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Metformin attenuates high glucose-induced cardiomyocytes apoptosis

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Abstract
Intracellular accumulation of excess reactive oxygen species (ROS) have been implicated to play an important role in the pathogenesis of type 2 diabetes (T2D)-associated cardiovascular disease (CVD) including the deleterious effects in the cardiovascular system such as cardiomyocyte apoptosis and necrosis. Thus, in the current study, we explored the cellular mechanisms whereby cardiomyocytes (H9C2) exposed to either with normal glucose (NG, 5.5 mM) or high glucose (HG, 25 mM) and the expressions of cell survival/apoptotic markers were accessed by immuno blotting. These results revealed that HG exposure caused a significant decrease in anti-apoptotic Sirtuin 1 (SIRT1), Bcl-2 expressions with a concomitant increase in the Ac-p53 expression (pro-apoptotic). Interestingly, treatment with the anti-diabetic drug, metformin prevented the deleterious effects (apoptosis) by up-regulating the SIRT1 expression and its downstream signalling in HG-exposed cardiomyocytes. These results indicate that in a diabetic milieu, persistent HG exposure enhances the activation of p53, but down regulates the expression of SIRT1 and Bcl-2 proteins. Furthermore, metformin attenuated the detrimental effects of HG-exposure suggesting that in addition to its anti-hyperglycaemic action, metformin has therapeutic effects in cardiovascular function, which is more beneficial in T2D associated co-morbid conditions.

Keywords: Metformin attenuates, glucose-induced, cardiomyocytes apoptosis, ROS, T2D, CVD

Introduction
Clinical studies revealed that cardiovascular diseases (CVDs) are the major cause for the type 2 diabetes (T2D)-related morbidity and mortality. Particularly, cardiomyocytes apoptosis is an important pathogenic event that significantly plays a critical role in T2D-associated myocardial failure and dysfunction [1]. The elevated level of glucose (hyperglycemia) can cause the production of excessive reactive oxygen species (ROS) and induce the cardiac apoptosis, leading to progression of diabetic heart failure. T2D-associated cardiovascular perturbations including ischemic heart disease, myocardial infarction and cardiomyopathy are often concomitant with cardiomyocytes apoptosis, leading to adverse cardiac consequences [2].
Excessive oxidative stress has been implicated for the induction of apoptosis in cardiomyocytes exposed to high glucose (HG) conditions via mitochondrial cytochrome c-activated caspase 3 pathway (including activation of p53). HG impairs intracellular Ca^{2+} balance, leading to ventricular dilatation and induces systolic dysfunction, all of which affect the contractile function of cardiomyocytes. Previous studies have shown that HG treatment (30 mM) for 24 h leads to a prominent loss of viability in H9C2 cells through the induction of ROS, mitochondrial damage and apoptosis mediated cell death [3, 4].
Silent information regulator 1 (SIRT1), referred to as an anti-ageing gene, has been described as a novel regulatory "switch" in cardiovascular homoeostasis [5]. SIRT1 can deacetylate histones and several transcription regulators in the nucleus as well as specific proteins in the cytoplasm and mitochondria including the inhibition of transcription factors (NF-κB, MMP-9, FOXO3a and p53), eNOS, PGC-1α and AMPK. Results from in-vitro and in-vivo studies also indicate that SIRT1 plays a central role in the regulation of senescence, apoptosis and necrosis (5, 6). Metformin, a biguanide, is the most widely prescribed oral hypoglycaemic drug for the treatment of T2D [5]. SIRT1 activation by metformin significantly attenuates ROS-mediated activation of the transcription factor and regulator of inflammatory responses in vascular endothelial cells [5] and in diabetic rats [7]. However, whether metformin activates SIRT1 expression, thus the attenuation of cardiomyocyte apoptosis is not clearly understood. In the current study, we have tested hypothesis that metformin attenuates the HG-induced cardiomyocytes apoptosis, thus the similar condition seen in T2D.

Methods

Chemicals

Unless otherwise stated all chemicals used were of analytical grade and purchased from Sigma-Aldrich, USA.

Cell Culture

H9C2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). When cell populations reached 40-50% confluence, the cultures were exposed to D-glucose (Sigma) in a final concentration of 5.5 (normal glucose, NG) or 25 mmol/l (high glucose, HG) for 24 h alone and HG exposure in combination with metformin (100 µM).

Immuno-blotting

Immuno-blotting was used to detect the expression of SIRT1, Bcl-2, Ac-p53, p53 and β-actin as previous described [5]. Briefly, cellular protein (50 µg) was electrophoresed on SDS-PAGE gel and transblotted on to nitrocellulose membrane. Membranes were blocked with 5% (w/v) non-fat milk or bovine serum albumin in PBS (phosphate buffered saline) containing 0.1% (v/v) Tween 20 and incubated with the appropriate primary antibody (1:1,000 dilution). After washing, bound antibody was detected using anti-rabbit/mouse antibody (1:2,000 dilution) linked to horseradish peroxidase and bound complexes were detected and documented using enhanced chemiluminescence method (Bio-Rad, USA) and Geliance Imaging system (PerkinElmer, USA).

Statistical Analysis

All data were analysed by statistical software 'GraphPad Prism 5.0' (San Diego, USA). Statistical analysis was performed by using one-way analysis of variance (ANOVA). Post-hoc comparisons between the groups were performed by Tukey's Multiple Comparisons Test. Results are presented as mean ± SEM with $p<0.05$ used to indicate statistical significance.

Results

High Glucose induce apoptosis in cardiomyocytes

To access the effects of HG exposure, cardiomyocytes were exposed with culture media consisting of either NG or HG for 24 h. As shown in Figure 1, HG-exposure significantly increased the expression of Ac-p53 together with a decrease in the expressions of SIRT1 and Bcl-2. This result showed that HG-exposure induce apoptosis in cardiomyocytes.

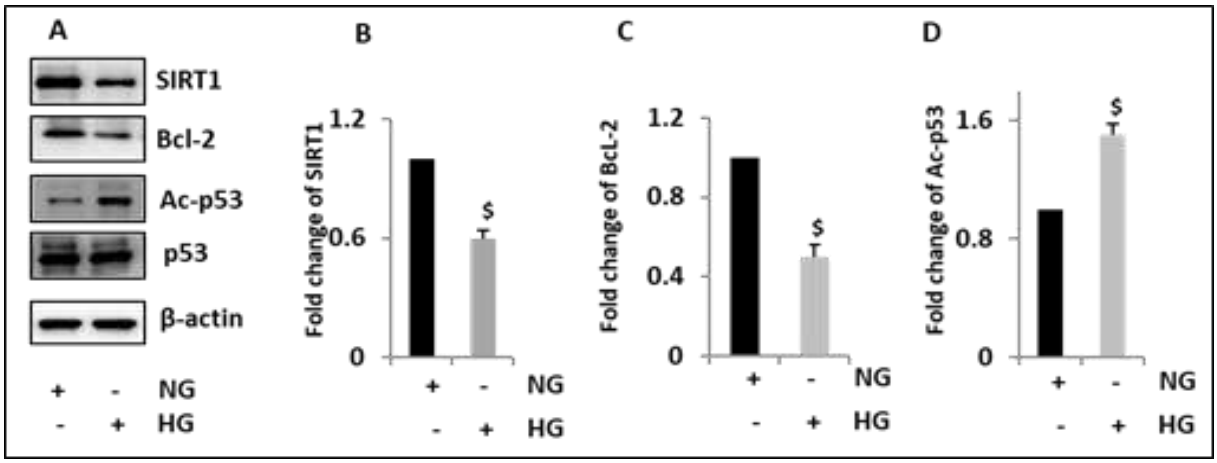

Fig 1: Effects of HG-exposure in cardiomyocytes

Cardiomyocytes cultured either in media consisting of normal glucose (NG, 5.5 mM) or high glucose (HG, 25 mM) for 24 h and SIRT1, Bcl-2, Ac-p53 and p53 protein expression levels were determined by immunoblotting (Panel A). Histograms represent the relative intensity of SIRT1, Bcl-2, Ac-p53 (B-D). Values represent Mean ± SEM (n=3 per group) and $^{\$}(p\leq0.05)$ significant when compared with NG

Metformin attenuates the High glucose induced cardiomyocyte apoptosis

We have previously reported that metformin attenuates HG-induced endothelial senescence and apoptosis via a mechanism that involves the up-regulation of SIRT1 and its downstream signalling pathway in microvascular endothelial cells [5]. However, it is not known whether metformin possesses direct cardio-protective properties. Thus, in the current study we investigated whether metformin can attenuate HG-induced apoptosis in cardiomyocytes. As illustrated in Figure 2, treatment with 100µM metformin significantly decreased the expression of Ac-p53 with a significant increase in the expressions of SIRT1 and Bcl-2 in HG-exposed cardiomyocytes. These results indicate that, metformin significantly attenuates the HG-induced apoptosis by up-regulating the SIRT1 and Bcl-2 expression in cardiomyocytes.

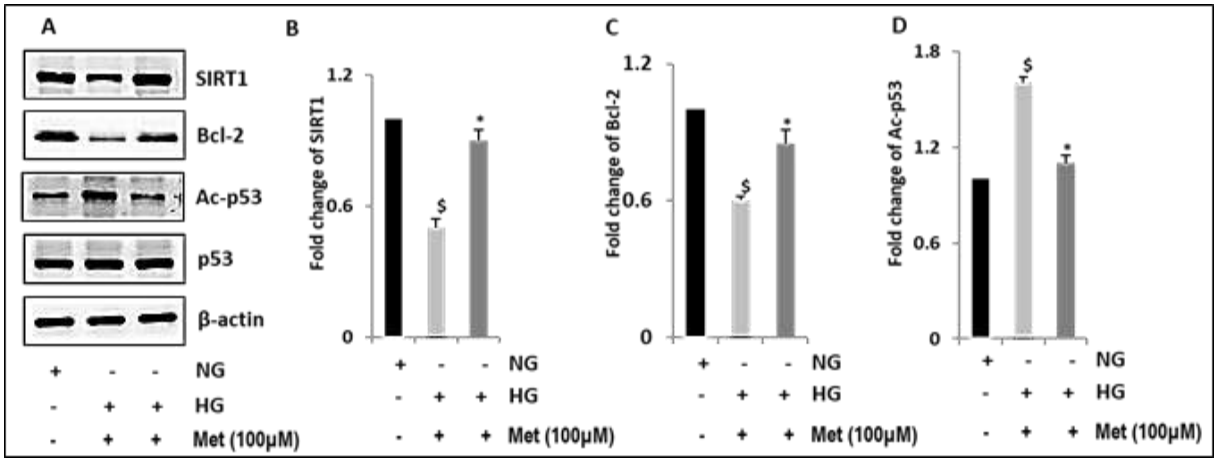

Fig 2: Protective effects of metformin in HG-induced cardiomyocytes apoptosis
Cardiomyocytes cultured either in media consisting normal glucose (NG, 5.5 mM) and high glucose (HG, 25 mM) along with metformin (100μM) for 24h and SIRT1, Bcl-2, Ac-p53 and p53 protein expression levels were determined by immunoblotting (Panel A). Histograms represent the relative intensity of SIRT1, Bcl-2, Ac-p53 (B-D). Values represent Mean ± SEM (n=3) and $($p\leq0.05$) significant when compared with NG. *($p\leq0.05$) significant when compared with HG.

Discussion

Progressive loss and death of cardiovascular cells (cardiomyocytes) through the alteration of molecular signalling pathways has been strongly associated with T2D-mediated CVDs, which is grouped as diabetic heart disease (DHD) [8, 9]. SIRT1 is highly expressed in the heart vasculature and has been shown to function as a deacetylase for a number of transcription factors including FoxO-1, p53 and thus potentially regulates cell differentiation, senescence and survival in response to cellular stress [5, 10, 11]. Metformin has been shown to improve endothelial function and protects the macro-and microvasculature in diabetes via mechanisms that appears to be independent of its hypoglycemic actions [5, 12, 13]. The main finding of the present study is the reduction of HG-induced cardiomyocytes apoptosis by metformin, which is possibly due to activation of SIRT1 and attenuation of apoptotic genes expression.

Apoptosis is the process of programmed cell death, which under physiological conditions, acts as a homeostasis mechanism to eliminate ageing, damaged, or mutated cells [14, 15]. Particularly, a chronic hyperglycaemic state such as diabetes increases the levels of oxidative stress, which has been demonstrated to activate the apoptotic pathways leading to excessive cardiomyocytes apoptosis [16]. In the current study, the cardiomyocytes exposed to HG showed a significant increase in p53 acetylation (pro-apoptotic event) along with reduction in SIRT1 and Bcl-2 expressions (anti-apoptotic event). Previous studies have also revealed a significant role of abnormal Bcl-2 expression during cardiomyocyte apoptosis in myocardial failure, as its expression rate has a direct effect on cardiomyocytes apoptosis and cardiac function. p53 is an important apoptotic associated gene, has been critically involved in the activation of death receptor pathways [17]. Earlier studies evidenced that p53 activation in turn affects the Bcl-2 expression and was direct correlation with induction of cardiomyocytes apoptosis under HG-exposure [17, 18]. Our previous study also demonstrated that hyperglycemia/oxidative stress- associated SIRT1 depletion results in accelerated senescence and apoptosis in vascular endothelial cells [5].

Interestingly, it has been reported that metformin decreases the intracellular ROS production and inhibits diabetes-induced renal hypertrophy via the activation of AMPK [7, 19]. In contrast, Zheng *et al*. have reported that under high glucose conditions, metformin increases SIRT1 level/activity directly, or in part, via the LKB1/AMPK pathway and thereby inhibited the intracellular ROS production, NF-κB activation and Bax-induced apoptosis in diabetic retinal endothelial cells [7].

In our study, we have found a significant decrease in anti-apoptotic Bcl-2 level in HG-exposed cardiomyocytes, that could possibly be due to an increase in p53 acetylation and the decrease in SIRT1 expression during the HG-exposure. However, upon metformin treatment in HG-exposed cardiomyocytes, the expressive levels of SIRT1 and Bcl-2 were significantly increased along with decrease in p53 acetylation, suggesting that metformin-mediated SIRT1 activation may be responsible for the attenuation of apoptotic p53 expression in HG-exposed cardiomyocytes. Activation of SIRT1 in the vasculature has been reported to inhibit pro-apoptotic Bad, caspase-3 activation and thus the apoptosis in HG-exposed cerebral endothelial cells and SIRT1-knockdown in these cells exacerbates HG-induced apoptosis [20, 21, 5].

Collectively, our data demonstrate that HG-induced oxidative stress reduces the SIRT1 and Bcl-2 expression with concomitant increase in p53-medicated cardiomyocytes apoptosis. Treatment with metformin attenuates the adverse effects of HG on SIRT1 expression and thereby reduces the hyperglyceamia-induced apoptosis in cardiomycytes. The results from this study provide molecular insights into the cellular actions of this important oral hypoglycemic drug, whereby metformin can protect the cardiomycytes against the deleterious effects of hyperglycemia via SIRT1-dependent molecular mechanisms thus suggesting new avenues and therapeutic targets for counteracting diabetes-associated CVDs.

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