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2',4'-Dihydroxy-6'-methoxy-3',5'-dimethylchalcone inhibits apoptosis of MIN6 cells via improving mitochondrial function

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Mitochondrial dysfunction due to oxidative stress and concomitant β-cell apoptosis may play a key role in type 2 diabetes. Inhibiting β-cell apoptosis through ameliorating oxidative mitochondrial dysfunction with specific natural products may have preventive or therapeutic potential. In this study, the anti-apoptotic effect of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (DMC), a isolated chalcone from the buds of *Cleistocalyx operculatus*, on H_2O_2 induced MIN6 cells damage was investigated. Exposure to H_2O_2 at 250 μM for 3 h, the viability of MIN6 cells was significantly decreased and the apoptosis apparently occurred. A pre-treatment with DMC at the concentrations of 12.5–25 μM, before H_2O_2 addition, reduced nucleus fragmentation, decreased endogenous reactive oxygen species (ROS) production, and improved mitochondrial potential (MMP), and consequently, inhibited apoptosis. Furthermore, decreased activities of caspase-3 and caspase-9 were observed. These results clearly demonstrated DMC protected MIN6 cells against apoptosis due to its highly protective effect on mitochondria, and thus, it has great potential as a candidate drug for the diabetes care.

1. Introduction

Both forms of diabetes mellitus, type I and type II. Both are characterized by progressive β-cell failure. Apoptosis is thought to be the main form of β-cell death in both types (Evans et al. 2002). Mitochondria dysfunction plays a key role in β-cell death. In type I diabetes, members of the Bcl-2 protein family on the mitochondria membrane regulate the release of cytochrome *c* from mitochondrial into cytosol, sequentially activate caspase-9 and caspase-3 and execute cell death (Friedlander 2003). While in type II, mitochondrial oxidant production associated with hyperlipemia and hyperglycemia disrupts glucose-stimulated insulin secretion and causes cellular damage through activating stress-sensitive signaling pathways, such as NF-κB, p38MAPK, JNK/SAPK, PKC, AGE/RAGE, and others (Cnop et al. 2005; Chandra et al. 2001).

Medicinal herbs have been used for diabetes treatment for a long history in many countries (Chan et al. 2009; Modak et al. 2007). Nowadays, continuous efforts are still made for searching bioactive compounds from natural origin. Modern pharmacological researches have revealed that many herbal extracts have great potential in diabetes care. Among the pharmacologically active natural products accounting for this effect are flavonoids. For instance, the isoflavone genistein increases insulin secretion both in the MIN6 cells (Ohno et al. 1993) and cultured islets from rats 8Sorenson et al. 1994). The isoflavone purarin could inhibit the H_2O_2 induced apoptosis in rat islet cells with an increase in the activities of antioxidant enzymes (Linag et al. 2006). Naringenin, a citrus flavonoid, increases muscle cell glucose uptake via AMPK (Zygmunt et al. 2010).

Cleistocalyx operculatus (Roxb.) Merr. et Perry (Myrtaceae) is a well known medicinal plant whose buds are commonly used as an ingredient for tonic drinks in Southern China (Ye et al. 2004a, b). Its main bioactive compounds comprise a variety of flavoniods, among which 2',4'-dihydroxy-6'-methoxy-3'-5'-dimethylchalcone (DMC) (Fig. 1) was found to be the most abundant (Ye et al. 2004 a,b). Accumulative evidence demonstrates that DMC exerts anti-inflammatory effects through blocking NF-κB activation and effectively reverses multi-drug resistance in resistant human hepatocellular carcinoma cell line BEL-7402/5-FU (Kim et al. 2010; Huang et al. 2011). Hepatoprotective effects of DMC against CCl_4-induced acute liver injury in mice have recently been found as well (Lu et al. 2011). In addition, DMC has been reported to have anti-diabetic (Ma et al. 2005), anti-bacterial (Gafner et al. 1996), spasmolytic (Amor et al. 2005) and anti-tumoral (Ye et al. 2005) properties. Although DMC has beneficial effects on various cells or tissues, it is unclear whether it plays a role in preventing β-cell failure. Therefore, in the present study we examined the anti-apoptotic effect of DMC on H_2O_2 induced MIN6 cell damage, and the cellular mechanisms related to this effect.

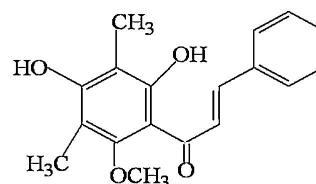


Fig. 1: Structure of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone

2. Investigations and results

2.1. Cell viability assay

To determine cell viability, the MTT method was used. MIN6 cell viability was significantly decreased by $41.82 \pm 1.89\%$

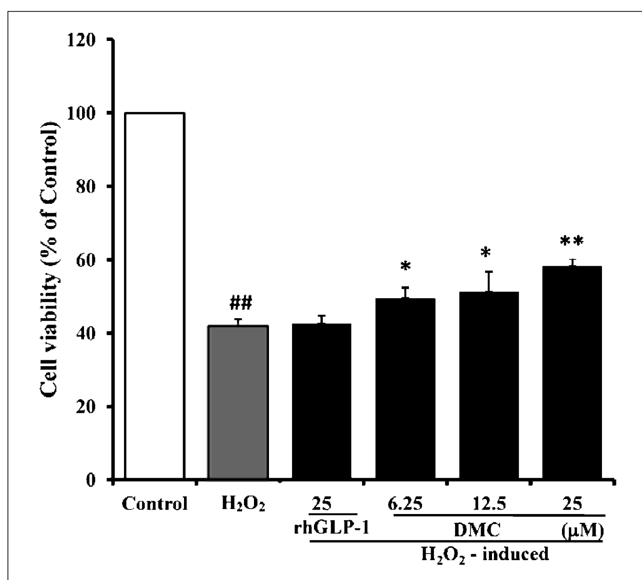


Fig. 2: Cell protective effect of DMC on H_2O_2 induced cytotoxicity on MIN6 cells. Cells were pre-treated with 25 μM rhGLP-1 or DMC (6.25∼25 μM) for 48 h, then incubated in the presence of 250 μM H_2O_2 for 3 h. The viability of control cells was defined as 100%. Data were presented as means ± SD and were representative of an average of three independent experiments per concentrations. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to H_2O_2 cells. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to control cells

when exposed to 250 μM H_2O_2 for 3 h (Fig. 2). Long term pre-treatment (48 h) of DMC and rhGLP-1 protected the MIN6 cells from H_2O_2 induced toxicity. Pre-treatment with DMC at 25 μM increased cell viability by 16.41%, while rhGLP-1 at the same concentration nearly had no increase. Cells pre-treated with DMC had no cytotoxicity between 6.25 and 25 μM (data not shown).

2.2. *Hoechst nuclear staining*

The morphological changes of MIN6 cells were examined when exposed to the pro-apoptotic agents H_2O_2. Control cells were regular and their fluorescence was well-distributed throughout the cell nuclei (Fig. 3A). In contrast, the nuclei of MIN6 cells exposed to 250 μM H_2O_2 exhibited cell shrinkage, chromatin condensation and fragmented bodies (Fig. 3B). Obviously, cells treated with 25 μM rhGLP-1(7∼36) (Fig. 3C) and 25 μM DMC (Fig. 3D) had less apoptotic cells than those exposed to H_2O_2 (Fig. 3B), and the protective effect of DMC was better than rhGLP-1(7∼36). The treatment with DMC and rhGLP-1 before exposure to H_2O_2 protected nucleus fragmentation and inhibited cell apoptosis.

2.3. *FACS analysis of apoptotic cells*

That H_2O_2-induced apoptosis was further documented using annexin-V and PI staining. Apoptotic cells were analyzed by a flow cytometer. Cells in the lower right quadrant indicate annexin-positive, early apoptotic cells (LR). The cells in the upper right quadrant indicate annexin-positive/PI-positive, late apoptotic cells (UR). The cells in the lower left quadrant indicate non-apoptotic (LL). The apoptosis was calculated by UR cells and LR cells.
The result of annexin-V and PI double staining is shown in Fig. 4. Long term pretreatment (48 h) with different concentrations of DMC decreased H_2O_2 induced apoptosis in MIN6 cells. H_2O_2 increased cell apoptosis by 70%, whereas DMC at 25 μM rescued theses cells from H_2O_2 induced apoptosis by 32.27%, which was better than the positive control rhGLP-1 at the same concentration.

2.4. *Measurement of endogenous reactive oxygen species (ROS) production*

Intracellular ROS formation was determined by using DCFH-DA, a probe which was oxidized to green fluorescent DCF by reactive oxygen and nitrogen species. The fluorescent intensity was measured by a FACS. As shown in Fig. 5, the exposure of MIN6 cells to 250 μM H_2O_2 significantly enhanced ROS formation (6.27-fold) compared with normal cells. The pre-incubation

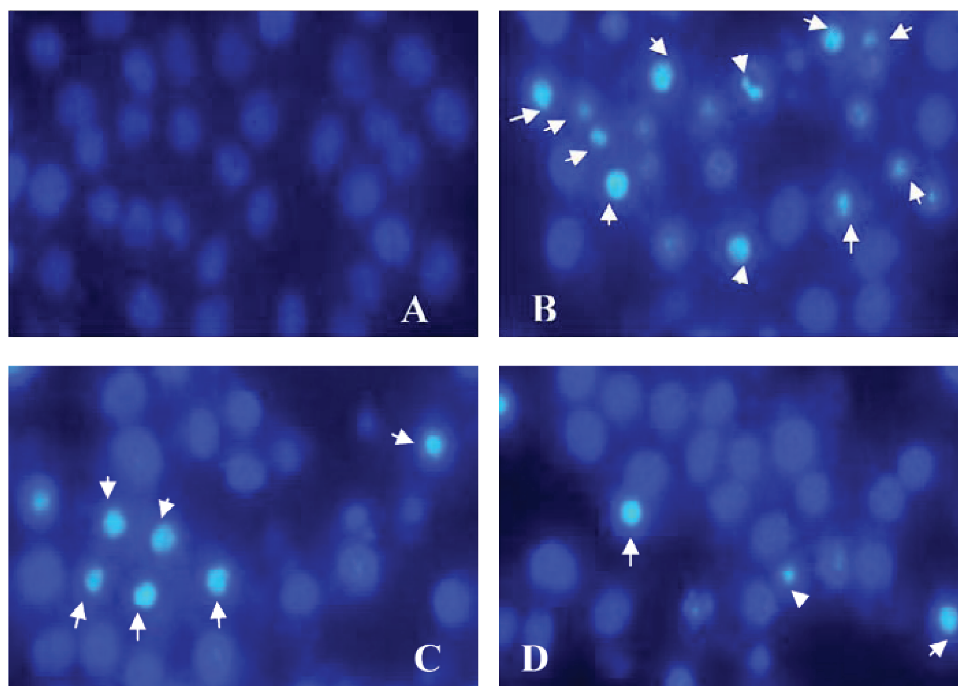
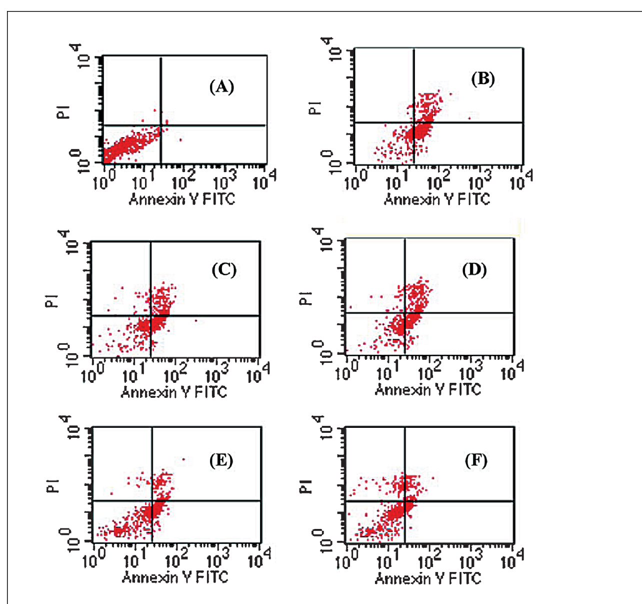


Fig. 3: Effect of DMC on the morphology of nuclear chromatin stained by Hoechst33342. Cells were pretreated with 25 μM rhGLP-1 and 25 μM DMC for 48 h, then exposed to 250 μM H_2O_2 for 3 h. After fixing the cells, they were stained with 10 μg/ml Hoechst 33342. Morphological changes of nuclear chromatin were then viewed under a fluorescence microscope. A. MIN6 cells cultured in regular medium; B. MIN6 cells exposed to 250 μM H_2O_2 for 3 h. C. MIN6 cells pretreated with 25 μM rhGLP-1 for 48 h, then exposed to 250 μM H_2O_2 for 3 h. D.MIN6 Cells pretreated with 25 μM DMC for 48 h, then exposed to 250 μM H_2O_2 for 3 h



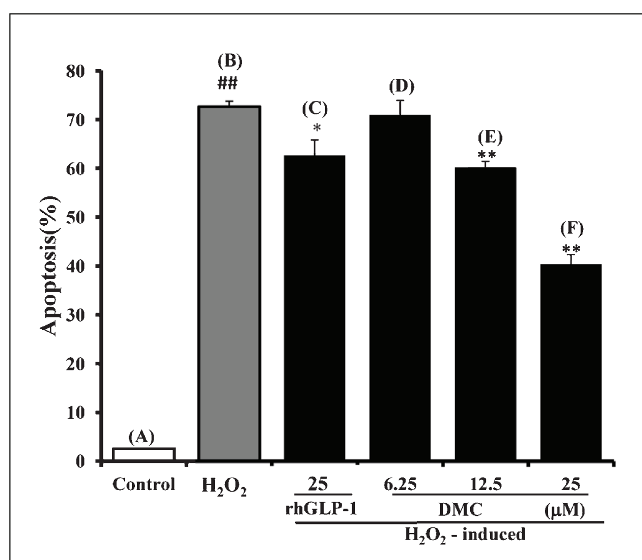


Fig. 4: Inhibitory effect of DMC on H_2O_2 induced apoptosis in MIN6 cells. FACS analysis via Annexin V-FITC/PI staining was used to observe the induction of apoptosis. (A), cells cultured in regular medium; (B), cells exposed to H_2O_2 (250 μM for 3 h); (C), cells pretreated with 25 μM rhGLP-1. (D)–(F), cells pretreated with DMC (6.25∼25 μM for 48 h). Cells in the lower right quadrant indicate Annexin-positive, early apoptotic cells (LR). The cells in the upper right quadrant indicate Annexin-positive/PI-positive, late apoptotic cells (UR). Data were presented as means ± SD and were representative of an average of three independent experiments per concentrations. $^*P < 0.05$, $^{**}P < 0.01$ compared to H_2O_2 cells. $\#P < 0.05$, $\#\#P < 0.01$ compared to control cells

of both 6.25∼25 μM DMC and 25 μM rhGLP-1 inhibited the H_2O_2 induced ROS formation in MIN6 cells. The ROS level was decreased gradually with the raise of the concentration of DMC.

2.5. *Evaluation of mitochondrial membrane potential (MMP)*

Exposure of MIN6 cells to 250 μM H_2O_2 leaded to 60% decrease of MMP. DMC not only prevented this decrease but also improved the MMP in a dose-dependent manner (Fig. 6). DMC significantly protected against the H_2O_2-induced decrease in MMP both at 12.5 μM and 25 μM ($P < 0.01$ vs H_2O_2 cells) as well as rhGLP-1 at 25 μM. DMC at 25 μM was better than

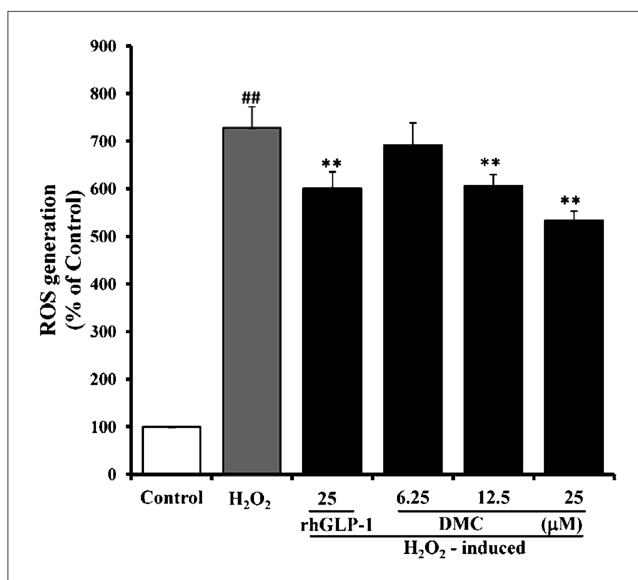


Fig. 5: Effect of DMC on reactive oxygen species in H_2O_2-induced MIN6 cells using ROS kit. Cells were pre-treated with 25 μM rhGLP-1 or DMC (6.25∼25 μM) for 48 h, then incubated in the presence of 250 μM H_2O_2 for 3 h. After that, loaded with DCFH-DA probe, and then analyzed by flow cytometry. Data were presented as means ± SD and were representative of an average of three independent experiments per concentrations. $^*P < 0.05$, $^{**}P < 0.01$ compared to H_2O_2 cells. $\#P < 0.05$, $\#\#P < 0.01$ compared to control cells

rhGLP-1 at the same concentration in enhancing MMP of H_2O_2-induced apoptotic cells.

2.6. *Caspase activity*

To evaluate whether specific caspase subtypes contributed to β-cell death, we measured caspase-3 and caspase-9 activities. H_2O_2 raised the caspase-3 and caspase-9 activities by 109.33% and 49.33% respectively both compared with control cells

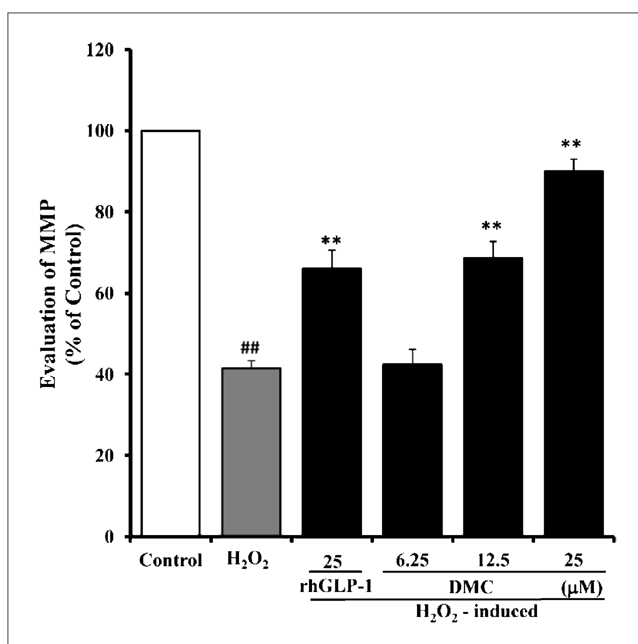


Fig. 6: Changes in mitochondrial membrane potential in MIN6 cells. Cells were pre-treated with 25 μM rhGLP-1 or DMC (6.25∼25 μM) for 48 h, then incubated in the presence of 250 μM H_2O_2 for 3 h. After that, the mitochondrial membrane potential was analyzed by flow cytometer using fluorescentdye Rho 123. Data were presented as means ± SD and were representative of an average of three independent experiments per concentrations. $^*P < 0.05$, $^{**}P < 0.01$ compared to H_2O_2 cells. $\#P < 0.05$, $\#\#P < 0.01$ compared to control cells

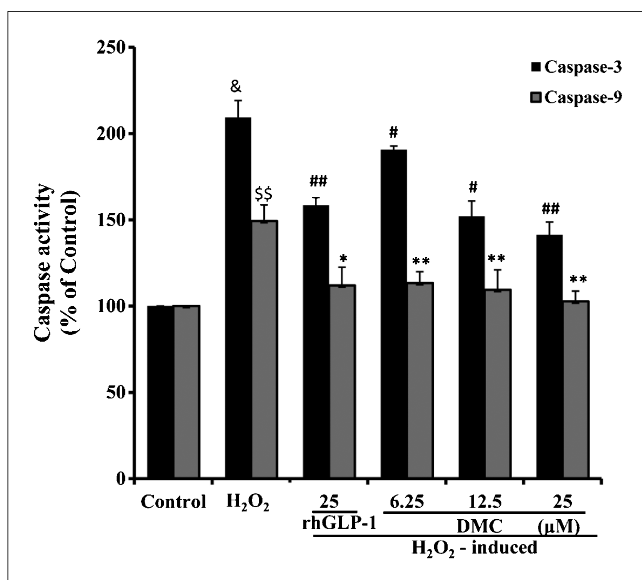


Fig. 7: DMC reduces caspase-3 and caspase-9 activities in H_2O_2-induced MIN6 cells apoptosis. Cells were pre-treated with 25 μM rhGLP-1 or DMC (6.25∼25 μM) for 48 h, then incubated in the presence of 250 μM H_2O_2 for 3 h. After that, the caspase-3 and caspase-9 activities in MIN6 cells were detected by caspase-3 and caspase-9 kits. Data were presented as means ± SD and were representative of an average of three independent experiments per concentrations. *P, #$P < 0.05$, **P, ##$P < 0.01$ compared to H_2O_2 cells in caspase-3, caspase-9 activity analysis respectively. &&P, $$$P < 0.01$ compared to control cells

(Fig. 7). DMC at 6.25∼25 μM decreased the caspase-3 activity as well as caspase-9 (compared with H_2O_2 cells). The results suggested that activation of caspase-3, 9 possibly contributed to H_2O_2 induced apoptosis.

3. Discussion

DMC, the main compound from buds of *C. operculatus*, exerts various biological actions. One of the effects is its significant ability to lower the blood glucose in alloxan-diabetic mice. In an oral glucose tolerance test, at a dosage of 1.0 mg/20 g mice, DMC can lower the blood glucose levels in glucose-hyperglycaemic mice when administered 15 min after a glucose load (Ma et al. 2005). The *in vitro* study proved that DMC owns the peroxisome proliferator-activated receptor-γ (PPAR-γ) ligand-binding activity, and can promote glucose uptake in 3T3-L1 adipocytes (Jin et al. 2008). These beneficial effects may contribute to its anti-diabetic activity. However, a direct effect of DMC on β-cells has not been reported.

In the present study, β-cell damage was induced by H_2O_2. It is generally believed that H_2O_2 as a cytotoxic molecule plays an important role attacking pancreatic β-cells leading to alteration of function and eventually β-cell destruction. It is released from activated macrophages during the early stages of insulitis, and it is also produced by agents like alloxan and streptozotocin which are commonly used to induce diabetes (Takasu et al. 1991). High doses of H_2O_2 can cause cell necrosis; while in low doses, it induces apoptosis in many cultured cell lines (Hui et al. 2003; Hampton and Orrenius 1997). We exposed MIN6 cells to H_2O_2 in different concentrations and incubation times to induce apoptosis. MIN6 cells cultured in 250 μM H_2O_2 for 3 h were capable of inducing cell apoptosis as demonstrated by FACS analysis for annexin-V/PI, internucleosomal DNA fragmentation and activation of caspase-3. Based on these data, exposure to H_2O_2 at 250 μM for 3 h was chosen to be our research model. The addition of rhGLP-1 to cells exposed to H_2O_2 could not increase their survival rate, while the pre-treatment of DMC at 25 μM before the exposure to H_2O_2 had a significant improvement of cell viability.

Mitochondria are the principal energy sources of the cell that convert nutrients into energy through cellular respiration (Wallace 2005). They are key organelles for β-cell function survival (Maechler and Wohlheim 2001). However, mitochondria also play a key role in triggering apoptosis (Newmeyer and Miller 2003). Particular proapoptotic signals such as respiratory chain failure and mitochondrial membrane potential loss rise in the cell and alter the permeability of their membranes until the mitochondrial membrane permeabilization is achieved, the cytochrome *c* is allowed to be released from the inner membrane, in the presence of dATP, caspase-9 is directly activated by Apaf-1 and cytochrome *c*. Active caspase-9, in turn, activates caspase-3, leading to activation of the caspase-3 cascade and apoptosis (Sampson et al. 2010). The caspases represent a novel class of cytoplasmic cysteine proteases mediating apoptosis. Caspase-3 has been identified as being a key mediator of apoptosis of mammalian cells. Our data showed that H_2O_2 caused a significant damage to MIN6 cells. Many cells characterized by cell shrinkage, chromatin condensation or fragmented bodies were clearly observed among H_2O_2 treated cells by Hoechst 33342. After pretreatment with DMC, fluorescent staining analysis revealed that the cell nucleus was nearly to the normal cells and less apoptotic cells were observed. The FACS analysis also showed DMC inhibited cell apoptosis. Furthermore, DMC significantly ($P < 0.01$) attenuated the caspase-3, 9 activities in MIN6 cells exposed to H_2O_2. These data indicated that anti-apoptotic effect of DMC was associated with the attenuation of caspase-3, 9 activities.

The protective effect of DMC against H_2O_2 induced apoptosis was also supported by higher MMP and lower ROS. The major site of ROS production in the cell is the mitochondrial respiratory chain. Mitochondrial radical production associated with hyperlipidemia and hyperglycemia disrupts glucose-stimulated insulin secretion and causes cellular damage (Cnop et al. 2005). The pancreatic β-cells are highly sensitive to oxidative stress for its lower antioxidant enzymes level (Lenzen et al. 1996). Hence strategies to decrease mitochondrial free radical production and oxidative damage may have therapeutic potential (Green et al. 2004). Pretreatment with resveratrol, a natural polyphenol with antioxidative properties, for 12 h at a concentration of 30 μM, inhibits streptozotocin-induced apoptosis of rat islets (Ku et al. 2011). Curcumin, a potent antioxidant/radical scavenging compound, shows significant inhibition of nitric oxide generation as compared to streptozotocin-induced islets without curcumin and protects islets against streptozotocin-induced death and dysfunction at 10 μM without affecting the normal function of these cellular structures (Meghana et al. 2007). Our studies also showed that DMC pretreatment at 12.5 μM and 25 μM attenuated H_2O_2 induced ROS production which is responsible for the mitochondrial dysfunction characterized by a decrease in membrane potential and prevented the loss of MMP.

The present study demonstrates the protective effects of DMC on H_2O_2 induced cell apoptosis by improving mitochondrial function. But the deep mechanisms responsible for this apoptosis are still unclear. In summary, the present study provides evidence for an anti-apoptotic effect of DMC on oxidative stress damaged pancreatic β-cells by protecting mitochondria function. Therefore, the results reported here support the potential of DMC to be used as an effective anti-apoptosis drug in diabetes therapy.

4. Experimental

4.1. Materials

DMC was isolated from *C. operculatus* in our lab as described by Ye et al. (2004 a,b), and its purity was above 98% (HPLC analysis). Recom-

bined human Glucagon-like peptide-1(7∼36) (rhGLP-1(7∼36)) was kindly given from Huay bio-lab Co. Ltd (Shanghai, China). Dulbecco's modified Eagle medium (DMEM) was obtained from Gibco (Los Angeles, US); Fetal bovine serum (FBS) was obtained from Hyclone (Losan, UT, US); Hoechst 33342 was purchased from Biyuntian institute of Biotechnology (Haimen, China); Caspase-3,Caspase-9 activity kits and Annexin V-FITC/PI staining kit were from Keygen (Nanjin, China); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) was from Amersco (Solon, OH, US); 2',7'-Dichlorofluorescein diacetate (DCFH-DA) was obtained from Sigma (St. Louis, MO); The other regents were analytic reagents (AR).

4.2. Cell lines and cell culture

Mouse insulinoma cell line, MIN6, was given from Huay bio-lab Co. Ltd (Shanghai, China) and cultured in DMEM with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37 °C under humidified condition of 5% CO_2/95% air.
DMC was dissolved in dimethylsulfoxide (DMSO) and stored at −20 °C before use. When needed, the stock solution was diluted with cell culture media for the required concentrations. The final DMSO concentration must be below 0.1% (v/v) which would not be harmful to cellular activity in previous experiments. The rhGLP-1(7∼36), taken as positive control, was obtained in the way of genetic engineering. During the gene recombination, GLP-1(1–37)'s 7 to 36 amino acid sequences which decided the bio-activity was retained. rhGLP-1(7∼36) was diluted in DMEM before each individual experiment.

4.3. Methods

4.3.1. Cell viability analysis

MIN6 cells were seeded into 96-well plates as 2×10^4 cells/well and pre-incubated for 4 h. After that, the cells were pre-treated at 37 for 48 h with or without DMC (6.25∼25 μM) or rhGLP-1(7∼36) (25 μM), Then they were exposed to 250 μM H_2O_2 for 3 h. Cell viability was measured by using MTT assay.
The final concentration of MTT was 0.5 mg/ml. After 4 h at 37 °C incubation of MTT in the dark, the MTT medium was replaced with DMSO. The absorbance of the dissolved formazan was measured at 570 and 630 nm as test and reference wavelengths, respectively in an Automated Microplate Reader (model3550-UV, Bio-Rad, US).

4.3.2. Hoechst nuclear staining

Hoechst 33342 was used to distinguish normal cell nuclei and apoptotic nuclei. MIN6 cells were seeded into six well plates as 5×10^5 cells/well and pre-incubated for 6 h. They were pretreated with DMC and rhGLP-1(7∼36) for 48 h, a final concentration of 250 μM H_2O_2 was added to each well except the control. After treatment, they were washed with PBS (pH7.3) twice and fixed with 0.5 ml 1% paraformaldehyde for 20 min at room temperature. After a remove of the fixing solution, the cells were washed twice with PBS (pH 7.3) and stained with 10 μg/ml Hoechst 33342 for 5 min at room temperature. Then, they were washed in PBS twice. The fluorescence of Hoechst was visualized under a fluorescence microscope (Olympus XB-51, Tokyo, Japan).

4.3.3. Flow cytometric assay of apoptosis

MIN6 cells were seeded into six well plates as 2×10^4 cells/well and pre-incubated for 6 h. After that, the cells were pretreated at 37 °C for 48 h with or without DMC (6.25∼25 μM) or rhGLP-1(7∼36) (25 μM), Then they were exposed to 250 μM H_2O_2 for 3 h. Cells were then harvested and suspended in Binding Buffer. Apoptotic cells were analyzed by a FACS flow cytometer (Becton Dickinson calibur, US) using the annexin V-FITC/PI staining kit.

4.3.4. Measurement of endogenous reactive oxygen species (ROS) production

DCFH-DA was used to measure intracellular oxidative activity by flow cytometric analysis. After treatment, cells were harvested and incubated in 10 μM DCFH-DA for 30 min at 37 °C in dark condition. Then they were washed in PBS for several times, the endogenous ROS production were measured by flow cytometer (Becton Dickinson calibur, US).

4.3.5. Evaluation of mitochondrial membrane potential (MMP)

MMP was monitored by rhodamine123 fluorescence in MIN6 cells. Briefly, Cells were loaded with 10 μg/ml rhodamine123 for 30 min at 37 °C in dark place. After centrifugation, the cells were washed twice and resuspended with 500 μl PBS, followed by analysis on flow cytometer (Becton Dickinson calibur, US) with an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

4.3.6. Caspase activity

Activation of caspases was measured by caspase-3 and caspase-9 activity assay kits. Cells were collected by centrifugation, placed on ice after adding lysis buffer for 40 min. The supernatant from lysed cells was mixed with reaction buffer and substrate for 4 h at 37 °C in dark place. The substrates of caspase-3 and caspase-9 were Ac-DEVD-pNA and Ac-LEHD-pNA respectively. The absorbance of both caspase-3 and caspase-9 was measured at 405 nm in an Automated Microplate Reader (model3550-UV, Bio-Rad, US).

4.4. Statistical analysis of data

Data were presented as the mean ± s.e.m. Statistical significance was tested with an unpaired two-tailed Student t-test. Statistical data were from at least three independent experiments. $P < 0.05$ was recognized as statistically significant.

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