis has not yet been elucidated.

Connective tissue growth factor (CTGF) is known to play an important role in the myocardial fibrosis process and has emerged

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as a new target for therapeutic intervention in fibrotic diseases.[17–19] Its synthesis mechanism involves Ras/MEK/ERK (kinase), RhoA/ROCK, and reactive oxygen species (ROS)-related signalling pathways.[17,20–22] Although the antioxidant properties of TMZ were recognized in ventricular myocytes with ischaemia or hypoxia,[6,7,23,24] whether TMZ could reduce ROS production and CTGF synthesis in cardiac fibroblasts with angiotensin II (Ang II) stimulation without ischaemia or hypoxia has not been tested.

Thus, we aim to investigate whether TMZ has an effect on cardiac fibrosis from pressure overload by downregulating CTGF expression and its potential signalling targets.

2. Methods

The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The experiments were approved by the Committee on Ethics of Animal Experiments and were conducted in accordance with the Guidelines for Animal Experiments, Peking University Health Science Center.

2.1 Animal model preparation

Male Sprague–Dawley (SD) rats (8 weeks old, 180–220 g) were obtained from the Animal Department of Peking University Health Science Center. All surgical procedures were performed with animals anaesthetized with 5% tribromoethanol (250 mg/kg intraperitoneally). Transverse aortic constriction (TAC, $n = 18$) between the innominate artery and the left carotid artery was performed. Sham animals ($n = 14$) were prepared in the same way except for the aortic constriction. Four rats died from acute heart failure after TAC operation and none died in sham group. Twenty weeks after surgery, rats were examined by echocardiography (Supplementary Data). Then sham and TAC rats were randomly subdivided into two groups each for the treatment: one group received TMZ (10 mg/kg/day) and the other group received physiological saline by intraperitoneal injection (each day) for 5 weeks.[25] The peak concentration of circulating TMZ was in the range from 1.5 to 1.9 μM. Echocardiography was performed 1 day before rats were sacrificed. The free wall of left ventricles was fixed or immediately embedded in tissue freezing medium and the rest samples were stored in liquid nitrogen.

2.2 Histological analysis

Left ventricles were fixed with 4% paraformaldehyde, dehydrated, and embedded in paraffin. Sections (6 μm thick) were stained with Sirius-red reagent for detecting collagen. To determine the degree of cardiac fibrosis, 10 fields were randomly selected, and the cardiac collagen volume fraction was calculated as the ratio of Sirius red-stained fibrosis area to total myocardium area with the use of Image Pro-plus 5.0 software.

2.3 Hydroxyproline and malondialdehyde measurement

The content of hydroxyproline and malondialdehyde (MDA) in myocardial tissue was assayed by the use of two commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) for detecting hydroxyproline (hydrolysis with alkali) and MDA (TBA colorimetric determination) according to the manufacturer's instructions.

2.4 Quantitative real-time PCR

Total RNA was isolated by the use of Trizol Reagent (Invitrogen). Relative quantitation by real-time PCR involved SYBR-green detection of PCR products in real time with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). In each experiment, the rat GAPDH RNA was amplified as a reference standard. Reactions were prepared in triplicate and heated to 95°C for 5 min, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The primer sequences are shown in Supplementary Data.

2.5 Cell culture

Cardiac fibroblasts were isolated and cultured from 1-day-old neonatal SD rats as described previously.[26,27] All experiments were performed in fibroblasts at passages 2–3. The purity of these cultures was greater than 98% cardiac fibroblasts, as determined by positive staining for vimentin and negative staining for von Willebrand factor. Studies involved cardiac fibroblasts grown to 80% confluence and serum starved for 24 h in serum-free medium before treatment.

2.6 BrdU ELISA

Neonatal rat cardiac fibroblasts (passage 2) were pre-treated with TMZ (0.1–10 μM) for 30 min, followed by stimulation of Ang II (0.1 μM) for 24 h. Next assay was performed according to the manufacturer's instructions of BrdU ELISA kit (Roche).

2.7 Western blot analysis

Protein expression was examined by western blot analysis as previously described.[28] Briefly, samples were separated on 8–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred by electroblotting to a nitrocellulose membrane. The membrane was blocked in 5% bovine serum albumin and then incubated with anti-CTGF, anti-ERK1/2, anti-collagen I, anti-collagen III (Santa Cruz, CA, USA), anti-phospho-ERK1/2 (Cell Signaling, USA), and anti-Rac1 antibody (Upstate, USA) in 5% BSA overnight at 4°C, then washed and incubated with horseradish peroxidase-labelled second antibodies for 1 h at room temperature. Bands were visualized by the use of a super-western sensitivity chemiluminescence detection system (Pierce). Autoradiographs were quantitated by densitometry (Science Imaging System, Bio-Rad). GAPDH was the internal control for protein normalization.

2.8 Membrane and cytosolic protein preparation

Cells were washed with ice-cold phosphate-buffered saline (PBS), dissolved in lysis buffer B, which contained 12.5 mM Tris–HCl (pH 7.4), 2.5 mM EGTA, 5 mM DTT, 50 mM NaF, 1 mM EDTA and protease inhibitors, collected, and sonicated. After centrifugation (30 min, 20 000 *g*, 4°C), the supernatant represented the cytosolic fraction. The resulting pellet was dissolved in lysis buffer B including 2% Triton X-100. After centrifugation at 20 000 *g* at 4°C for 5 min, the supernatant represented the membrane fraction.[29]

2.9 NADPH oxidase activity assay

Quiescent cells were starved by serum deprivation for 24 h and treated as indicated, washed twice with ice-cold PBS, pH 7.4. Then NADPH oxidase activity was measured by lucigenin-enhanced chemiluminescence with a commercial kit (Genmed Scientifics Inc., China) according to the manufacturer's instructions.

2.10 Quantification of intracellular ROS

Intracellular ROS production was detected by the use of fluorescent probe dye, CM-H2DCFDA, and dihydroethidium (DHE) (Invitrogen Molecular Probes, Eugene, OR, USA). Briefly, frozen tissue sections (7 μM) or cardiac fibroblasts were incubated with 10 μM DHE at 37°C for 30 min according to the manufacturer's instructions. Red fluorescence was assessed by confocal laser scanning microscopy as described.[30] Fluorescence intensity per cell was analysed by the use of WCIF ImageJ software. In addition, cells were incubated with 10 μM CM-H2DCFDA for 30 min at 37°C before treatment, and DCF fluorescence were detected by flow cytometry (BD FACSCalibur) equipped with a laser lamp (emission 480 nm; band pass filter 530 nm). For each sample, 10 000 events were collected.

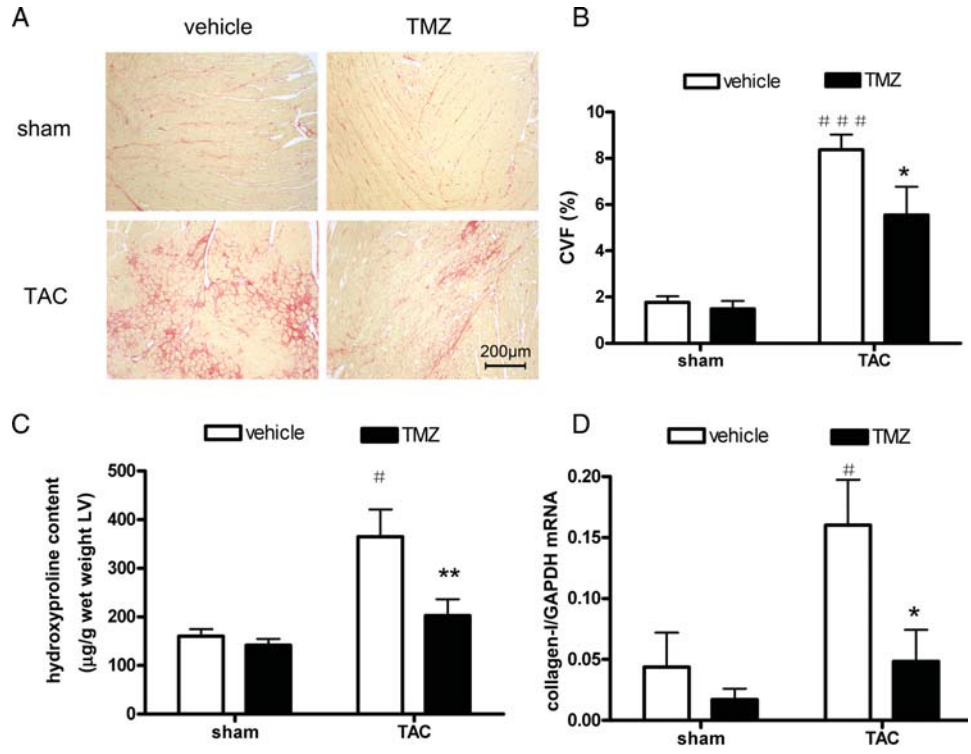

Figure 1 Effect of TMZ on cardiac fibrosis induced by pressure overload. (*A*) Representative micrographs of Sirius red-stained sections in left ventricles (LVs) of vehicle+sham, vehicle+TAC, TMZ+sham and TMZ+TAC rats 5 weeks after treatment (bar = 200 μm). (*B*) Interstitial collagen volume fraction (CVF) calculated in Sirius red-stained cross-sections of the LVs in rats ($n = 5$). (*C*) Collagen concentration in LVs determined by hydroxyproline assay; results are expressed as g/mg wet weight LV ($n = 6$). (*D*) Expression of collagen I mRNA normalized to that of GAPDH ($n = 3$). All values are the mean ± SEM. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared with vehicle+sham; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle+TAC.

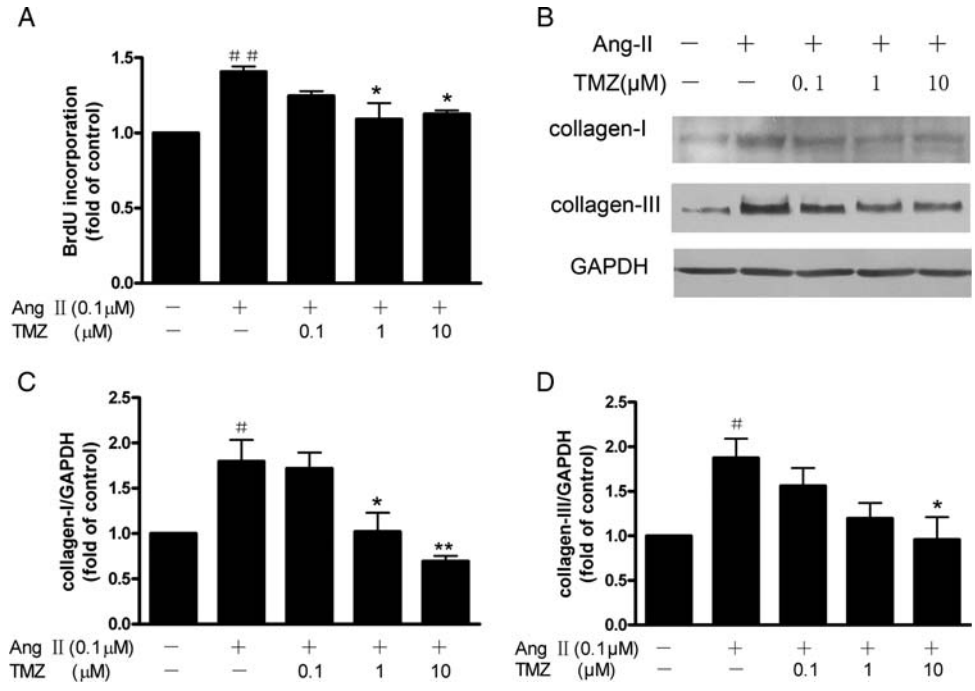

Figure 2 Effect of TMZ on cardiac fibroblasts proliferation and collagen synthesis. (*A*) Cardiac fibroblasts proliferation detected by BrdU ELISA ($n = 3$). (*B*) Collagen-I and collagen-III expression examined by western blot analysis. (*C* and *D*) Quantification of collagen-I and collagen-III protein expression standardized to GAPDH. Results were calculated as fold of the control value. All values are the mean ± SEM ($n = 3$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Ang II group.

ROS production was expressed as mean fluorescence intensity, which was calculated by the use of Cell Quest software.[28]

2.11 Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). One-way ANOVA or two-way ANOVA with a Bonferroni *post hoc* test was used as applicable. $P < 0.05$ was considered statistically significant.

3. Results

3.1 TMZ inhibited cardiac fibrosis from pressure overload

Heart sections were stained with Sirius red for the examination of the extent of interstitial fibrosis 5 weeks after treatment with TAC and TMZ (*Figure 1A*). Morphologically, collagen deposition appeared to increase in response to TAC but was attenuated by TMZ. On quantitative analysis of interstitial fibrosis in the heart by collagen volume fraction, TMZ markedly reduced intermuscular interstitial fibrosis by 34% when compared with control TAC rats, with no significant difference between the vehicle + sham group and TMZ + sham groups (*Figure 1B*). Consistent with the results of collagen volume fraction, the LV hydroxyproline content was increased in TAC rats (365.10 ± 56.19 μg/g wet weight LV, $n = 6$), whereas the content was significantly reduced in TMZ-treated rats (202.81 ± 33.42 μg/g wet weight LV, $n = 6$) (*Figure 1C*). Quantitative real-time RT–PCR analysis demonstrated that in TAC rats, relative mRNA content of myocardial collagen-I was significantly increased when compared with sham-treated rats, but was inhibited by 70% with TMZ (*Figure 1D*).

3.2 TMZ inhibited neonatal rat cardiac fibroblasts proliferation and collagen synthesis

To detect the direct effects of TMZ on cardiac fibroblasts proliferation and collagen synthesis, fibroblasts were incubated with Ang II

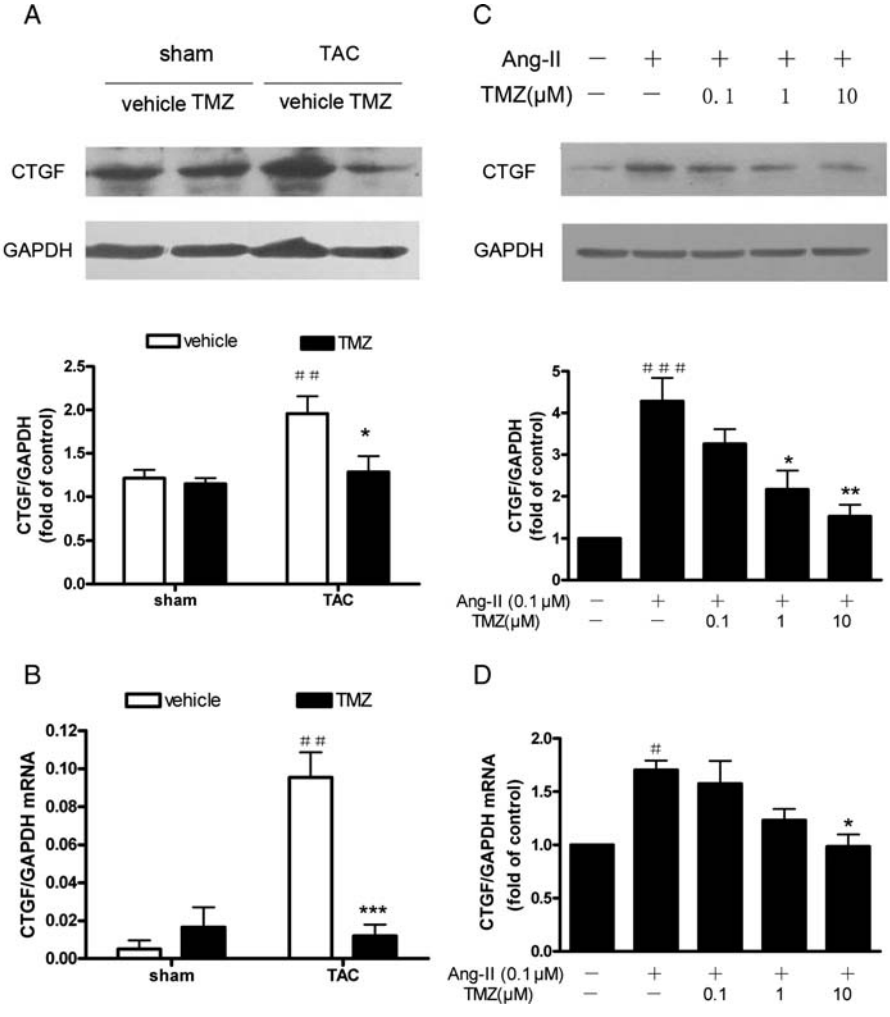

Figure 3 Effect of TMZ on CTGF expression in myocardial tissue and neonatal rat cardiac fibroblasts. (*A* and *B*) CTGF protein and mRNA expression examined by western blot analysis ($n = 5$ in each group) and real-time PCR ($n = 3$ in each group), respectively. Expression of CTGF was standardized to that of GAPDH. All values are the mean ± SEM. $^{\#\#}P < 0.01$ compared with vehicle+sham; $^{*}P < 0.05$, $^{***}P < 0.001$ compared with vehicle+TAC. (*C* and *D*) Cells were pre-incubated with TMZ (0.1, 1 and 10 μM) for 30 min and then treated with Ang II (0.1 μM, 24 h), harvested for detecting CTGF expression by western blot ($n = 4$) and real-time RT–PCR ($n = 3$). Results were calculated as fold of the control value. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. control; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Ang II.

(0.1 μM) for up to 24 h after pre-treatment with TMZ (0.1–10 μM) for 30 min. Then Cell proliferation was detected by BrdU ELISA, and collagen synthesis was assayed by western blot. The BrdU ELISA result showed that Ang II-induced fibroblasts proliferation was dose relatively inhibited by TMZ, and 23% inhibition was observed with 1 μM TMZ treatment (1.09 ± 0.18 vs. 1.41 ± 0.06-fold of control, $P < 0.05$, $n = 3$) (*Figure 2A*). Collagen-I and collagen-III protein synthesis was significantly upregulated by Ang II (1.80 ± 0.41 and 1.87 ± 0.21-fold of control), and dose relatively decreased with TMZ pre-treatment. One micrometer TMZ could inhibit collagen-I protein level by 53% (1.02 ± 0.21 vs. 1.80 ± 0.24-fold of control, $P < 0.05$, $n = 3$) and 10 μM TMZ could inhibit collagen-III protein by 49% (0.96 ± 0.25 vs. 1.87 ± 0.21-fold of control, $P < 0.01$, $n = 3$) (*Figure 2B–D*).

3.3 TMZ inhibited CTGF expression in myocardial tissue and neonatal rat cardiac fibroblasts

In vivo, CTGF protein level was significantly upregulated in vehicle+TAC group by 1.61-fold when compared with the control (1.21 ± 0.096 vs. 1.96 ± 0.20-fold of control, $P < 0.01$) and markedly reduced in the TMZ+TAC group by 34% (1.29 ± 0.18 vs. 1.96 ± 0.20-fold of control, $P < 0.05$) (*Figure 3A*). As well, the change in CTGF mRNA expression was consistent with its protein level (*Figure 3B*).

To discriminate between the direct and indirect effects of TMZ, regulation of CTGF was further investigated *in vitro* in neonatal rat fibroblasts. After pre-treatment with TMZ (0.1–10 μM) for 30 min, fibroblasts were incubated with Ang II (0.1 μM) for up to 24 h, and CTGF protein expression was detected by the western blot analysis. Upregulation of the CTGF level under Ang II stimulation was dose relatively inhibited by TMZ, with 1 μM TMZ significantly inhibiting CTGF expression (2.17 ± 0.45 vs. 4.29 ± 0.55-fold of control, $P < 0.05$, $n = 4$) (*Figure 3C*). Cells were harvested after incubation with Ang II for 12 h, and the change in mRNA expression was consistent with western blot findings (*Figure 3D*).

3.4 TMZ reduced MDA level and ROS formation *in vivo*

To explore the mechanism of the downregulation of CTGF by TMZ, we investigated the ROS formation and level of MDA in tissue. DHE fluorescence intensity, indicating increased tissue ROS production, was increased by 1.3-fold in the vehicle+TAC group and inhibited by 19% in the TMZ+TAC group (*Figure 4A* and *B*). Cardiac MDA level, an indicator of lipid peroxide content, was increased 1.5-fold in the vehicle+TAC group and reduced by 39% in the TMZ+TAC group when compared with the vehicle+sham group (*Figure 4C*). Thus, TMZ had an antioxidant effect on the progression of pressure overload-induced cardiac fibrosis.

3.5 Effect of TMZ on ROS production in neonatal rat cardiac fibroblasts

We detected ROS production in fibroblasts, which is important to the synthesis of CTGF. First, the time phase of intracellular ROS generation stimulated by Ang II was tested in neonatal rat cardiac fibroblasts (see Supplementary material online, *Figure S5*). DHE fluorescence microscopy revealed that ROS release significantly increased at 15 min. Then we treated fibroblasts with Ang II (0.1 μM) for 15 min after pre-treatment with TMZ (1 μM) for 30 min. Fluorescence microscopy illustrated that TMZ inhibited Ang II-induced ROS release. TMZ alone had no significant effect on basal ROS production (*Figure 5A*). In addition, intracellular ROS production from fibroblasts was detected by flow cytometry with the incubation of 10 μM CM-H2DCFDA before treatment (*Figure 5B*). Ang II significantly increased ROS generation in fibroblasts by 2.15 ± 0.01-fold that of controls at 15 min and was reduced to 1.6 ± 0.08-fold that of controls by 1 μM TMZ ($P < 0.05$ vs. Ang II). The results suggested that TMZ inhibited ROS generation in cardiac fibroblasts.

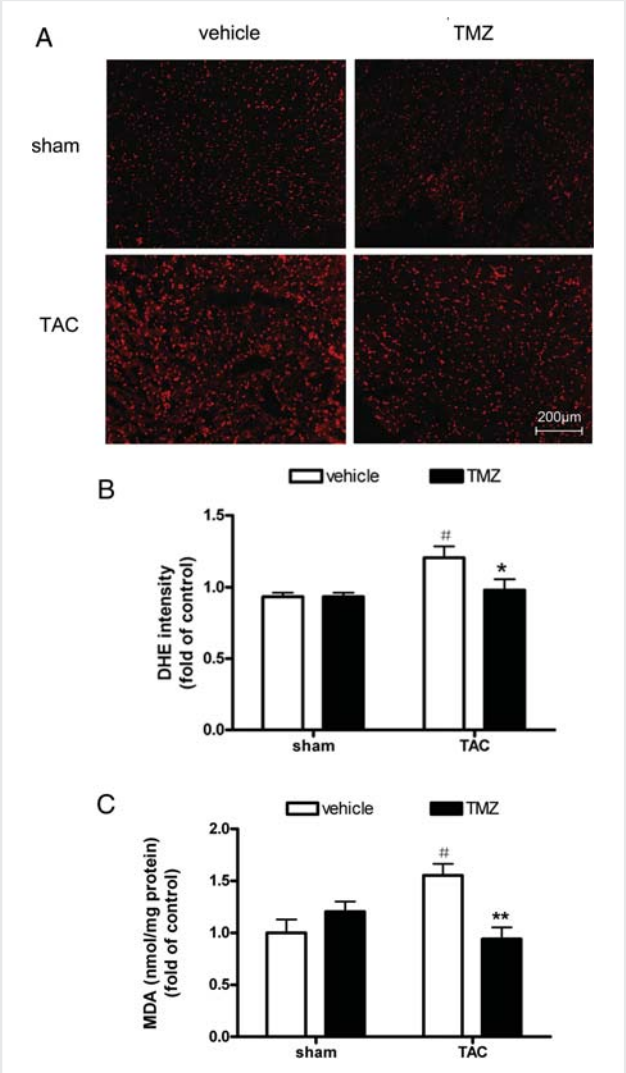

Figure 4 Effect of TMZ on oxidative stress in myocardial tissue. (*A*) DHE staining in frozen sections by fluorescent microscopy (bar = 200 μm). (*B*) Fluorescence intensity of DHE in each group ($n = 3$). (*C*) MDA content in myocardial tissue in each group ($n = 6$). Data are expressed as fold of control. The values are means ± SEM. $^{\#}P < 0.05$ compared with vehicle+sham; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle+TAC.

3.6 TMZ inhibited upstream signalling of ROS-NADPH oxidase activity in cardiac fibroblasts

The results obtained in previous experiments prompted us to evaluate whether TMZ was a free radical scavenger or directly affected

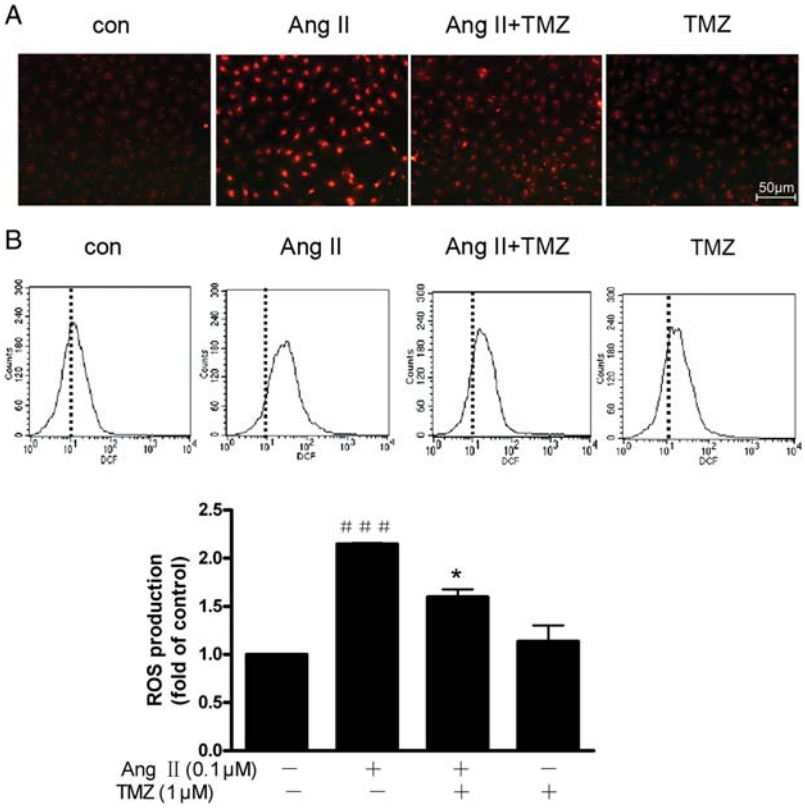

Figure 5 Effect of TMZ on ROS generation in neonatal rat cardiac fibroblasts. (*A* and *B*) The intracellular ROS generated was detected by fluorescence microscopy (A, bar = 50 μm) and flow cytometry (B). Cells were pre-incubated with TMZ (1 μM) for 30 min after incubation with DHE (10 μM) for 30 min or CM-H2DCFDA (10 μM) for 15 min and then stimulated with Ang II (0.1 μM) for 15 min. Lower panel shows mean cell fluorescence for CM-H2DCFDA. The fluorescence intensities in untreated control cells are expressed as 1. Results are as mean ± SEM of triplicate experiments. $^{###}P < 0.001$ vs. control; $^{*}P < 0.05$ vs. Ang II.

NADPH oxidase activity. We prepared cell homogenates and assayed NADPH oxidase activity by lucigenin-enhanced chemiluminescence. The time course of the oxidase with Ang II treatment showed an increase in activity at 15 min (3.00 ± 0.56-fold, $P < 0.01$ vs. control) that continued to increase for 30 min (2.71 ± 0.19-fold, $P < 0.05$ vs. control) (*Figure 6A*). Pre-treatment with 1 μM TMZ significantly decreased NADPH oxidase activity when compared with Ang II stimulation for 15 min (1.29 ± 0.19 vs. 2.18 ± 0.18-fold of control, $P < 0.05$) (*Figure 6B*).

Because the translocation of the cytosolic subunit of NADPH oxidase, Rac1, is necessary for its activity, we detected the change in Rac1 translocation from the cytosol to membrane with TMZ treatment. Western blot analysis demonstrated a time-dependent upregulation of Rac1 translocation to the membrane with 0.1 μM Ang II treatment ($P < 0.01$ at 15 min) (*Figure 6C*). Pre-treatment with 1 μM TMZ inhibited Rac1 translocation to the membrane when compared with Ang II stimulation alone (1.38 ± 0.11 vs. 3.29 ± 0.61-fold of control, $P < 0.05$) (*Figure 6D*). These results indicated that TMZ had a direct effect on NADPH oxidase activity in cardiac fibroblasts.

4. Discussion

Although TMZ has been generally accepted as a metabolic-related drug, its molecular basis and non-metabolic effects remained to be elucidated. Previous studies have shown that TMZ has both metabolic and non-metabolic effects in patients with heart failure.[8,16] In these patients, metabolic status was improved and EF was preserved. However, little research has focused in the effect of TMZ on fibrosis in compensated cardiac hypertrophy induced by pressure overload. In the present study, we found that myocardial interstitial fibrosis from pressure overload, which appeared before EF descended, was attenuated by treatment with TMZ.

Pressure overload induces renin–angiotensin–aldosterone system (RAAS) activation which is characterized by the elevation of Ang II in circulating system and local myocardium. Elevated Ang II stimulates multiple pro-fibrotic factors secretion in heart, such as TGF-β1, which promotes pressure overload-induced cardiac fibrosis and activates fibroblasts appearing proliferation and increasing collagen synthesis.[31,32] Therefore, we selected Ang II as a stimulator of cardiac fibroblasts and detected the effect of TMZ on cardiac fibroblast proliferation and collagen synthesis *in vitro*. The cell study demonstrated that TMZ could ameliorate Ang II-induced fibroblast proliferation and collagen synthesis. In order to elucidate the effect of TMZ on pressure overload-induced cardiac fibrosis further, we used another important pro-fibrotic factor-TGF-β1 to stimulate cardiac fibroblasts, and also found that TMZ could inhibit TGF-β1-induced cardiac fibroblasts proliferation and collagen synthesis (Supplementary material online, *Figure S4B*, *C* and *D*). Thus the present study demonstrated

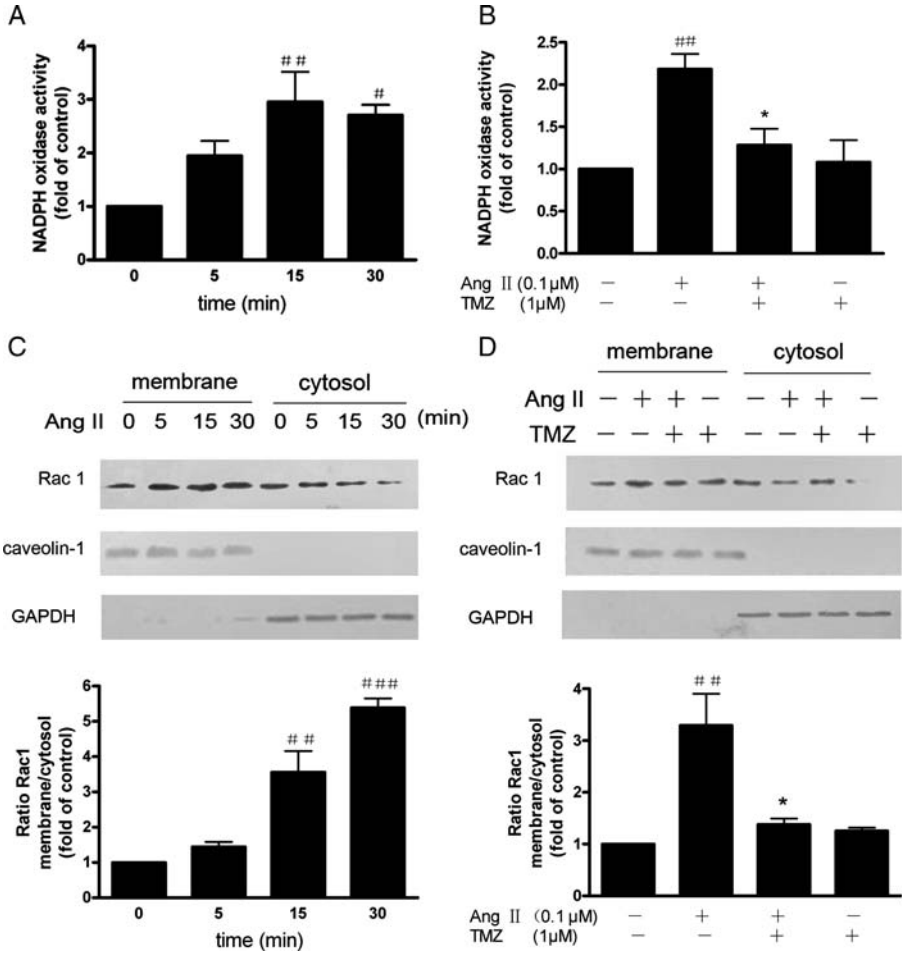

Figure 6 Effect of TMZ on Ang II-increased NADPH oxidase activity and subcellular translocation Rac1 in cardiac fibroblasts. (*A*) Cells were stimulated with Ang II (0.1 μM) for various times. (*B*) Cells were pre-incubated with TMZ (1 μM, 30 min) and then stimulated with Ang II (0.1 μM) for 15 min. Cardiac fibroblasts after treatment were lysed and measured by lucigenin-enhanced chemiluminescence in cellular homogenates after the addition of NADPH as a substrate. Values represent the percentage increase of chemiluminescence over control. (*C*) Subcellular localization of Rac1 in Ang II-stimulated cells for various times. (*D*) Cells were pre-incubated with TMZ (1 μM, 30 min) and then stimulated with Ang II (0.1 μM) for 15 min. Rac1 level was, respectively, standardized to caveolin-1 and GAPDH in membrane and cytosolic fraction. The below panels show the densitometric analysis of the western blots of the ratio of Rac1 translocation to membrane. Data represent mean ± SEM from three independent experiments. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. control; $^{*}P < 0.05$ vs. Ang II alone.

that TMZ could attenuate pressure overload-induced cardiac fibrosis *in vivo* and *in vitro*.

Concerning the molecular basis of TMZ effects on fibrosis, we hypothesized that the ECM synthesis was inhibited by TMZ through targeting the common mediator of Ang II and TGF-β1 induced fibrosis. During the past decade, CTGF was recognized to play an important role in fibrogenic processes.[18,33] Many *in vitro* studies have shown that CTGF stimulates the proliferation and differentiation of fibroblasts and enhances ECM production, and direct *in vivo* evidence suggests that it orchestrates the pro-fibrotic actions of important local factors such as Ang II and TGF-β1 and regulates their activity in cardiac fibrosis.[17,18] In the present study, we first evaluated the effect of TMZ on CTGF level in pressure overload-induced cardiac fibrosis. Upregulated CTGF synthesis induced by TAC was inhibited by TMZ treatment. However, TMZ did not inhibit the expression of TGF-β1 in the TAC model (Supplementary material online, *Figure S3*). Although TGF-β1 is a powerful pro-fibrotic factor promoting myocardial fibrosis, blockade of CTGF synthesis can effectively inhibit fibroblast differentiation and ECM production induced by TGF-β1.[34] Therefore, we speculated that the anti-fibrotic effect of TMZ mainly contributed to inhibiting CTGF synthesis.

To define the exact molecular target of TMZ, we detected the effect of TMZ on the upstream signals of CTGF in fibroblasts. Studies have demonstrated that ROS and ERK1/2 are key intracellular signals for regulating CTGF production.[20,22] In previous studies, TMZ significantly decreased superoxide anion generation and MDA level in patients with chronic heart failure or in rat hearts under ischaemia/reperfusion.[8,35] Although the antioxidant properties of TMZ were recognized in the heart, its target was myocytes with ischaemia or hypoxia. In our study, we first detected whether TMZ could reduce ROS production in fibroblasts. TMZ inhibited ROS formation in pressure overload-induced hypetrophy or in Ang II-induced cardiac fibroblast proliferation.

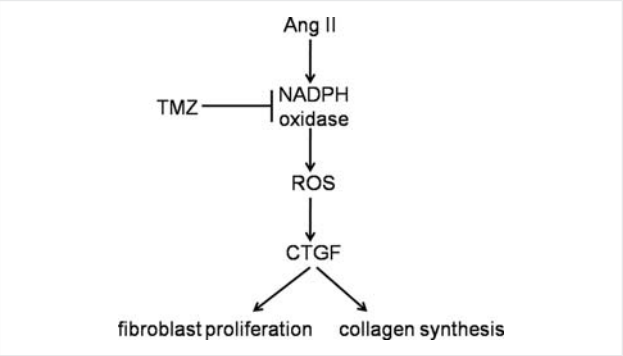

Figure 7 Model for TMZ inhibiting cardiac fibrosis. TMZ suppresses Ang II-induced NADPH oxidase activation which is the main source of ROS generation, decreases its downstream-CTGF synthesis, and attenuates cardiac fibroblast proliferation and collagen synthesis.

ERK1/2 signalling may be involved in fibroblast proliferation and CTGF synthesis.[36] In the present study, we evaluated the effect of TMZ on ERK1/2 activation (Supplementary material online, *Figure S6*). Interestingly, TMZ did not affect the immediate activation of ERK1/2 with Ang II stimulation. This finding suggested that TMZ-mediated inhibition of CTGF synthesis might not be attributed to ERK signalling transduction. The findings are consistent with those of a previous study in which TMZ and its derivatives did not affect ERK1/2 activation in an ischaemia/reperfusion model (*Figure 7*).[35] A previous study also showed that p38 mitogen-activated protein kinase (MAPK) and c-Jun NH2-terminal kinase (JNK) signalling cascades were not involved in the TMZ-mediated cardioprotective effect.[23] Thus, MAPK cascades might not be the molecular basis of the TMZ-mediated cardioprotective role.

Although many studies have shown that TMZ has antioxidant properties, its mechanism is still not thoroughly understood. A few studies have demonstrated that TMZ reduced oxidative stress by increasing antioxidative key enzymes such as superoxide dismutase and glutathione peroxidase levels.[37] TMZ reduced the formation of oxidative damage by preserving cardiac mitochondrial function, whereas pre-incubating rat heart mitochondria with 0.1–10 μM TMZ did not affect NADH oxidase, NADH dehydrogenase, NADH-cytochrome c reductase, succinate oxidase, and cytochrome c oxidase activities.[38] However, our study showed that TMZ played an antioxidant role by downregulating NADPH oxidase activity, which resulted in decreased ROS level in cultured cardiac fibroblasts. The possible reason for the differing results may be that we pre-incubated fibroblasts with Ang II instead of using isolated mitochondria of ischaemic hypertrophied rat myocardia. In addition, we detected the NADPH oxidase activity in the membrane and cytosolic fractions rather than in mitochondria. Because NADPH oxidase plays an important role in ROS production[39,40] and its activity is mainly regulated by the translocation of the cytosolic subunit Rac1 to the membrane,[41] we analysed the TMZ effect on Rac1 translocation. Pre-treatment with 1 μM TMZ inhibited Rac1 translocation to the membrane when compared with Ang II stimulation alone. However, whether TMZ has a direct or secondary effect on Rac1 activity still needs further study.

In summary, the present study suggests that TMZ plays an important role in preventing myocardial fibrosis *in vivo* or *in vitro*. The NADPH oxidase–ROS–CTGF signalling pathway is a central mechanism for the anti-fibrotic effect of TMZ. Because cardiac fibrosis is the main risk factor for heart failure and sudden death, these results suggest a novel pharmacological approach to treat cardiac fibrosis in patients for whom TMZ therapy may not be indicated. However, supporting evidence from large clinical trials is still required.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: none declared.

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