Metformin protects high glucose-cultured cardiomyocytes from oxidative stress by promoting NDUFA13 expression and mitochondrial biogenesis via the AMPK signaling pathway

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Abstract. Tissue damage in diabetes is at least partly due to elevated reactive oxygen species production by the mitochondrial respiratory chain during hyperglycemia. Sustained hyperglycemia results in mitochondrial dysfunction and the abnormal expression of mitochondrial genes, such as NADH:Ubiquinone oxidoreductase subunit A13 (NDUFA13). Metformin, an AMP-activated protein kinase (AMPK) activator, protects cardiomyocytes from oxidative stress by improving mitochondrial function; however, the exact underlying mechanisms are not completely understood. The aim of the present study was to investigate the molecular changes and related regulatory mechanisms in the response of H9C2 cardiomyocytes to metformin under high glucose conditions. H9C2 cells were subjected to CCK-8 assay to assess cell viability. Reactive oxygen species generation was measured with DCFH-DA assay. Western blotting was used to analyze the expression levels of NDUFA13, AMPK, p-AMPK and GAPDH. Reverse transcription-quantitative PCR was used to evaluate the expression levels of mitochondrial genes and transcription factors. It was observed that metformin protected H9C2 cardiomyocytes by suppressing high glucose (HG)-induced elevated oxidative stress. In addition, metformin stimulated mitochondrial biogenesis, as indicated by increased expression levels of mitochondrial genes (NDUFA1, NDUFA2, NDUFA13 and manganese superoxide dismutase) and mitochondrial biogenesis-related transcription factors [peroxisome proliferator-activated receptor-gamma coactivator-1α, nuclear respiratory factor (nrF)-1, and nrF-2] in the metformin + HG group compared with the HG group. Moreover, metformin promoted mitochondrial NDUFA13 protein expression via the AMPK signaling pathway, which was abolished by pretreatment with the AMPK inhibitor, Compound C. The results suggested that metformin protected cardiomyocytes against HG-induced oxidative stress via a mechanism involving AMPK, NDUFA13 and mitochondrial biogenesis.

Introduction

Diabetic patients have an notably increased incidence of heart failure according to the Framingham study (1); however, the molecular mechanisms underlying the pathogenesis of diabetic cardiomyopathy are not completely understood. Among the hypothesized mechanisms, hyperglycemia-induced oxidative stress is recognized as a critical participant in the pathogenesis and progression of diabetes (2). Increased levels of oxidative stress lead to harmful modifications of macromolecules, including DNA, proteins and lipids (3), which can result in cardiomyocyte apoptosis, hypertrophy and fibrosis, ultimately leading to cardiac remodeling and dysfunction (3). Therefore, developing an effective treatment to suppress increased oxidative stress levels and subsequent cardiomyocyte injury in patients with diabetes is important.

Metformin is widely used as a first-line hypoglycemic drug (4). Previous studies identified cardioprotective effects of metformin beyond its antihyperglycemic effects (5-7). The United Kingdom Prospective Diabetes Study demonstrated that early and intensive metformin intervention could reduce the incidence of myocardial infarction and increase the survival rate in patients with type 2 diabetes (5). Metformin
exerts cardioprotective effects partly by suppressing high glucose (HG)-induced excessive reactive oxygen species production and inflammatory responses (8); however, the exact underlying mechanisms are not completely understood.

AMP-activated protein kinase (AMPK), a key mediator of the downstream effects of metformin, can be activated by cytokines, hormones and oral hypoglycemic agents that are commonly used to treat type 2 diabetes (9). Activated AMPK promotes the production of ATP by regulating glucose and fatty acid metabolism (9), indicating that AMPK may serve as an innate survival mechanism for the heart. For example, AMPK activation during myocardial ischemia reduces the infarct size in the hearts of diabetic model rats (10). In addition, upregulation of AMPK signaling in the vasculature improves microvascular function in the hearts of diabetic model mice (11).

Based on its role in regulating cellular energy status, AMPK is a major regulator of mitochondrial function. Hydroxytyrosol, a natural antioxidant, reduces oxidative stress and improves mitochondrial function, presumably by activating AMPK signaling in the brain of db/db mice (12). Therefore, it was hypothesized that AMPK may serve as a critical modulator of mitochondrial biogenesis and function, and enhance resistance to oxidative stress. However, the exact role and mechanisms underlying AMPK in the cardioprotective effects of metformin in response to oxidative stress are not completely understood.

Mitochondria are the primary energy generating organelles, but are also the main source of ROS (13). NADH:Ubiquinone oxidoreductase subunit A13 (NDUFA13) is an indispensable assembly factor of complex I (14). The primary role of NDUFA13 in the antioxidant effect of resveratrol has been previously reported (15). Downregulated NDUFA13 expression increases mitochondrial ROS generation in H9C2 cardiomyocytes (16), and sustained high glucose decreases NDUFA13 expression levels (15).

Based on the aforementioned studies, it was hypothesized that NDUFA13 may be associated with alleviating oxidative stress in diabetic cardiomyopathy, and metformin may prevent diabetic myocardial injury by suppressing hyperglycemia-induced ROS overproduction. The present study aimed to investigate whether the protective effects of metformin were mediated by promoting mitochondrial biogenesis and NDUFA13 expression. Additionally, the exact role of the AMPK signaling pathway in metformin-induced effects was investigated.

Materials and methods

Cell culture and treatments. H9C2 cells (The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified incubator with 5% CO₂ and 95% air.

H9C2 cells cultured to approximately 80% confluence were then incubated for 12, 24, 36 and 48 h at 37°C. These included 5.5 and 33.3 mM glucose and 25 mM glucose plus 8.3 mM mannitol. According to changes in cell viability, 5.5 mM glucose was selected to mimic normal conditions and 33.3 mM glucose was selected to mimic the diabetic condition. Subsequently, cells were treated with low concentrations of metformin (0.5 or 1 mM; Sigma-Aldrich; Merck KGaA) for 36 h at 37°C. For subsequent experiments, 1 mM metformin was used, which was proven to be an effective in previous studies (17,18). The cardiomyocytes were divided into the following experimental groups: i) Control, medium containing 5.5 mM glucose; ii) HG, medium containing 33.3 mM glucose; iii) mannitol, medium containing 25 mM glucose + 8.3 mM mannitol; iv) metformin 0.5 mM, medium containing 33.3 mM glucose + 0.5 mM metformin; vi) Met 1, medium containing 33.3 mM glucose + 1 mM metformin; and vii) Compound C, medium containing 33.3 mM glucose + 1 mM metformin + 10 µM Compound C (Sigma-Aldrich; Merck KGaA).

Cell Counting Kit-8 (CCK-8) assay for cell viability. Cell viability was assessed using a CCK-8 assay kit (Shanghai Yeasen Biotechnology Co., Ltd.) according to the manufacturer's protocol. H9C2 cells were seeded (5x10⁴ cells/well) into 96-well plates. Subsequently, CCK-8 reagent was added to each well and incubated for 1 h at 37°C. The absorbance of each well was measured at a wavelength of 450 nm using a microplate reader (BioTek Instruments, Inc.).

Lactate dehydrogenase (LDH) release. Cell death was assessed using an LDH cytotoxicity assay kit (Shanghai Yeasen Biotechnology Co., Ltd.) according to the manufacturer's instructions. Following treatment for 36 h, the cell medium was collected. The absorbance of each sample was measured at a wavelength of 450 nm using a microplate reader.

Measurement of ROS, malondialdehyde (MDA) and superoxide dismutase (SOD). Intracellular ROS levels were determined using a ROS assay kit (Shanghai Yeasen Biotechnology Co., Ltd.) according to the manufacturer's protocol. Dichlorofluorescein fluorescence was detected using an Olympus IX71 fluorescence microscope (Olympus Corporation; magnification, x200). MDA levels were assessed using an MDA assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The absorbance of each sample was measured at a wavelength of 535 nm using a microplate reader. SOD activity was measured using a total SOD assay kit (Shanghai Yeasen Biotechnology Co., Ltd.) according to the manufacturer's protocol. The absorbance of each sample was measured at a wavelength of 450 nm using a microplate reader.

Western blotting. The H9c2 cells were lysed with RIPA buffer (Beyotime Institute of Biotechnology) for 45 min at 4°C. Total protein was quantified using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein were separated via 10-12% SDS-PAGE and transferred electrophoretically to PVDF membranes (Invitrogen; Thermo Fisher Scientific, Inc.). The membranes were blocked with Tris buffer (Beyotime Institute of
Biotechnology) containing 0.1% Tween-20 (TBST) in 5% milk for 1 h at room temperature and then incubated over-night with primary antibodies targeted against: nduFa13 (17 kDa; 1:1,000; cat. no. ab110240; Abcam), AMPK (62 KD; 1:1,000; cat. no. 5831; Cell Signaling Technology, Inc.), phosphorylated (p)-AMPK (62 KD; 1:1,000; cat. no. 50081; Cell Signaling Technology, Inc.) and GAPDH (37 Kd; 1:10,000; cat. no. 5174; Cell Signaling Technology, Inc.). Following primary incubation, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000; cat. no. H a1001; HuaBio inc.) for 2 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence system (EMD Millipore). ImageJ software (version 1.41; National Institutes of Health) was used to quantify the bands of each protein and GAPDH was used as the loading control.

Immunofluorescence assay. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.1% Triton X-100 for 10 min. Subsequently, cells were blocked with 3% bovine serum albumin (Beyotime Institute of Biotechnology) for 2 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence system (EMD Millipore). ImageJ software (version 1.41; National Institutes of Health) was used to quantify the bands of each protein and GAPDH was used as the loading control.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA at 42°C for 1 h then 90°C for 5 min using a cDNA synthesis kit (Takara Biotechnology Co., Ltd.). Subsequently, qPCR was performed using SYBR green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 2 min; and 40 cycles of 95°C for 15 sec and 60°C for 1 min. The sequences of the primers used for qPCR are presented in Table I. mRNA expression levels were quantified using the 2^(-ΔΔCq) method (19) and normalized to the internal reference gene β-actin.

Statistical analysis. Data are expressed as the mean ± standard deviation of at least three independent experiments. Comparisons among multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. Statistical analyses were performed using GraphPad Prism software (version 7; GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Metformin protects H9C2 cells from HG-induced cytotoxicity. To investigate the effect of metformin on cardiomyocyte survival in various concentrations of glucose (5.5-50 mM), H9C2 cell viability was assessed. Cell viability was significantly decreased following incubation with 33.3 mM glucose (Fig. 1A) for at least 24 h (Fig. 1B) compared with the control group. Subsequently, cardiomyocytes were pretreated with metformin and then incubated with 33.3 mM glucose for 36 h. Metformin (0.5 and 1 mM) significantly increased

| Gene          | Sequence (5‘→3’) Product size (bp) |
|---------------|------------------------------------|-------------------------------|
| β-actin       | F: GGTCCACCCGGCAGATCAAA            | 118                           |
|               | R: ACATGCCGGAGCCGGTTGC             |                               |
| NDUFA1        | F: TGCTGCCGAAGAGCCGGTGA            | 189                           |
|               | R: TCCTTGGCCTTGGTGTA               |                               |
| NDUFA2        | F: ACTGAGCAGCTGAAACCCACCA          | 175                           |
|               | R: GCGACATCCAGCCGGTACG             |                               |
| NDUFA13       | F: CTAAGGAGAATAATGAGGTGAAC         | 156                           |
|               | R: CCAGGGGCACATCCTTCA              |                               |
| Mn-SOD        | F: GTGTCCTGTGGAGGTCAAGG            | 149                           |
|               | R: TGCTCCACCATCAATCC               |                               |
| PGC-1α        | F: GGGGCACATCTGTCCCTCA             | 156                           |
|               | R: GCTTGACTGGAGTGACCGAA            |                               |
| NRF1          | F: ACAACAGCATGCCCATCTCG            | 226                           |
|               | R: GGTCATTTCCGCGCCGCTGA            |                               |
| NRF2          | F: ACGAAGACTTTGGGGCCACTTA          | 112                           |
|               | R: TCTGGCCTTCTGCTCCTTGG            |                               |

NDUF, NADH: Ubiquinone oxidoreductase subunit; Mn-SOD, manganese superoxide dismutase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; NRF, nuclear respiratory factor.
cell viability (Fig. 1C) and decreased LDH release (Fig. 1D) under HG conditions compared with the HG group. The results indicated that metformin rescued cardiomyocytes from HG-induced injury. Mannitol was used as an osmotic control and did not mimic the effects of 33.3 mM glucose (Fig. 1). For subsequent experiments, 33.3 mM glucose was used to induce a HG situation and 1 mM metformin was applied, unless stated otherwise.
Clinically, the median plasma concentration of metformin is 330 µM (20); however, the concentration of metformin is several times higher in tissues compared with in the blood (20). Therefore, intracellular metformin concentrations are 10-15% of the drug present in the medium (21), meaning the dose used in the present study was higher than the therapeutic dose.

**Metformin suppresses HG-induced oxidative stress in H9C2 cells.** Markers of oxidative stress, including ROS, MDA and SOD, were detected. Compared with the control and mannitol groups, ROS and MDA levels were significantly increased in the high glucose group. However, metformin pretreatment significantly decreased ROS (Fig. 2A and B) and MDA (Fig. 2C) levels compared with the HG group. By contrast, HG significantly inhibited SOD activity compared with the control and mannitol groups, whereas metformin reversed HG-mediated inhibition of SOD activity in H9C2 cells (Fig. 2D).

**Metformin prevents HG-mediated downregulation of NDUFAl3.** To examine the effects of metformin on NDUFAl3 expression, immunofluorescence and western blotting assays were performed. The immunofluorescence results suggested that the expression of NDUFAl3 was significantly decreased in the HG group compared with the control and mannitol groups, but this effect was partly reversed by pretreatment with metformin (Fig. 3A and B). The western blotting results were consistent with the immunofluorescence results (Fig. 3C and D). Mannitol was used as the osmotic control and did not mimic the effects of 33.3 mM glucose (Fig. 3).

**Metformin activates AMPK and induces mitochondrial biogenesis.** The activity of AMPK was evaluated by detecting the phosphorylation of Thr-172 (p-AMPK) via western blotting. p-AMPK expression levels were significantly decreased in the HG group compared with the control group, but metformin reversed HG-mediated effects (Fig. 4A and B).

RT-qPCR was performed to evaluate the effects of metformin on the mRNA expression levels of mitochondrial genes (NDUFAl, NDUFa2, NDUFa13 and Mn-SOD) and transcription factors (PGC-1α, NRF-1 and NRF-2). The results suggested that, compared with the control group, the expression levels of mitochondrial genes related to intracellular ROS production were significantly decreased in the HG group, which was reversed by metformin (Fig. 4C). Therefore, the results provided a potential explanation for the ability of metformin to reduce ROS production. Additionally, the inhibitory effect of HG on mitochondria biogenesis-related transcription factor expression was reversed by metformin pretreatment (Fig. 4D), which may be associated with metformin-mediated promotion of cardiomyocyte survival under high glucose conditions.
Mannitol was used as an osmotic control and did not mimic the effects of 33.3 mM glucose (Fig. 4).

**Compound C reduced the expression of NDUFA13 and mitochondrial biogenesis via suppressing AMPK pathway.** To examine the dependency of AMPK in metformin-induced mitochondrial biogenesis and NDUFA13 expression, a selective AMPK chemical inhibitor (Compound C) was used. The results indicated that metformin-induced AMPK activation, as evidenced by an increase in p-AMPK expression levels, was blocked by pretreatment with Compound C (Fig. 5A and B). Compared with the HG + Met group, mitochondrial gene and mitochondrial biogenesis-related transcription factor expression levels were also significantly decreased in the HG + Met + Comp C group (Fig. 5D and E). In addition, metformin-induced NDUFA13 upregulation reversed by Compound C under HG conditions (Fig. 5A and C). The results suggested that the roles of metformin in NDUFA13 expression and mitochondrial biogenesis were AMPK signaling pathway-dependent.

**Discussion**

In the present study, metformin exerted protective effects on H9C2 cardiomyocytes by suppressing HG-induced oxidative stress, as evidenced by ameliorating HG-induced decreases in cell viability and SOD levels, and increases in ROS and MDA levels. mRNA expression levels of the mitochondrial genes (NDUFA1, NDUFA2, NDUFA13, Mn-SOD) were upregulated by metformin under HG conditions compared with the HG group. Furthermore, under HG conditions, the expression levels of the mitochondrial protein NDUFA13 were increased by metformin compared with the HG group. The results also indicated that mitochondrial biogenesis-related transcription factors (PGC-1α, NRF1 and NRF2) were targets of metformin. The present study identified the role of the AMPK signaling pathway in the mechanism underlying metformin-mediated regulation of NDUFA13 and mitochondrial biogenesis based on the effects of the AMPK inhibitor, Compound C. The results indicated that metformin protected cardiac cells against HG-induced oxidative stress via a mechanism involving p-AMPK, NDUFA13 and mitochondrial biogenesis.

Although the association between mitochondrial biogenesis and ROS production is not completely understood, it has been reported that hyperglycemia-induced ROS impairs mitochondrial biogenesis and mitochondrial function (12,22). Metformin is an orally administered biguanide that is widely used to treat type 2 diabetes (4). The cardioprotective effects of metformin have received increasing attention and may be related to the ability of metformin to regulate oxidative stress; however, the exact mechanism is not completely understood (23).
A previous study identified the role of metformin in mitochondrial biogenesis, which is an endogenous protective response to stress (8). Mitochondrial biogenesis is significantly impaired in cardiomyocytes from db/db mice, as indicated by reduced mitochondrial DNA content and mitochondrial respiratory chain activity (24). Metformin administration promotes mitochondrial biogenesis and improves mitochondrial function in the skeletal muscle and human umbilical vein endothelial cells by activating PGC-1α (25). Activated PGC-1α is a powerful inducer of NRF-1, NRF-2 and mitochondrial transcription factor a, which initiates the expression of nuclear and mitochondrial genes encoding mitochondrial respiratory chain proteins (26). Although the role of metformin in mitochondrial biogenesis has been investigated, the effects of metformin in cardiomyocytes have not been fully elucidated.

The results of the current study suggested that pretreatment with metformin increased the expression of mitochondrial complex I subcomplexes, including NDUFA1, NDUFA2 and NDUFA13, under HG conditions. Additionally, the expression levels of mitochondrial biogenesis-related transcription factors, such as PGC-1α, NRF-1 and NRF-2, and the antioxidant enzyme, Mn-SOD, were regulated by metformin under HG conditions. The results suggested that metformin stimulated cardiomyocyte mitochondrial biogenesis, and may serve a critical role in cellular defense and cell survival responses to HG-induced oxidative stress.

The present study investigated the role of AMPK activation in metformin-mediated effects in cardiomyocytes. AMPK is a highly conserved sensor of cellular energy homeostasis and is the most recognized factor that mediates multiple effects of metformin (27). AMPK activation causes mitochondrial biogenesis (28). Mice expressing a dominant-negative form of AMPK in skeletal muscle are unable to increase mitochondrial biogenesis in response to energy deprivation (29). Activated AMPK stimulates PGC-1α, NRF1 and NRF2, which in turn promote mitochondrial biogenesis (30). In the present study, metformin activated AMPK and enhanced the expression of PGC-1α, NRF1 and NRF2 under HG conditions compared with the HG group. The expression of mitochondrial genes NDUFA1, NDUFA2, NDUFA13 and Mn-SOD was also regulated by metformin. The effects of metformin on expression were reversed by Compound C; therefore, the results suggested that AMPK served an important role in promoting mitochondrial biogenesis and the expression of mitochondrial genes in response to metformin. Additionally, alternative signaling pathways involving AMPK, Mn-SOD and PGC-1α, and those independent of mitochondrial genes should be investigated. Tumor suppressor p53-mediated PGC-1α upregulation suppresses increased levels of oxidative stress via nuclear factor (erythroid-derived 2)-like2-mediated expression of Mn-SOD and γ-glutamyl cysteine ligase without modulating mitochondrial biogenesis (31).
The present study suggested that the expression of the mitochondrial protein NDUFA13, a newly identified accessory subunit of mitochondrial complex I, was increased by metformin under HG conditions. Furthermore, the results indicated that NDUFA13 was regulated by metformin via an AMPK signal-dependent pathway, as evidenced by the inhibition of NDUFA13 induction in HG conditions by Compound C.

NDUFA13 was originally identified as a death activator in tumor cells and was recognized as an indispensable subunit of mitochondrial complex I (32). Elimination of NDUFA13 prevents the assembly and electron transfer activity of complex I, and also influences other complexes in the mitochondrial respiratory chain (14). The role of NDUFA13 in H9C2 cardiomyocytes and the relationship among NDUFA13, metformin and AMPK are not completely understood. However, mutations in the genes encoding subunits of complex I and complex I increase ROS generation (33). NDUFA13 downregulation increases basal ROS generation, which may serve as a survival signal by activating the STAT3/Bcl-2 signaling pathway (34). In the present study, NDUFA13 expression levels were decreased in HG-induced H9C2 cardiomyocytes compared with control H9C2 cardiomyocytes. Therefore, it was hypothesized that reduced expression levels of NDUFA13 in H9C2 cardiomyocytes were responsible for elevated ROS production under HG conditions, which may explain the antioxidant effects of metformin.

Moreover, it was observed that mitochondrial Mn-SOD was upregulated by metformin under HG conditions. Mn-SOD is a major antioxidant mitochondrial enzyme that serves as a primary ROS regulator. Mn-SOD-deficient mitochondria are more susceptible to oxidative stress and exhibit ultrastructural damage (35). The present study demonstrated that increased Mn-SOD expression might be involved in the mechanism underlying metformin-mediated amelioration of oxidative stress.

The present study had several limitations. AMPK kinase inhibitor Compound C can also inhibit vascular endothelial growth factor and bone morphogenetic proteins receptors (36,37). Therefore, further investigations into the effects of AMPK deficiency, for example, are required to verify the results of the present study. In addition, only the H9c2 cell line was used in the present study; therefore, in vivo studies or in vitro studies involving additional cell lines are required to verify the conclusions of the present study.

In conclusion, the present study suggested that metformin protected cardiomyocytes against HG-induced oxidative stress. The results indicated that metformin-mediated protection occurred at least in part via promoting mitochondrial biogenesis and NDUFA13 expression. In addition, the present study indicated that the AMPK signaling pathway was associated with the mechanisms underlying metformin-mediated regulation of NDUFA13 and mitochondrial biogenesis. The results suggested that AMPK may serve as a potential therapeutic target for normalizing mitochondrial function in diabetes, and NDUFA13 may serve as a useful target for designing novel pharmacological approaches to prevent diabetic complications.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
QYZ, LLW and XDL designed the study. YGL, GYW, YZ, MLY and YGB performed the experiments. XDL and YGL drafted the manuscript. MW and XBL helped with the statistical analysis. QYZ, MW and XBL revised the paper. All authors reviewed the manuscript and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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