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

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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. Metfromin markedly lowered isoproterenol-induced elevation in the levels of TLR4 mRNA, myeloid differentiation protein 88 (MyD88), tumor necrosis factor-alpha (TNF-α), and interleukin 6 (IL-6) in the heart tissues. Similar changes were also seen in the serum levels of TNF-α and IL-6. However, the lower doses of 25 and 50 mg/kg were more effective than 100 mg/kg. Phosphorylated AMPKα (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.

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-κB, transcription of pro-inflammatory genes, and increased production of TNF-α 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

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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-kB activation in response to inflammatory cytokines such as TNF-α (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 ± 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 ± 1 °C and 50 ± 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-AMPKα (Thr172), AMPKα 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 10,000*g* 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 μg) were loaded into lanes of polyacrylamide-SDS gels using a Mini-PROTEAN® 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-AMPKα, AMPKα, 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-AMPKα were normalized to total AMPKα and in the case of MyD88 the values were normalized to GAPDH.