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Original Contribution

N-Acetylcysteine and allopurinol up-regulated the Jak/STAT3 and PI3K/Akt pathways via adiponectin and attenuated myocardial postischemic injury in diabetes

Tingting Wang [a,b], Xiaowen Mao [a], Haobo Li [a], Shigang Qiao [a], Aimin Xu [c], Junwen Wang [d], Shaoqing Lei [a], Zipeng Liu [a], Kwok F.J. Ng [a,c], Gordon T. Wong [a], Paul M. Vanhoutte [c], Michael G. Irwin [a,*], Zhengyuan Xia [a,c,**]

[a] Department of Anesthesiology, The University of Hong Kong, Hong Kong SAR, China
[b] Department of Anesthesiology and Critical Care, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[c] Department of Pharmacology and Pharmacy, The University of Hong Kong, Hong Kong SAR, China
[d] Department of Biochemistry, The University of Hong Kong, Hong Kong SAR, China

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ABSTRACT

N-Acetylcysteine (NAC) and allopurinol (ALP) synergistically reduce myocardial ischemia reperfusion (MI/R) injury in diabetes. However, the mechanism is unclear. We postulated that NAC and ALP attenuated diabetic MI/R injury by up-regulating phosphatidylinositol 3-kinase/Akt (PI3K/Akt) and Janus kinase 2/signal transducer and activator of transcription-3 (JAK2/STAT3) pathways subsequent to adiponectin (APN) activation. Control (C) or streptozotocin-induced diabetic rats (D) were untreated or treated with NAC and ALP followed by MI/R. D rats displayed larger infarct size accompanied by decreased phosphorylation of Akt, STAT3 and decreased cardiac nitric oxide (NO) and APN levels. NAC and ALP decreased MI/R injury in D rats, enhanced phosphorylation of Akt and STAT3, and increased NO and APN. High glucose and hypoxia/reoxygenation exposure induced cell death and Akt and STAT3 inactivation in cultured cardiomyocytes, which were prevented by NAC and ALP. The PI3K inhibitor wortmannin and Jak2 inhibitor AG490 abolished the protection of NAC and ALP. Similarly, APN restored posthypoxic Akt and STAT3 activation and decreased cell death in cardiomyocytes. Gene silencing with AdipoR2 siRNA or STAT3 siRNA but not AdipoR1 siRNA abolished the protection of NAC and ALP. In conclusion, NAC and ALP prevented diabetic MI/R injury through PI3K/Akt and Jak2/STAT3 and cardiac APN may serve as a mediator via AdipoR2 in this process.

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Introduction

Myocardial infarction is a major cause of sudden death and is also one of the most common perioperative complications particularly prevalent in diabetes mellitus. Diabetes-induced oxidative stress has been suggested to be the major mechanism contributing to the development and progression of myocardial infarction [1,2]. Restoring blood flow to the ischemic heart is clearly necessary for myocardial salvage. However, reperfusion can further exacerbate myocardial ischemia/reperfusion (MI/R) injury due to aggravated oxidative stress injury. Therefore, intensive research has been focused on the various pathophysiological mechanisms related to MI/R injury and on the development of potential therapeutic strategies. Our previous study found that antioxidants *N*-acetylcysteine (NAC) and allopurinol (ALP) can confer synergy in combating MI/R injury in diabetic rats [3]. However, the underlying mechanism is unclear.

Nitric oxide (NO) synthesized by endothelial nitric oxide synthase (eNOS) represents one of the most important defense mechanisms against MI/R injury. Activation of the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway and the Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) pathway plays critical roles in the activation of eNOS and the

Abbreviations: ALP, allopurinol; APN, adiponectin; C, control; D, streptozotocin-induced diabetic rats; CK-MB, creatinine kinase-MB; eNOS, endothelial nitric oxide synthase; 15-F2t-IsoP, 15-F2t-isoprostane; H/R, hypoxia/reoxygenation; IS, infarct size; LAD, left anterior descending; LDH, lactate dehydrogenase MI/R, myocardial ischemia reperfusion; NAC, *N*-acetylcysteine; NO, nitric oxide; PI3K/Akt, phosphatidylinositol 3-kinase/Akt; JAK2/STAT3, Janus kinase 2/signal transducer and activator of transcription-3

* Corresponding author at: 21 Sassoon Road, 10/F, University of Hong Kong, Li Ka Shing Faculty of Medicine, Laboratory Block, Hong Kong SAR, China. Fax: +852 2819 9791.
** Corresponding author at: Room K424, Block K, Queen Mary Hospital, 102 Pokfulam Road, Hong Kong SAR, China. Fax: +852 2855 1654.
E-mail addresses: zyxia@hku.hk (M.G. Irwin), mgirwin@hku.hk (Z. Xia).

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subsequent attenuation of MI/R injury [4,5]. However, NO is decreased in diabetic myocardium [6,7], potentially due to reduced Akt and STAT3 activation and subsequent reduction of eNOS activation. This makes the diabetic heart more sensitive to MI/R [8,9]. Thus, strategies that can activate Akt and STAT3 in diabetes may also be effective in attenuating MI/R injury. The antioxidant compound H-2693, a modified mexiletine derivative, can attenuate MI/R injury by inducing Akt activation [10]. Similarly, NAC and ALP can restore Akt and STAT3 activation before MI/R in the myocardium of diabetic rats and this was associated with significant attenuation of postischemic myocardial infarction [3]. It is plausible that activation of Akt and STAT3 may represent the major mechanism by which NAC and ALP confer synergy in attenuating MI/R injury in diabetes.

Oxidative stress can down-regulate adiponectin (APN), an adipose-specific plasma protein that possesses insulin-sensitizing, antiapoptotic and anti-inflammatory properties [11,12]. Plasma APN levels are reduced in diabetic patients [13] and STZ-induced diabetic rats [3], which may be the major factor that has rendered the diabetic heart less resistant to ischemic insult given that APN deficiency can lead to increased myocardial damage in response to ischemic insult while APN supplementation increased NO production and attenuated MI/R injury [14–16]. Also, oxidative stress decrease APN production and reduces the activation of both Akt and JAK/STAT, two important signaling pathways involved in MI/R injury [17]. These investigations collectively suggest that antioxidant treatment may activate both Akt and JAK/STAT, potentially in part via APN activation, and attenuate MI/R injury in diabetes.

We have shown that NAC and ALP confer a synergistic effect on restoration of cardiac APN content and APN receptor 2 (AdipoR2) expression with concomitant increase in eNOS activation before MI/R insult, which could be a potential mechanism for enhancing myocardial resistance to MI/R injury [3]. However, since MI/R is the major cause of increased oxidative stress which can cause disruptions in cellular signaling and contribute to tissue injury, whether or not antioxidants can change the postischemic APN content and APN-related signaling pathways as well as the underlying mechanism remains unclear. Therefore, in the present study, we focused on the postischemic pathophysiological changes and using PI3K/Akt or Jak2/STAT3 inhibitors/siRNAs and/or APN adenovirus to test the hypothesis that NAC and ALP confer synergy in reducing MI/R injury in diabetes, primarily by activating PI3K/Akt and Jak2/STAT3 pathways and that APN activation plays a critical role in this process. This hypothesis was tested both in in vivo models of MI/R in STZ-induced diabetic rats and in vitro in cultured rat cardiomyocytes exposed to high glucose and subjected to hypoxia/reoxygenation (H/R).

Materials and methods

Induction of diabetes

Male Sprague-Dawley rats (250 ± 10 g) supplied by the Laboratory Animal Service Center (University of Hong Kong) were used. The experiments were performed after obtaining approval from the Committee on the Use of Live Animals in Teaching and Research (CULATR). Diabetic rats were induced by a single intraperitoneal injection of streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO) as described [3].

Experimental protocol

Rats were randomly divided into five groups:

- Control (C)
- Diabetes (D)
- Diabetes treated with NAC and ALP (D+N+A)
- D+N+A rats intravenously treated with the putative PI3K inhibitor wortmannin (15 μg/kg [18], D+N+A+Wort) 10 min before inducing indexed ischemia
- D+N+A rats intravenously treated with the JAK2 inhibitor AG490 (3 mg/kg [19], D+N+A+AG) 10 min before inducing indexed ischemia.

NAC (1.5 g/kg/day) and ALP (100 mg/kg/day) were dissolved in drinking water for 4 weeks starting at 1 week after induction of diabetes as previous described [3]. Water intake and food consumption were recorded daily, while plasma glucose and body weight were monitored weekly. At termination, rats were weighed and subjected to MI/R as described below. Before and after MI/R, plasma was extracted from blood samples and stored at -80 °C until assay. The ventricular tissue was removed and immediately frozen in liquid nitrogen and stored at -80 °C until being analyzed.

In some experiments, control (C) rats and STZ-induced D rats were untreated or treated with NAC and/or ALP for 4 weeks starting at 5 weeks after induction of diabetes. The rats were then subjected to MI/R and myocardial infarction was determined as described below.

Cardiac function assessment by echocardiography

Echocardiographic studies were performed at termination using a 17.5 MHz liner array transducer system (Vevo 770TM High Resolution Imaging System; VisualSonics) as described [20]. Rats were anaesthetized with 3% isopentane. Left ventricular contractile and diastolic functions were measured as we described [20].

In vivo model of myocardial IRI and myocardial infarct size determination

At the end of treatments, rats in the respective subgroups ($n=7$/group) were anesthetized and subjected to MI/R achieved by occluding the left anterior descending (LAD) artery for 30 min followed by reperfusion for 2 h as we described [3]. Myocardial infarct size (IS) was measured using TTC (1% 2,3,5-triphenyltetrazolium chloride) staining and expressed as a percentage of the anatomic area at risk (AAR). Hemodynamics was monitored as we described [21]. In brief, direct arterial blood pressure monitoring was achieved using a polyethylene catheter that was inserted into the carotid artery. Electrocardiogram (lead II) monitoring was performed via subcutaneous stainless steel electrodes that were connected via a cable to a PowerLab monitoring system (AD Instruments, Colorado Springs, CO). The rate pressure product (RPP) was calculated to evaluate the myocardial oxygen demand of the animals. The in vivo MI/R model of 30 min LAD occlusion and 2 h was chosen based on our preliminary study which showed that the majority of the diabetic rats that we used in the current study could not survive 60 min LAD occlusion and that although the diabetic rats could withstand 45 min of LAD occlusion the postischemic myocardial infarct sizes were much higher than those in the control rats (Fig. 1A) and as such part of the diabetic rats which were subjected to 45 min of LAD occlusion could not survive 2 h of postischemic reperfusion (data not shown).

Measurement of plasma creatinine kinase-MB levels

Creatinine kinase-MB (CK-MB) isoenzyme is a major biomarker for myocardial cellular injury. After 2 h reperfusion, blood samples were collected for measurement of CK-MB by enzyme immunoassay using a commercial kit (Uscn Life Science Inc., Wuhan, China).

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