Amelioration of streptozotocin-induced pancreatic β cell damage by morin: Involvement of the AMPK-FOXO3-catalase signaling pathway

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Abstract. Pancreatic β cells are sensitive to oxidative stress, which is one of the predominant causes of cell damage and the emergence of diabetes. The identification of effective therapeutic strategies to protect pancreatic cells from oxidative stress has increased interest in the screening of antioxidants from natural products. The present study aimed to investigate the protective effects of morin against streptozotocin (STZ)-induced cell damage in a rat insulinoma cell line (RINm5F pancreatic β cells) and to identify the underlying mechanisms. The results indicated that morin inhibited the increase in intracellular reactive oxygen species, attenuated the activity of poly (ADP-ribose) polymerase, restored intracellular nicotinamide adenine dinucleotide levels and reduced the apoptotic cell death of STZ-treated pancreatic β cells. Treatment with morin significantly upregulated catalase in pancreatic β cells, and ameliorated the STZ-induced loss of catalase at the genetic, protein and enzymatic level. In further experiments, morin induced the phosphorylation of 5' adenosine monophosphate-activated protein kinase (AMPK), which subsequently promoted the translocation of forkhead box O3 (FOXO3) to the nucleus. Specific small interfering RNAs (siRNAs) against AMPK and FOXO3 suppressed morin-induced catalase expression. Furthermore, catalase-specific siRNA abolished the protective effects of morin against STZ-stimulated cell death. Taken together, these results indicated that morin protected RINm5F cells from STZ-induced cell damage by triggering the phosphorylation of AMPK, thus resulting in subsequent activation of FOXO3 and induction of catalase.

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Introduction

Previous studies have revealed that oxidative stress is associated with almost every aspect promoting diabetes and its complications, thus suggesting the importance of antioxidant therapy in the development of novel treatments for this disease (1,2). Particularly, pancreatic β cells are vulnerable to oxidative stress, as they have a high metabolic activity and low antioxidant capacity. Once the overproduction of reactive oxygen species (ROS) exceeds the available antioxidant defense systems, it may result in cellular membrane, protein, RNA and DNA damage, dysregulation of ROS signaling pathways and activation of inflammatory responses, which may eventually lead to the dysfunction or apoptosis of pancreatic islet β cells (3). Scavenging of ROS by antioxidants and the re-establishment of redox homeostasis in pancreatic cells offers a promising strategy to alleviate the suffering of patients with diabetes. Notably, ROS neutralization by antioxidants has not yielded the expected outcomes (4). Numerous studies have suggested that it would be more beneficial to target the pathways involved in ROS generation (5,6). Therefore, the identification of antioxidants and the characterization of their associated signaling pathways are critical for future antidiabetes drug design.

2',3,4',5,7-Pentahydroxyflavone (morin) has exhibited novel activity in reducing ROS production and increasing the antioxidative capacity of cells; therefore, it has received increasing attention with regards to its clinical applications. Morin is a dietary flavonol found in fruits or dietary plants, including figs, apples, tea and cereal grains (7,8). Morin treatment has not exhibited any lethal toxicity to experimental animals, even at high doses (9,10). In addition, the beneficial effects of morin against oxidative stress-induced cell damage have been demonstrated in cardiovascular cells (11), hepatocytes (12) and neurons (13) . Morin is effective in retaining the normal histological appearance of pancreatic islets, as well as in preserving insulin-positive β cells in streptozotocin (STZ)-induced diabetic rats (14). Furthermore, morin has been reported to increase protein expression and enzyme activity of catalase in lung fibroblast cells (15). However, in pancreatic cells, the detailed molecular mechanisms responsible for the protective effects of morin have yet to be elucidated.

Increasing evidence has indicated that the 5' adenosine monophosphate-activated protein kinase (AMPK) pathway exerts protective effects against oxidative stress-induced cell damage (16-18). The AMPK pathway is a key energy metabolic signaling pathway involved in ROS regulation (19). It has been reported that AMPK activation can promote cell survival by inducing autophagy, mitochondrial biogenesis and the expression of genes, which are involved in antioxidant defense in response to oxidative stress (20,21). Forkhead box O (FOXO) transcription factors are prime candidates that are regulated by AMPK. Previous studies have revealed the pivotal roles of FOXO3 in cell resistance to oxidative stress and highlighted the clinical potential to develop novel therapeutic strategies through approaches that target FOXO3 (22,23). Inactivation of FOXO3 and its downstream target genes contributes to oxidative stress in early diabetic nephropathy, and accelerates renal disease (24). In cardiomyocytes, activated FOXO3 may increase downstream target antioxidant enzymes, including catalase, effectively manage ROS and alleviate diabetic cardiomyopathy (25,26). Regulating the transcription of antioxidant enzymes based on targeting the AMPK-FOXO3 signaling pathway has been suggested as a promising approach to limit the progression of oxidative stress-mediated diseases and has merited intensive investigation (16,27,28).

Although there is some evidence to suggest that the antidiabetic effects of morin may be due to its capacity to decrease oxidative stress, few studies have focused on its cytoprotective effects on pancreatic β cells and the underlying molecular mechanism. Due to the potential role of the AMPK-FOXO3 pathway in protecting cells against oxidative stress, it is of great interest to determine whether morin may activate this pathway, and thus upregulate the antioxidant enzyme catalase. Therefore, the present study aimed to investigate the protective effects of morin against STZ-induced β cell damage and to identify the underlying molecular mechanism. The purpose of the present study was to characterize a potential antioxidant with precise targeting for the development of novel therapeutic strategies for the treatment of diabetes and associated complications.

Materials and methods

Reagents. Morin, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), MTT, Hoechst 33342 and STZ were all purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Catalase (sc-271803) and β-actin (sc-8432) primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Phosphorylated-AMPKα (Thr-172; cat. no. 2531), AMPKα (cat. no. 5831) and FOXO3 (cat. no. 2497) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-TATA binding protein (TBP; ab818) antibody was obtained from Abcam (Cambridge, MA, USA). Anti-poly (ADP-ribose) (PAR; cat. no. 4335-MC-100) antibody was purchased from Trevigen (Gaithersburg, MD, USA). All other chemicals and reagents were of analytical grade.

Cell culture. RINm5F rat pancreatic β cells were obtained from the American Type Culture Collection (Manassas, VA, USA). All the cells used in this study were maintained at 37˚C in a humidified atmosphere containing 5% CO_2, and were cultured in Roswell Park Memorial Institute-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, streptomycin (100 μg/ml) and penicillin (100 U/ml).

Determination of the toxicity of STZ. Cells were seeded into 96-well plates at a density of $1x10^5$ cells/ml. After 16 h, cells were incubated for 24 h with various concentrations of STZ dissolved in 0.1 M citrate buffer (pH 4.5); the final concentrations were as follows: 1, 2, 4, 6, 8 and 10 mM. The toxicity of STZ on RINm5F cells was examined using the MTT assay, which is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenase in viable cells (29).

Cell viability assay. To evaluate the effects of morin on STZ-induced cell death, cell viability was examined using the MTT assay. Cells were seeded into 96-well plates at a density of $1x10^5$ cells/ml. After 16 h, the cells were treated with various concentrations of morin (10, 25 and 50 μM) for 1 h, followed by exposure to STZ for 24 h at 37˚C. Subsequently, 50 μl MTT stock solution (2 mg/ml) was added to each well and incubated for a further 4 h at 37˚C. The medium containing MTT was aspirated and the formazan crystals in the viable cells were then dissolved using 150 μl dimethyl sulfoxide. The absorbance of each well was determined using a Multiskan GO Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 490 nm.

Determination of intracellular ROS. Levels of intracellular ROS were determined using the DCFH-DA method as previously described (15). Briefly, RINm5F cells were seeded in 6-well plates at $1x10^5$ cells/ml. After 16 h, the cells were treated with 25 μM morin for 1 h. Following exposure to STZ for 12 h, cells were collected and incubated with 10 μM DCFH-DA at 37˚C for 30 min. Subsequently, the cells were rinsed twice and resuspended in PBS, and fluorescence intensity was measured by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). The data were analyzed using CellQuest (version 7.5.3; BD Biosciences). The relative fluorescence intensity was recorded as the mean value from three repeated experiments. Image analysis for the generation of intracellular ROS was also conducted. Briefly, cells were seeded onto cover slips in 6-well plates at $1x10^5$ cells/ml and were incubated with 5 μM DCFH-DA for 30 min at 37˚C as previously described. After washing with PBS, the stained cells were mounted onto a microscope slide in mounting medium (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). Microscopic images were captured under a laser confocal microscope (Zeiss GmbH, Jena, Germany).

Determination of cellular nicotinamide adenine dinucleotide (NAD^+). Intracellular NAD^+ content was measured using the EnzyChrom™ NAD^+/NADH quantification kit (BioAssay Systems, Hayward, CA, USA), according to the manufacturer's protocol.

Detection of sub-G_1 hypodiploid cell. The proportion of apoptotic sub-G_1 hypodiploid cells was determined by flow cytometry (30). Cells were initially treated with 25 μM morin for 1 h. Subsequently, STZ was added and cells were incubated for a further 24 h. Harvested cells were washed twice

with PBS and fixed in 70% ethanol for 30 min at 4˚C. Cells were then rinsed twice with PBS and were incubated for 30 min in the dark at 37˚C in 1 ml PBS containing 100 μg propidium iodide (PI) and 100 μg RNase A. Flow cytometric analysis was performed using a flow cytometer (FACSCalibur; BD Biosciences). The proportion of sub-G_1 hypodiploid cells was assessed using histograms generated by the computer programs CellQuest (version 7.5.3; BD Biosciences) and ModFit (version 3.0; Verity Software House, Inc., Topsham, ME, USA).

Nuclear staining with Hoechst 33342. Cells were plated in 24-well plates at 1x10^5 cells/ml. After 16 h, cells were treated with 25 μM morin for 1 h and then with STZ at a final concentration of 6 mM for 24 h. Subsequently, a DNA-specific fluorescent dye, Hoechst 33342, at a final concentration of 15 μg/ml, was added to each well and incubated for 10 min at 37˚C. The stained cells were then observed using a fluorescence microscope to examine the extent of nuclear condensation.

Quantitative polymerase chain reaction (qPCR) analysis. Total RNA was isolated from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA synthesis was performed with 2 μg isolated RNA using SuperScript III Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR was performed in 96-well optical plates using a CFX-96 Touch™ Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PCR reaction was conducted in a total volume of 20 μl: 10 μl 2X SYBR *Premix Ex Taq* II kit (Takara Bio, Inc., Otsu, Japan), 1 μl diluted template cDNA (~10 ng) and 10 μM each forward and reverse primers. The PCR protocol was conducted as follows: Pre-incubation at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec and annealing and extension at 60˚C for 34 sec. Primers used in the present study were as follows: β-actin, forward 5'-GAAGATCCTGACCGAGCGTG-3', reverse 5'-CGAAGTCTAGGGCAACATAGCA-3'; and catalase, forward 5'-AGGTGCTTTTGGATACTTTGAGG-3' and reverse, 5'-CGACTGTGGAGAATCGGACGG-3'. The quantification cycle (Cq) method was employed to evaluate relative alterations in gene expression and the $2^{-\Delta\Delta Cq}$ value was calculated after β-actin normalization (31).

Western blot analysis. Cells were rinsed twice with PBS and lysed in 100 μl lysis buffer [40 mM Tris (pH 8.0), 120 mM NaCl, 0.1% NP-40] on ice for 30 min, after which they were centrifuged at 13,000 x g for 15 min at 4˚C. Supernatants were collected and total protein concentrations were determined using the bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Haimen, China). Aliquots (40 μg total protein) of the collected supernatants were boiled for 5 min, separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk for 1 h, and then incubated with primary antibodies (targeting catalase (1:1,000), β-actin (1:5,000), p-AMPKα (1:1,000), AMPKα (1:1,000), FOXO3 (1:1,000), TBP (1:2,000), and PAR (1:1,000) overnight at 4˚C), washed with TTBS, and incubated with horseradish peroxidase-conjugated anti-immunoglobulin-G secondary antibodies (cat. nos. 31430 and 31460; 1:5,000 Pierce; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. The blots were visualized using an enhanced chemiluminescence western blotting detection kit (GE Healthcare Life Sciences, Little Chalfont, UK).

Nuclear extract preparation. Cells were seeded in culture dishes at a density of 1.5x10^5 cells/ml for 16 h. The cultured cells were then treated with morin for a series of time periods. Cells were harvested and lysed on ice with 1 ml lysis buffer [10 mM Tris-HCl (pH 7.9), 10 mM NaCl, 3 mM $MgCl_2$ and 1% NP-40] for 4 min. Cell pellets were collected by centrifugation at 3,000 x g for 10 min at 4˚C, suspended in 5 μl extraction buffer [20 mM HEPES (pH 7.9), 20% glycerol, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT and 1 mM PMSF] and incubated on ice for 30 min. The extraction solution was centrifuged at 13,000 x g for 5 min at 4˚C, and the supernatants were harvested as nuclear protein extracts for further blotting analysis and stored at -70˚C.

Catalase activity assay. Cells were seeded into Petri dishes at a density of 1.5x10^5 cells/ml. After 16 h, cells were treated with morin for a series of time periods. Catalase activity was determined using a catalase assay kit (S0051; Beyotime Institute of Biotechnology) according to the manufacturer's protocol. In this assay, catalase reacts with hydrogen peroxide (H_2O_2) to produce water and oxygen; unconverted H_2O_2 is then converted to a chromogenic product, which is measured at 520 nm using a spectrophotometer. Catalase activity is presented as U/mg, and 1 unit catalase activity is defined as the amount of enzyme catalyzing the degradation of 1 μmol H_2O_2/min under the conditions of 25˚C and pH 7.0. In order to detect the effects of morin on STZ-induced suppression of catalase activity, cells were pretreated with morin for 1 h, and were then incubated with STZ for 24 h. Catalase activity was assessed using the aforementioned catalase assay kit.

Transient transfection of small interfering RNA (siRNA). Cells were seeded at 1.5x10^5 cells/ml in 24-well plates and reached ~50% confluence prior to transfection with AMPK siRNA (siAMPK), FOXO3 siRNA (siFOXO3) (both from Invitrogen; Thermo Fisher Scientific, Inc.), catalase siRNA (siCatalase) or control mismatched siRNA (both from Santa Cruz Biotechnology, Inc.). Cells were transfected with 10-50 nM siRNA using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of 24 h post-transfection, cells were treated with or without morin and/or STZ for 24 h and were subjected to western blot analysis and MTT assay.

Statistical analysis. All experiments were performed in triplicate and all values are presented as the means ± standard error of the mean. The results were analyzed by one-way analysis of variance in the SigmaStat 3.5 and the post hoc Tukey's test was used to assess statistical significance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cytotoxicity of STZ in RINm5F cells. The effects of various concentrations of STZ on RINm5F cell viability are presented

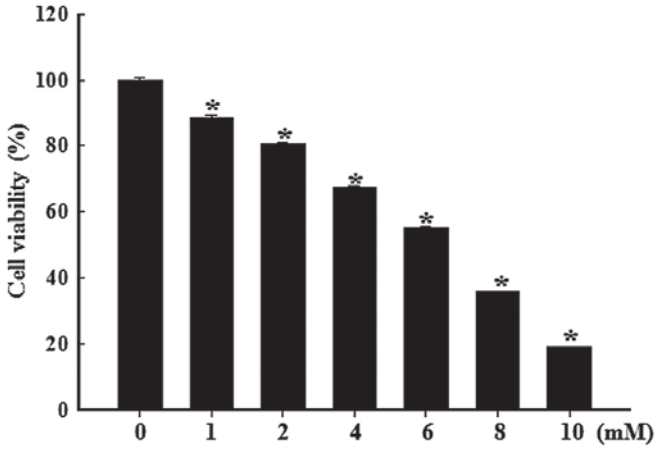

Figure 1. Toxic effects of STZ on RINm5F cells. RINm5F cells were treated with various concentrations of STZ and cell viability was determined after 24 h by MTT assay. Data are presented as the means ± standard error of the means from three experiments. *P<0.05 vs. control cells. STZ, streptozotocin.

in Fig. 1. A dose-dependent decrease in cell viability was observed when cells were exposed to various concentrations of STZ for 24 h. Following treatment with 6 mM STZ, ~50% inhibition of RINm5F cell viability occurred, and at a concentration of 10 mM, cell viability was markedly reduced to 20%. Based on these results, 6 mM was selected as the appropriate dose of STZ for further experiments.

Effects of morin on the viability of STZ-damaged RINm5F cells. The protective effects of morin on the viability of STZ-treated cells were measured using the MTT assay. As shown in Fig. 2A, exposure to 6 mM STZ for 24 h markedly reduced the viability of RINm5F cells, whereas pretreatment with morin at 10, 25 and 50 μM for 1 h resulted in amelioration of cell viability. Pretreatment with 25 μM morin exhibited the highest efficiency with regards to the protection of RINm5F cells. In addition, treatment with various concentrations of morin alone did not exhibit any adverse effects on cell viability (Fig. 2B); therefore, morin at 25 μM was chosen as the appropriate dose for subsequent experiments. These results indicated that morin may be capable of protecting RINm5F cells from STZ-induced cell damage.

Effects of morin on intracellular STZ-induced ROS generation in RINm5F cells. To confirm the effects of morin on STZ-induced ROS production, ROS fluorescence intensity was detected by flow cytometry. The results of flow cytometric analysis revealed that STZ increased ROS generation; however, pretreatment with morin ameliorated the STZ-stimulated increase in ROS content (Fig. 3A). In addition, intracellular ROS levels were observed under a fluorescence microscope. As presented in Fig. 3B, exposure to STZ for 12 h markedly increased red fluorescence intensity in RINm5F cells, whereas pretreatment with morin reduced red fluorescence intensity upon STZ treatment, thus reflecting a reduction in ROS generation. These results indicated that morin significantly decreased STZ-induced elevated ROS levels, thus suggesting that morin conferred resistance to oxidative stress by suppressing the increase in intracellular ROS levels.

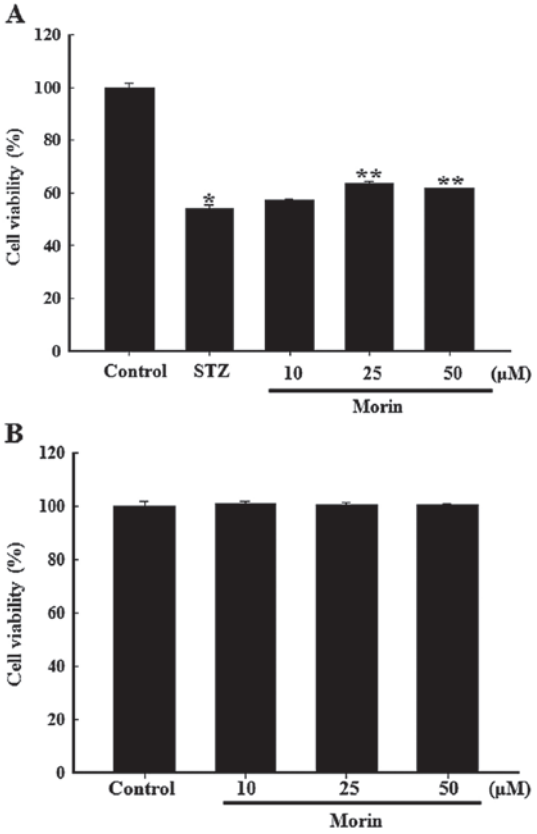

Figure 2. Effects of morin on the viability of STZ-treated RINm5F cells. (A) Cells were treated with morin at various concentrations. After 1 h, 6 mM STZ was added to the cells and cell viability was determined after 24 h by MTT assay. *P<0.05 vs. control cells; **P<0.05 vs. STZ-treated cells. (B) Cells were treated with various concentrations of morin. Following a 24 h incubation, cell viability was determined by MTT assay. STZ, streptozotocin.

Effects of morin on STZ-induced alterations in PAR polymerase (PARP) activity and NAD$^+$ levels. PARP is known to be activated under conditions of oxidative stress and has been well demonstrated in the STZ-induced model of diabetes (32). PARP activity may be examined by western blotting of the PAR protein, as previously described (33). As shown in Fig. 4A, the expression levels of PAR were markedly increased in STZ-treated cells compared with in the control cells, whereas pretreatment with morin inhibited STZ-induced PAR protein expression. As an important substrate for PARP, NAD$^+$ is a crucial component in the modulation of cellular metabolism. Therefore, intracellular NAD$^+$ levels were measured in the present study. As expected, pretreatment with morin significantly ameliorated the loss of NAD$^+$ levels, which was induced by STZ treatment (Fig. 4B). These results indicated that PARP may be activated in response to STZ stimulation, which was accompanied by NAD$^+$ depletion; however, morin effectively reversed the increase in PARP activity and the depletion of NAD$^+$ levels in STZ-treated cells.

Effects of morin on STZ-induced apoptosis of RINm5F cells. The protective effects of morin against STZ-induced apoptotic cell death were assessed. As shown in Fig. 5A, the combination of morin and STZ markedly increased cell survival rate compared with in STZ-treated cells. A previous study reported that STZ treatment stimulated pancreatic β cells to

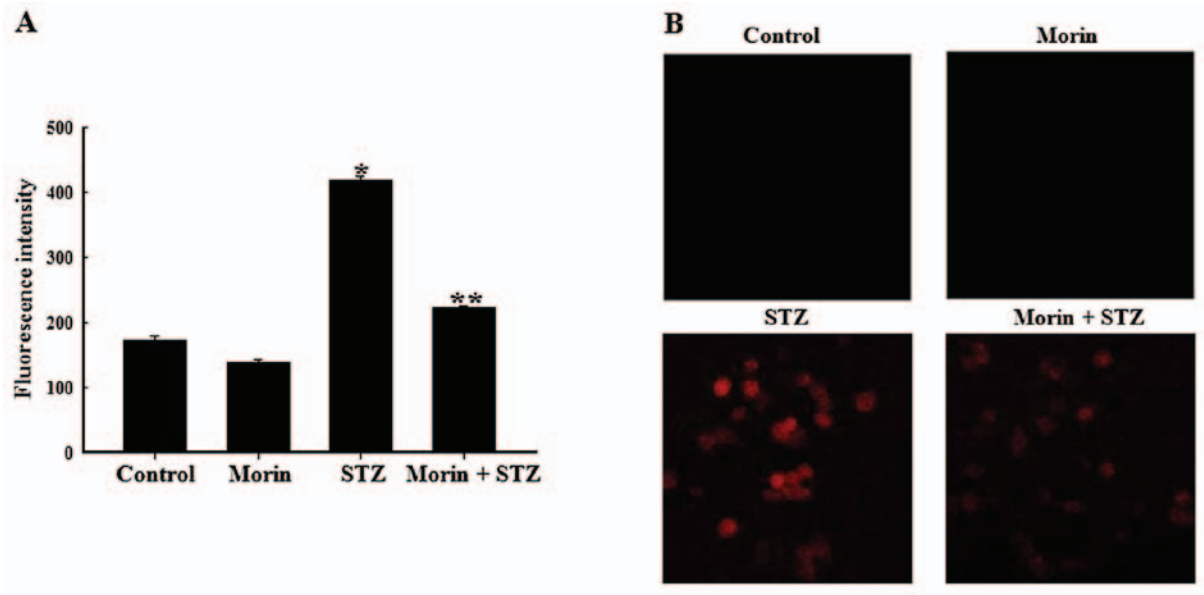

Figure 3. Effects of morin on STZ-induced intracellular ROS production. Cells were treated with morin (25 μM) for 1 h, after which 6 mM STZ was added to the cells for an additional 12 h. Intracellular ROS generation was detected by (A) flow cytometry following 2',7'-dichlorodihydrofluorescein diacetate treatment. Measurements were conducted in triplicate and the values are presented as the means ± standard error of the mean. *P<0.05 vs. control cells; **P<0.05 vs. STZ-treated cells. (B) Representative confocal images illustrating the increase in red fluorescence intensity produced by ROS in STZ-treated cells compared with in the control cells, and the reduced fluorescence intensity in STZ-treated cells pretreated with morin. ROS, reactive oxygen species; STZ, streptozotocin.

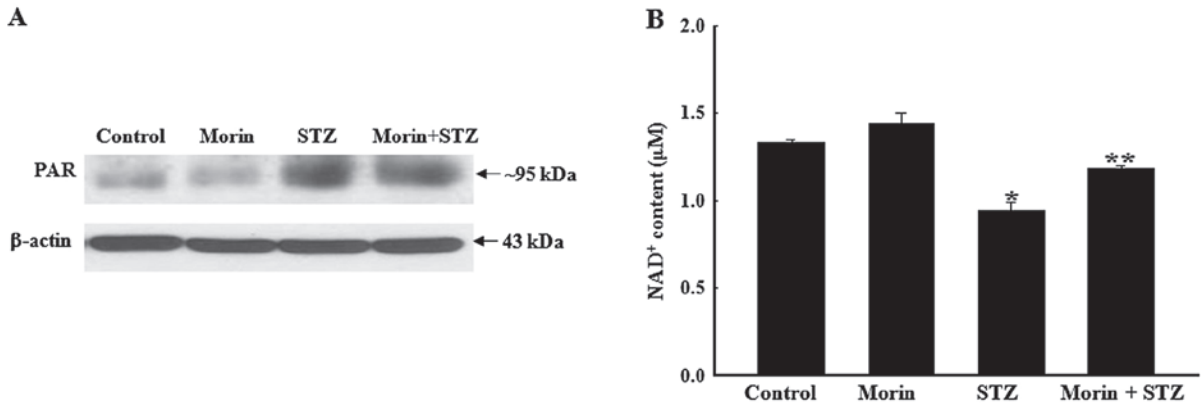

Figure 4. Effects of morin on STZ-induced increases in PARP activity and depletion of NAD^+ levels. Cells were treated with 25 μM morin for 1 h and were then incubated with 6 mM STZ for 24 h. (A) Cell lysates were electrophoresed and the protein expression levels of PAR were examined by western blot analysis. (B) Intracellular NAD^+ content was measured using the NAD^+/NADH quantification kit. The measurements were made in triplicate and the values are presented as the means ± standard error of the mean. *P<0.05 vs. control cells; **P<0.05 vs. STZ-treated cells. NAD^+, nicotinamide adenine dinucleotide; PARP, poly (ADP-ribose) polymerase; ROS, reactive oxygen species; STZ, streptozotocin.

produce large amount of ROS, which in turn induced cell apoptosis (34). To determine the cytoprotective effects of morin on STZ-induced pancreatic cell apoptosis, cell nuclei were stained with Hoechst 33342 for microscopic examination. The microscopic images presented in Fig. 5B indicated that the nuclei of the control cells were intact, whereas the STZ-treated cells exhibited marked nuclear fragmentation, which is a typical feature of apoptosis. However, when the cells were pretreated with morin, a marked decrease in nuclear fragmentation was observed. In addition, the anti-apoptotic effects of morin on STZ-treated cells were confirmed by flow cytometric analysis with PI staining. As shown in Fig. 5C, apoptotic sub-G_1 DNA content in STZ-treated cells was increased compared with in the control cells. However, prior treatment with morin reduced apoptotic sub-G_1 DNA content in STZ-treated cells. These results indicated that morin reduced apoptotic cell death, thus suggesting that the cytoprotective effects of morin on RINm5F cells may be due to the inhibition of apoptosis.

Effects of morin on catalase mRNA transcription, protein expression and enzyme activity. It is well known that the pancreas is particularly susceptible to STZ-induced free radical damage, due to the low levels of endogenous antioxidant enzymes responsible for scavenging free radicals (35). Since catalase serves an important role in the cellular defense against ROS, and morin has been reported to significantly increase protein expression and enzyme activity of catalase in lung fibroblast cells (15), the effects of morin on catalase expression and activity in RINm5F cells were investigated

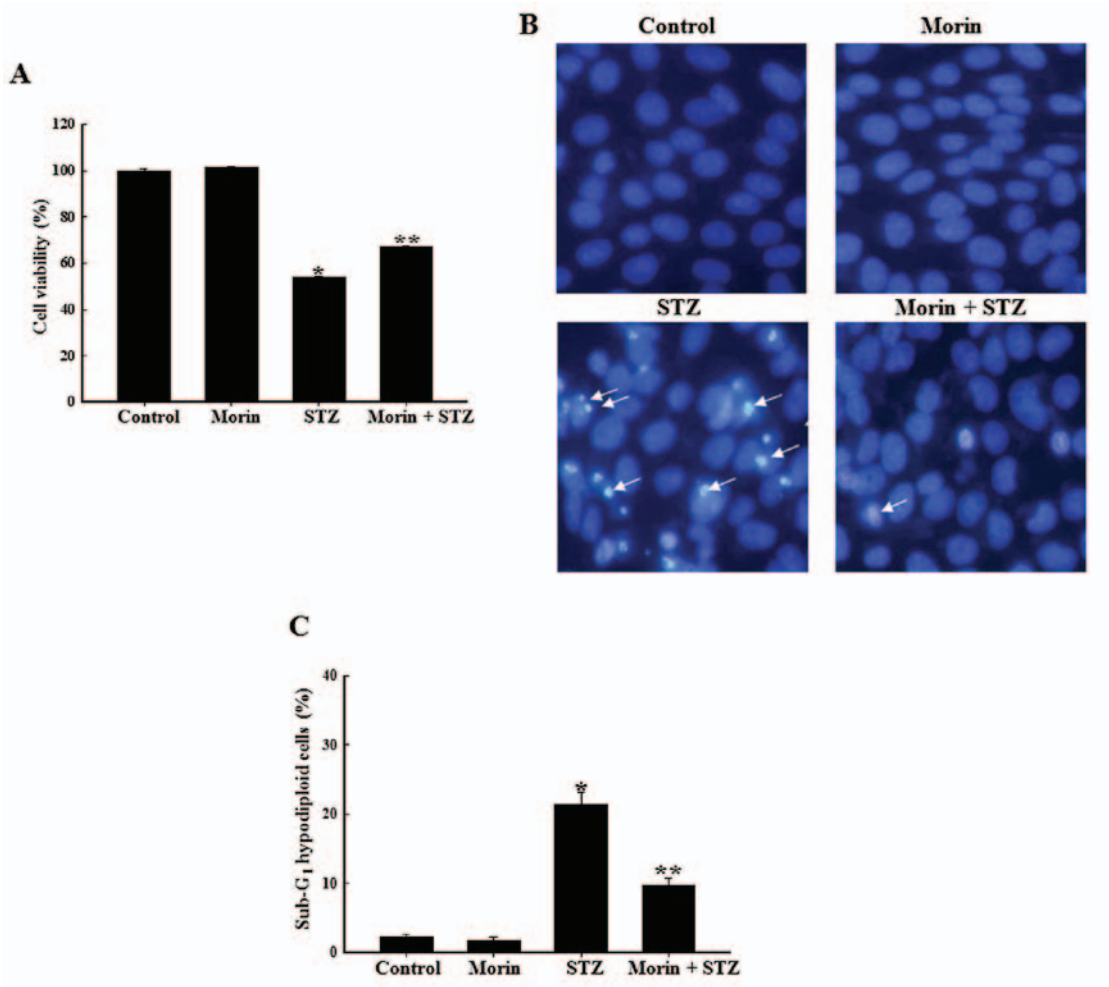

Figure 5. Protective effects of morin on STZ-induced damage in RINm5F cells. (A) Cell viability was determined by MTT assay. (B) Apoptotic body formation was observed under a fluorescence microscope following Hoechst 33342 staining; apoptotic bodies are indicated by arrows. (C) Apoptotic sub-G_1 DNA content was detected by flow cytometry following propidium iodide staining. Measurements were made in triplicate and the values are presented as the means ± standard error of the mean. *P<0.05 vs. control cells; **P<0.05 vs. STZ-treated cells. STZ, streptozotocin.

in the present study. The present results revealed that morin treatment alone induced catalase mRNA transcription in a time-dependent manner (Fig. 6A). In addition, catalase protein expression and enzyme activity were time-dependently increased following treatment with morin (Fig. 6B and C). Treatment with STZ alone for 24 h significantly attenuated the mRNA transcription, protein expression and enzyme activity of catalase in RINm5F cells. Compared with these findings, pretreatment with morin reduced STZ-induced attenuation of catalase mRNA transcription and protein expression (Fig. 6D and E), leading to a significant increase in catalase activity (Fig. 6F).

Effects of morin on AMPK activation and FOXO3a subcellular translocation. Since AMPK is an upstream kinase that regulates FOXO3 activation, and the AMPK-FOXO3 signaling pathway has been suggested to be responsible for the induction of antioxidant enzymes (16,36), the effects of morin on AMPK-FOXO3 activation were examined by western blot analysis. As shown in Fig. 7A, 25 μM morin induced phosphorylation of the AMPK catalytic unit AMPKα in a time-dependent manner, thus promoting the nuclear accumulation of FOXO3 (Fig. 7B).

Role of the AMPK-FOXO3 pathway in regulation of catalase expression. Since catalase is a downstream transcriptional target of FOXO3, the present study aimed to determine whether the AMPK-FOXO3 signaling pathway is involved in the induction of catalase by morin. Following transfection of the pancreatic cells with siAMPK or siFOXO3, cells were treated with morin for 24 h and the protein expression levels of catalase were determined. Silencing of AMPK or FOXO3 expression markedly suppressed the morin-dependent increase in catalase protein expression (Fig. 8A and B). To determine whether morin-enhanced catalase activity confers cytoprotection against oxidative stress, cells were transfected with siCatalase. siCatalase reduced the protective effects of morin against STZ-induced cytotoxicity (Fig. 8C). Taken together, these results revealed that AMPK may be involved in the activation of FOXO3 and the upregulation of catalase.

Discussion

In the present study, STZ treatment was employed to mimic the diabetes-associated oxidative stress environment in pancreatic β cells. STZ is a widely used chemical, which is able to induce experimental diabetes in animals and can induce cells to produce

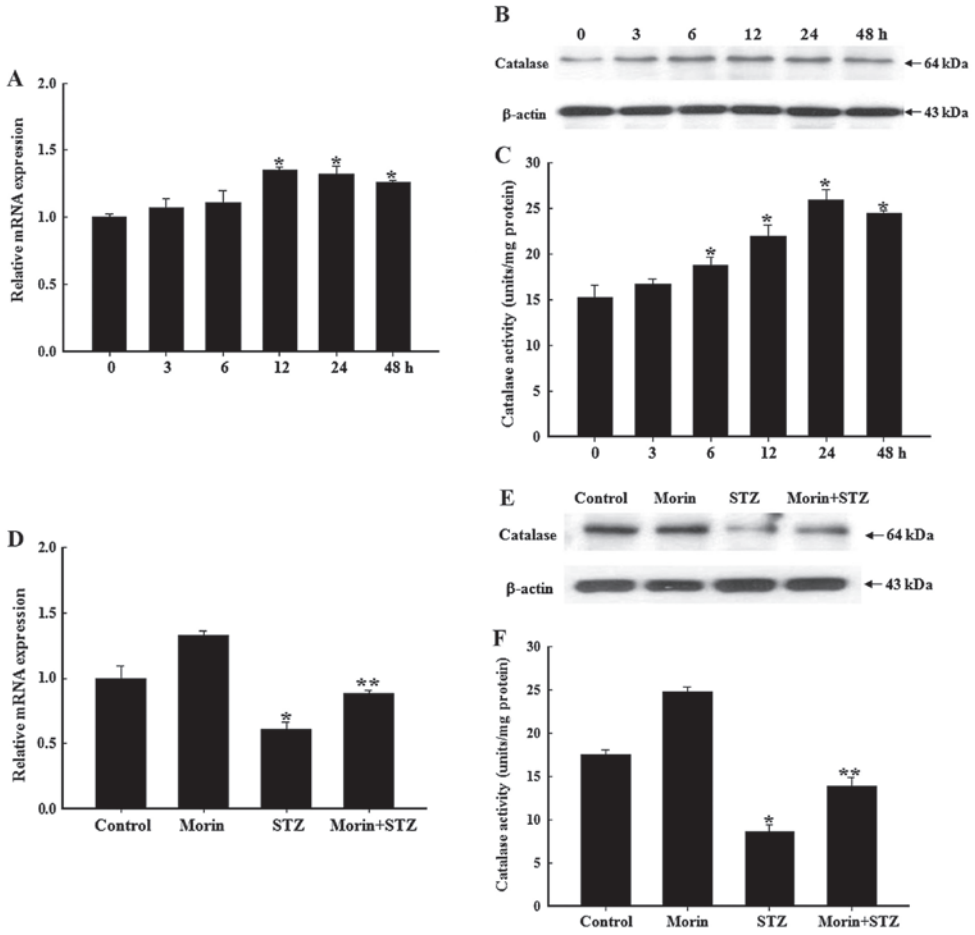

Figure 6. Effects of morin on catalase mRNA transcription, protein expression and enzyme activity. (A) Cells were treated with 25 μM morin for a series of time periods, total RNA was extracted and catalase mRNA expression was analyzed by qPCR. β-actin was used as the internal reference. (B) Cells were treated with 25 μM morin for the indicated time periods. Cell lysates were electrophoresed and the expression of catalase protein was detected using a catalase-specific antibody. β-actin was used as a loading control. (C) Cells were treated with morin for the indicated time periods. Catalase activity was measured using a colorimetric assay kit. *P<0.05 vs. control cells. (D) Cells were treated with 25 μM morin for 1 h, after which 6 mM STZ was added for 12 h. Total RNA was extracted and catalase mRNA expression was analyzed by qPCR. (E) Cells were treated with 25 μM morin for 1 h, 6 mM STZ was then added for 24 h. Cell lysates were electrophoresed and the expression of catalase protein was detected by catalase-specific antibody. (F) Catalase activity was measured using a colorimetric assay kit. *P<0.05 vs. control cells; **P<0.05 vs. STZ-treated cells. qPCR, quantitative polymerase chain reaction; STZ, streptozotocin.

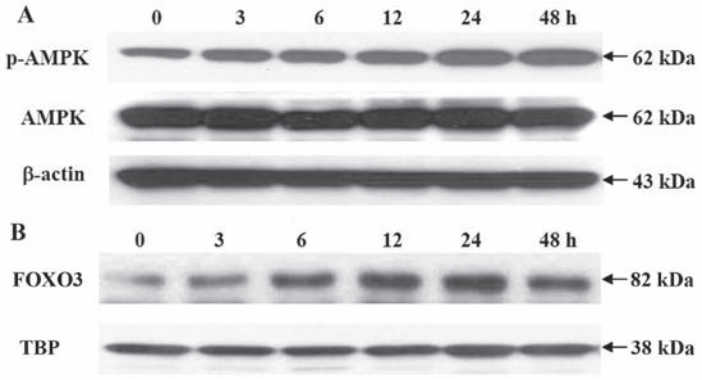

Figure 7. Effects of morin on AMPK activation and FOXO3a translocation. Cells were treated with 25 μM morin for the indicated time periods. (A) Cell lysates underwent western blotting with primary antibodies against p-AMPKα and AMPKα. (B) Nuclear fractions were prepared and FOXO3a protein expression levels were examined by western blot analysis. TBP was used as a loading control. AMPK, 5' adenosine monophosphate-activated protein kinase; FOXO3, forkhead box O3; p-AMPK, phosphorylated-AMPK; TBP, TATA binding protein.

several types of ROS, including superoxide anion, hydroxyl radical and H_2O_2 (37). Previous studies have demonstrated that exposure to STZ may result in β cell dysfunction and apoptosis (38,39). In addition, it has been suggested that STZ may enter β cells and cause alkylation of DNA; DNA damage induces activation of PARP. Hyperactivation of PARP is able

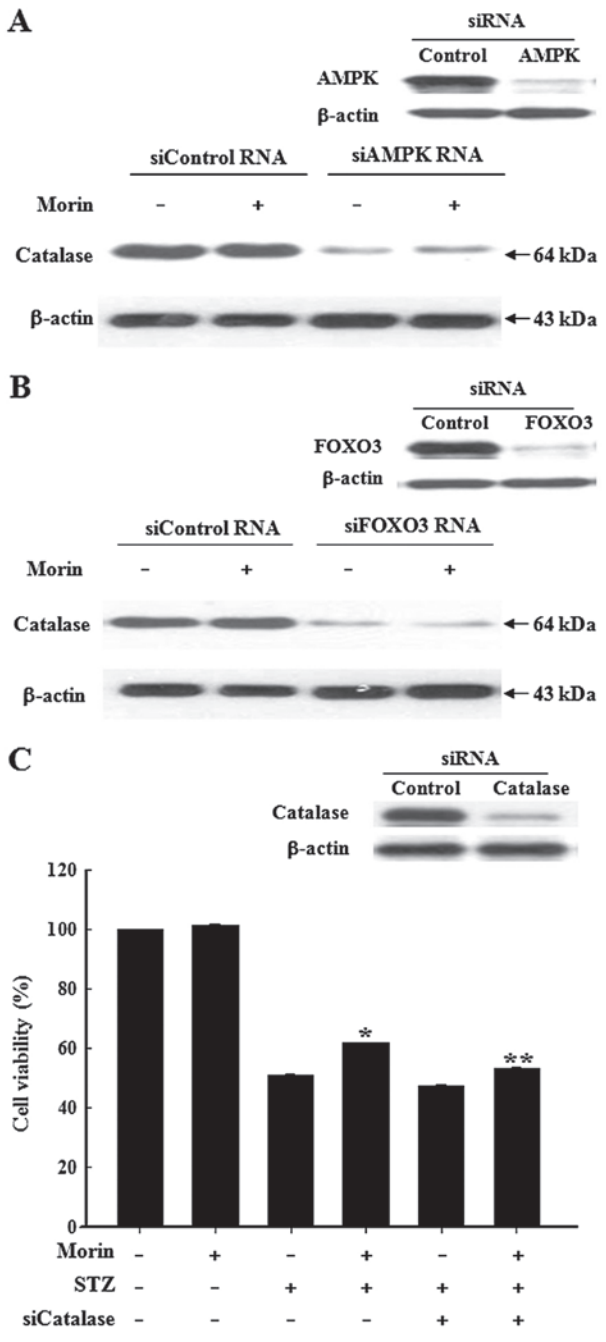

Figure 8. Induction of catalase by morin via the AMPK-FOXO3a signaling pathway. (A) Cells were transfected with 10-50 nM siControl or siAMPK. A total of 24 h post-transfection, cells were treated with 25 μM morin for 24 h. The protein expression levels of catalase were then evaluated in cell lysates by western blot analysis. (B) Cells were transfected with 10-50 nM siControl or siFOXO3a. A total of 24 h post-transfection, cells were treated with 25 μM morin for 24 h, and the protein expression levels of catalase were evaluated in cell lysates by western blot analysis. (C) Cells were transfected with 10-50 nM siControl or siCatalase, followed by 1 h treatment with morin and exposure to 6 mM STZ for 24 h. Cell viability was measured using the MTT assay. *P<0.05 vs. STZ + siControl-transfected cells; **P<0.05 vs. morin + STZ + siControl-transfected cells. AMPK, 5' adenosine monophosphate-activated protein kinase; FOXO3, forkhead box O3; si/siRNA, small interfering RNA; STZ, streptozotocin.

to initiate the programmed cell death pathway, resulting in the depletion of cellular NAD^+ (37). In the present study, exposure of RINm5F cells to STZ significantly increased intracellular ROS production, which was accompanied by a marked decrease in cell viability, augmentation of PARP activity, attenuation of NAD^+ levels and an increase in apoptosis. Flavonoids, including morin, have been reported to possess potent antioxidant and antidiabetic activity in animal models of experimental diabetes (40,41). Therefore, the present study further investigated the protective roles of morin in STZ-treated RINm5F cells.

The present results suggested that morin could effectively protect pancreatic cells by augmenting cellular catalase levels. Pretreatment with morin significantly reduced ROS levels, restored cell viability, reduced PARP activity, ameliorated intracellular NAD^+ levels and inhibited apoptosis induced by STZ. Generally, cells can be protected from excessive ROS via the augmentation of endogenous antioxidant enzymes. A previous study reported that stable transfection of insulin-producing RINm5F cells with catalase resulted in defense against cytokine toxicity (42). Furthermore, β cell-specific transgenic expression of catalase has been revealed to shield isolated islets from H_2O_2 and reduce the effects of STZ treatment (43,44). Although a previous study indicated that morin possesses antioxidant and antidiabetic activities (45), few studies have elucidated the molecular mechanisms by which morin may protect pancreatic β cells and regulate endogenous antioxidant enzymes. The present study investigated the effects of morin on the regulation of catalase expression and activity. Notably, morin time-dependently increased the gene transcription, protein expression and enzyme activity of catalase, and alleviated the downregulation of catalase in β cells induced by exposure to STZ. In addition, knockdown of catalase using specific siCatalase abolished the protective effects of morin against STZ-induced cell death, providing evidence verifying the vital role of catalase in the cytoprotective mechanism of morin in RINm5F cells. These results suggested that the cytoprotective effects of morin may be attributed to augmentation of the gene transcription, protein expression and enzyme activity of catalase.

The present study also demonstrated that augmentation of catalase was associated with activation of the AMPK-FOXO3 signaling pathway. Morin treatment time-dependently increased the phosphorylation of AMPK, thus suggesting that morin may activate AMPK and function via its downstream network of signaling pathways. It has previously been reported that AMPK activation is involved in the antioxidant defense system in response to oxidative stress (21). Disruption of AMPK activation in cells under oxidative stress may trigger cell death due to accumulation of ROS. In addition, knockdown of the catalytic AMPK-α1 subunit in human umbilical vein endothelial cells attenuated the expression of key components in the antioxidant defense system, including catalase (27).

Regarding how the AMPK pathway upregulates catalase, the present study examined FOXO3 expression. Morin induced FOXO3 expression, and its subsequent translocation into the nucleus. It has previously been reported that AMPK may directly phosphorylate and activate FOXO3 (46). Activated FOXO3 is subsequently translocated into the nucleus and regulates antioxidant enzymes in response to oxidative stress (16). In mouse hematopoietic stem cells, FOXO3 has been identified as a critical mediator of cell resistance to physiological oxidative stress. Furthermore, the conditional deletion of FOXO3 can lead to mass ROS generation (46,47). A previous study indicated that resveratrol could upregulate FOXO proteins and provide photoreceptor cells with effective protection against oxidative stress (48). It has also been reported that

FOXO3 may be involved in the induction of catalase (49). The AMPK-FOXO3 signaling pathway has been confirmed to be responsible for the induction of catalase in vascular endothelial cells (28). In the present study, transfection of cells with siAMPK or siFOXO3 reduced morin-induced protein expression of catalase. These findings suggested that activation of the AMPK-FOXO3 signaling pathway may be critically involved in morin-induced catalase expression and enzyme activity.

The FOXO3 transcription factor is not the only transcriptional regulator of catalase. The transcription factor NF-E2-related factor 2 (Nrf2) has also been reported to be involved in the regulation of catalase in pancreatic β cells (50). Therefore, future studies may focus on the association between FOXO3 and Nrf2 in the regulation of morin-induced catalase. Furthermore, targeting the phosphoinositide 3-kinase/protein kinase B/FOXO3 signaling pathway may lead to the development of novel approaches for the possible treatment of diabetic neuropathy (51). In addition, it will be interesting to determine the signaling network responsible for the regulation of FOXO3. These findings suggested that the identification of potential FOXO3 activators may be promising to limit the progression of oxidative stress-mediated diseases and merits intensive investigation.

Besides upregulation of catalase, AMPK has been reported to mediate the expression of manganese superoxide dismutase, in order to reduce mitochondrial ROS production in human umbilical vein endothelial cells (52) and Chang liver cells (36). Therefore, it may be hypothesized that the AMPK-FOXO3 signaling pathway serves an essential role in the expression of various antioxidant enzymes, including catalase, in pancreatic β cells. Future studies should aim to investigate the role of the AMPK pathway in the regulation of other antioxidant enzymes for the protection of β cells from oxidative stress-induced cell damage.

In conclusion, the present study demonstrated that morin may suppress STZ-induced intracellular ROS production and apoptosis, which may be ascribed, at least partly, to the upregulation of catalase via the induction of FOXO3 expression and its subsequent translocation into the nucleus via AMPK activation. Therefore, morin-induced activation of the AMPK-FOXO3-catalase pathway may be considered one of the pathways that potentially mediates the antioxidative functions of morin. Morin may be considered a promising strategy for the amelioration and/or prevention of pancreatic β cell dysfunction and diabetes.

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References

1. Kaneto H and Matsuoka TA: Involvement of oxidative stress in suppression of insulin biosynthesis under diabetic conditions. Int J Mol Sci 13: 13680-13690, 2012.
2. Pérez S, Pereda J, Sabater L and Sastre J: Redox signaling in acute pancreatitis. Redox Biol 5: 1-14, 2015.
3. Keane KN, Cruzat VF, Carlessi R, de Bittencourt PI Jr and Newsholme P: Molecular events linking oxidative stress and inflammation to insulin resistance and β-cell dysfunction. Oxid Med Cell Longev 2015: 181643, 2015.
4. Mishra V: Oxidative stress and role of antioxidant supplementation in critical illness. Clin Lab 53: 199-209, 2007.
5. Manna P and Jain SK: Obesity, oxidative stress, adipose tissue dysfunction, and the associated health risks: causes and therapeutic strategies. Metab Syndr Relat Disord 13: 423-444, 2015.
6. Yadav UC, Rani V, Deep G, Singh RK and Palle K: Oxidative stress in metabolic disorders: pathogenesis, prevention, and therapeutics. Oxid Med Cell Longev 2016: 9137629, 2016.
7. Xie MX, Long M, Liu Y, Qin C and Wang YD: Characterization of the interaction between human serum albumin and morin. Biochim Biophys Acta 1760: 1184-1191, 2006.
8. Dhanasekar C and Rasool M: Morin, a dietary bioflavonol suppresses monosodium urate crystal-induced inflammation in an animal model of acute gouty arthritis with reference to NLRP3 inflammasome, hypo-xanthine phospho-ribosyl transferase, and inflammatory mediators. Eur J Pharmacol 786: 116-127, 2016.
9. Prahalathan P, Kumar S and Raja B: Morin attenuates blood pressure and oxidative stress in deoxycorticosterone acetate-salt hypertensive rats: a biochemical and histopathological evaluation. Metabolism 61: 1087-1099, 2012.
10. Abuohashish HM, Al-Rejaie SS, Al-Hosaini KA, Parmar MY and Ahmed MM: Alleviating effects of morin against experimentally-induced diabetic osteopenia. Diabetol Metab Syndr 5: 5, 2013.
11. Kok LD, Wong YP, Wu TW, Chan HC, Kwok TT and Fung KP: Morin hydrate: a potential antioxidant in minimizing the free-radicals-mediated damage to cardiovascular cells by anti-tumor drugs. Life Sci 67: 91-99, 2000.
12. Singh MP, Jakhar R and Kang SC: Morin hydrate attenuates the acrylamide-induced imbalance in antioxidant enzymes in a murine model. Int J Mol Med 36: 992-1000, 2015.
13. Dilshara MG, Jayasooriya RG, Lee S, Choi YH and Kim GY: Morin downregulates nitric oxide and prostaglandin E_2 production in LPS-stimulated BV2 microglial cells by suppressing NF-κB activity and activating HO-1 induction. Environ Toxicol Pharmacol 44: 62-68, 2016.
14. Vanitha P, Uma C, Suganya N, Bhakkiyalakshmi E, Suriyanarayanan S, Gunasekaran P, Sivasubramanian S and Ramkumar KM: Modulatory effects of morin on hyperglycemia by attenuating the hepatic key enzymes of carbohydrate metabolism and β-cell function in streptozotocin-induced diabetic rats. Environ Toxicol Pharmacol 37: 326-335, 2014.
15. Zhang R, Kang KA, Piao MJ, Maeng YH, Lee KH, Chang WY, You HJ, Kim JS, Kang SS and Hyun JW: Cellular protection of morin against the oxidative stress induced by hydrogen peroxide. Chem Biol Interact 177: 21-27, 2009.
16. Li XN, Song J, Zhang L, LeMaire SA, Hou X, Zhang C, Coselli JS, Chen L, Wang XL, Zhang Y, *et al*: Activation of the AMPK-FOXO3 pathway reduces fatty acid-induced increase in intracellular reactive oxygen species by upregulating thioredoxin. Diabetes 58: 2246-2257, 2009.
17. Zheng A, Li H, Xu J, Cao K, Li H, Pu W, Yang Z, Peng Y, Long J, Liu J, *et al*: Hydroxytyrosol improves mitochondrial function and reduces oxidative stress in the brain of db/db mice: role of AMP-activated protein kinase activation. Br J Nutr 113: 1667-1676, 2015.
18. Guan Y, Cui ZJ, Sun B, Han LP, Li CJ and Chen LM: Celastrol attenuates oxidative stress in the skeletal muscle of diabetic rats by regulating the AMPK-PGC1α-SIRT3 signaling pathway. Int J Mol Med 37: 1229-1238, 2016.
19. Ceolotto G, Gallo A, Papparella I, Franco L, Murphy E, Iori E, Pagnin E, Fadini GP, Albiero M, Semplicini A, *et al*: Rosiglitazone reduces glucose-induced oxidative stress mediated by NAD(P)H oxidase via AMPK-dependent mechanism. Arterioscler Thromb Vasc Biol 27: 2627-2633, 2007.
20. Essick EE and Sam F: Oxidative stress and autophagy in cardiac disease, neurological disorders, aging and cancer. Oxid Med Cell Longev 3: 168-177, 2010.
21. Sid B, Verrax J and Calderon PB: Role of AMPK activation in oxidative cell damage: implications for alcohol-induced liver disease. Biochem Pharmacol 86: 200-209, 2013.
22. Maiese K, Chong ZZ and Shang YC: OutFOXOing disease and disability: the therapeutic potential of targeting FoxO proteins. Trends Mol Med 14: 219-227, 2008.
23. Nho RS and Hergert P: FoxO3a and disease progression. World J Biol Chem 5: 346-354, 2014.
24. Kato M, Yuan H, Xu ZG, Lanting L, Li SL, Wang M, Hu MC, Reddy MA and Natarajan R: Role of the Akt/FoxO3a pathway in TGF-beta1-mediated mesangial cell dysfunction: a novel mechanism related to diabetic kidney disease. J Am Soc Nephrol 17: 3325-3335, 2006.

25. Sundaresan NR, Gupta M, Kim G, Rajamohan SB, Isbatan A and Gupta MP: Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. J Clin Invest 119: 2758-2771, 2009.
26. Turdi S, Li Q, Lopez FL and Ren J: Catalase alleviates cardiomyocyte dysfunction in diabetes: role of Akt, forkhead transcriptional factor and silent information regulator 2. Life Sci 81: 895-905, 2007.
27. Colombo SL and Moncada S: AMPKalpha1 regulates the antioxidant status of vascular endothelial cells. Biochem J 421: 163-169, 2009.
28. Zrelli H, Matsuoka M, Kitazaki S, Zarrouk M and Miyazaki H: Hydroxytyrosol reduces intracellular reactive oxygen species levels in vascular endothelial cells by upregulating catalase expression through the AMPK-FOXO3a pathway. Eur J Pharmacol 660: 275-282, 2011.
29. Carmichael J, DeGraff WG, Gazdar AF, Minna JD and Mitchell JB: Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res 47: 936-942, 1987.
30. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F and Riccardi C: A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 139: 271-279, 1991.
31. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
32. Chandak PG, Gaikwad AB and Tikoo K: Gallotannin ameliorates the development of streptozotocin-induced diabetic nephropathy by preventing the activation of PARP. Phytother Res 23: 72-77, 2009.
33. Guzyk MM, Tykhomyrov AA, Nedzvetsky VS, Prischepa IV, Grinenko TV, Yanitska LV and Kuchmerovska TM: Poly(ADP-ribose) polymerase-1 (PARP-1) inhibitors reduce reactive gliosis and improve angiostatin levels in retina of diabetic rats. Neurochem Res 41: 2526-2537, 2016.
34. Chen F, Xiong H, Wang J, Ding X, Shu G and Mei Z: Antidiabetic effect of total flavonoids from *Sanguis draxonis* in type 2 diabetic rats. J Ethnopharmacol 149: 729-736, 2013.
35. Lenzen S: Oxidative stress: the vulnerable beta-cell. Biochem Soc Trans 36: 343-347, 2008.
36. Kim AD, Kang KA, Piao MJ, Kim KC, Zheng J, Yao CW, Cha JW, Hyun CL, Kang HK, Lee NH, *et al*: Cytoprotective effect of eckol against oxidative stress-induced mitochondrial dysfunction: involvement of the FoxO3a/AMPK pathway. J Cell Biochem 115: 1403-1411, 2014.
37. Szkudelski T: The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol Res 50: 537-546, 2001.
38. Lei H, Han J, Wang Q, Guo S, Sun H and Zhang X: Effects of sesamin on streptozotocin (STZ)-induced NIT-1 pancreatic β-cell damage. Int J Mol Sci 13: 16961-16970, 2012.
39. Zhang R, Kim JS, Kang KA, Piao MJ, Kim KC and Hyun JW: Protective mechanism of KIOM-4 in streptozotocin-induced pancreatic β-cells damage is involved in the inhibition of endoplasmic reticulum stress. Evid Based Complement Alternat Med 2011: 1-10, 2011.
40. Bansal P, Paul P, Mudgal J, Nayak PG, Pannakal ST, Priyadarsini KI and Unnikrishnan MK: Antidiabetic, antihyperlipidemic and antioxidant effects of the flavonoid rich fraction of *Pilea microphylla* (L.) in high fat diet/streptozotocin-induced diabetes in mice. Exp Toxicol Pathol 64: 651-658, 2012.
41. Sendrayaperumal V, Iyyam Pillai S and Subramanian S: Design, synthesis and characterization of zinc-morin, a metal flavonol complex and evaluation of its antidiabetic potential in HFD-STZ induced type 2 diabetes in rats. Chem Biol Interact 219: 9-17, 2014.
42. Lortz S, Tiedge M, Nachtwey T, Karlsen AE, Nerup J and Lenzen S: Protection of insulin-producing RINm5F cells against cytokine-mediated toxicity through overexpression of antioxidant enzymes. Diabetes 49: 1123-1130, 2000.
43. Benhamou PY, Moriscot C, Richard MJ, Beatrix O, Badet L, Pattou F, Kerr-Conte J, Chroboczek J, Lemarchand P and Halimi S: Adenovirus-mediated catalase gene transfer reduces oxidant stress in human, porcine and rat pancreatic islets. Diabetologia 41: 1093-1100, 1998.
44. Xu B, Moritz JT and Epstein PN: Overexpression of catalase provides partial protection to transgenic mouse beta cells. Free Radic Biol Med 27: 830-837, 1999.
45. Ola MS, Aleisa AM, Al-Rejaie SS, Abuohashish HM, Parmar MY, Alhomida AS and Ahmed MM: Flavonoid, morin inhibits oxidative stress, inflammation and enhances neurotrophic support in the brain of streptozotocin-induced diabetic rats. Neurol Sci 35: 1003-1008, 2014.
46. Greer EL, Oskoui PR, Banko MR, Maniar JM, Gygi MP, Gygi SP and Brunet A: The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor. J Biol Chem 282: 30107-30119, 2007.
47. Tothova Z, Kollipara R, Huntly BJ, Lee BH, Castrillon DH, Cullen DE, McDowell EP, Lazo-Kallanian S, Williams IR, Sears C, *et al*: FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell 128: 325-339, 2007.
48. Huang W, Li G, Qiu J, Gonzalez P and Challa P: Protective effects of resveratrol in experimental retinal detachment. PLoS One 8: e75735, 2013.
49. Alcendor RR, Gao S, Zhai P, Zablocki D, Holle E, Yu X, Tian B, Wagner T, Vatner SF and Sadoshima J: Sirt1 regulates aging and resistance to oxidative stress in the heart. Circ Res 100: 1512-1521, 2007.
50. Bhakkiyalakshmi E, Shalini D, Sekar TV, Rajaguru P, Paulmurugan R and Ramkumar KM: Therapeutic potential of pterostilbene against pancreatic beta-cell apoptosis mediated through Nrf2. Br J Pharmacol 171: 1747-1757, 2014.
51. Liu MH, Yuan C, He J, Tan TP, Wu SJ, Fu HY, Liu J, Yu S, Chen YD, Le QF, *et al*: Resveratrol protects PC12 cells from high glucose-induced neurotoxicity via PI3K/Akt/FoxO3a pathway. Cell Mol Neurobiol 35: 513-522, 2015.
52. Kukidome D, Nishikawa T, Sonoda K, Imoto K, Fujisawa K, Yano M, Motoshima H, Taguchi T, Matsumura T and Araki E: Activation of AMP-activated protein kinase reduces hyperglycemia-induced mitochondrial reactive oxygen species production and promotes mitochondrial biogenesis in human umbilical vein endothelial cells. Diabetes 55: 120-127, 2006.