Cardiovascular Research (2010) **87**, 504–513
doi:10.1093/cvr/cvq066

Metformin attenuates cardiac fibrosis by inhibiting the TGFβ_1–Smad3 signalling pathway

Han Xiao[1,4], Xiaowei Ma[1,4], Wei Feng[1,4], Yongnan Fu[1,4], Zhizhen Lu[1,4], Ming Xu[1,4], Qiang Shen[3,4], Yi Zhu[2,4], and Youyi Zhang[1,4]*

[1]Institute of Vascular Medicine, Peking University Third Hospital, Beijing 100191, China; [2]Department of Physiology and Pathophysiology, Peking University, Health Science Center, Beijing 100191, China; [3]Institute of Cardiovascular Sciences, Peking University, Beijing 100191, China; and [4]Key Laboratory of Molecular Cardiovascular Sciences, Ministry of Education, Beijing 100191, China

Received 4 November 2009; revised 21 February 2010; accepted 23 February 2010; online publish-ahead-of-print 3 March 2010

Time for primary review: 19 days

Aims The mechanism of the cardioprotective action of metformin is incompletely understood. We determined the role of metformin in cardiac fibrosis and investigated the mechanism.

Methods and results Ten-week-old male mice (C57BL/6) were subjected to left ventricular pressure overload by transverse aortic constriction. Mice received metformin (200 mg/kg/day) or normal saline for 6 weeks. Metformin inhibited cardiac fibrosis (fibrosis area/total heart area: 0.6 ± 0.3 vs. $3.6 \pm 0.9\%$, $P < 0.01$) induced by pressure overload and improved cardiac diastolic function (left ventricular end-diastolic pressure: 5.2 ± 0.9 vs. 11.0 ± 1.6 mmHg, $P < 0.05$). Metformin inhibited the pressure overload-induced transforming growth factor (TGF)-β_1 production in mouse hearts and the TGF-β_1-induced collagen synthesis in cultured adult mouse cardiac fibroblasts (CFs). Metformin suppressed the phosphorylation of Smad3 in response to TGF-β_1 in CFs. Metformin also inhibited the nuclear translocation and transcriptional activity of Smad3 in CFs.

Conclusion Metformin inhibited cardiac fibrosis induced by pressure overload *in vivo* and inhibited collagen synthesis in CFs probably via inhibition of the TGF-β_1–Smad3 signalling pathway. These findings provide a new mechanism for the cardioprotective effects of metformin.

Keywords Metformin • Cardiac fibrosis • TGF-β_1 • Cardiac fibroblast • Collagen

1. Introduction

Metformin has been widely used for the treatment of type-2 diabetes without causing overt hypoglycaemia. The United Kingdom Prospective Diabetes Study (UKPDS) demonstrated that metformin had cardiovascular protective actions beyond its antihyperglycaemic properties.[1,2] The possible mechanisms include reducing insulin resistance, improving lipid metabolism, and decreasing hypercoagulation, as well as favourable actions on vascular smooth muscle, endothelial function, and intracellular calcium handling within cardiomyocytes.[3] Recent studies demonstrated that metformin has a cardioprotective effect against ischaemia–reperfusion injury through the activation of adenosine monophosphate-activated protein kinase (AMPK).[4,5] Metformin decreased cardiomyocyte apoptosis and improved cardiac function in failing canine hearts along with the activation of AMPK.[6] These studies suggested that metformin may have a therapeutic potential for heart failure. Nevertheless, the exact mechanism of the cardioprotective action of metformin remains unclear.

Metformin has cardioprotective effects beyond its antihyperglycaemic properties,[1] so it may have cardioprotective properties in non-diabetic patients. Biguanides and the Prevention of the Risk of Obesity (BIGPRO) trials supports the conclusion that metformin favourably affects several cardiovascular risk factors (fasting plasma insulin, total cholesterol, and Apo B) in non-diabetic subjects.[7] Besides these effects on the cardiovascular risk factors, whether metformin has a direct effect on the heart has not been studied.

Myocardial fibrosis is an important pathophysiological process that contributes to the conversion from hypertrophy to heart failure by increasing myocardial stiffness and reducing pumping capacity. Preventing myocardial fibrosis improves cardiac function. Metformin attenuated the increment of diastolic chamber stiffness and diastolic dysfunction in diabetic myocardium.[8] Metformin can improve

* Corresponding author. Tel: +86 10 82802306; fax: +86 10 62361450, Email: zhangyy@bjmu.edu.cn

cardiac function in the failing heart.[5,6] Thus, we hypothesized that metformin may have a cardioprotective effect against myocardial fibrosis and improve diastolic dysfunction.

Transforming growth factor (TGF)-β_1 plays a causal role in myocardial fibrosis induced by pressure overload, and blocking of TGF-β_1 activity ameliorates diastolic dysfunction by preventing myocardial fibrosis.[9] TGF-β_1 signal was transduced through its downstream effectors, the Smad proteins. Activation of the TGF-β_1–Smad2/3 cascade plays an essential part in the gene expression of extracellular matrix proteins. Smad3 signalling critically regulates fibrotic remodelling of the infarcted ventricle.[10] Whether or not metformin affects TGF-β_1–Smad signalling pathway is unknown.

In the present study, we planned to answer these questions: (i) does metformin inhibit cardiac fibrosis induced by pressure overload? (ii) What is the mechanism of the action of metformin on cardiac fibrosis? (iii) What is the effect of metformin on TGF-β_1–Smad signalling?

2. Methods

2.1 Materials

Metformin was purchased from Sigma-Aldrich (St Louis, MO, USA). Aminoimidazole carboxamide ribonucleotide (AICAR) was from Toronto Research Chemicals (Toronto, ON, Canada). Compound C was purchased from Calbiochem (EMD Biosciences, Incorporated, San Diego, CA, USA). Recombinant human TGF-β_1 was from Peprotech (London, UK).

2.2 Animals, surgery, and drug treatment

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). Male C57BL/6 mice (age, 10 weeks) were provided by the Animal Department of Peking University Health Science Center (Beijing, China). All experimental protocols were approved by the Peking University Institutional Committee for Animal Care and Use. Thoracic aortic constriction (TAC) was carried out as described previously.[11] Briefly, mice were anaesthetized with tribromoethanol (200 mg/kg, ip; Sigma-Aldrich) and ventilated (pressure-controlled ventilator; Kent Scientific, CT, USA). A longitudinal cut was made in the proximal portion of the sternum. A 7-0 silk suture was placed around the aorta between the right innominate artery and left common carotid artery. The suture was tied around a 26-gauge needle and the aorta. After ligation, the needle was promptly removed. The sham procedure was identical except that the aorta was not ligated. Three days after aortic constriction, mice were injected with metformin (200 mg/kg/day, sc) or normal saline (control) every day for 6 weeks. No mouse died before data collection in each group.

2.3 Echocardiography and evaluation of left ventricular haemodynamics

Mice were anaesthetized with 1.5% isoflurane (Baxter Healthcare Corporation, New Providence, NJ, USA). Echocardiographic images were obtained with a Visualsonics high-resolution Vevo 770 system (VisualSonics, Incorporated, Toronto, ON, Canada). For measurement of aortic and left ventricular (LV) pressure, a 1.4-F micromanometer conductance catheter (SPR-835; Millar Instruments, Incorporated, Houston, TX, USA) was introduced through the right common carotid artery into the ascending aorta and then advanced into the left ventricle as described previously.[12]

2.4 Histological analyses

Hearts were excised and retrograde-perfused with phosphate-buffered saline (PBS). They were fixed with 4% paraformaldehyde overnight and embedded in paraffin. Serial LV cross-sections (6 μm thickness) were stained with picric acid Sirus red. Tissue morphometry was evaluated in a blinded fashion using the Leica Q550 IW imaging workstation (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK). Interstitial fibrosis was revealed with picric acid Sirius red staining. The positively stained (red) fibrotic area was expressed as a percentage of total area.

2.5 Enzyme-linked immunosorbent assay (ELISA)

Collagen I and TGF-β_1 protein levels in the ventricles were measured using an ELISA kit (collagen I: USCN Life Science Incorporated, Wuhan, China; TGF-β_1: R&D Systems Incorporated, Minneapolis, MN, USA). Insulin levels in serum were measured using an ELISA kit from Linco Research, Incorporated (Billerica, MA, USA). All values were in the linear range and were normalized for protein concentrations.

2.6 Preparation of cardiac fibroblasts

Adult cardiac fibroblasts (CFs) were isolated from 8- to 10-week-old male C57BL/6 mice as described.[13] Minced ventricles were digested with 0.01% collagenase II (Worthington, Columbia, NJ, USA). Cells were collected and plated for 2 h at 37°C. Unattached cardiomyocytes were removed. CFs were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS; Hyclone Laboratories, Incorporated, Omaha, NE, USA) at 37°C. Cells in the second passage were used in this experiment.

2.7 Measurement of collagen synthesis

Collagen synthesis was determined by measuring incorporation of ^{3}H-proline as described.[14] In brief, CFs in 24 wells were cultured in serum-free DMEM for 24 h. Cells were then exposed to metformin (1 mmol/L) in the presence or absence of TGFβ$_1$ (5 ng/mL). L-[2,3-^{3}H] proline (Perkin Elmer, Incorporated Waltham, MA, USA) (0.5 μCi/well) were then supplied for 48 h. Cell extracts were analysed in a liquid scintillation counter. The count represented the amount of newly synthesized type-I collagen.

2.8 Proliferation assay

Proliferation was measured by bromodeoxyuridine (BrdU) incorporation. As previously described,[15] Cells were plated on 96-well flat clear bottom microplates (Corning Incorporated, Corning, NY, USA). After 24 h of serum starvation, cells were incubated for 18 h with angiotensin II (1 μmol/L), TGFβ$_1$ (5 ng/mL), and 10% FBS combined with or without metformin (1 mmol/L). BrdU labelling solutions (10 μmol/L) were added and cells reincubated for an additional 6 h. Incorporated BrdU was detected by an anti-BrdU ELISA kit (Roche Diagnostics, Mannheim, Germany).

2.9 Western blot analysis

Cell lysates and heart extracts were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted on nitrocellulose membranes. Membranes were then incubated with antibodies directed against phosphorylated Smad3, Smad2, AMPK, and acetyl CoA carboxylase (ACC) and then against the respective total level of each protein (antibodies from Cell Signaling Technology Incorporated, Danvers, MA, USA). Protein bands were visualized with Supersignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, Rockford, IL, USA). Blots were subsequently reprobed with the following antibodies to confirm equal loading: eIF5, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-tubulin, or lamin A (antibodies from Santa Cruz Biotechnology, Incorporated, Santa Cruz, CA, USA).

2.10 Immunofluorescent detection of Smad3

Quiescent mouse CFs were pretreated with metformin (1 mmol/L) or vehicle for 2 h and then exposed to TGF-β_1 (5 ng/mL) for an additional 1 h. CFs were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100 in PBS. Fixed CFs were stained with selective anti-Smad3 monoclonal antibody overnight at 4°C and then with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG secondary antibody (Jackson Immunoresearch Laboratories, Incorporated, West Grove, PA, USA) for 1 h at 37°C to assess the nuclear translocation of Smad3 using confocal fluorescence microscopy.

2.11 Isolation of nuclear and cytoplasmic extract

Nuclear and cytoplasmic protein fractions were separated by a commercially available separation kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Protein concentrations were determined using a bicinchoninic acid protein assay (Thermo Fisher Scientific).

2.12 Measurement of Smad3-directed transcriptional activity

The Smad3-responsive reporter pGL3-$(CAGA)_{12}$-luc was transfected into CFs by using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Cells were cotransfected with pGL3-$(CAGA)_{12}$-Luc and pRL-TK (Promega, Madison, WI, USA), a control reporter vector which express synthetic renilla luciferase to normalize transfection efficiency. After treatment with TGF-β_1 and metformin for 24 h, cytosolic luciferase activity was detected with Dural-luciferase reporter assay reagents (Promega) using a GloMax 96 Microplate Luminometer (Promega). Experimental data were normalized to Renilla luciferase activity/well to control for differences in transfection efficiency, and then expressed as the multiple of difference (*x*-fold) relative to basal conditions.

2.13 Adenoviral infection

A recombinant adenovirus expressing a dominant negative mutant of AMPK (Ad-AMPK-DN) was generated by subcloning the cDNA encoding AMPKα1-DN-(D159A) into an adenoviral vector pJM17.[16] CFs at a confluence of 60–70% were infected with recombinant adenoviruses at a multiplicity of infection of 5, and incubated for another 24 h before experimentation.

2.14 Statistical analysis

Data are means ± SEM. Differences in data between groups were compared using Prism 4 (GraphPad Software Incorporae, La Jolla, CA, USA) with Student's paired two-tailed *t*-test or two-way ANOVA (if appropriate). For the ANOVA, if a significant variance was found, the Tukey or Bonferroni test was used as the *post hoc* analysis. $P < 0.05$ was considered significant.

3. Results

3.1 Metformin reverses cardiac fibrosis induced by TAC and improves cardiac diastolic function

To determine if metformin plays a part in cardiac fibrosis induced by TAC, we treated TAC-operated mice with metformin (200 mg/kg/day) or with normal saline for 6 weeks after surgery. Anatomic and functional data for sham- and TAC-operated mice are shown in *Table 1*. There was a significant increase in heart weight and the ratio of heart weight to tibia length (HW/tibia length) in the TAC-operated mice compared with the sham-operated controls ($P < 0.001$). Echocardiography data showed that LV anterior and posterior wall dimension in diastole (LVAWd and LVPWd) increased greatly in TAC-operated mice ($P < 0.001$ vs. sham-operated group). However, LV internal diameter, fractional shortening (FS), and mid-wall FS (mFS) were similar in each group. Haemodynamic data showed that left ventricular end-diastolic pressure (LVEDP) and the relaxation time constant (Tau) increased after 6 weeks of TAC ($P < 0.05$ vs. sham-operated group) with no change in the rise of the first derivative of pressure ($+dp/dt$). These results indicated that TAC for 6 weeks induced concentric hypertrophy with isolated diastolic dysfunction but without systolic dysfunction.

Metformin treatment had no effect on any echocardiographic or haemodynamic parameters in sham-operated mice. When compared with TAC mice, mice who were treated with TAC + metformin had a lower HW/tibia length ($P < 0.05$), smaller LV wall dimension (LVAWd and LVPWd, $P < 0.05$) and a reduction in LVEDP and Tau value ($P < 0.05$; *Table 1*). Echocardiography data showed that metformin significantly inhibited cardiac hypertrophy after 5 weeks of TAC ($P < 0.05$). There is no statistical significant difference in FS between saline- and metformin-treated mice (*Table 1*, see Supplementary material online, *Figure S1*). Sustained treatment with metformin reduced cardiac fibrosis relative to saline-treated hearts (*Figure 1A* and *B*). Levels of collagen I in the hearts of TAC-operated mice were significantly reduced in the metformin-treated group when compared with the saline-treated group ($P < 0.05$; *Figure 1C*). Metfromin treatment also inhibited fibronectin composition or had tendency to inhibit Collagen III composition induced by TAC (see Supplementary material online, *Figure S2A and B*). Taken together, these results indicated that metformin attenuated the development of TAC-induced fibrosis and diastolic dysfunction.

Metformin is a hypoglycaemic drug, so we measured the postoperative level of fasting blood glucose every week and the insulin level for 6 weeks in each group. There was no significant difference in the fasting blood glucose and insulin level in saline- and metformin-treated mice (*Table 2*, see Supplementary material online, *Figure S3*). These results suggested that metformin does not affect the level of blood glucose and insulin in non-diabetic mice.

3.2 Metformin inhibits the TGF-β_1 production and the phosphorylation of Smad3 induced by TAC

To investigate the mechanism for the attenuation of TAC-induced cardiac fibrosis by treatment with metformin, we measured the expression of TGF-β_1, a major profibrotic cytokines, in hearts by ELISA. The expression of TGF-β_1 protein was increased in the left ventricles of TAC mice compared with that in sham-operated controls (77.9 ± 6.6 vs. 48.0 ± 9.0 pg/mg tissue, $P < 0.05$). Metformin treatment inhibited TAC-induced TGF-β_1 production (54.3 ± 2.6 vs. 77.9 ± 6.6 pg/mg tissue, $P < 0.05$, *Figure 2A*). Western blotting demonstrated that TAC-operated mouse hearts had increased phosphorylated-Smad3 compared with those of sham hearts. Metformin treatment reduced the level of phosphorylated-Smad3 (*Figure 2B*).

3.3 Metformin inhibits TGF-β_1-induced collagen synthesis of mouse CFs *in vitro*

Another potential cause for the attenuated fibrotic response of metformin may be attenuated collagen synthesis or proliferation of CFs. Because TGF-β_1 appears to be a crucial mediator in the composition of the extracellular matrix during cardiac remodelling induced by

Table 1 Anatomic and functional data for sham and TAC-operated mice

	Sham 6 weeks		TAC 6 weeks	
	Saline	Metformin	Saline	Metformin
Anatomic data				
n	9	8	15	8
Body weight (g)	24.0 ± 2.0	24.8 ± 1.2	26.5 ± 1.3	24.9 ± 1.3
Heart weight (mg)	96.7 ± 4.1	94.9 ± 4.6	135.5 ± 3.8***	115.1 ± 6.6#
Heart weight/tibia length (mg/mm)	5.45 ± 0.25	5.32 ± 0.34	7.57 ± 0.21***	6.51 ± 0.39#
Echocardiographic data				
n	13	8	16	11
HR (bpm)	413 ± 9	412 ± 19	428 ± 8	434 ± 12
LVIDd (mm)	3.90 ± 0.08	3.83 ± 0.11	4.03 ± 0.07	3.81 ± 0.09
LVIDs (mm)	2.85 ± 0.14	2.74 ± 0.24	3.04 ± 0.11	2.70 ± 0.13
LVAWd (mm)	0.73 ± 0.02	0.76 ± 0.02	0.98 ± 0.03***	0.88 ± 0.02#
LVPWd (mm)	0.64 ± 0.01	0.66 ± 0.01	0.89 ± 0.01***	0.82 ± 0.02##
FS%	30.3 ± 2.7	31.8 ± 4.7	27.9 ± 1.6	30.5 ± 1.9
mFS%	21.5 ± 1.8	22.6 ± 3.7	18.7 ± 1.4	22.3 ± 1.6
Haemodynamic data				
n	9	8	15	8
HR (bpm)	480 ± 18	443 ± 14	478 ± 15	478 ± 16
Systolic BP(mmHg)	89.4 ± 6.5	93.6 ± 2.5	133.8 ± 5.1***	126.3 ± 7.9
Diastolic BP(mmHg)	59.5 ± 3.5	65.9 ± 3.7	78.0 ± 3.4*	78.5 ± 6.3
LVSP (mmHg)	84.7 ± 4.0	88.4 ± 3.1	135.7 ± 5.2***	130.2 ± 10.0
LVEDP (mmHg)	2.5 ± 1.0	3.9 ± 0.9	11.0 ± 1.6***	5.2 ± 0.9#
+d*p*/d*t* (mmHg/s)	7191 ± 843	6824 ± 413	6774 ± 382	7464 ± 387
(+d*p*/d*t*)/instant pressure	113.2 ± 13.3	130.6 ± 4.4	107.6 ± 3.6	141.0 ± 7.6#
−d*p*/d*t* (mmHg/s)	−6720 ± 745	−6238 ± 389	−7497 ± 319	−8874 ± 654
(−d*p*/d*t*)/instant pressure	−157.3 ± 9.3	−157.1 ± 19.6	−153.0 ± 6.5	−171.0 ± 8.3
Tau (ms)	13.2 ± 1.1	14.3 ± 0.7	23.5 ± 3.8*	11.5 ± 0.9##

Values are means ± SEM; TAC, thoracic aortic constriction; *n*, number of mice; HR, heart rate; LVIDd, left ventricular (LV) internal diameter in diastole; LVIDs, LV internal diameter in systole; LVAWd, LV anterior wall diameter in diastole; LVPWd, LV posterior wall diameter in diastole; FS, fractional shortening; mFS, mid-wall fractional shortening was calculated as {[LVIDd + (LVPWd + LVAWd)/4] − [LVIDs + (LVPWs + LVAWs)/4]}/[LVIDd + (LVPWd + LVAWd)/4]; BP, blood pressure recorded in the carotid; LVSP, LV peak systolic pressure; LVEDP, LV end-diastolic pressure; +d*p*/d*t* and −d*p*/d*t*, the rise and decline of the first derivative of pressure, respectively. (+d*p*/d*t*)/instant pressure and (−d*p*/d*t*)/instant pressure, LV maximum rate of rise of ventricular pressure divided by pressure at the moment such maximum occurs. Tau, relaxation time constant calculated by Glantz method (regression of d*p*/d*t* vs. pressure).
*$P < 0.05$, ***$P < 0.001$ vs. sham-operated group; #$P < 0.05$, ##$P < 0.01$ vs. TAC-operated group. Two-way ANOVA analysis.

pressure overload. Therefore, we investigated if metformin inhibits collagen synthesis induced by TGF-β_1 in CFs. We used [^{3}H]-proline uptake to represent collagen synthesis in CFs. TGF-β_1 (5 ng/mL) stimulation increased [^{3}H]-proline incorporation by 1.55 ± 0.08 times compared with control. When metformin was pretreated, dose-dependent reduction of [^{3}H]-proline incorporation was observed (*Figure 3A*). BrdU incorporation was used to determine whether metformin has inhibitory effects on the proliferation of CFs. Metformin had no effect on angiotensin II-, TGF-β_1-, and serum-stimulated proliferation in CFs (*Figure 3B*). To investigate if metformin promotes collagen degradation, we measured the expression of matrix metalloproteinase (MMP)2 and MMP9 in the left ventricles. We also measured their inhibitors, tissue inhibitor of metalloproteinase (TIMP)1 and TIMP2. Western blotting demonstrated that TAC-operated mouse hearts had increased levels of MMP2 (see Supplementary material online, *Figure S4A*) and TIMP2 (see Supplementary material online, *Figure S4B*) compared with those of sham hearts. Metformin treatment reduced the level of MMP2 without a change in TIMP2 level (see Supplementary material online, *Figure S4A and B*). The level of MMP9 and TIMP1 was similar in each group (see Supplementary material online, *Figure S4C and D*).

3.4 Metformin inhibits the TGF-β_1–Smad3 signalling pathway in cultured adult mouse CFs

To determine the mechanism underlying inhibition of TGF-β_1-induced collagen synthesis by metformin, we studied signal downstreams of TGF-β_1 activation. In fibroblasts, the intracellular mediators of TGF-β_1 signalling are the Smad2 and Smad3.[17] Western blot analysis demonstrated that TGF-β_1 (5 ng/mL for 1 h) treatment significantly increased phosphorylated Smad3 levels in CFs. Pretreatment with metformin (1 mmol/L for 2 h) inhibited this process (*Figure 4A*). Neither TGF-β_1 nor metformin altered total Smad3 levels in CFs. Metformin did not inhibit TGF-β_1-induced Smad2 phosphorylation (*Figure 4B*).

Smad nuclear translocation is one of the key components modifying the TGF-β–Smads pathway. Western blot analysis showed that metformin inhibited the induction of Smad3 levels induced by TGF-β_1 in the

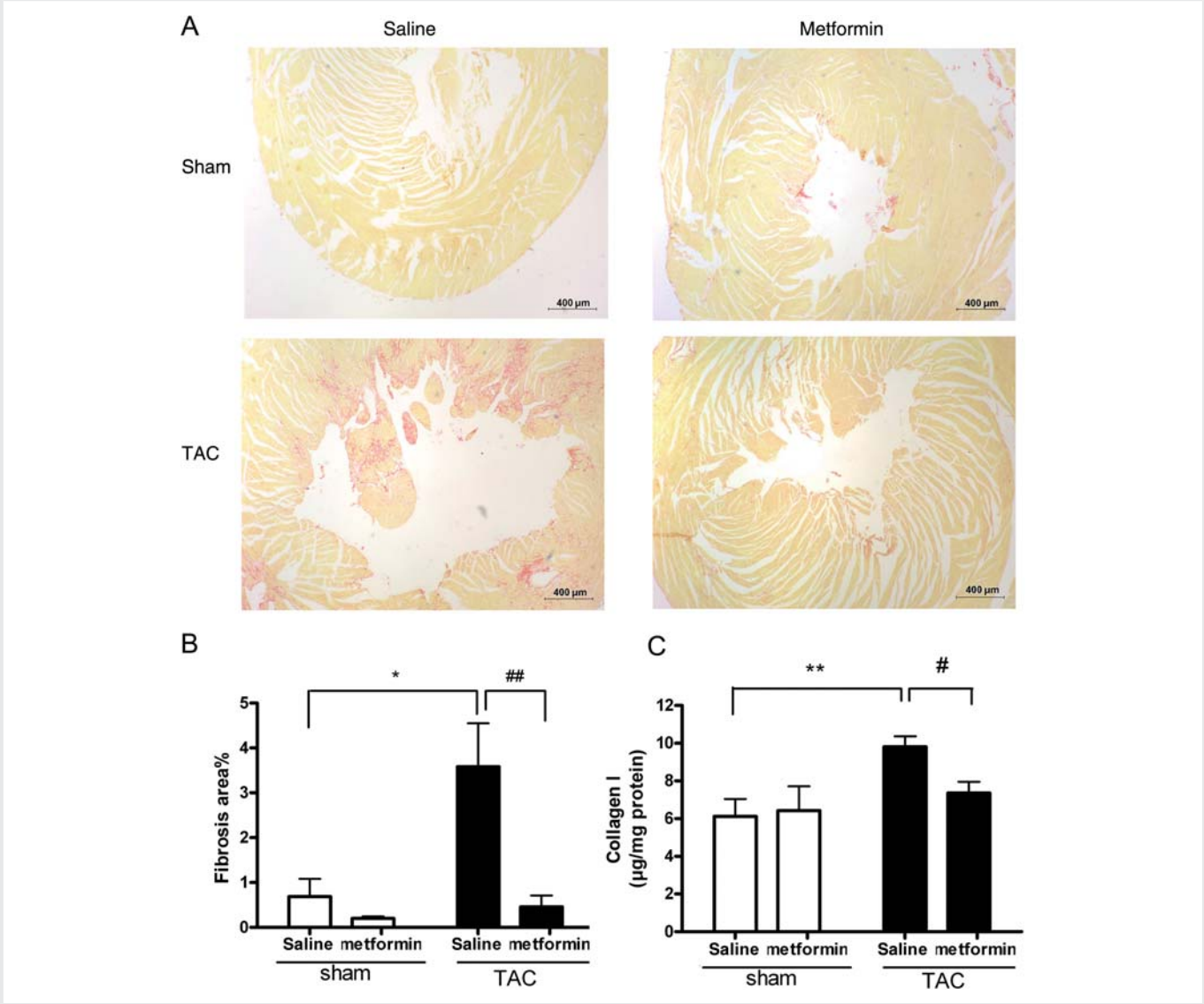

Figure 1 Metformin inhibits cardiac fibrosis induced by transverse aortic constriction (TAC). (*A*) Images show Sirius red-stained collagen in cardiac interstitium (×40), bar = 400 μm, red parts represent collagen. (*B*) Percent LV interstitial collagen content quantified from Sirius red-stained sections with results expressed as percent collagen content ($n = 5$). (*C*) Collagen I deposition in the hearts measured by ELISA (sham + saline $n = 9$; sham + metformin $n = 8$; TAC + saline $n = 13$; TAC + metformin $n = 8$); data represent the mean ± SEM. $^{*}P < 0.05$ $^{**}P < 0.01$, $^{***}P < 0.001$ TAC + saline vs. sham + saline; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ TAC + metformin vs. TAC + saline.

nucleus, and reversed the reduction of Smad3 levels induced by TGF-β_1 in the cytoplasm (*Figure 4C*). Immunofluorescent analysis was also used to assess if metformin blocks Smad3 nuclear translocation. In vehicle-treated cells, immunostaining of Smad3 (*Figure 4D*) was distributed evenly in the cytoplasm and nucleus. This suggested that significant nuclear translocation of Smad3 did not occur. Treatment with TGF-β_1 (5 ng/mL for 1 h) significantly stimulated nuclear translocation of Smad3 as indicated by strong Smad3 staining in the nucleus (*Figure 4D*). The nucleus/cytoplasm ratios of Smad3 density increased from 1.9 ± 0.6 to 6.2 ± 0.6 ($P < 0.01$, *Figure 4E*). Pretreatment with metformin (1 mmol/L for 2 h) inhibited TGF-β_1-induced Smad3 translocation (*Figure 4D*). When compared with TGF-β_1-treated CFs, the nucleus/cytoplasm ratios of Smad3 density in metformin + TGFβ_1-treated CFs decreased significantly (3.6 ± 0.2 vs. 6.2 ± 0.6, $P < 0.05$; *Figure 4E*).

To investigate the effect of metformin on Smad3-mediated transcription, we employed the Smad3-responsive reporter pGL3-$(CAGA)_{12}$-luc. TGF-β_1 increased luciferase reporter activity by 18.7-fold compared with control (*Figure 4F*). Metformin blocked the increase in luciferase activity in CFs exposed to TGF-β1 (7.1-fold vs. 18.7-fold, $P < 0.05$; *Figure 4F*).

3.5 Inhibitory effect of metformin on collagen synthesis is independent of AMPK

It is well established that metformin is an AMPK activator. Is the inhibitory effect of metformin on collagen synthesis dependent on AMPK activation? Western blot analysis showed that metformin treatment increased the phosphorylation of AMPK and ACC which served as indicators of AMPK activity in mice hearts (*Figure 5A*). Another AMPK activator, AICAR, did not inhibit collagen synthesis induced by TGF-β_1 in cultured mouse CFs (*Figure 5B*). The AMPK inhibitor Compound C did not reverse the inhibitory effect of metformin on collagen synthesis

Table 2 Fasting blood glucose and serum insulin level in sham- and TAC-operated mice

	Sham 6 weeks		TAC 6 weeks	
	Saline	Metformin	Saline	Metformin
n	9	8	15	8
Fasting blood glucose (mmol/L)	8.4 ± 1.1	7.8 ± 1.4	8.8 ± 1.0	8.3 ± 0.7
Serum insulin (ng/mL)	0.62 ± 0.18	0.57 ± 0.20	0.66 ± 0.16	0.45 ± 0.18

Values are means ± SEM; *n*, number of mice. Two-way ANOVA analysis.

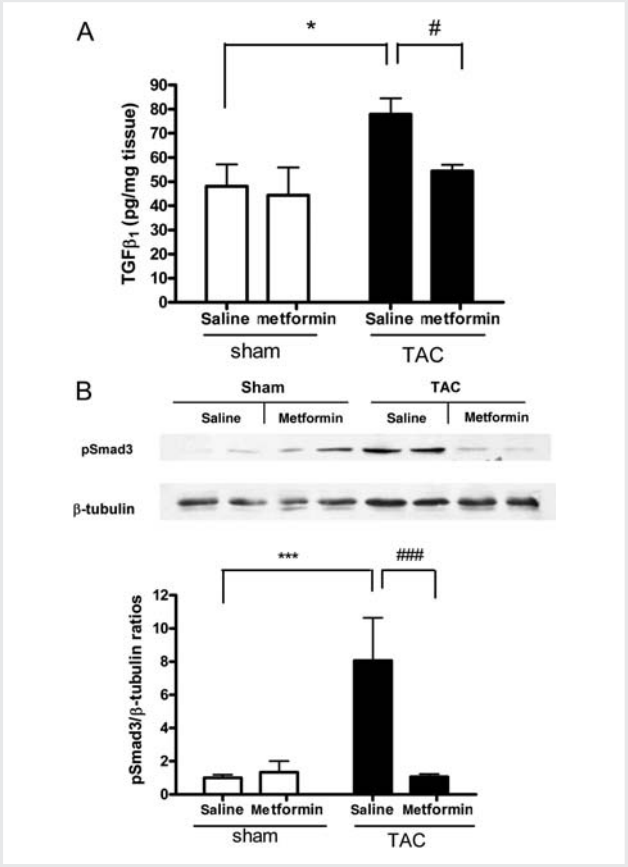

Figure 2 Metformin inhibits the TGFβ$_1$ production and the phosphorylation of Smad3 induced by transverse aortic constriction (TAC). (*A*) TGFβ$_1$ protein levels in the hearts of mice (sham + saline $n = 8$; sham + metformin $n = 6$; TAC + saline $n = 11$; TAC + metformin $n = 8$). (*B*) Representative western blots and averaged bar graphs of pSmad3 (phosphorylated Smad3) demonstrate that metformin inhibits the phosphorylation of Smad3 induced by TAC in the hearts ($n = 6$). β-Tubulin protein levels were measured to show protein loading. Data represent the mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$ TAC + saline vs. sham + saline; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ TAC + metformin vs. TAC + saline.

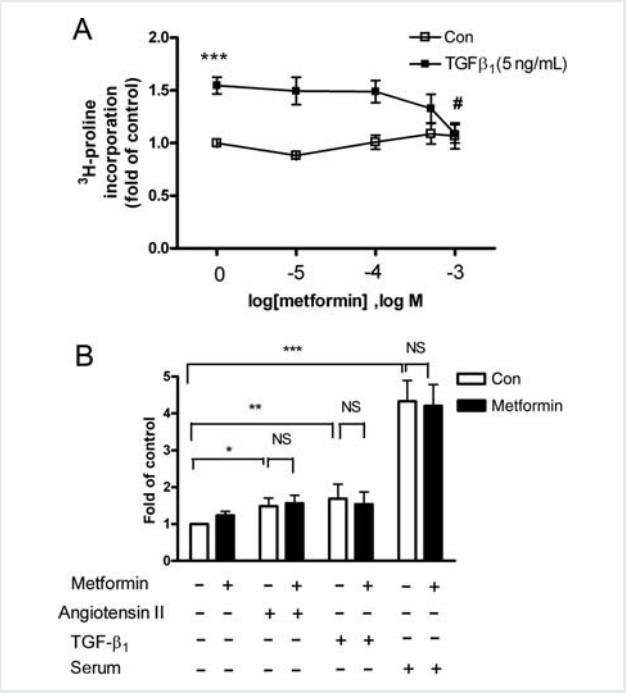

Figure 3 Metformin dose-dependently inhibits TGFβ$_1$-induced collagen synthesis in cultured adult mouse cardiac fibroblast (CFs) but has no inhibitory effect on CFs proliferation. (*A*) Collagen synthesis of CFs treated with or without metformin was examined by [^{3}H] proline incorporation in response to 5 ng/mL TGF-β$_1$. Data represent the mean ± SEM. $^{***}P < 0.001$ vs. con; $^{\#}P < 0.05$ vs. TGFβ$_1$, $n = 7$. (*B*) CFs were exposed to either angiotensin II (1 μmol/L), TGFβ$_1$ (5 ng/mL), 10% FBS or vehicle combined with or without metformin (1 mmol/L) for 24 h. BrdU incorporation of vehicle-treated cells was arbitrarily set at 1. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle-treated cells. NS means no significant. $n = 4$

(*Figure 5C*). Collagen synthesis induced by TGF-β$_1$ was inhibited by metformin with Ad-null transfection (*Figure 5D*) but was also inhibited by metformin with dominant negative AMPKα1 mutant expression (*Figure 5E*). Western blot analysis showed that both metformin (1 mmol/L for 2 h) and AICAR (100 μmol/L for 30 min) activated AMPK in cultured CFs (see Supplementary material online, *Figure S4A*). Compound C (pretreated with 1 μmol/L for 30 min) inhibited AMPK phosphorylation induced by metformin (1 mmol/L for 2 h) (see Supplementary material online, *Figure S4B*). Ad-AMPK-DN also inhibited ACC phosphorylation (which serves as an indicator of AMPK activity) (see Supplementary material online, *Figure S4C*). These results suggested that the inhibitory effect of metformin on collagen synthesis is not dependent on AMPK.

4. Discussion

The key findings in the present study were: (i) metformin protects against cardiac fibrosis induced by pressure overload; (ii) the mechanism of the antifibrotic action of metformin may well be attributed

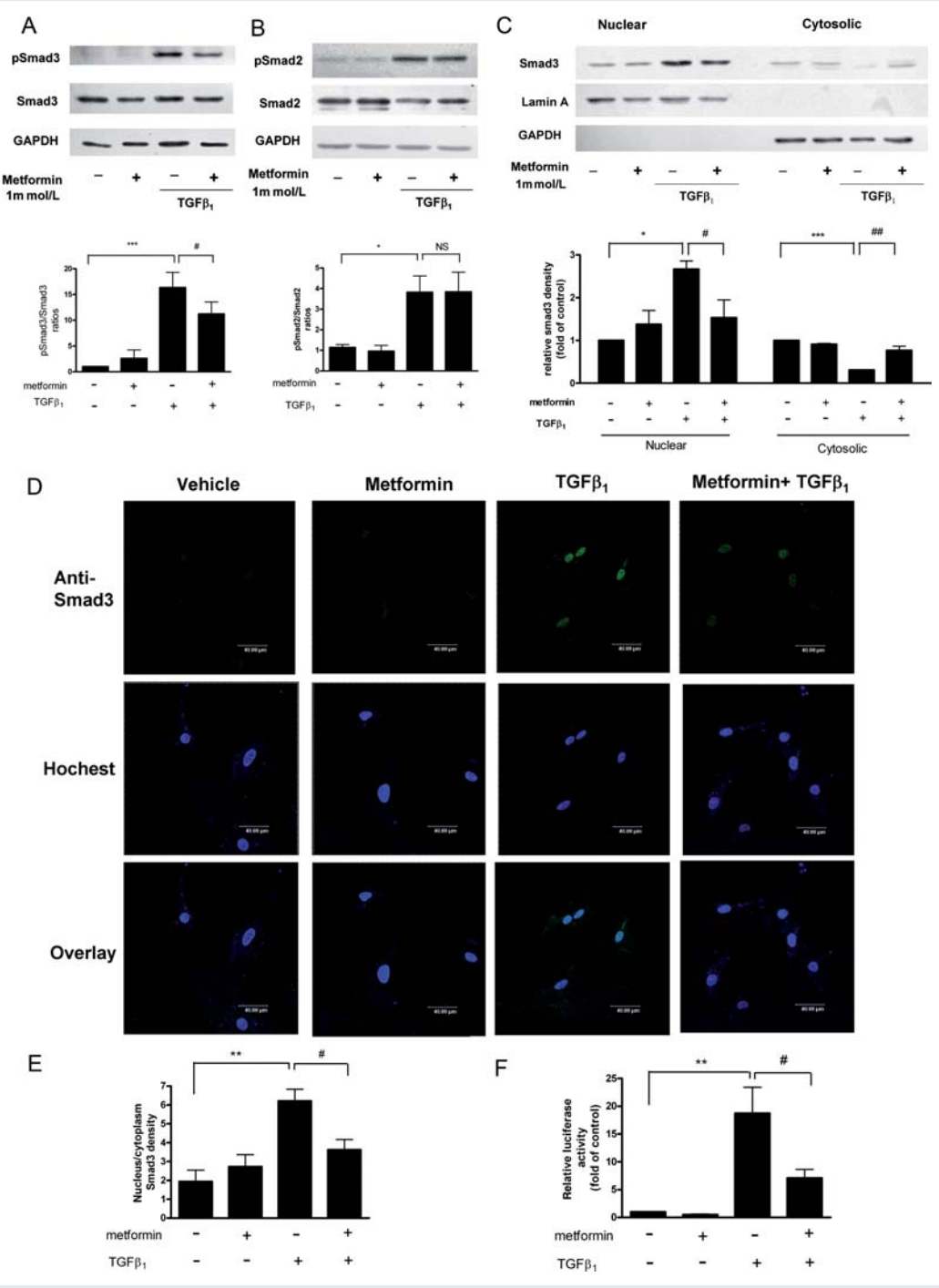

Figure 4 Metformin inhibits TGFβ$_1$-Smad3 signalling pathway in cultured adult mouse cardiac fibroblast (CFs). (*A*) Representative western blots and averaged bar graphs of pSmad3 (phosphorylated Smad3) show that metformin blocks TGFβ$_1$-induced phosphorylation of Smad3 in cultured adult mouse CFs. (*B*) Representative western blots and averaged bar graphs of pSmad2 (phosphorylated Smad2) show that metformin does not block TGFβ$_1$-induced phosphorylation of Smad2 in cultured adult mouse CFs. (*C*) Western blotting results show that metformin blocks the increase of Smad3 in the nuclear induced by TGFβ$_1$. Quiescent CFs were stimulated for 2 h with 1 mmol/L metformin, 5 ng/mL TGFβ$_1$, or metformin plus TGFβ$_1$. Subcellular cytosolic and nuclear fractions were analysed by western blotting for the indicated proteins. The blots were reprobed with GAPDH or lamin-A to confirm equal protein loading and to assess the relative purity of the cytosolic and nuclear fractions. Results are representative of three independent experiments; (*D*) Immunofluroescence images show that metformin blocks nuclear translocation of Smad3 induced by TGFβ$_1$. Green fluorescence signals represent Smad3 protein. Blue signals represent nuclear, bar = 40 μm; (*E*) Intensity ratios of Smad3 staining measured in nucleus and cytoplasm of CFs treated with vehicle, metformin (1 mmol/L), and/or TGFβ$_1$ (5 ng/mL) as in (*D*). Results are means ± SEM [$n = 3$; a total of 20 fields (10 in nucleus and 10 in cytoplasm) in each sample were scanned and averaged]. (*F*) Metformin inhibits Smad3 transcriptional activity induced by TGFβ$_1$. CFs were cotransfected with Smad3-responsive reporter pGL3-$(CAGA)_{12}$-luc and renilla luciferase vector for 24 h, and then treated with TGFβ$_1$ (5 ng/mL) and metformin (1 mmol/L), Data represent the mean ± SEM. $^{**}P < 0.01$ vs. control, $^{\#}P < 0.05$ vs. TGFβ$_1$, $n = 3$.

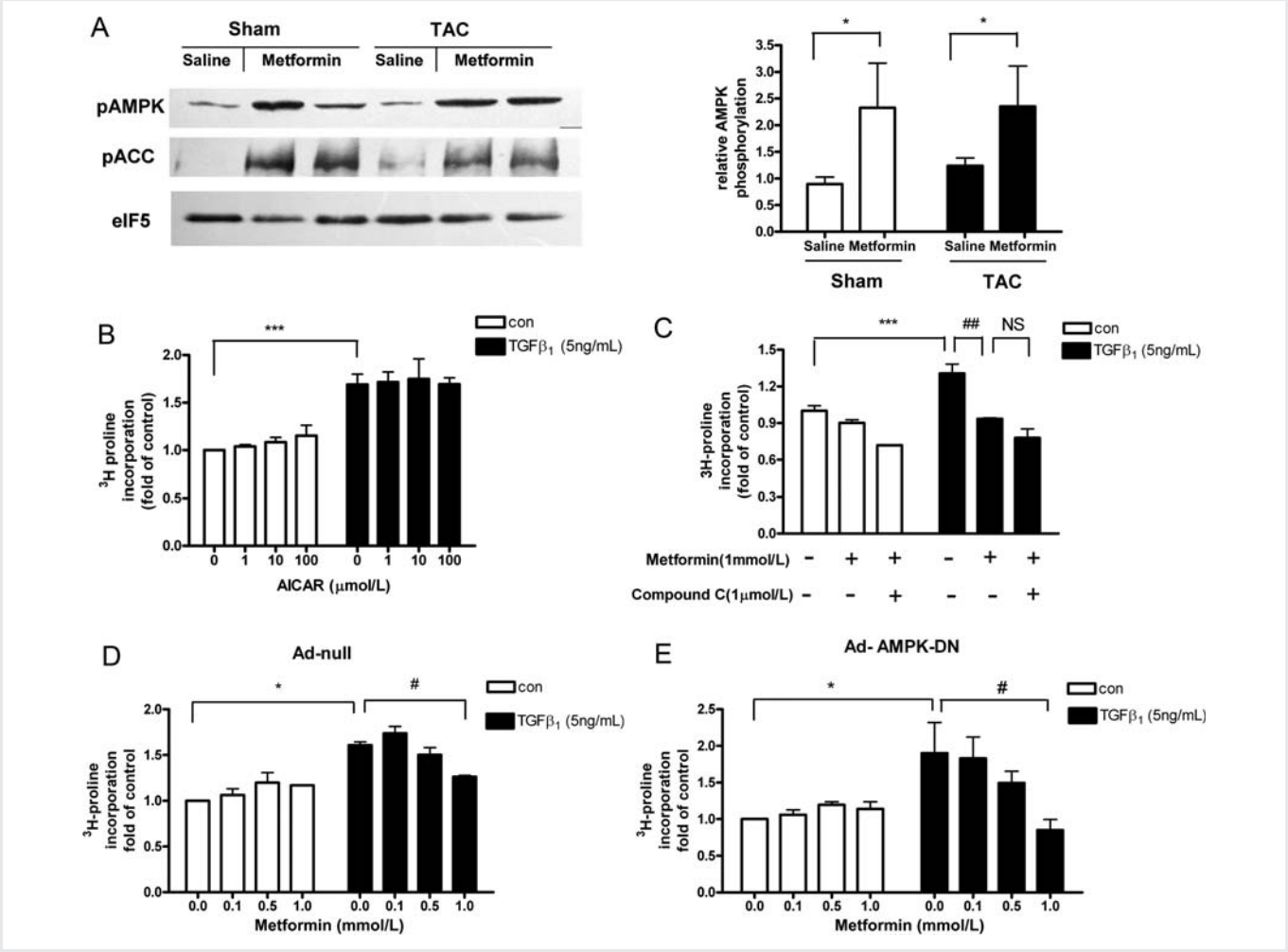

Figure 5 The inhibitory effect of metformin on collagen synthesis is independent of adenosine monophosphate-activated protein kinase (AMPK) activation. (*A*) Effect of metformin on the phosphorylation of AMPK and acetyl CoA carboxylase (ACC) in the transverse aortic constriction (TAC) or sham-operated animals after surgery 6 weeks. Heart extracts were analysed with the western blot for phosphorylated AMPK and ACC. The data presented are representative of a minimum of five separate experiments. (*B*) Another AMPK activator, 5-aminoimidazole-4-carboxamide riboside (AICAR), does not inhibit collagen synthesis induced by TGFβ$_1$. ***$P < 0.001$ TGFβ$_1$ vs. control, $n = 4$. (*C*) AMPK inhibitor (compound C) does not reverse the inhibitory effect of metformin. ***$P < 0.001$ TGFβ$_1$ vs. control, $^{\#\#}P < 0.01$ metformin+ TGFβ$_1$ vs. TGFβ$_1$, NS means no significant, $n = 3$. (*D*) Collagen synthesis induced by TGFβ$_1$ is inhibited by metformin with Ad-null transfection. *$P < 0.05$ TGFβ$_1$ vs. control, $^{\#}P < 0.05$ metformin + TGFβ$_1$ vs. TGFβ$_1$, $n = 3$; (*E*) Collagen synthesis induced by TGFβ$_1$ is also inhibited by metformin with recombinant adenovirus expressing a dominant negative mutant of AMPK (Ad-AMPK-DN), *$P < 0.05$ TGFβ$_1$ vs. control, $^{\#}P < 0.05$ metformin + TGFβ$_1$vs. TGFβ$_1$, $n = 3$.

to the inhibition on collagen synthesis; (iii) metformin inhibits the TGF-β$_1$–Smad3 signalling pathway (including TGF-β$_1$ production, phosphorylation of Smad3, and nuclear translocation of Smad3); and (iv) the antifibrotic action of metformin may be independent of AMPK activation.

In the present study, TAC for 6 weeks induced concentric hypertrophy with isolated diastolic dysfunction and fibrosis but without systolic dysfunction. Metformin (200mg/kg/day) treatment reduced cardiac fibrosis and improved diastolic dysfunction. In the preliminary experiments, we also found that metformin (50 mg/kg/day) inhibited cardiac fibrosis without significantly decrease in LVEDP and HW/tibia length. Therefore, we think that metformin inhibited cardiac fibrosis in a dose-related manner. The dosage of 200 mg/kg/day we used in this study was effective on fibrosis and had no side-effect of hypoglycaemia. Although the dose of metformin we used in this study is higher than that used in diabetes patients (10–40 mg/kg), previous reports investigating the anti-diabetic and anti-tumour effects of metformin in the mouse model used more higher amount of metformin (250–350 mg/kg) due to the difference of drug sensitivity between rodent and human.[18–20] Thus we think the dose of 200 mg/kg/day metformin in the mouse model is acceptable.

Sasaki *et al.*[6] also found that metformin attenuated fibrosis in failing canine hearts after 4 weeks of continuous rapid pacing. The antifibrotic effect of metformin in the study by Sasaki *et al.*[6] may be attributed to prevention of the progression of heart failure. In the present study, the cardiac systolic function was normal in each group, so metformin may have a direct inhibitory effect on cardiac fibrosis rather than a secondary effect due to the improvement of cardiac systolic function. To determine whether the anti-fibrotic effect was due to a lower pressure overload in metformin-treated mice, we measured aortic and LV pressure in each group. TAC mice had markedly increased systolic arterial pressure ($P < 0.001$),

diastolic arterial pressure ($P < 0.05$), and LV systolic pressure ($P < 0.001$) (*Table 1*) compared with sham-operated mice. There was no significant difference in the TAC-induced increments of these parameters in both saline- and metformin-treated mice, indicating a similar degree of pressure overload. Clinical trials also suggested that metformin has (if any) only a minor clinically insignificant effect on blood pressure in non-diabetic hypertensives.[21] As a hypoglycaemic drug, metformin prevented collagen glycation and diastolic dysfunction in the diabetic myocardium.[8] In the present study, we used non-diabetic mice. Metformin did not affect fasting blood glucose level and insulin level in TAC- or sham-operated mice (*Table 2*, see Supplementary material online, *Figure S2*). The present study suggested that metformin directly inhibited cardiac fibrosis and improved cardiac diastolic function without a change in blood glucose and insulin level in non-diabetic mice.

Cardiac fibrosis involves an increase in collagen synthesis, the proliferation of CFs, or a decrease in collagen degradation. To investigate the mechanism for the antifibrotic effect of metformin, we measured the effect of metformin on collagen synthesis or proliferation of CFs. TGF-β_1 has been shown to regulate the expression of pro-collagen genes and promote the synthesis of extracellular matrix components, thereby contributing to myocardial fibrosis.[22] In the present study, metformin inhibited production of TAC-induced myocardial TGF-β_1. Similarly, Sasaki *et al.*[6] also found that metformin suppressed the increase of TGF-β_1 mRNA expression in the failing canine hearts. Furthermore, we found that metformin dose-dependently inhibited collagen synthesis induced by TGF-β_1 in CFs. In contrast, some studies demonstrated that metformin increased expression of type-I collagen in osteoblast-like cells,[23] and increased type-I collagen synthesis in bone marrow progenitor cells.[24] Cortes *et al.*[25] found that metformin did not alter collagen metabolism in mesangial cells. This variability probably reflects the underlying cell- or tissue-specific characteristics of metformin. Although metformin has been shown to inhibit proliferation of airway smooth muscle cells[26] and the growth of breast cancer cells,[27] we found that metformin had no inhibitory effect on the proliferation of CFs. With regard to collagen degradation, we found that the level of MMP2 was lower in the metformin-treated group than that of the saline-treated group. These results did not indicate that metformin inhibited TAC-induced cardiac fibrosis by promoting collagen degradation. In hypertensive patients, response to increased synthesis of extracellular matrix (ECM) components is to increase the levels and activity of enzymes (MMPs) that degrade the ECM. Our results showed that TAC-operated mouse hearts had increased levels of MMP2. MMP activation can contribute to the fibrotic process by participating in a vicious circle in which ECM degradation promotes ECM protein synthesis and fibrosis. This pathway is particularly detrimental.[28] Our results showed that metformin did not activate MMP pathway. The reduced MMP2 level in the metformin-treated group might be secondary to the reduced cardiac fibrosis in the metformin-treated group. Thus, the mechanism of the antifibrotic action of metformin may be attributed to the inhibition of collagen synthesis.

The most important finding in the present study was that metformin inhibits cardiac fibrosis through inhibiting the TGF-β_1–Smad3 signalling pathway. The primary TGF-β_1 signal transduction pathway is the highly conserved Smad pathway.[29] Upon stimulation of receptors of the TGF-β_1, receptor regulated Smads (e.g. Smad2 and Smad3) become phosphorylated and activated, undergoing dimerization and thereafter forming heterotrimers with Co-Smad (Smad4).[29,30] These active Smad complexes translocate into the nucleus, where they accumulate and bind to target genes to directly regulate their transcription. The key components modifying the TGF-β–Smads pathway include receptor–receptor interactions, receptor-activated phosphorylation/dephosphorylation, Smad–Smad interactions, Smad shuttling and nuclear transportation, and accumulation of Smad complexes that bind to specific promoter elements to alter gene transcription. In fibroblasts, the intracellular mediators of TGF-β_1 signalling are Smad2 and Smad3.[17] Only Smad3 has a DNA-binding and transactivation domain, so it is the most likely Smad to be directly involved in transcription.[17] Studies have revealed that Smad3 plays a pivotal role in mediating TGF-β_1-induced stimulation of collagen gene expression in normal fibroblasts.[31] We showed that metformin inhibited TGF-β_1-induced phosphorylation of Smad3 rather than Smad2 in CFs. Nuclear translocation of Smad3 was blocked by metformin. Metformin blocked TGF-β_1-stimulated transactivation of the pGL3-$(CAGA)_{12}$-luc reporter, which can be specifically activated by Smad3. This suggested that metformin protects against cardiac fibrosis through interference of downstream Smad3 signalling from TGF-β_1. Smad3 signalling is essential for fibrotic remodelling of the infarcted ventricle, and Smad3 inhibition is likely to attenuate fibrotic remodeling.[10] Our findings provide a new therapeutic target of metformin in cardiac fibrosis.

Metformin is known to activate AMPK,[32] which is expressed in various tissues. Studies have demonstrated that metformin confers cardioprotection against myocardial ischaemia–reperfusion injury,[4,5,33] and inhibits cardiac hypertrophy[34] through AMPK activation. Surprisingly, we found that the AMPK inhibitor (Compound C) and adenovirus transduction with AMPKα1-DN did not reverse the inhibitory effect of metformin on collagen synthesis. These results suggested that the inhibitory effect of metformin on collagen synthesis may be independent of AMPK activation. Recent reports also suggest that metformin exerts its effects in a manner independent of AMPK activation. Guigas *et al.*[35] have found that metformin inhibited hepatic glucose phosphorylation by an AMPK-independent effect on glucokinase translocation. Saeedi *et al.*[36] have demonstrated that metformin altered fatty acid oxidation and glucose utilization in the heart independent of changes in AMPK activity.

In summary, the present study demonstrated that metformin inhibited cardiac fibrosis induced by pressure overload *in vivo* and inhibited collagen synthesis in CFs. The underlying mechanisms were that metformin inhibits the TGF-β_1–Smad3 signalling pathway. These findings provide a new mechanism for the cardioprotective effects of metformin and a new therapeutic target of metformin.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

The authors thank Dr Ye-Guang Chen (Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing, China) for his generous gift of pGL3-$(CAGA)_{12}$-luc reporter plasmids. The authors thank Dr Nanping Wang (Institute of Cardiovascular Sciences, Peking University, Beijing, China) for his suggestions on the manuscript.

Conflict of interest: none declared.

Funding

This work was supported by the Key grant Project of Chinese Ministry of Education [307001]; the Foundation of National Key Basic Research and Development Project [2006CB503806]; and the National Natural Science Foundation of China [30672466, 30821001].

References

1. UK Prospective Diabetes Study (UKPDS) Group. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). *Lancet* 1998;**352**:854–865.
2. Holman RR, Paul SK, Bethel MA, Matthews DR, Neil HA. 10-year follow-up of intensive glucose control in type 2 diabetes. *N Engl J Med* 2008;**359**:1577–1589.
3. Kirpichnikov D, McFarlane SI, Sowers JR. Metformin: an update. *Ann Intern Med* 2002; **137**:25–33.
4. Solskov L, Lofgren B, Kristiansen SB, Jessen N, Pold R, Nielsen TT *et al*. Metformin induces cardioprotection against ischaemia/reperfusion injury in the rat heart 24 h after administration. *Basic Clin Pharmacol Toxicol* 2008;**103**:82–87.
5. Gundewar S, Calvert JW, Jha S, Toedt-Pingel I, Ji SY, Nunez D *et al*. Activation of AMP-activated protein kinase by metformin improves left ventricular function and survival in heart failure. *Circ Res* 2009;**104**:403–411.
6. Sasaki H, Asanuma H, Fujita M, Takahama H, Wakeno M, Ito S *et al*. Metformin prevents progression of heart failure in dogs: role of AMP-activated protein kinase. *Circulation* 2009;**119**:2568–2577.
7. Charles MA, Eschwege E, Grandmottet P, Isnard F, Cohen JM, Bensoussan JL *et al*. Treatment with metformin of non-diabetic men with hypertension, hypertriglyceridaemia and central fat distribution: the BIGPRO 1.2 trial. *Diabetes Metab Res Rev* 2000;**16**:2–7.
8. Jyothirmayi GN, Soni BJ, Masurekar M, Lyons M, Regan TJ. Effects of metformin on collagen glycation and diastolic dysfunction in diabetic myocardium. *J Cardiovasc Pharmacol Ther* 1998;**3**:319–326.
9. Kuwahara F, Kai H, Tokuda K, Kai M, Takeshita A, Egashira K *et al*. Transforming growth factor-beta function blocking prevents myocardial fibrosis and diastolic dysfunction in pressure-overloaded rats. *Circulation* 2002;**106**:130–135.
10. Bujak M, Ren G, Kweon HJ, Dobaczewski M, Reddy A, Taffet G *et al*. Essential role of Smad3 in infarct healing and in the pathogenesis of cardiac remodeling. *Circulation* 2007;**116**:2127–2138.
11. Tarnavski O, McMullen JR, Schinke M, Nie Q, Kong S, Izumo S. Mouse cardiac surgery: comprehensive techniques for the generation of mouse models of human diseases and their application for genomic studies. *Physiol Genomics* 2004;**16**:349–360.
12. Wang J, Xu N, Feng X, Hou N, Zhang J, Cheng X *et al*. Targeted disruption of Smad4 in cardiomyocytes results in cardiac hypertrophy and heart failure. *Circ Res* 2005;**97**: 821–828.
13. Du JH, Xu N, Song Y, Xu M, Lu ZZ, Han C *et al*. AICAR stimulates IL-6 production via p38 MAPK in cardiac fibroblasts in adult mice: a possible role for AMPK. *Biochem Biophys Res Commun* 2005;**337**:1139–1144.
14. Chen K, Li D, Zhang X, Hermonat PL, Mehta JL. Anoxia-reoxygenation stimulates collagen type-I and MMP-1 expression in cardiac fibroblasts: modulation by the PPAR-gamma ligand pioglitazone. *J Cardiovasc Pharmacol* 2004;**44**:682–687.
15. Teunissen BE, Smeets PJ, Willemsen PH, De Windt LJ, Van der Vusse GJ, Van Bilsen M. Activation of PPARdelta inhibits cardiac fibroblast proliferation and the transdifferentiation into myofibroblasts. *Cardiovasc Res* 2007;**75**:519–529.
16. Zhang Y, Lee TS, Kolb EM, Sun K, Lu X, Sladek FM *et al*. AMP-activated protein kinase is involved in endothelial NO synthase activation in response to shear stress. *Arterioscler Thromb Vasc Biol* 2006;**26**:1281–1287.
17. Massague J. Wounding Smad. *Nat Cell Biol* 1999;**1**:E117–E119.
18. Buzzai M, Jones RG, Amaravadi RK, Lum JJ, DeBerardinis RJ, Zhao F *et al*. Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth. *Cancer Res* 2007;**67**:6745–6752.
19. Zou MH, Kirkpatrick SS, Davis BJ, Nelson JS, Wiles WG, Schlattner U *et al*. Activation of the AMP-activated protein kinase by the anti-diabetic drug metformin in vivo - Role of mitochondrial reactive nitrogen species. *J Biol Chem* 2004;**279**:43940–43951.
20. Bergheim I, Guo L, Davis MA, Lambert JC, Beier JI, Duveau I *et al*. Metformin prevents alcohol-induced liver injury in the mouse: critical role of plasminogen activator inhibitor-1. *Gastroenterology* 2006;**130**:2099–2112.
21. Snorgaard O, Kober L, Carlsen J. The effect of metformin on blood pressure and metabolism in nondiabetic hypertensive patients. *J Intern Med* 1997;**242**:407–412.
22. Khan R, Sheppard R. Fibrosis in heart disease: understanding the role of transforming growth factor-beta in cardiomyopathy, valvular disease and arrhythmia. *Immunology* 2006;**118**:10–24.
23. Cortizo AM, Sedlinsky C, McCarthy AD, Blanco A, Schurman L. Osteogenic actions of the anti-diabetic drug metformin on osteoblasts in culture. *Eur J Pharmacol* 2006; **536**:38–46.
24. Molinuevo MS, Schurman L, McCarthy AD, Cortizo AM, Tolosa MJ, Gangoiti MV *et al*. Effect of metformin on bone marrow progenitor cell differentiation: *in vivo* and *in vitro* studies. *J Bone Miner Res* 2010;**25**:211–221.
25. Cortes P, Riser BL, Asano K, Rodriguez-Barbero A, Narins RG, Yee J. Effects of oral antihyperglycemic agents on extracellular matrix synthesis by mesangial cells. *Kidney Int* 1998;**54**:1985–1998.
26. Ratnovsky A, Mellema M, An SS, Fredberg JJ, Shore SA. Airway smooth muscle proliferation and mechanics: effects of AMP kinase agonists. *Mol Cell Biomech* 2007;**4**: 143–157.
27. Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M. Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. *Cancer Res* 2006;**66**: 10269–10273.
28. Berk BC, Fujiwara K, Lehoux S. ECM remodeling in hypertensive heart disease. *J Clin Invest* 2007;**117**:568–575.
29. Schmierer B, Hill CS. TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. *Nat Rev Mol Cell Biol* 2007;**8**:970–982.
30. Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003;**113**:685–700.
31. Chen SJ, Ning H, Ishida W, Sodin-Semrl S, Takagawa S, Mori Y *et al*. The early-immediate gene EGR-1 is induced by transforming growth factor-beta and mediates stimulation of collagen gene expression. *J Biol Chem* 2006;**281**:21183–21197.
32. Zhou GC, Myers R, Li Y, Chen YL, Shen XL, Fenyk-Melody J *et al*. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 2001;**108**: 1167–1174.
33. Calvert JW, Gundewar S, Jha S, Greer JJ, Bestermann WH, Tian R *et al*. Acute metformin therapy confers cardioprotection against myocardial infarction via AMPK-eNOS-mediated signaling. *Diabetes* 2008;**57**:696–705.
34. Chan AY, Soltys CL, Young ME, Proud CG, Dyck JR. Activation of AMP-activated protein kinase inhibits protein synthesis associated with hypertrophy in the cardiac myocyte. *J Biol Chem* 2004;**279**:32771–32779.
35. Guigas B, Viollet B, Taleux N, Foretz M, Vaulont S, Hue L. 5-Aminoimidazole-4-carboxamide riboside (AICAR) and metformin inhibit hepatic mitochondrial oxidative phosphorylation by two different AMPK-independent mechanisms. *FEBS J* 2005;**272**:306–306.
36. Saeedi R, Parsons HL, Wambolt RB, Paulson K, Sharma V, Dyck JR *et al*. Metabolic actions of metformin in the heart can occur by AMPK-independent mechanisms. *Am J Physiol Heart Circ Physiol* 2008;**294**:H2497–H2506.