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## Cardiovascular pharmacology

## Chronic treatment with metformin suppresses toll-like receptor 4 signaling and attenuates left ventricular dysfunction following myocardial infarction

Hamid Soraya<sup>a</sup>, Alexander S. Clanachan<sup>b</sup>, Maryam Rameshrad<sup>c</sup>, Nasrin Maleki-Dizaji<sup>c</sup>, Mahmoud Ghazi-Khansari<sup>d</sup>, Alireza Garjani<sup>c,\*</sup><sup>a</sup> Department of Pharmacology, Faculty of Pharmacy, Urmia University of Medical Sciences, Urmia, Iran<sup>b</sup> Department of Pharmacology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Canada<sup>c</sup> Department of Pharmacology and Toxicology, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran<sup>d</sup> Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

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## ABSTRACT

Acute treatment with metformin has a protective effect in myocardial infarction by suppression of inflammatory responses due to activation of AMP-activated protein kinase (AMPK). In the present study, the effect of chronic pre-treatment with metformin on cardiac dysfunction and toll-like receptor 4 (TLR4) activities following myocardial infarction and their relation with AMPK were assessed. Male Wistar rats were randomly assigned to one of 5 groups ( $n=6$ ): normal control and groups were injected isoproterenol after chronic pre-treatment with 0, 25, 50, or 100 mg/kg of metformin twice daily for 14 days. Isoproterenol (100 mg/kg) was injected subcutaneously on the 13th and 14th days to induce acute myocardial infarction. Isoproterenol alone decreased left ventricular systolic pressure and myocardial contractility indexed as  $LV dp/dt_{max}$  and  $LV dp/dt_{min}$ . The left ventricular dysfunction was significantly lower in the groups treated with 25 and 50 mg/kg of metformin. Metformin markedly lowered isoproterenol-induced elevation in the levels of TLR4 mRNA, myeloid differentiation protein 88 (MyD88), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin 6 (IL-6) in the heart tissues. Similar changes were also seen in the serum levels of TNF- $\alpha$  and IL-6. However, the lower doses of 25 and 50 mg/kg were more effective than 100 mg/kg. Phosphorylated AMPK $\alpha$  (p-AMPK) in the myocardium was significantly elevated by 25 mg/kg of metformin, slightly by 50 mg/kg, but not by 100 mg/kg. Chronic pre-treatment with metformin reduces post-myocardial infarction cardiac dysfunction and suppresses inflammatory responses, possibly through inhibition of TLR4 activities. This mechanism can be considered as a target to protect infarcted myocardium.

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## 1. Introduction

Myocardial infarction is a major cause of morbidity and mortality worldwide. One of the main determinants of patients' outcomes following myocardial infarction is left ventricle failure that is characterized by myocardial hypertrophy, necrosis, and left ventricle mechanical dysfunction, ultimately leading to congestive heart failure (Timmers et al., 2008). Myocardial infarction is an important consequence of ischemic heart disease that occurs more often in patients with diabetes mellitus. While short periods of cardiac ischemia impair myocardial energy metabolism and

mechanical function, sustained ischemia/reperfusion initiates profound inflammatory responses that worsen irreversible tissue damage (Frangogiannis et al., 2002; Steffens et al., 2009). Such inflammatory responses are partly activated by toll-like receptors that initiate an intracellular signaling cascade via myeloid differentiation protein 88 (MyD88) activation, nuclear translocation of NF- $\kappa$ B, transcription of pro-inflammatory genes, and increased production of TNF- $\alpha$  and IL-6 (Arumugam et al., 2009; Frantz and Bauersachs, 2010). The role of toll-like receptor 4 (TLR4) in myocardial ischemia/reperfusion are well established (Arslan et al., 2009; Oyama et al., 2004) and activation of TLR4 mediates left ventricular remodeling and contributes to left ventricular dysfunction after myocardial infarction (Timmers et al., 2008).

Metformin is an oral anti-diabetic drug that is commonly prescribed for the treatment of type 2 diabetes. The United Kingdom Prospective Diabetes Study (1998) showed that metformin was

\* Correspondence to: Department of Pharmacology, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.

Tel.: +98 411 3341315, +98 914 3014818; fax: +98 411 3344798.

E-mail addresses: [garjania@tbzmed.ac.ir](mailto:garjania@tbzmed.ac.ir), [garjania2002@yahoo.com](mailto:garjania2002@yahoo.com) (A. Garjani).

associated with a decreased risk of diabetes-related cardiovascular endpoints when compared with conventional therapies. These findings were confirmed by experimental studies that demonstrated metformin improves cardiac function in post-MI heart failure and after ischemia *in vivo* (Legtenberg et al., 2002; Soraya et al., 2012a; Yin et al., 2011), and in hearts from diabetic or non-diabetic subjects (Bhamra et al., 2008; Paiva et al., 2010; Solskov et al., 2008). Thus, it seems that the cardioprotective effects of metformin are not solely due to its anti-hyperglycemic properties (Kirpichnikov et al., 2002; Zhang et al., 2011).

Recent experimental studies suggest that the cardioprotective effects of metformin may be mediated via activation of the stress kinase, AMP-activated protein kinase (AMPK) (Gundewar et al., 2009; Yin et al., 2011). Interestingly, activation of AMPK by metformin prevents endothelial NF- $\kappa$ B activation in response to inflammatory cytokines such as TNF- $\alpha$  (Zhao et al., 2008). We have recently reported that acute administration of metformin suppresses inflammatory markers and inhibits myocardial infarction-associated left ventricular dysfunction (Soraya et al., 2012a, 2012b). The question remains whether chronic administration of metformin, such as that used in diabetic patients, also possesses cardioprotective effectiveness. Since inflammatory processes play a crucial role in myocardial ischemia-reperfusion injury, and also because of reported anti-inflammatory effects of AMPK activation, this study tested the hypothesis that chronic metformin treatment protects the myocardium against isoproterenol-induced infarction by attenuation of TLR signaling. Our objectives were to examine the effects of chronic pre-treatment with metformin on left ventricular dysfunction and inflammatory responses associated with isoproterenol-induced myocardial infarction in rats. To explore the effect of metformin on AMPK activation and its relation to TLR4 signaling, myocardial levels of phosphorylated AMPK, TLR4 mRNA, and MyD88 protein were also measured.

## 2. Materials and methods

### 2.1. Animals

Healthy adult male Wistar rats, weighing  $260 \pm 20$  g were used in this study. Rats were housed in polyethylene cages, six per cage, with food and water available *ad libitum* at controlled ambient temperature of  $22 \pm 1$  °C and  $50 \pm 10\%$  relative humidity under a 12-h light/12-h dark cycle. The study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of Tabriz University of Medical Sciences, Tabriz-Iran and in accordance with the National Institutes of Health Publication No. 85-23, revised 1996.

### 2.2. Chemical reagents

Metformin and isoproterenol were obtained from Sigma Chemicals Co. Phospho-AMPK $\alpha$  (Thr172), AMPK $\alpha$  and MyD88 rabbit monoclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). GAPDH mouse monoclonal antibody, peroxidase-conjugated goat anti-rabbit and rabbit anti mouse secondary antibodies were obtained from Abcam (Cambridge, MA, USA). The other reagents were of a commercial analytical grade.

### 2.3. Experimental protocol

The animals were assigned into 5 groups consisting of 6 rats in each. Rats in group 1 (normal control) received a subcutaneous injection of sterile saline (0.5 ml) and were left untreated for the entire experimental period. Rats in group 2 were received oral administration of saline (twice daily) for 14 days and on the 13th

and 14th days were injected subcutaneously with isoproterenol (100 mg/kg; animals with myocardial infarction without metformin pre-treatment). Rats in the last three groups were treated orally with metformin at 25, 50, 100 mg/kg/12 h for 14 days and then like group 2, to induce myocardial infarction were injected isoproterenol (100 mg/kg, SC) on the day thirteenth for two consecutive days (animals with myocardial infarction plus metformin pre-treatment). The animals in all groups were prepared for hemodynamic measurements, serum collection, and tissue harvesting on day 15. Metformin was dissolved in saline and was gavaged at a volume of 0.25–0.5 ml based on body weight.

### 2.4. Hemodynamic measurements

On day 15, hemodynamic measurements were recorded. Animals were anesthetized with 0.1 ml/100 g body weight of a ketamine, xylazine, and acepromazine mixture (65, 13, 1.5 mg/kg, respectively). After induction of anesthesia when rats no longer responded to external stimuli, a polyethylene cannula (Protex; OD 0.98 mm, ID 0.58 mm) connected to a pressure transducer (PowerLab system; AD Instruments, Bella Vista, NSW, Australia) was inserted into the left common carotid artery to measure arterial blood pressure. Mean arterial blood pressure was calculated from systolic and diastolic blood pressure measurements. To assess left ventricular function, a Mikro-Tip catheter transducer (Millar Instruments, Inc.) was advanced into the lumen of left ventricular to measure the left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), maximum rates of increases and decreases in left ventricular pressure ( $LV dp/dt_{max}$  and  $LV dp/dt_{min}$ ), as well as the rate of pressure change at a fixed left ventricular pressure ( $LV dp/dt/P$ ) (Garjani et al., 2011). All parameters were continuously recorded (PowerLab system, AD Instruments, Bella Vista, NSW, Australia).

### 2.5. Western immunoblot analysis

Western immunoblot analysis was performed as previously described (Kewalramani et al., 2007; Omar et al., 2012) with minor modifications. Myocardial tissue samples were homogenized in lysis buffer containing 50 mM Tris-HCl, 150 mM sodium chloride, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol (DTT), 0.1% sodium dodecyl sulfate (w/v), 1% Triton X-100 (v/v), and protease inhibitor cocktail (Roche, Mannheim, Germany). Insoluble materials were removed by centrifugation of the tissue homogenate at 10,000g for 10 min at 4 °C. The protein concentration of the supernatant was quantified using a Bradford Protein Assay Kit with bovine serum albumin as standard. Samples were mixed with sample loading buffer, and equal amounts of protein (50  $\mu$ g) were loaded into lanes of polyacrylamide-SDS gels using a Mini-PROTEAN<sup>®</sup> Tetra Cell system (Hercules, CA). Gels were electrophoresed and then transferred to an Immobilon-P membrane (Millipore, Billerica, MA). The membranes were blocked in 5% non-fat milk in Tris-buffered saline containing Tween-20 and were probed with primary antibodies raised against phospho-AMPK $\alpha$ , AMPK $\alpha$ , MyD88, as well as GAPDH (1:1000 dilution) overnight at 4 °C. Then the immunoblots were processed with peroxidase-conjugated goat anti-rabbit and rabbit anti-mouse secondary antibodies (1:5000 dilution). After washing, antibodies were visualized using the BM chemiluminescence kit (Roche, Mannheim, Germany). Densitometric analysis of the immunoblots was performed using Image J software (Wayne Rasband, National Institute of Health, USA). Densitometric values for phospho-AMPK $\alpha$  were normalized to total AMPK $\alpha$  and in the case of MyD88 the values were normalized to GAPDH.

## 2.6. Measurement of TLR4 mRNA expression in myocardium by quantitative real-time PCR

TRI reagent was used for total RNA extraction (Sigma Chemicals Co, St. Louis, USA) according to the manufacturer's instructions. Agarose electrophoresis was used to evaluate the integrity of the extracted RNA and optical density measurement (A260/A280 ratio) with a nanodrop instrument (ND 1000, Wilmington, USA) was used to determine the purity of total RNA. Real time PCR was performed by random hexamer primer and RevertAid™ H Minus M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany). All reactions were performed in a total volume of 25 µl containing: 1 µl cDNA, 1 µl primer (100 nM each primer), 12.5 µl 2X Power SYBR Green PCR Master Mix (Applied Biosystems, Foster city, USA), and 10.5 µl RNase/DNase free water. All reactions were performed in triplicate and negative controls as well as no template control (NTC) included in each experiment using the iQ5 optical system (Bio-Rad Laboratories, Inc, Hercules, CA). The cycling conditions were as follow: 1 cycle at 94 °C for 10 min, 40 cycles at 95 °C for 15 s, 53 °C (annealing temperature) for 30 s and 72 °C for 25 s.

The primers were designed for detection of TLR4 gene expression, as given below:

For TLR4,

forward: 5'-AAGTTATTGGTGGTGTCTAG-3';

reverse: 5'-GAGGTAGGTGTTCTGCTAAG-3'.

For 18S rRNA,

forward: 5'-ACACGGACAGGATTGACAGATTG-3';

reverse: 5'-GCCAGAGTCTCGTTCGTTATCG-3'.

TLR4 gene abundance was normalized to 18S as an internal standard gene and interpretation of the result was performed using the Pfaffle method (Pfaffl, 2001).

## 2.7. Measurement of serum and cardiac TNF-α and IL-6 levels by enzyme-linked immunosorbent assay (ELISA)

We quantified cardiac and serum levels of TNF-α and IL-6 with the use of ELISA kits (Rat TNF-α and IL-6 kit, IBL, Hamburg, Germany). Briefly, cardiac tissue samples were homogenized in lysis buffer containing 50 mM Tris-HCl, 150 mM sodium chloride, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol (DTT), 0.1% sodium dodecyl sulfate (w/v), 1% Triton X-100 (v/v), and protease inhibitor cocktail (Roche, Mannheim, Germany). For removing insoluble materials, samples were centrifuged twice at 10,000g for 10 min at 4 °C. The resulting supernatants were used for assay and concentration of the cytokines was expressed as pg/100 mg cardiac tissue or pg/ml of serum.

## 2.8. Statistics

Data are presented as mean ± S.E.M. One way ANOVA was used to make comparisons among the groups. If the ANOVA analysis indicated significant differences, a Student–Newman–Keuls post-test was performed to compare the mean values between the treatment groups and the control group. Differences between groups were considered significant if  $P < 0.05$ .

## 3. Results

### 3.1. Effect of chronic treatment with metformin on hemodynamic responses to isoproterenol-induced myocardial infarction

Mean arterial blood pressure was significantly decreased from  $108 \pm 6$  mmHg in normal control to  $65 \pm 5$  mmHg in the group

with myocardial infarction ( $P < 0.01$ ; Table 1). There was a slight improvement in the mean arterial blood pressure of rats pre-treated with 25, 50, and 100 mg/kg to  $82 \pm 9$ ,  $74 \pm 8$ , and  $71 \pm 8$  mmHg, respectively. Isoproterenol depressed LVSP from  $110 \pm 4$  mmHg in the normal control to  $72 \pm 8$  mmHg in the group that received isoproterenol alone ( $P < 0.01$ ). LVSP depression was significantly lower in the rats that were administered metformin at 25 and 50 mg/kg ( $101 \pm 8$  and  $98 \pm 6$ , respectively;  $P < 0.05$ ) while, pre-treatment with 100 mg/kg of metformin had no effect on the depressed LVSP ( $73 \pm 5$  mmHg vs  $72 \pm 8$  mmHg; Table 1). Isoproterenol alone elevated LVEDP by 81% from  $5.8 \pm 0.8$  mmHg in the normal animals to  $10.5 \pm 2.0$  mmHg, indicating left ventricular debility. LVEDP was as low as healthy normal control rats ( $P < 0.01$ ) in all three metformin treated groups ( $5.3 \pm 0.7$ ,  $4.9 \pm 0.3$ , and  $5.0 \pm 0.1$  mmHg, respectively; Table 1). When compared with the normal control group, both LV  $dP/dt_{max}$  and LV  $dP/dt_{min}$  ( $P < 0.01$ , Fig. 1) as well as LV  $dP/dt/P$  ( $P < 0.05$ , Table 1) were depressed by isoproterenol treatment. As for the changes in LVSP, these indices of myocardial contractility showed marked improvements ( $P < 0.05$ ) in the groups pre-treated with 25 and 50 mg/kg of metformin. Whilst, compared with the group without treatment

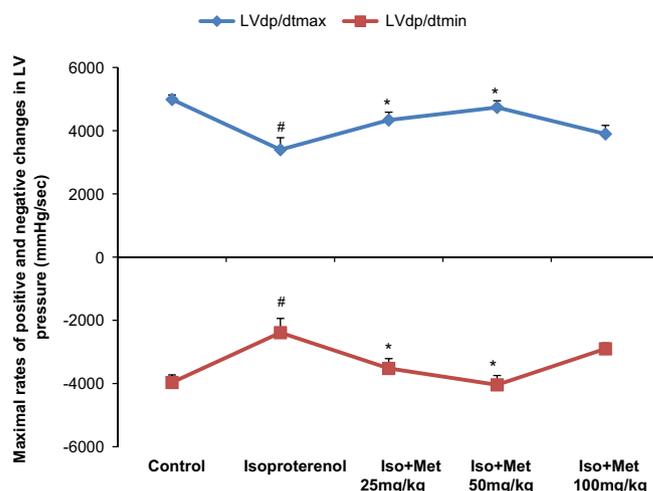
**Table 1**

Hemodynamic changes and left ventricular function in the normal control and in the groups treated with isoproterenol alone or with isoproterenol plus 25, 50, and 100 mg/kg of metformin.

Hemodynamic parameters	Control	Iso	Met (25 mg/kg)+Iso	Met (50 mg/kg)+Iso	Met (100 mg/kg)+Iso
MAP (mmHg)	$108 \pm 6$	$65 \pm 5^a$	$82 \pm 9$	$74 \pm 7$	$71 \pm 8$
Heart rate (bpm)	$240 \pm 7$	$336 \pm 18^b$	$319 \pm 7$	$299 \pm 27$	$266 \pm 23$
LVSP (mmHg)	$110 \pm 4$	$72 \pm 8^a$	$101 \pm 8^d$	$98 \pm 6^d$	$73 \pm 5$
LVEDP (mmHg)	$5.8 \pm 0.8$	$10.5 \pm 2^a$	$5.3 \pm 0.7^c$	$4.9 \pm 0.3^c$	$5.0 \pm 0.1^c$
LV $dP/dt/P$ (1/s)	$85 \pm 4$	$58 \pm 7^b$	$83 \pm 5^c$	$86 \pm 4^c$	$73 \pm 5$

Data are expressed as mean ± S.E.M. Iso: isoproterenol; Met: metformin; MAP: mean arterial pressure; LVSP: left ventricular systolic pressure; LVEDP: left ventricular end diastolic pressure.

- <sup>a</sup>  $P < 0.01$  vs normal control.  
<sup>b</sup>  $P < 0.05$  vs normal control.  
<sup>c</sup>  $P < 0.01$  vs isoproterenol-injected group.  
<sup>d</sup>  $P < 0.05$  vs isoproterenol-injected group.



**Fig. 1.** Maximal rates of positive and negative changes in LV pressure (LV  $dP/dt_{max}$ ; LV  $dP/dt_{min}$ ) in the normal control group and in rats treated with isoproterenol alone or with isoproterenol plus 25, 50, and 100 mg/kg of metformin. Iso: isoproterenol; Met: metformin. Values are mean ± S.E.M. ( $n=6$ ). <sup>#</sup> $P < 0.01$  from respective control value; <sup>\*</sup> $P < 0.05$  as compared with isoproterenol treated group using one way ANOVA with Student–Newman–Keuls *post-hoc* test.

the contractility and relaxation statistically remained unchanged by 100 mg/kg of metformin (Fig. 1).

### 3.2. Effect of chronic treatment with metformin on the levels of TNF- $\alpha$ in the myocardium and serum after isoproterenol-induced myocardial infarction

Compared with normal control group, isoproterenol profoundly increased TNF- $\alpha$  concentration by 240% ( $P < 0.001$ ) in myocardium and by 178% ( $P < 0.001$ ) in serum (Fig. 2). The isoproterenol-mediated increases in the levels of TNF- $\alpha$  were markedly decreased by pre-treatment with metformin, both in the myocardium and in serum. The concentration of TNF- $\alpha$  was considerably reduced from  $212 \pm 14$  pg/100 mg of left ventricular wet tissue in the group with myocardial infarction to  $130 \pm 10$ ,  $84 \pm 8$  ( $P < 0.001$ ), and  $159 \pm 6$  pg/100 mg ( $P < 0.01$ ) in the groups that pre-treated with 25, 50, and 100 mg/kg of metformin, respectively. Similarly, chronic pre-treatment with all three doses of metformin remarkably reduced the serum level of TNF- $\alpha$  from  $587 \pm 37$  pg/ml to  $370 \pm 12$ ,  $266 \pm 21$  ( $P < 0.001$ ), and  $400 \pm 45$  pg/ml ( $P < 0.01$ ), respectively (Fig. 2). However, the high dose of 100 mg/kg was less effective.

### 3.3. Effect of chronic treatment with metformin on the levels of IL-6 in the myocardium and serum after isoproterenol-induced myocardial infarction

Induction of myocardial infarction by isoproterenol has almost tripled the level of IL-6 both in the myocardium and serum ( $P < 0.001$ , Fig. 3). The elevated levels of IL-6 in the myocardium were significantly declined by pre-treatment with all three doses of metformin from  $60 \pm 2.1$  pg/g to  $15 \pm 0.3$  ( $P < 0.001$ ),  $14.6 \pm 0.6$  ( $P < 0.001$ ), and  $20 \pm 0.9$  pg/g ( $P < 0.001$ ), respectively. Serum levels of IL-6 were also reduced from  $117 \pm 10.5$  pg/ml to  $77 \pm 3.9$  ( $P < 0.01$ ),  $82 \pm 8.9$  ( $P < 0.05$ ) pg/ml following pre-treatment with 25 and 50 mg/kg of metformin. While, the high dose of 100 mg/kg statistically did not produce a significant effect ( $93 \pm 7.5$  vs  $117 \pm 10.5$  pg/ml;  $P > 0.05$ ; Fig. 3).

### 3.4. Effect of chronic treatment with metformin on the TLR4 expression in the myocardium after isoproterenol-induced myocardial infarction

To determine potential mechanisms for the metformin-induced inhibition of TNF- $\alpha$  and IL-6 elevation in the heart tissue after

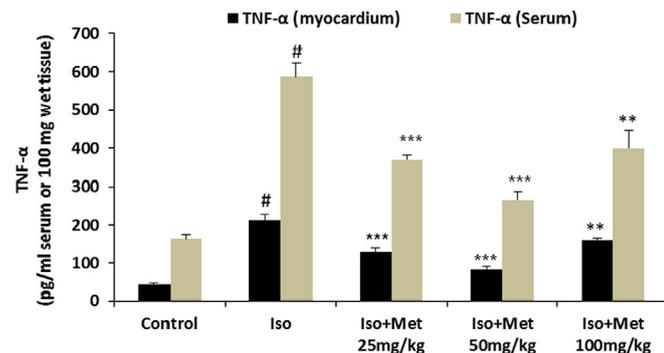


Fig. 2. The effects of chronic pre-treatment with metformin on TNF- $\alpha$  concentration in the heart tissue and in serum. Values are mean  $\pm$  S.E.M ( $n=6$ ) in normal control group (Control) and in groups after isoproterenol-induced myocardial infarction in the absence (Iso) or presence of pre-treatment with graded doses of metformin (25, 50, and 100 mg/kg/12 h for 14 days). Iso: isoproterenol; Met: metformin. # $P < 0.001$  from respective control value; \*\*\* $P < 0.001$ , \*\* $P < 0.01$  as compared with isoproterenol-treated group using one way ANOVA with Student–Newman–Keuls *post-hoc* test.

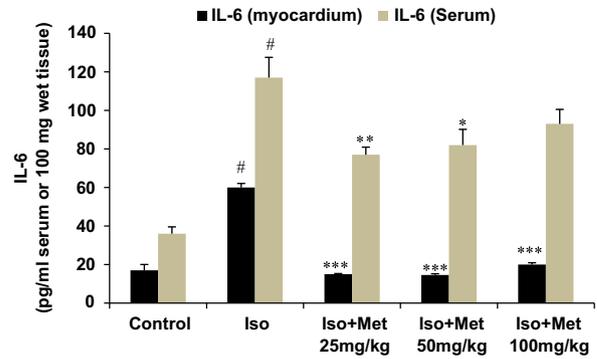


Fig. 3. The effects of chronic pre-treatment with metformin on IL-6 concentration in the heart tissue and in serum. Values are mean  $\pm$  S.E.M ( $n=6$ ) in normal control group (Control) and in groups after isoproterenol-induced myocardial infarction in the absence (Iso) or presence of pre-treatment with graded doses of metformin (25, 50, and 100 mg/kg/12 h for 14 days) in the absence (Iso) or presence of pre-treatment with graded doses of metformin (25, 50, and 100 mg/kg, twice daily for 14 days). Iso: isoproterenol; Met: metformin. # $P < 0.001$  from respective control value; \*\*\* $P < 0.001$ , \*\* $P < 0.01$  and \* $P < 0.05$  as compared with isoproterenol-treated group using one way ANOVA with Student–Newman–Keuls *post-hoc* test.

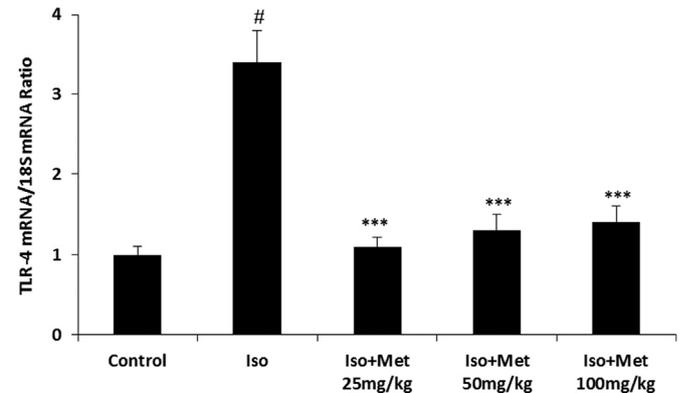


Fig. 4. The effects of chronic pre-treatment with metformin on TLR4 mRNA expression in the heart tissue. Values are mean  $\pm$  S.E.M ( $n=5$ ) for the ratio of TLR4 mRNA to 18S mRNA in the normal control group (Control) and in groups after isoproterenol-induced myocardial infarction in the absence (Iso) or presence of pre-treatment with graded doses of metformin (25, 50, and 100 mg/kg/12 h for 14 days). Iso: isoproterenol; Met: metformin. # $P < 0.001$  vs control, \*\*\* $P < 0.001$  as compared with isoproterenol-treated group using one way ANOVA with Student–Newman–Keuls *post-hoc* test.

myocardial infarction, TLR4 mRNA content in the myocardium were assessed. Real-time quantitative PCR showed that the level of TLR4 mRNA in the myocardium was increased ( $P < 0.001$ ) significantly by isoproterenol (by 170%) compared with the normal control group (Fig. 4). Chronic pre-treatment with all three doses of metformin significantly ( $P < 0.001$ ) abolished the increase of TLR4 mRNA levels (Fig. 4) in the heart. The lowest dose of 25 mg/kg of metformin produced the greatest effect.

### 3.5. Effect of chronic treatment with metformin on MyD88 protein expression in the myocardium after isoproterenol-induced myocardial infarction

MyD88, a universal adapter protein which is critical for many toll-like receptors activities, stimulates NF- $\kappa$ B transcription factor and therefore increases systemic and tissue levels of TNF- $\alpha$  and IL-6. To determine whether chronic treatment with metformin could suppress toll-like receptor activity by MyD88 downregulation, we measured the level of MyD88 protein in the myocardium following isoproterenol-induced myocardial infarction. Compared with the normal control group, isoproterenol profoundly ( $P < 0.001$ ) increased the content of MyD88 protein in the heart by 370%

(Fig. 5). The raise in MyD88 protein was significantly reduced by pre-treatment with all three doses of metformin. Again, the high dose of 100 mg/kg of metformin created minimal effect.

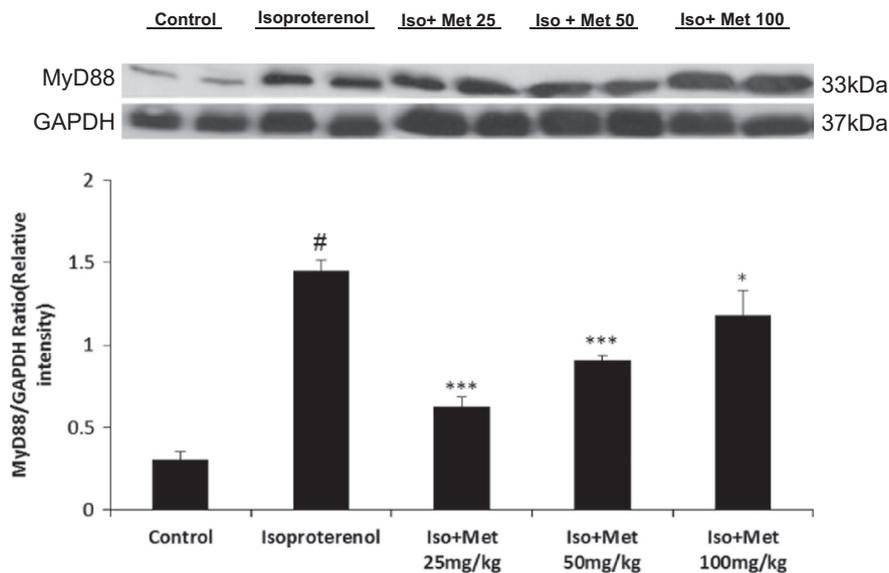
### 3.6. Effect of chronic treatment with metformin on AMPK $\alpha$ phosphorylation in the myocardium after isoproterenol-induced myocardial infarction

Since metformin activates AMPK, a stress kinase that may have anti-inflammatory properties, we determined myocardial AMPK $\alpha$  phosphorylation status. Relative to the normal control group, the ratio of phosphorylated  $\alpha$  subunit of AMPK (at threonine residue 172) to total AMPK $\alpha$  was slightly lowered by isoproterenol (Fig. 6). Phospho-AMPK $\alpha$  in the heart tissue was significantly augmented

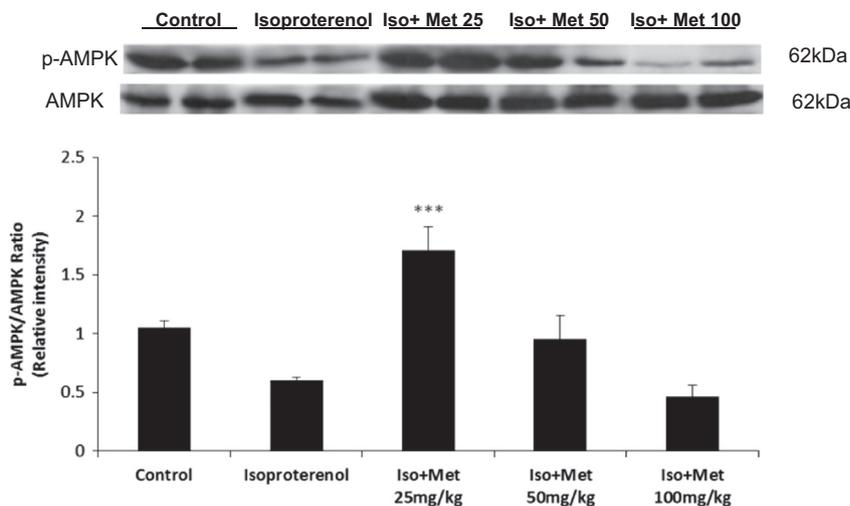
only by 25 mg/kg of metformin ( $P < 0.001$ ). Pre-treatment with metformin at 50 mg/kg also increased the p-AMPK $\alpha$ /AMPK $\alpha$  ratio but, the increase did not attain a significant level. Metformin at 100 mg/kg had no effect on the AMPK phosphorylation.

## 4. Discussion

Our key findings in the present study are: (1) chronic pre-treatment for 14 days with metformin at 25 and 50 mg/kg/12 h, but not 100 mg/kg/12 h, attenuates left ventricular contractile dysfunction in a rat model of isoproterenol-induced myocardial infarction, (2) chronic pre-treatment with metformin reduces serum and heart tissue levels of inflammatory markers, TNF- $\alpha$  and IL-6, which are normally elevated in isoproterenol-induced



**Fig. 5.** The effects of chronic pre-treatment with metformin on MyD88 protein content in the heart tissue. **Upper:** Representative immunoblots of MyD88 protein content in the normal control heart tissues (Control) and in the hearts after isoproterenol-induced myocardial infarction in the absence (Isoproterenol) or presence of pre-treatment with graded doses of metformin (25, 50, and 100 mg/kg/12 h for 14 days). **Lower:** Densitometric analysis of MyD88. Values are mean  $\pm$  S.E.M ( $n=5$ ) for the ratio of MyD88 to GAPDH. Iso: isoproterenol; Met: metformin. <sup>#</sup> $P < 0.001$  vs normal control, <sup>\*\*\*</sup> $P < 0.001$ , <sup>\*</sup> $P < 0.05$  as compared with isoproterenol-treated group using one way ANOVA with Student–Newman–Keuls *post-hoc* test.



**Fig. 6.** The effects of chronic pre-treatment with metformin on the phosphorylation of AMPK at residue threonine 172 in the heart tissue. **Upper:** Representative immunoblots of phosphorylated AMPK $\alpha$  at residue threonine 172 (p-AMPK $\alpha$ ) and total AMPK $\alpha$  following in the normal control hearts (Control) and the hearts with isoproterenol-induced myocardial infarction in the absence (Isoproterenol) or presence of pre-treatment with graded doses of metformin (25, 50, and 100 mg/kg/12 h for 14 days). **Lower:** Densitometric analysis of phosphorylated AMPK<sup>Thr172</sup>. Values are mean  $\pm$  S.E.M ( $n=5$ ) for the ratio of phosphorylated AMPK $\alpha$  to total AMPK $\alpha$ . Iso: isoproterenol; Met: metformin. <sup>\*\*\*</sup> $P < 0.001$  as compared with isoproterenol-treated group using one way ANOVA with Student–Newman–Keuls *post-hoc* test.

myocardial infarction, and (3) beneficial effects of chronic pre-treatment with low doses of metformin are associated with reductions in TLR4 signaling as indicated by depression of myocardial levels of TLR4 mRNA and MyD88 protein. Therefore, these results demonstrated that the chronic pre-treatment with metformin improved cardiac function following an isoproterenol-induced myocardial infarction. The improvement was associated with inhibition of TLR4 activity and reduction of inflammatory responses. Previously, we have reported that short-term administration of metformin, starting along with induction of myocardial infarction by isoproterenol, also produced marked beneficial and dose dependent effects against cardiac dysfunction and toll-like receptor activation (Soraya et al., 2012a, 2012b). The results suggested that administration of metformin immediately after myocardial infarction can be cardioprotective. The question that remains to be answered is whether taking metformin for a long-time before a heart attack may have a protective role. Comparing the results of the present study with that of our previous studies shows that the acute treatment with metformin was much more effective than the chronic pre-treatment. Besides, when a high dose of metformin was used for a long time, prior to incidence of infarction, the cardioprotective effects were reversed.

Metformin is an effective anti-hyperglycemic drug that is commonly used in the management of type 2 diabetes and its anti-hyperglycemic effects are well-established. Experimental studies on the potential beneficial actions of metformin on cardiomyocytes *in vitro* have generally used acute exposures to a wide range of concentrations (50  $\mu$ M to 100 mM) where actions have been noted to be both time-dependent as well as concentration dependent (Fischer et al., 1995; Owen et al., 2000). *in vivo* studies have investigated the cardiovascular consequences of both acute and chronic exposure to metformin and several, including our own (Bhamra et al., 2008; Solskov et al., 2008; Yin et al., 2011) have shown that metformin has protective effects on the cardiovascular system, independent of changes in blood glucose (Whittington et al., 2013). In contrast, other studies have detected no improvement in outcomes with chronic metformin exposures following experimental myocardial infarction (Noyan-Ashraf et al., 2009). Thus, the effectiveness and the precise mechanisms action of metformin on the heart, particularly when administered chronically, are still not fully understood.

Administration of high doses of the  $\beta$ -adrenoceptor agonist, isoproterenol, is a commonly used experimental approach to produce myocardial infarction that is characterized subendocardial ischemia and necrosis (Korkmaz et al., 2009), and leads to infarction-like alterations that are similar to human myocardial infarction. Such treatment, as demonstrated in the present study, causes significant hemodynamic impairment and left ventricular mechanical dysfunction. Using this model, we have shown previously that short-term administration of metformin strongly protects the jeopardized myocardium and completely reverses indices of left ventricular dysfunction and hemodynamic disturbances, with each of the three doses (25, 50 or 100 mg/kg) having equivalent benefits (Soraya et al., 2012a). Although a similar degree of left ventricular contractile dysfunction following isoproterenol administration was observed in the present study, protection by the same doses of metformin when administered chronically (14 days) prior to the isoproterenol challenge was less effective. While changes in the measured indices of left ventricular contractility,  $LVDp/dt_{max}$  and  $LVDp/dt_{min}$ , were similar for acute and chronic treatment protocols, mean arterial blood pressure (MAP) and left ventricular systolic pressure (LVSP) were only partially improved. Unexpectedly, the lowest dose of metformin exerted the greatest benefit while the highest dose was the least effective. It is noteworthy that beneficial effects of metformin were observed in a dose range that approximates the clinical situation (14–35 mg/kg),

whereas the dose level of 100 mg/kg, which is higher than that used clinically, has no beneficial effect.

Isoproterenol-induced myocardial infarction, as well as human myocardial infarction, is accompanied by a marked inflammatory response that contributes to additional myocardial injury (Frangogiannis et al., 2002; Soraya et al., 2012b). We have shown that acute treatment with metformin attenuates the isoproterenol-induced inflammatory response via inhibition of toll-like receptor signaling (Soraya et al., 2012b). Toll-like receptors are an important component of the innate immune system that is expressed on antigen-presenting cells and also on non-antigen-presenting cells such as cardiomyocytes. To date 10 functional toll-like receptors in human and 13 types of toll-like receptors in mice have been identified. Toll-like receptors have been implicated in a number of cardiovascular diseases including myocardial infarction. Endogenous signals (DAMPs) which appear after tissue injury activate toll-like receptors which causes detrimental inflammatory responses (Timmers et al., 2012). Among the toll-like receptor family, the roles of TLR4 and TLR2 in cardiovascular disorders are well established. Several studies show that targeted disruption or deletion of TLR4 and TLR2 or the adapter protein, MyD88, improves cardiac function and reduces infarct size (Arumugam et al., 2009; Arslan et al., 2009; Feng et al., 2008; Oyama et al., 2004). On the contrary, activation of TLR4 signaling induces maladaptive cardiac remodeling and left ventricular dysfunction (Timmers et al., 2008). In this study, using a chronic 14-day metformin treatment protocol, we have determined that metformin retains its ability to suppress isoproterenol-induced activation of toll-like receptor signaling. Inhibition of toll-like receptor signaling by metformin was mediated via both a lower upregulation of TLR4 mRNA by isoproterenol as well as a reduction of the levels of the TLR4 adaptor protein, MyD88. This suggests that suppression of TLR4 activation and inhibition of its consequent inflammatory responses is an effective target for the protection of the heart from post-MI mechanical dysfunction and remodeling. Interestingly, acute treatment with metformin (25, 50 and 100 mg/kg) caused a dose-related inhibition of the isoproterenol-mediated increase in TLR4 mRNA, whereas in the present chronic study, a similar suppression of TLR4 mRNA back to baseline values was seen with each dose. This suggests that maximal benefit is obtained even with the lowest metformin dose and no additional benefits on TLR4 mRNA can be achieved with higher doses.

Isoproterenol-induced myocardial infarction is also accompanied by significant increases in levels of the toll-like receptor adaptor protein, MyD88, which enhances toll-like receptor signaling. Again, while acute metformin treatment causes a dose-dependent decrease in MyD88 (with 25 mg/kg having no significant effect), the dose-response relationship with chronic metformin was markedly different with the lowest dose (25 mg/kg) has the greatest effect, and the highest dose (100 mg/kg) causing only a minor suppression of MyD88 protein. This is in agreement with our earlier observations of a lesser cardioprotective effectiveness, with chronic therapy with high doses of metformin.

Consistent with our results on TLR4 signaling, metformin, particularly at the lowest dose (25 mg/kg), markedly lowered cardiac and serum levels of the pro-inflammatory cytokines, TNF- $\alpha$  and IL-6. TNF- $\alpha$  is recognized as a major contributor to cardiac injury as it promotes apoptosis, stimulates excessive reactive oxygen species (ROS) production and depresses cardiac mechanical function (Nian et al., 2004). Thus, the ability of chronic therapy with metformin to attenuate the elevations in TNF- $\alpha$  and IL-6 following isoproterenol-induced injury suggests their suppression is a key component of metformin-induced cardioprotection. As for the measured indices of toll-like receptor signaling, there is a lack of a clear dose-related suppression of these inflammatory cytokines as beneficial effects are higher with the

lower doses. The basis of this inverse dose–response relationship was not investigated. However, the loss of effectiveness of chronic use of the high dose of metformin, despite almost equal suppression of TLR4 expression by all three doses, may be due to the desensitization of additional downstream pathways involved in the cardioprotective action of metformin. Alternatively, the high doses of metformin elicit diverse effects such as promotion of apoptosis, acceleration of fatty acid oxidation which potentially can be deleterious to the ischemic heart, and excessive inhibition of mitochondrial respiratory-chain complex 1 that indirectly diminishes cardioprotection (Jiang et al., 2014; Viollet et al., 2012; Vecchio et al., 2014).

Numerous studies have identified activation of the stress kinase, AMPK, as a mediator involved in metformin actions (Gundewar et al., 2009; Sasaki et al., 2009). AMPK is a heterotrimeric protein kinase which acts as an intracellular energy sensor. Upon activation, AMPK has been reported to activate ATP-generating catabolic pathways such as glycolysis, glucose uptake, and fatty acid oxidation and to inhibit ATP-consuming anabolic pathways such as protein and glycogen synthesis (Hardie et al., 2006; Paiva et al., 2010). Its role is particularly relevant in tissues with high metabolic demands and little energy reserves, such as cardiac tissue and its role as energy sensor is particularly important during low energy states such as cardiac ischemia and myocardial infarction (Dyck and Lopaschuk, 2006). As cardioprotection arising from AMPK activation has been noted in some (Gundewar et al., 2009; Russell et al., 2004; Solskov et al., 2008), but not all studies (Folmes et al., 2009; Omar et al., 2012) we examined the relationship between metformin-induced AMPK activation and metformin-mediated cardioprotection. While isoproterenol decreased levels of phospho-AMPK by about 50% (possibly due to  $\beta$ -adrenoceptor-mediated increases in glucose and fatty acid availability), phospho-AMPK was increased to levels above baseline by the lowest dose of metformin, while the mid and highest doses of metformin had no significant effect. This inverse dose–response relationship contrasts with that observed with acute metformin dosing where only the highest dose was effective. The absence of any correlation between the effects of chronic metformin on phospho-AMPK and on isoproterenol-induced left ventricular dysfunction suggests that, while the acute beneficial actions of metformin may involve AMPK, the benefits of chronic metformin may no longer be dependent on the activation of AMPK.

## 5. Conclusion

In summary, our findings demonstrate that chronic pre-treatment with a low dose of metformin suppresses TLR4 signaling, inhibits the release of inflammatory mediators and reverses left ventricular contractile dysfunction in the setting of myocardial infarction in an AMPK-independent manner. However, it appears that the cardioprotective effects of metformin against myocardial infarction are time and dose dependent. Suppression of toll-like receptor 4 signaling may be a useful target to protect infarcted cardiac tissue.

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