

Original Article

# Metformin attenuates pressure overload-induced cardiac hypertrophy via AMPK activation

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**Aim:** To identify the role of metformin in cardiac hypertrophy and investigate the possible mechanism underlying this effect.

**Methods:** Wild type and AMPK $\alpha$ 2 knockout (AMPK $\alpha$ 2<sup>-/-</sup>) littermates were subjected to left ventricular pressure overload caused by transverse aortic constriction. After administration of metformin (200 mgkg<sup>-1</sup>d<sup>-1</sup>) for 6 weeks, the degree of cardiac hypertrophy was evaluated using echocardiography and anatomic and histological methods. The antihypertrophic mechanism of metformin was analyzed using Western blotting.

**Results:** Metformin significantly attenuated cardiac hypertrophy induced by pressure overload in wild type mice, but the antihypertrophic actions of metformin were ablated in AMPK $\alpha$ 2<sup>-/-</sup> mice. Furthermore, metformin suppressed the phosphorylation of Akt/protein kinase B (AKT) and mammalian target of rapamycin (mTOR) in response to pressure overload in wild type mice, but not in AMPK $\alpha$ 2<sup>-/-</sup> mice.

**Conclusion:** Long-term administration of metformin may attenuate cardiac hypertrophy induced by pressure overload in nondiabetic mice, and this attenuation is highly dependent on AMPK activation. These findings may provide a potential therapy for patients at risk of developing pathological cardiac hypertrophy.

**Keywords:** metformin; cardiac hypertrophy; AMP-activated protein kinase; protein synthesis; transverse aortic constriction; Akt/protein kinase B; mammalian target of rapamycin

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## Introduction

Metformin is one of the most commonly prescribed antihyperglycemic drugs for the treatment of type 2 diabetes that does not cause hypoglycemia. Apart from its antihyperglycemic effect, metformin has been believed to have other potential effects, as indicated in two large-scale clinical trials<sup>[1, 2]</sup>. Those trials suggested that metformin could significantly decrease the risk of diabetes-related cardiovascular end points and all cause deaths compared with conventional therapies when lowering blood glucose to similar levels. However, the exact mechanism associated with the cardioprotective role of metformin is still elusive.

AMPK is a serine-threonine kinase that acts as an energy sensor in various cell types<sup>[3]</sup>. It also plays an important role in cardioprotective effects<sup>[4, 5]</sup>. Zhou *et al*<sup>[6]</sup> demonstrated that metformin could activate AMPK. Recent studies have sug-

gested that metformin could inhibit cardiomyocyte apoptosis and improve cardiac function through AMPK activation<sup>[7, 8]</sup>. Pathological left ventricular hypertrophy is a crucial pathological condition that triggers several serious cardiac events, including arrhythmias, heart failure, and sudden death<sup>[9, 10]</sup>. However, whether metformin has an inhibitory effect on cardiac hypertrophy has not been elucidated. Thus, we hypothesized that metformin might have a direct antihypertrophic effect via AMPK activation.

In this study, we aim to determine whether metformin inhibits cardiac hypertrophy induced by pressure overload and how metformin exerts its antihypertrophic effect.

## Materials and methods

### Animals

The research protocol was designed in compliance with the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health of the United States of America (NIH Publication No 85–23, revised 1996). All experimental protocols were approved by the Institutional Committee for Animal Care and Use of Peking University. Heterozygous

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AMPK $\alpha$ 2 knockout C57BL/6 mice were kindly donated by Professor Benoit VIOLLET (Institute National de la Santé et de la Recherche Médicale U567, Paris, France) and bred in an specific pathogen free (SPF) environment with a 12 h/12 h light-dark cycle and received standard rodent food. Male AMPK $\alpha$ 2<sup>-/-</sup> mice and wild-type (WT) littermates (10 weeks old) were bred from the heterozygotes.

#### TAC and drug treatment

Transverse aortic constriction (TAC) was carried out as previously described<sup>[11]</sup>. Briefly, the mice were anesthetized with tribromoethanol (200 mg/kg, ip; Sigma-Aldrich, Milwaukee, WI, USA) and ventilated with a 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 the 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 the aorta was not ligated. Three days after aortic constriction, the mice were injected with metformin (200 mg·kg<sup>-1</sup>·d<sup>-1</sup>, sc, Sigma-Aldrich, St Louis, MO, USA) or saline (as a control) every day for 6 weeks.

#### Echocardiography and evaluation of left ventricular (LV) hemodynamics

After anesthetizing the mice with 1.5% isoflurane (Baxter Healthcare Corporation, New Providence, NJ, USA), we obtained echocardiographic images with a VisualSonics high-resolution Vevo 770 system (VisualSonics, Toronto, ON, Canada). For measurement of the aortic and LV pressure, a 1.4-F micromanometer conductance catheter (SPR-835; Millar Instruments, Houston, TX, USA) was introduced through the right common carotid artery into the ascending aorta and then advanced into the LV as previously described<sup>[12]</sup>.

#### Histological analysis

The mice were anesthetized with tribromoethanol, and the hearts were harvested, retrograde-perfused with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde overnight, and then embedded in paraffin. Serial 6- $\mu$ m-thick LV cross sections were stained with hematoxylin and eosin (HE). Morphometric evaluation of the tissue was performed in a blinded fashion using the Leica Q550 IW imaging workstation (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK). The mean myocyte cross-sectional area was calculated by measuring 100 cells in the HE-stained sections.

#### Quantitative real-time PCR

Total RNA was isolated from the heart tissue with Trizol Reagent (Invitrogen, Carlsbad, CA, USA). 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). The PCR used the following primers: atrial natriuretic factor (ANF), 5'-GCCCTGAGTGAGCAGACTG-3' (forward) and 5'-CG-

GAAGCTGTTGCAGCCTA-3' (reverse);  $\beta$ -myosin heavy chain ( $\beta$ -MHC), 5'-ACCTAC CAGACAGAGGAAGA-3' (forward) and 5'-TTGCAAAGAGTCCAGGTCTGAG-3' (reverse); and GAPDH, 5'-TCCTGGTATGACAATGAATACGGC-3' (forward) and 5'-TCTTGCTCAGTGTCCCTTGCTGG-3' (reverse). The GAPDH RNA was amplified as a reference standard. The reactions were conducted in triplicate by heating the reactant to 95 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s.

#### Western blot analysis

The heart extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on nitrocellulose membranes. The membranes were incubated with antibodies against phosphorylated AMPK, acetyl CoA carboxylase (ACC), eukaryotic elongation factor-2 (eEF2), mammalian target of rapamycin (mTOR) and Akt/protein kinase B (AKT) and then against the respective total level of each protein (antibodies from Cell Signaling Technology Incorporated, Danvers, MA, USA). The protein bands were visualized with Supersignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, Rockford, IL, USA). The blots were subsequently reprobed with the antibody eIF5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to confirm equal loading.

#### Statistical analysis

The data are expressed as the means $\pm$ SEM. Intergroup comparisons were performed using Prism 4 (GraphPad Software Incorporated, La Jolla, CA, USA) with Student's paired two-tailed *t*-test or two-way ANOVA. 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 statistically significant.

## Results

#### Metformin attenuates cardiac hypertrophy induced by TAC in WT mice

To determine whether metformin plays a part in cardiac hypertrophy induced by TAC, we treated TAC-operated mice with metformin (200 mg·kg<sup>-1</sup>·d<sup>-1</sup>) or saline for 6 weeks after surgery. Echocardiographic data showed no significant difference in the WT mice before sham or TAC operation (data not shown). The mice that received TAC developed cardiac hypertrophy with normal cardiac systolic function 6 weeks after the operation (Figure 1, Table 1). Sustained treatment with metformin decreased the LV posterior wall dimension in diastole (LVPWd) compared with saline-treated hearts (Figure 1A, 1B). The heart weight/tibial length (HW/TL) of the TAC+saline group increased by 26% compared with the sham+saline group (*P*<0.01; Figure 1C). In contrast, the HW/TL of TAC+metformin group increased by only 11.7% compared with the sham+saline group. It could therefore be calculated that metformin suppressed the TAC-induced increase in HW/TL by 55% (*P*<0.05; Figure 1C). The cardiomyocyte cross-sectional area also decreased in the metformin-treated

**Table 1.** Anatomic and functional data for WT mice and AMPK $\alpha$ 2<sup>-/-</sup> littermates 6 weeks after sham or TAC operation. Values are means $\pm$ SEM; in WT groups, <sup>c</sup>*P*<0.01 vs sham+saline; <sup>e</sup>*P*<0.05 vs TAC+saline; in AMPK $\alpha$ 2<sup>-/-</sup> groups, <sup>h</sup>*P*<0.05 and <sup>i</sup>*P*<0.01 vs sham+saline; <sup>j</sup>*P*>0.05 vs TAC+saline.

	WT				AMPK $\alpha$ 2 <sup>-/-</sup>			
	Sham		TAC		Sham		TAC	
	Saline	Metformin	Saline	Metformin	Saline	Metformin	Saline	Metformin
<b>Anatomic data</b>								
<i>n</i>	9	7	9	9	8	6	9	8
Body weight (g)	25.9 $\pm$ 0.96	25.3 $\pm$ 1.02	27.0 $\pm$ 1.03	25.0 $\pm$ 0.75	26.1 $\pm$ 0.82	27 $\pm$ 0.99	27.6 $\pm$ 0.78	26.7 $\pm$ 0.80
Heart weight (mg)	114.1 $\pm$ 3.7	109.4 $\pm$ 4.4	150.0 $\pm$ 7.2 <sup>c</sup>	126.4 $\pm$ 4.9 <sup>e</sup>	117.5 $\pm$ 3.4	112.8 $\pm$ 4.7	152.5 $\pm$ 5.7 <sup>i</sup>	158.2 $\pm$ 6.3 <sup>j</sup>
HW/BW (mg/g)	4.42 $\pm$ 0.07	4.31 $\pm$ 0.09	5.59 $\pm$ 0.18 <sup>c</sup>	5.04 $\pm$ 0.13 <sup>e</sup>	4.47 $\pm$ 0.14	4.18 $\pm$ 0.05	5.62 $\pm$ 0.17 <sup>i</sup>	5.9 $\pm$ 0.25 <sup>j</sup>
<b>Echocardiographic data</b>								
<i>n</i>	9	7	9	9	8	6	9	8
HR (bpm)	408 $\pm$ 8	440 $\pm$ 12	421 $\pm$ 15	431 $\pm$ 19	425 $\pm$ 11	411 $\pm$ 10	447 $\pm$ 17	428 $\pm$ 10
LVIDd (mm)	4.02 $\pm$ 0.10	3.93 $\pm$ 0.13	4.14 $\pm$ 0.11	3.79 $\pm$ 0.10	3.92 $\pm$ 0.09	3.91 $\pm$ 0.12	4.04 $\pm$ 0.11	4.16 $\pm$ 0.08
LVIDs (mm)	2.79 $\pm$ 0.13	2.62 $\pm$ 0.17	3.02 $\pm$ 0.15	2.60 $\pm$ 0.11	2.61 $\pm$ 0.12	2.66 $\pm$ 0.13	2.91 $\pm$ 0.14	3.01 $\pm$ 0.14
LVAWd (mm)	0.82 $\pm$ 0.03	0.76 $\pm$ 0.02	1.02 $\pm$ 0.03 <sup>c</sup>	0.89 $\pm$ 0.02 <sup>e</sup>	0.83 $\pm$ 0.03	0.80 $\pm$ 0.02	1.07 $\pm$ 0.05 <sup>i</sup>	1.08 $\pm$ 0.05 <sup>j</sup>
FS%	31.0 $\pm$ 1.76	33.5 $\pm$ 2.33	31.7 $\pm$ 1.08	32.9 $\pm$ 1.29	32.8 $\pm$ 1.55	32.1 $\pm$ 1.95	29.8 $\pm$ 1.54	30.7 $\pm$ 1.22
<b>Hemodynamic data</b>								
<i>n</i>	8	7	8	9	8	6	9	8
HR (bpm)	479 $\pm$ 15	475 $\pm$ 26	491 $\pm$ 29	474 $\pm$ 20	493 $\pm$ 18	479 $\pm$ 19	494.2 $\pm$ 21	471.6 $\pm$ 16
Systolic BP (mmHg)	81.2 $\pm$ 5.4	82.8 $\pm$ 2.4	127.5 $\pm$ 7.0 <sup>c</sup>	124.2 $\pm$ 9.2	89.5 $\pm$ 2.5	91.4 $\pm$ 4.1	131.7 $\pm$ 8.3 <sup>i</sup>	133.1 $\pm$ 7.1
Diastolic BP (mmHg)	55.0 $\pm$ 4.5	57.7 $\pm$ 3.5	67.9 $\pm$ 6.9	61.3 $\pm$ 5.5	57.4 $\pm$ 3.1	62.5 $\pm$ 5.8	70.9 $\pm$ 4.9	66.3 $\pm$ 4.6
LVSP (mmHg)	81.8 $\pm$ 4.1	83.6 $\pm$ 3.4	132.8 $\pm$ 6.7 <sup>c</sup>	130.8 $\pm$ 8.1	91.4 $\pm$ 3.0	92.3 $\pm$ 5.2	132.5 $\pm$ 8.8 <sup>i</sup>	135.7 $\pm$ 5.5
LVEDP (mmHg)	1.2 $\pm$ 0.8	1.9 $\pm$ 1.3	8.7 $\pm$ 1.0 <sup>c</sup>	5.2 $\pm$ 1.5 <sup>e</sup>	2.0 $\pm$ 1.0	2.5 $\pm$ 0.8	9.9 $\pm$ 1.6 <sup>h</sup>	7.8 $\pm$ 1.4
+dp/dt (mmHg/s)	8414 $\pm$ 514	7318 $\pm$ 435	8259 $\pm$ 679	7548 $\pm$ 790	8619 $\pm$ 543	8618 $\pm$ 651	8577 $\pm$ 755	7892 $\pm$ 328
-dp/dt (mmHg/s)	-7973 $\pm$ 364	-7124 $\pm$ 286	-8498 $\pm$ 673	-8657 $\pm$ 471	-8582 $\pm$ 303	-7928 $\pm$ 306	-8720 $\pm$ 768	-7190 $\pm$ 753

TAC, thoracic aortic constriction; *n*, number of mice; HW, heart weight; BW, body weight; HR, heart rate; LVIDd, left ventricular (LV) internal diameter in diastole; LVIDs, LV internal diameter in systole; LVAWd, LV anterior wall diameter in diastole; FS, fractional shortening; LVSP, LV peak systolic pressure; LVEDP, LV end-diastolic pressure; +dp/dt and -dp/dt, the rise and decline of the first derivative of pressure, respectively.

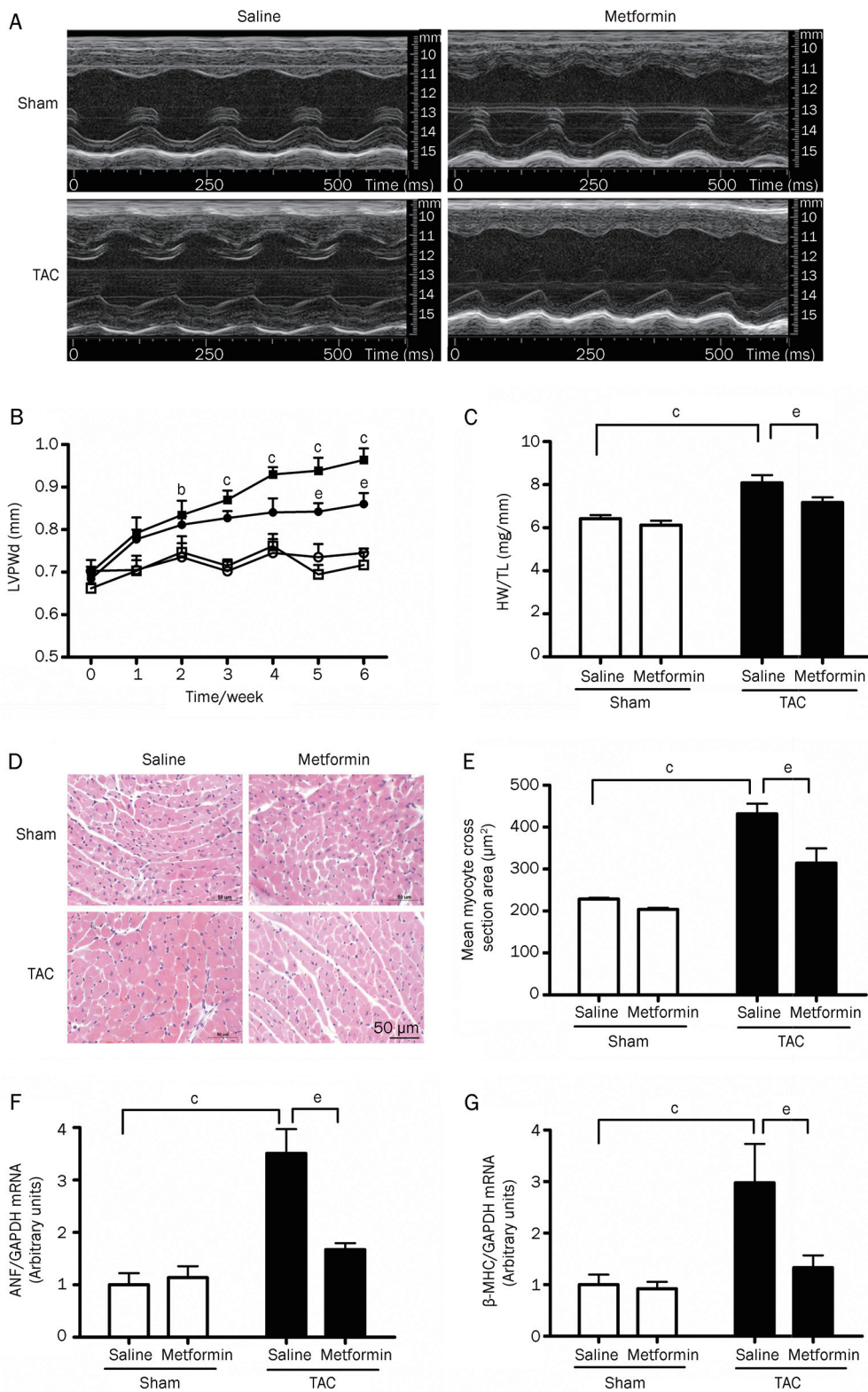
group compared with the saline-treated group (*P*<0.01; Figure 1D, 1E). Echocardiographic and hemodynamic data showed that sustained treatment with metformin did not affect the cardiac systolic function, but mitigated the diastolic function impairment induced by TAC (*P*<0.05; Table 1). The treatment with metformin did not affect the systolic blood pressure (SBP) or the LV peak systolic pressure (LVSP) (Table 1). Additionally, metformin treatment inhibited the TAC-induced increase in mRNA levels of ANF and  $\beta$ -MHC (Figure 1F, 1G). Taken together, these results indicate that metformin attenuated the development of TAC-induced cardiac hypertrophy independent of hemodynamics. Moreover, there was no significant difference in anatomic and functional data (Table 1) between the sham+saline group and the sham+metformin group; therefore, metformin did not affect normal cardiac structure or function.

#### Metformin does not attenuate cardiac hypertrophy induced by TAC in AMPK $\alpha$ 2<sup>-/-</sup> mice

To investigate whether AMPK was critical for the inhibitory effect of metformin on cardiac hypertrophy, we subjected AMPK $\alpha$ 2<sup>-/-</sup> littermates to pressure overload caused by TAC. Similar to what was observed in WT mice, TAC induced

cardiac hypertrophy without deteriorating cardiac systolic function. However, sustained treatment with metformin did not decrease the LVPWd compared with saline-treated hearts (Figure 2A, 2B). The HW/TL did not decrease in the TAC+metformin group compared with the TAC+saline group (Figure 2C, Table 1). The cardiomyocyte cross-sectional area was similar in the TAC+metformin group and the TAC+saline group (Figure 2D, 2E). Echocardiographic and hemodynamic data showed that sustained metformin treatment did not affect cardiac systolic function (Table 1). Contrary to the findings in WT mice, metformin did not improve diastolic function that was impaired by TAC (*P*>0.05, Table 1). Moreover, metformin treatment in the AMPK $\alpha$ 2<sup>-/-</sup> littermates did not inhibit the TAC-induced increase in mRNA levels of ANF and  $\beta$ -MHC (Figure 2F, 2G). It can therefore be concluded that the inhibitory effect of metformin on cardiac hypertrophy is dependent on AMPK.

We also measured the postoperative level of fasting blood glucose every week for 6 weeks in each group. There was no significant difference in the fasting blood glucose between saline- and metformin-treated mice (data not shown). These results suggest that metformin did not affect the level of fasting blood glucose in nondiabetic mice.

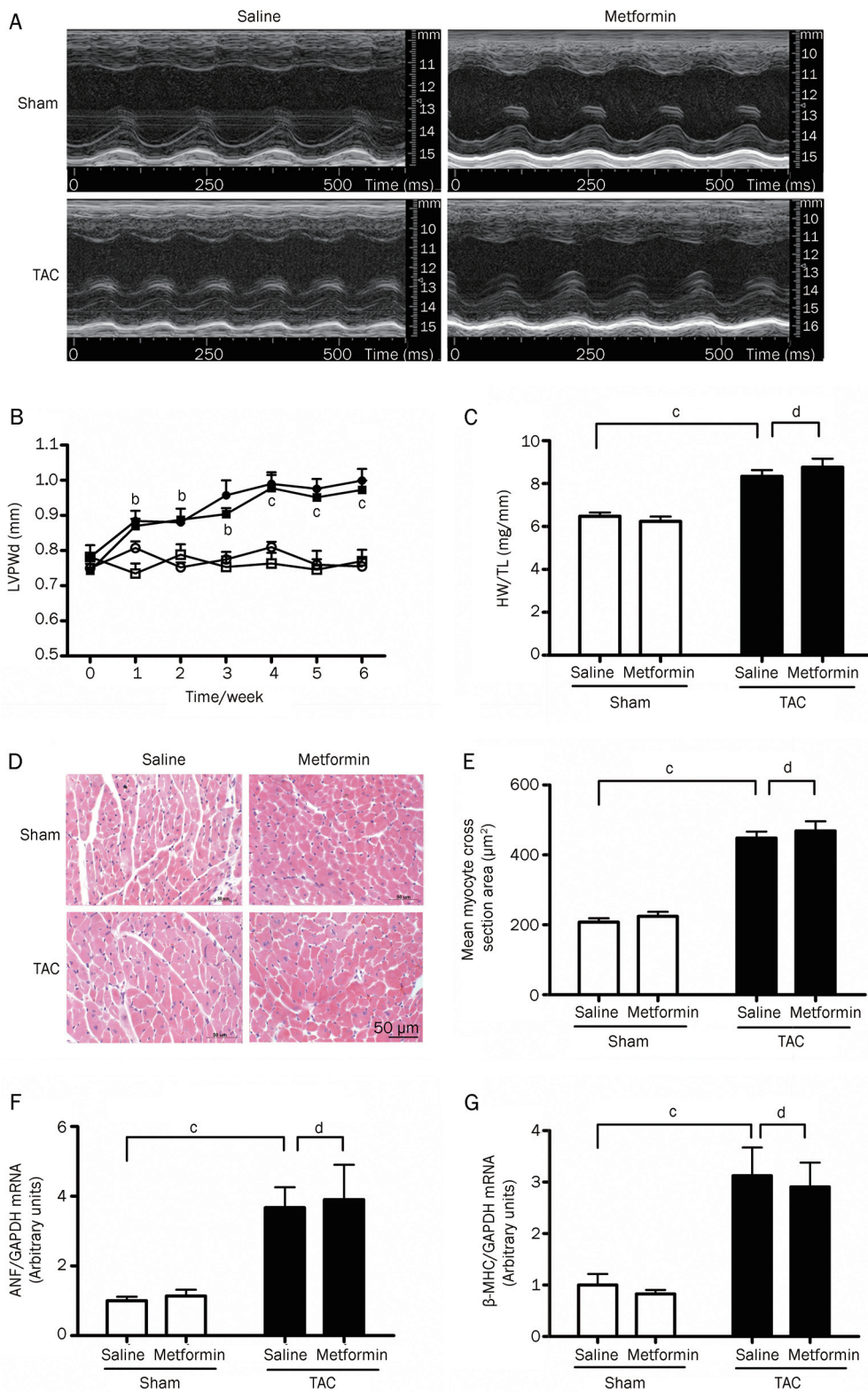


**Figure 1.** Metformin attenuates cardiac hypertrophy induced by TAC in WT mice. (A) Representative M-mode echocardiograms of the parasternal short-axis view at the level of the midpapillary muscle obtained in each group 6 weeks after sham or TAC operation. (B) Left ventricular posterior wall diameter in diastole (LVPWd) before the surgery and every week for 6 weeks after the surgery (□, sham+saline, ○, sham+metformin, ■, TAC+saline, and ●, TAC+metformin;  $n=7-9$ ). (C) The HW/TL was calculated 6 weeks after the sham or TAC operation ( $n=7-9$ ). (D) HE staining of heart cross-sections ( $\times 400$ , bar=50  $\mu\text{m}$ ) (E) Assessment of the myocyte cross-sectional cell area from left ventricular histological sections ( $n=6$ , each with a minimum of 100 cells counted). Quantitative real-time PCR analyses of ANF (F) and  $\beta$ -MHC (G) mRNA levels ( $n=4$ ). The data are presented as means $\pm$ SEM. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs sham+saline; <sup>e</sup> $P<0.05$  vs TAC+saline.

#### Effect of metformin on AMPK phosphorylation and protein synthesis.

To examine the role of AMPK in the antihypertrophic action of metformin, we assessed the extent of phosphorylated AMPK in WT mouse hearts. There was a significant increase in the

phosphorylation of AMPK at threonine residue 172 in the metformin-treated group compared with the saline-treated group ( $P<0.05$ ; Figure 3). Metformin treatment also increased the amount of phosphorylated acetyl coenzyme A (CoA) carboxylase (p-ACC), which serves as an indicator of AMPK activity.

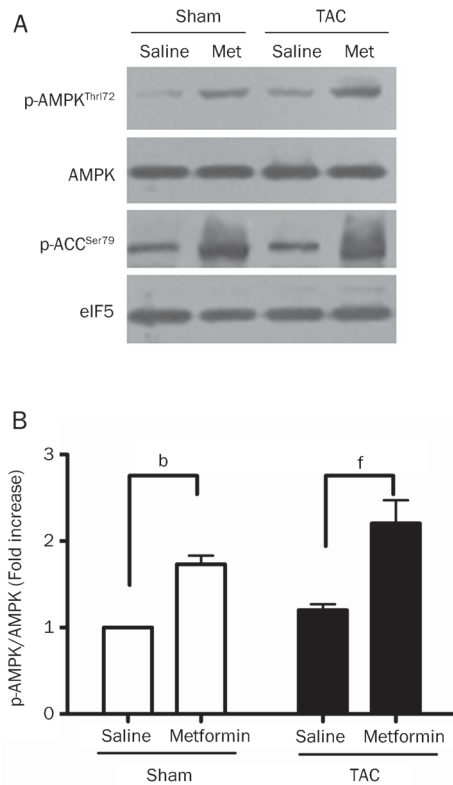


**Figure 2.** Metformin could not attenuate cardiac hypertrophy induced by TAC in AMPK $\alpha$ 2<sup>-/-</sup> mice. (A) Representative M-mode echocardiograms of the parasternal short-axis view at the level of the midpapillary muscle obtained in AMPK $\alpha$ 2<sup>-/-</sup> mice 6 weeks after the sham or TAC operation. (B) Left ventricular posterior wall diameter in diastole (LVPWd) before surgery and every week for 6 weeks after the surgery (□, sham+saline, ○, sham+metformin, ■, TAC+saline, and ●, TAC+metformin;  $n=6-9$ ). (C) HW/TL was calculated 6 weeks after the sham or TAC operation ( $n=6-9$ ). (D) HE staining of heart cross-sections ( $\times 400$ ), bar = 50  $\mu$ m. (E) Assessment of myocyte cross-sectional cell area from left ventricular histological sections ( $n=6$ , each with a minimum of 100 cells counted). Quantitative real-time PCR analyses of ANF (F) and  $\beta$ -MHC (G) mRNA levels ( $n=4$ ). The data are presented as means $\pm$ SEM. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs sham+saline; <sup>d</sup> $P>0.05$  vs TAC+saline.

Total myocardial AMPK $\alpha$  and phosphorylated AMPK $\alpha$  were significantly decreased in AMPK $\alpha$ 2<sup>-/-</sup> mice, both under control conditions and after TAC, compared with the WT mice (Figure 4). After TAC for 6 weeks, the increase in total AMPK $\alpha$  protein indicated a compensatory increase in

AMPK $\alpha$ 1 in AMPK $\alpha$ 2<sup>-/-</sup> mice, yet it was unable to compensate for the AMPK $\alpha$ 2 deficiency, as demonstrated by significantly lower levels of p-ACC (Figure 4).

LVH is characterized by an increased myocardial cell size in which protein synthesis is a necessary mediator. mTOR,



**Figure 3.** The effect of metformin on the phosphorylation of AMPK and ACC in WT mice 6 weeks after the sham or TAC operation. (A) Western blot of heart extracts for phosphorylated AMPK, phosphorylated ACC, total AMPK, and eIF5. (B) The ratio of phosphorylated AMPK to total AMPK is shown as means $\pm$ SEM of 6 animals per group. <sup>b</sup> $P < 0.05$  vs sham+saline; <sup>f</sup> $P < 0.01$  vs TAC+saline.

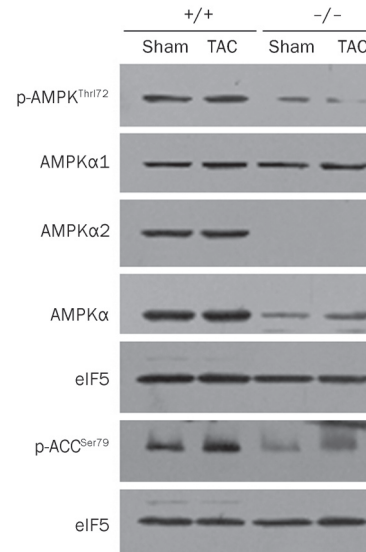
AKT, and eEF2 all play essential roles in the process of protein synthesis and cell growth. Western blot analysis showed a significant increase in phosphorylated mTOR and AKT in the TAC+saline group, but this increase was significantly inhibited in the TAC+metformin group ( $P < 0.05$ ; Figure 5A, 5B). However, the phosphorylation level of eEF2 did not show a significant change in any of the groups (Figure 5).

Next we tested the phosphorylation level of these proteins in AMPK $\alpha 2^{-/-}$  mice hearts. Similar to the effect in WT mice, TAC induced a significant increase in phosphorylated mTOR and AKT ( $P < 0.05$ ; Figure 5A, 5C). Interestingly, metformin did not inhibit the TAC-induced increase of phosphorylated mTOR and AKT in AMPK $\alpha 2^{-/-}$  mice (Figure 5A, 5C), suggesting that the inhibitory effect of metformin on protein synthesis may rely on AMPK.

## Discussion

This study demonstrated that long-term (6 weeks) administration of metformin, a widely used antidiabetic agent, attenuated pressure-overload-induced cardiac hypertrophy in nondiabetic mice, and the mechanism of this antihypertrophic action was dependent on AMPK activation.

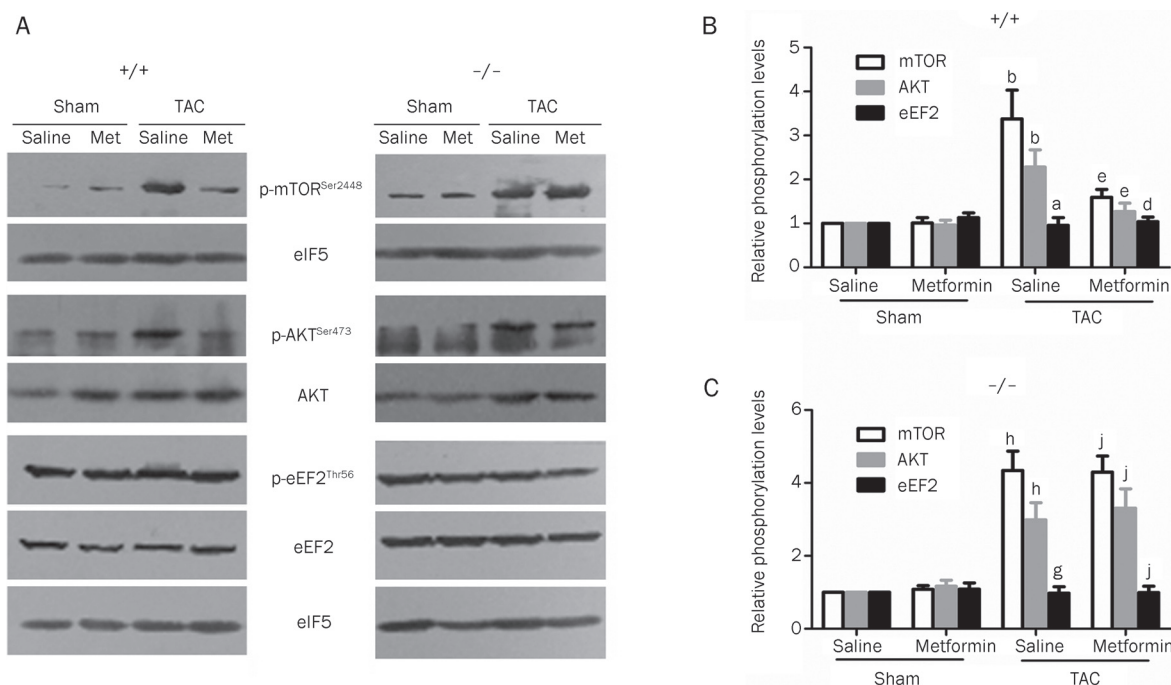
Chan *et al*<sup>[13]</sup> have shown that metformin inhibits cardio-



**Figure 4.** The myocardial AMPK $\alpha$  activity was decreased in AMPK $\alpha 2^{-/-}$  mice. Western blot analysis of myocardial phosphorylated AMPK $\alpha$  (total AMPK $\alpha 1$ , AMPK $\alpha 2$ , and AMPK $\alpha$ ), phosphorylated ACC and eIF5 in WT and AMPK $\alpha 2^{-/-}$  mice 6 weeks after the sham or TAC operation.

myocyte hypertrophy induced by phenylephrine. However, it is still unclear whether metformin has an inhibitory effect on cardiac hypertrophy *in vivo* and what dose is necessary for this inhibitory effect, if it exists. In this study, we used a cardiac hypertrophy mouse model in which cardiac hypertrophy without systolic dysfunction was induced by treatment with TAC for 6 weeks. Metformin treatment (200 mg $\cdot$ kg<sup>-1</sup> $\cdot$ d<sup>-1</sup>) was found to significantly alleviate the cardiac hypertrophy induced by TAC. In the preliminary examination, we also tested metformin at a dosage of 50 mg $\cdot$ kg<sup>-1</sup> $\cdot$ d<sup>-1</sup> in the wild type mice and found that this dosage of metformin had no significant effect on cardiac hypertrophy (date not shown). 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-tumor effects of metformin in the mouse model used a much higher amount of metformin (250–350 mg/kg) due to the difference in drug sensitivity between rodents and humans<sup>[14–16]</sup>. The dosage of 200 mg $\cdot$ kg<sup>-1</sup> $\cdot$ d<sup>-1</sup> that we used in this study was effective on hypertrophy and had no side effect of hypoglycemia. Thus, we believe that the dose of 200 mg $\cdot$ kg<sup>-1</sup> $\cdot$ d<sup>-1</sup> is appropriate.

To demonstrate that metformin has a direct inhibitory effect on cardiac hypertrophy rather than a secondary effect due to lowering pressure overload, we measured the aortic and LV pressure in each group. TAC mice had a marked increase in the SBP and LVSP ( $P < 0.001$ ; Table 1) compared with sham-operated mice. There was no significant difference in the SBP or LVSP (Table 1) between the TAC+saline and the TAC+metformin group. Our study indicated that metformin did not affect pressure overload, and it had a direct inhibitory effect on cardiac hypertrophy. Similarly, a clinical trial also suggested that metformin had only a clinically insignificant



**Figure 5.** The inhibitory effect of metformin on the protein synthesis pathway is dependent on AMPK activation. (A) Western blot of heart extracts for phosphorylated mTOR, phosphorylated AKT, phosphorylated eEF2, total AKT, total eEF2, and eIF5. Quantitative densitometry for mTOR, AKT, and eEF2 phosphorylation in the WT groups (B) and AMPK $\alpha 2^{-/-}$  groups (C). The data are presented as means $\pm$ SEM of 6 animals per group. In the WT groups, <sup>a</sup> $P > 0.05$ , <sup>b</sup> $P < 0.05$  vs sham+saline group; <sup>c</sup> $P > 0.05$ , <sup>d</sup> $P < 0.05$  vs TAC+saline group; in AMPK $\alpha 2^{-/-}$  groups, <sup>e</sup> $P > 0.05$ , <sup>f</sup> $P < 0.05$  vs sham+saline group; <sup>g</sup> $P > 0.05$  vs TAC+saline group.

effect on blood pressure in non-diabetic hypertensives<sup>[17]</sup>.

We previously reported that metformin inhibited cardiac fibrosis in the TAC-mouse model and demonstrated that it inhibited collagen synthesis, likely via inhibition of the TGF- $\beta_1$ -Smad3 signaling pathway<sup>[18]</sup>. Some studies have suggested an important role for TGF- $\beta_1$  in regulating cardiac hypertrophy. Transgenic mice overexpressing TGF- $\beta_1$  have been shown to develop cardiac hypertrophy<sup>[19]</sup>. Conversely, wild-type mice, but not TGF- $\beta_1$ -deficient mice, treated chronically with angiotensin II manifested cardiac hypertrophy<sup>[20]</sup>. However, the effect of TGF- $\beta_1$  signaling on TAC-induced cardiac hypertrophy is still not fully understood. Some studies have shown that a change in the TGF- $\beta$  signaling pathway did not affect TAC-induced cardiac hypertrophy. Kuwahara *et al*<sup>[21]</sup> found that an anti-TGF- $\beta$  neutralizing antibody inhibited myocardial fibrosis, but not myocyte hypertrophy. Another study also found that candesartan (an ACE inhibitor) suppressed the induction of TGF- $\beta$  and fibroblast proliferation in pressure-overloaded hearts, but did not affect myocyte hypertrophy<sup>[22]</sup>. In this study, we focused on AMPK signaling cascades, and we did not investigate whether the TGF- $\beta_1$ -Smad3 signaling pathway was involved in the metformin mediated antihypertrophic effect.

#### The inhibitory effect of metformin on cardiac hypertrophy via AMPK activation

Many studies have recently suggested that the cardioprotective

mechanism of metformin is mediated by AMPK. Metformin protects the heart against ischemia-reperfusion injury through AMPK activation<sup>[7]</sup>. Sasaki *et al*<sup>[8]</sup> demonstrated that metformin inhibited cardiomyocyte apoptosis induced by H<sub>2</sub>O<sub>2</sub> and prevented the progression of heart failure in dogs with the activation of AMPK. Tian *et al*<sup>[23]</sup> reported that the activity of AMPK was increased in rat hearts 12 weeks after TAC operation. Zhang *et al*<sup>[24]</sup> found that AMPK $\alpha 2$  deficiency exacerbated pressure-overload-induced left ventricular hypertrophy and dysfunction in mice. Those findings indicate that AMPK $\alpha 2$  exerts a cardiac protective effect against ventricular hypertrophy and dysfunction triggered by pressure overload. In this study, we found that long-term metformin treatment significantly increased the phosphorylation of AMPK and attenuated cardiac hypertrophy induced by TAC. Interestingly, the antihypertrophic actions of metformin were ablated in AMPK $\alpha 2^{-/-}$  mice. These results suggest that the chronic activation of AMPK during the development of cardiac hypertrophy is a critical mechanism that mediates the beneficial actions of metformin.

Although our data demonstrated that AMPK $\alpha 2$  had a cardiac antihypertrophic effect in TAC-operated mice, we did not find a significant increase in the phosphorylation of AMPK in the TAC+saline group compared with the sham+saline group. Previous reports disagree on this issue. Stuck *et al*<sup>[25]</sup> reported that the phosphorylation of AMPK decreased 30 min after TAC operation, while in the study by Lei *et al*<sup>[26]</sup>,

the phosphorylation of AMPK decreased the second week after TAC, followed by a minor increase by the sixth week. In addition, we did not find that a selective deletion of AMPK $\alpha$ 2 exacerbated the development of TAC-induced cardiac hypertrophy and LV dysfunction. These results were inconsistent with those of Zhang *et al*<sup>[24]</sup>. The reason underlying this discrepancy is not clear; one possible explanation is the degree of aortic constriction by the TAC operation. The wild type mice developed heart failure during the third week after TAC in the work of Zhang *et al*<sup>[24]</sup>. However, in the present study, both AMPK $\alpha$ 2<sup>-/-</sup> and WT mice had normal cardiac systolic function after TAC for 6 weeks. Thus, the degree of aortic constriction in the TAC models in the work of Zhang *et al*<sup>[24]</sup> was greater than that of ours.

AMPK is an endogenous protective protein. AMPK may have no or a minor effect on cardioprotection when the heart is subjected to a mild stimulus. This concept was confirmed by transgenic mouse models in which the AMPK gene was deleted<sup>[27]</sup> or the dominant mutation gene of AMPK was overexpressed<sup>[28]</sup>. These transgenic mice live without abnormalities. AMPK is activated and plays a role in the cardiac protective response to high-intensity stimulation, as demonstrated by many studies<sup>[5, 29, 30]</sup>. Thus, we suppose that the induction of AMPK-related cardiac protective activity is significantly related to the stimulus intensity.

#### Metformin inhibited signaling pathways that regulate protein synthesis through AMPK activation

Cardiac hypertrophy involves an increase in protein synthesis. To investigate the mechanism for the antihypertrophic effect of metformin, we measured the action of metformin on the signaling pathways that regulate protein synthesis. It was reported that AKT/PKB, an important signaling pathway in regulating protein synthesis, regulates the physiological growth of the heart. Akt1-null mice had a 20% reduction in body size, with a concomitant reduction in heart size. These mice were shown to be defective in exercise-induced cardiac hypertrophy<sup>[31]</sup>. Sasaki *et al*<sup>[8]</sup> recently reported that metformin and 5-aminoimidazole 4-carboxamide ribonucleotide (AICAR) inhibited the increase in AKT that was induced by pacing. In the present study, we found that TAC induced a significant increase in the phosphorylation of AKT, which was inhibited by metformin in an AMPK-dependent manner. Although it was also reported that Akt1-null mice developed an exacerbated cardiac dysfunction in response to TAC<sup>[31]</sup>, the decreased phosphorylation of AKT may be involved in the attenuation of hypertrophy.

mTOR can regulate protein synthesis through two pathways. It can activate p70/85 S6 kinase-1 (S6K1) and p54/56 S6K2, which increase ribosomal biosynthesis and protein translation. It also triggers the release of 4E-binding protein-1 from eIF4E; eIF4E can then bind to other initiation factors such as eIF4G, leading to the initiation of translation<sup>[32]</sup>. mTOR has been proposed to regulate pathological hypertrophy of the heart. The inhibition of mTOR by rapamycin was found to attenuate pathological cardiac hypertrophy and reverse myo-

cardial dysfunction<sup>[33]</sup>. Dowling *et al*<sup>[34]</sup> recently reported that metformin inhibited mTOR in a TSC2 and LKB1 dependent manner, which in turn decreased protein synthesis and inhibited cancer cell growth. Another study also demonstrated that metformin inhibited the increase in the phosphorylation of p70s6 kinase induced by phenylephrine *in vitro*<sup>[13]</sup>. Similarly, we found that the phosphorylation level of mTOR was significantly increased in the TAC group, but almost reversed to a normal state by 6 weeks of metformin treatment. This effect of metformin was not observed in AMPK $\alpha$ 2<sup>-/-</sup> mice, suggesting that the metformin-induced mTOR inhibition was AMPK-dependent. It has also been reported that mTOR could be activated by AKT<sup>[35]</sup>. Although both AKT and mTOR phosphorylation were increased in TAC-operated mice in our study, whether mTOR was activated by AKT in the TAC model needs further study to be validated.

AMPK activation can lead to an increase in eEF2 phosphorylation, which inhibits the translocation step during elongation. Chan *et al*<sup>[13]</sup> found that metformin and AICAR inhibited the decrease of eEF2 phosphorylation. However, we did not find a significant decrease in eEF2 phosphorylation in TAC-operated mice hearts, and the level of eEF2 phosphorylation was similar in saline- and metformin-treated groups. These data are in agreement with the findings of Zhang *et al*<sup>[24]</sup>. Therefore, eEF2 may not be involved in TAC-induced cardiac hypertrophy in mice.

#### Conclusion

Our findings in the present study demonstrate that long-term administration of metformin attenuates cardiac hypertrophy induced by pressure overload in nondiabetic mice. The antihypertrophic effect of metformin may be dependent on AMPK activation. Long-term metformin treatment could therefore be a potential therapy for patients at risk of developing pathological cardiac hypertrophy.

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#### Author contribution

You-yi ZHANG and Han XIAO conceived and designed the experiments. Yong-nan FU, Han XIAO, Xiao-wei MA, and Sheng-yang JIANG performed the experiments. Yong-nan FU and Han XIAO analyzed the data. Yong-nan FU, You-yi ZHANG, Han XIAO, and Ming XU wrote the paper.

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