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Morin activates the Nrf2-ARE pathway and reduces oxidative stress-induced DNA damage in pancreatic beta cells

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ABSTRACT

Oxidative stress is an important factor contributing to the pathogenesis of diabetes and its complications. In our earlier study, we demonstrated the antidiabetic efficacy of morin by regulating key enzymes of carbohydrate metabolism in diabetic rats. The present study was designed to assess the antigenotoxic potential of morin in pancreatic β-cells, using the COMET assay. To explore its potential mechanisms of action, three genotoxic agents, H_2O_2 which induces DNA damage by the generation of reactive oxygen species, streptozotocin (STZ) by RNS and Methyl methanesulfonate (MMS) by DNA alkylation was used. We found that STZ and H_2O_2- induced genotoxicity was dose dependently reduced by morin as assessed by DNA tail length, tail moment, DNA content and olive moment. Since the protective property was found to be specific against oxidative DNA damage, we explored the molecular mechanism underlying morin-induced Nuclear factor erythroid 2-related factor 2 (Nrf2) activation in pancreatic β-cells as assessed by ARE-driven downstream target genes with Luciferase reporter assay. In addition, morin inhibited intracellular free radical generation as assessed by using DCFDA and increased the intra cellular antioxidants viz, superoxide dismutase and catalase in INS-1E cells. In addition, morin attenuated glucose-stimulated insulin secretion following exposure to oxidative stress by STZ ($P < 0.05$). Collectively, our data provide evidence that morin protects pancreatic β-cells against oxidative stress-induced DNA damage by activating the Nrf2 signaling pathway

1. Introduction

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (American Diabetes, 2010). As a major public health problem, its prevalence is increasing globally and causing substantial morbidity and mortality, mainly from diabetic cardiovascular complications, neuropathy, renal failure and blindness. There are numerous evidences that hyperglycemia results in the excessive generation of free radicals, ultimately leading to increased oxidative stress in a variety of tissues, and playing an important role in diabetic complications (Dizdaroglu et al., 2002; Karunakaran and Park, 2013; Maritim et al., 2003). Increased levels of DNA damage have been reported in both clinical and experimental diabetes (Blasiak et al., 2004; Pacal et al., 2011). Moreover it has been reported as a critical mediator of β-cell apoptosis (Meares et al., 2013; Vasu et al., 2013). Moreover, due to its low level of antioxidant gene expression and deficient oxidative repair mechanisms, pancreatic β-cells are more vulnerable to DNA damage (Lenzen, 2008). Indeed, the prevention of oxidative stress mediated DNA damage is essential to preserve sufficient mass and to sustain insulin levels for the maintenance of glucose homeostasis.

More recently, the activation of transcription factor Nuclear factor-erythroid 2-related factor 2 (Nrf2), to bolster the cytoprotective genes remains as a promising strategy to combat against various stressful insults. In normal conditions, Nrf2 is associated with Kelch-like ECH-associated protein 1 (Keap1), which negatively regulates Nrf2 pathway. However, when the cell encounter stress, it favours the translocation of Nrf2 to nucleus and bind with Antioxidant Response Element (ARE) thereby triggers the Phase II (detoxifying and antioxidant) enzymes such as NAD(P)H quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1), γ-gutamylcysteine synthetase, glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) which aids in cell survival and maintaining cellular redox homeostasis. Moreover, recent evidence indicates that Nrf2 activation potential rescue pancreatic β-

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cells against various insults. In our earlier studies, we found that pterostilbene as a potent Nrf2 activator as assessed by Split Luciferase Complementation Assay (Bhakkiyalakshmi et al., 2016; Ramkumar et al., 2013) and its protective property against oxidative stress in pancreatic β-cells (Bhakkiyalakshmi et al., 2014).

Morin (2',3,4',5,7-pentahydroxyflavone), a potent natural antioxidant found in guava leaves, onion, apple, and other *Moracea* family that are traditionally used as dietary herbal medicines. Morin exhibited several pharmacological properties including anti-inflammatory, chemoprotective and anticancer activities (Kapoor and Kakkar, 2012; Wu et al., 1993; Yu et al., 2006). Moreover, we reported that morin prevents the destruction of β-cells in streptozotocin (STZ) -induced diabetic rats thereby exert its antihyperglycemic effect (Vanitha et al., 2014). In the present study, we examined the ability of morin to protect cells against DNA damage by underlying the potential activation of Nrf2 elucidated using comet assay in pancreatic β-cell line INS-1E.

2. Materials and methods

2.1. Chemicals and reagents

Morin, streptozotocin, methyl methanesulfonate (MMS) were purchased from Sigma–Aldrich Company. Materials for cell culture and all other chemicals were obtained from GE Healthcare Life Sciences (Logan, Utah).

2.2. Cell culture

INS-1E, insulin-secreting rat insulinoma β-cell line was obtained as a kind gift from Dr. Pierre Maechler, Department of Cell Physiology and Metabolism University Medical Center, Geneva, Switzerland. The cells were grown at 37 °C under a humidified, 5% CO_2 atmosphere in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 50 mM 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, and 100 mg/ml streptomycin. All experiments were carried out at passages 27–34 at a confluence rate of 70–80%.

2.3. Cell viability assay

Cell viability was assessed using the MTT reduction assay, which determines the ability of viable cells to convert a soluble tetrazolium salt into an insoluble formazan precipitate (Riss et al., 2004). The ability of cells to reduce MTT provides an indication of mitochondrial integrity and activity which, in turn, may be interpreted as a measure of viability. INS-1E cells (1×10^4 cells/well) were cultured in 96-well plates at 37 °C for 24 h, treated with morin (5–100 μM) in Dimethyl sulfoxide (DMSO), and incubated for another 24 h. After the incubation period, MTT solution was added (10 μg/100 μl medium), and then the plates were further incubated at 37 °C for 4 h in humidified 5% CO_2 atmosphere. After incubation, 100 μl of DMSO was added to each well, mixed thoroughly to dissolve the stain crystals and absorbance was read at 570 nm using a microplate ELISA reader (Bio-Rad). The cell viability was not affected up to 100 μM of morin and hence further studies were limited below this dosage.

The cytoprotective property of morin was tested against three genotoxic agents having different mechanisms of action such as H_2O_2 (100 μM) which induces the generation of reactive oxygen species, STZ (5 μM) induces Reactive Nitrogen Species and MMS (100 μM) induces DNA alkylation. The cells were pretreated with varying doses of morin (0–50 μM) for 24 h followed by treatment with the genotoxic agent followed by MTT assay was performed. Percentage inhibition of cytotoxicity was calculated as a fraction of the control, and expressed as relative percentage of cell viability respect to the control.

2.4. Antigenotoxic effect of morin in INS-1E cell line

To assess the antigenotoxic effect of morin, 24 h cultures of the INS-1E cells were pretreated with morin (0, 5, 10, and 20 μM) for 24 h. After replacing the medium with serum free medium, the cells were exposed to either H_2O_2 for 5 min or STZ for 1 h or MMS for 30 min and then subjected for the comet assay. The cells cultured in RPMI-1640 medium only were used as the control.

2.4.1. Alkaline comet assay (pH > 13)

The comet assay was performed under alkaline conditions following the procedure of Nandhakumar et al. with minor modifications (Nandhakumar et al., 2011). All the steps of the comet assay were conducted under yellow light to prevent the occurrence of additional DNA damage. The control and treated cells were centrifuged and resuspended in preheated 1% low melting point agarose maintained at 45 °C and pipette onto 1% normal melting point agarose pre-coated slides, which had been dried overnight, and covered with a cover slip. After keeping the slides on a chilled plate for 10 min for polymerization of agarose, the cover slips were removed and the slides were lowered into freshly made ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA-Na, 10 mM Tris, 10% DMSO, 1% Triton X-100, pH 10) and kept at 4 °C in dark for 60 min. After draining the lysis solution, the slides were rinsed with double distilled water for 15 min. After repeating the water wash, the slides were placed in horizontal electrophoresis tank, containing freshly made electrophoresis buffer (300 mM NaOH, and 1 mM EDTA-Na, pH > 13), for 30 min. Electrophoresis was performed in the same buffer for 25 min by applying an electric field of 25 V (0.8 V/cm) and adjusting the current to 300 mA by slowly changing the buffer level in the tank. After draining the electrophoresis buffer drop wise, the slides were rinsed gently with 0.4 M Tris-HCl buffer (pH 7.5) for 5 min. This step was repeated twice. Then the slides were dried at room temperature and kept in a refrigerator in a sealed container until analysis. For staining, the slides were immersed in double distilled water for 30 min then stained with 200 μl of EtBr. Then the slides were viewed on a Nikon microscope and images were transferred to a computer with a digital camera. Images of 50 randomly selected non-overlapping cells on the captured microphotographs were analyzed using and varying parameters like DNA tail length, tail moment, DNA content and olive moment were measured to assess extent and distribution of DNA damage. Comet images were analyzed with the Comet Score software (TriTek Corporation, USA).

2.5. Measurement of intracellular reactive oxygen species formation

Intracellular peroxides were measured using a non-fluorescent compound, 2',7'-dichlorofluorescin diacetate (DCFDA), which is de-esterified within the cells by endogenous esterases to the ionized fluorescent free acid, 2',7'-dichlorofluorescin (Rota et al., 1999). In brief, the INS-1E cells were seeded in a 96-well plate at 2×10^4 cells/well. At 16 h after plating, the cells were treated with 5 and 10 μM of morin for 24 h followed by treatment with 100 μM H_2O_2 for 5 min. Then 25 μM of DCFDA solution was added, incubated for 10 min, and then the fluorescence of 2',7'-dichlorofluorescein was measured at 502 nm excitation and 550 nm emission using a fluorescence spectrophotometer.

2.6. Detection of nitric oxide by DAF-FM

To measure nitric oxide, the INS-1E cells were seeded in a 96-well plate at 2×10^4 cells/well. At 16 h after plating, the cells were treated with 5 and 10 μM of morin for 24 h followed by STZ and incubated for 1 h. Then 1 μM of DAF-FM DA solution was added, incubated for 10 min and then the fluorescence was measured using a fluorescence spectrophotometer (Kojima et al., 1999).