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Inhibitory Effects of Crocetin On High Glucose-induced Apoptosis in Cultured Human Umbilical Vein Endothelial Cells and Its Mechanism

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Dysfunction of endothelial cell is considered as a major cause of vascular complications in diabetes. Crocetin has been shown to have strong antioxidant activities. In present study, we tested whether crocetin inhibited high glucose-induced apoptosis in cultured human umbilical vein endothelial cells ($HUVEC_S$) and to explore its possible mechanism. Exposure to high glucose (33 mM) for 72h induced a pronounced increase in apoptosis compared with normal glucose (5.5 mM),as evaluated by cell chromatin staining with Hoechst 33,258 and cell death detection ELISA. High glucose attenuated activation of Akt and endothelial nitric oxide synthase (eNOS). Crocetin (0.1 μM, 1.0 μM) prevented high glucose-induced apoptosis, which correlates with the increase of activation of p-Akt, following the up-regulation of eNOS and NO production. Pretreatment with phosphatidylinositol 3' kinase (PI3K) inhibitor LY294002 or eNOS inhibitor N^G-nitro- arginine methyl ester (LN or L-NAME) inhibited crocetin' effect on p-Akt or eNOS, respectively. For the first time, results of our study suggest that crocetin inhibits high glucose-induced apoptosis, at least partly, via PI3K/Akt/eNOS pathway in $HUVEC_S$ and crocetin may exert a beneficial effect in preventing diabetes-associated cardiovascular complications.

Keywords: High glucose, Crocetin, Endothelial cell, Apoptosis, Akt, eNOS

INTRODUCTION

Diabetes is associated with vascular complications. Its macrovascular and microvascular diseases are the most common causes of morbidity and mortality in diabetic patients. Endothelial dysfunction is a pivotal early event in diabetic atherosclerosis. And cell apoptosis plays an important role in the endothelium dysfunction. Evidences suggest that high glucose would induce generation of reactive oxygen species (ROS) in endothelial cells, which can cause cellular dysfunction and even cell death (Tesfamariam and Cohen, 1992; Baumgartner-Parzer *et al*., 1995). A wide variety of studies suggested that glucose induced apoptosis of human umbilical vein endothelial cells (Ido *et al.*, 2002; Meei *et al.*,2005; Sophie *et al.*, 2006; Ho *et al.*, 2006; Wei *et al.*, 2007). Therefore, prevention of glucose-mediated endothelial cell apoptosis may have important implications for pharmacological attempts at preventing diabetes-associated vascular complications.

It is well known that through activation of the downstream serine/threonine kinase Akt, PI3K plays an important role in preventing cell death produced by many death stimuli (Ahmed *et al.*, 1997; Dudek *et al.*, 1997; Datt *et al.*, 1999; Scheid *et al.*, 2000; Kim *et al.*, 2000). Akt activation has been reported to promote endothelium cell survival. Akt can activate endothelial nitric oxide synthase (eNOS), and thereby results in nitric oxide (NO) production, which prevents superoxide anion from forming its dismutation product (Fulton *et al.*, 1999). Therefore, PI3K/Akt/NO pathway plays a key role in preventing ROS-induced endothelial cell injury (Chavakis *et al.*, 2001).

Crocetin (Fig. 1) (a gift from professor Zhiyu Qian, Department of Pharmacology, China Pharmaceutical University, Nanjing 210009, China), an extract from *Gardenia jasminoides* Ellis, has antiatherosclerosis (He *et al.*, 2005), strong antioxidant effect (Gong *et al.*, 2001) and effect of

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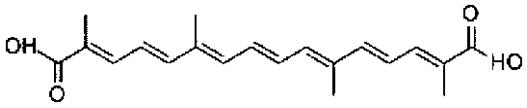

Fig. 1. chemical structure of crocetin

protection on myocardial cells (Yu *et al.*, 2003), etc.. Recent research found that crocin (an analog of crocetin) inhibited human endothelial cell apoptosis induced by trisol and prevented endothelial dysfunction (Liu *et al.*, 2005). Other studies showed that crocetin, possibly through intracellular ROS inhibition and $[Ca^{2+}]_i$ stabilization, prevented the bovine aortic endothelium cell apoptosis induced by OX-LDL or AGEs (advanced glycation end products, AGEs) (Tang *et al.*, 2005; Xiang *et al.*, 2006). These findings called us to test the hypothesis that crocetin may exert a beneficial effect in preventing glucose-induced endothelial cell apoptosis through the PI3K/Akt/eNOS pathway, to my knowledge, which remains unclear.

MATERIALS AND METHODS

Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were obtained by collagenase treatment of umbilical cord veins as described previously (Jaffe *et al.*, 1973; Mozaffarian *et al.*, 2000). Briefly, umbilical veins were rinsed with sterile saline and digested with 0.25% trypsin. Cells were cultured on gelatin-coated dishes and propagated in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 100 IU/mL of penicillin, and 0.1 ng/mL of streptomycin. Medium was refreshed every 2-3 days. These cells were tested positive for factor VIII antigen by immunohistochemical examination. Cells were incubated at 37°C in a humidified atmosphere of 95% air -5% carbon dioxide. HUVECs in passage 3 to 5 were used for experiments.

In experiments, HUVECs cultured in normal glucose (5.5 mM) served as normal control group (NG group). HUVECs were incubated with high glucose (33 mM, HG group) for different intervals (24-48 h). To observe the effect of crocetin on high glucose-mediated apoptosis, HUVECs were pretreated with crocetin (0.01 μM, 0.1 μM and 1.0 μM; C1 group, C2 group, C3 group, respectively) for 18 h before incubation with high glucose. To further determine the effect of crocetin on the role of the PI3K/Akt/eNOS pathway, another two groups of HUVECs were pretreated with the specific PI3K inhibitor LY294002 (10 μM; Sigma) or a non-specific eNOS inhibitor N^G-nitro-arginine methyl ester (L-NAME; 100 μM; Sigma) (LY294002 group, LY group; L-NAME group, LN group, respectively) for 30 min before crocetin was added. The cells were harvested at the predetermined time to evaluate cell apoptosis and the activation of Akt and eNOS.

Detection of apoptosis

Apoptosis of the treated HUVECs was detected by the method of cell death detection ELISA (Boehringer Mannheim) and morphological assessment through nuclei staining with Hoechst 33,258. ELISA for DNA fragmentation was described previously (Wang *et al.*, 1955; Ho *et al.*, 2000). Briefly, HUVECS (1×10^4) were plated in each well of 24-well plate. After predetermined treatment, the cells were washed once with PBS, and 0.5 mL lysis buffer was added. After 30-min incubation, the supernatant was recovered and assayed for DNA fragments according to manufacturer's instruction. Each treatment repeated in three times. For morphological assessment, cells were collected and fixed in methanol/acetone (1/3, vol/vol) solution for 5 min and washed with PBS. Then fixed cells were stained with 0.1 ng/mL Hoechst 33,258 for 10 min in the dark to counterstain nuclei. Hoechst-stained cells were visualized and photographed under confocal cell scanning microscope (Scanalytics).

Determination of intracellular ROS levels

DCF-DA as fluorochrome was used to measure intracellular ROS formation. Intracellular ROS levels were measured by the dichlorofluorescein assay as described by (Wang and Joseph, 1999; Yokozawa etal., 2007). In brief, Cells were plated on 96-well plates. As described above, $HUVEC_S$ were pre-treated with different concentration of crocetin (0.01 uM, 0.1 uM, 1 uM) for 18 h, then incubated with high glucose (33 mM) for 48 h. The cells were washed once with calcium- and magnesium-free PBS and incubated in 100 μM DCFH-DA-containing medium (DCFH-DA, sigma, U.S.A.). After 15 min, the medium was removed, and the cells were incubated with fresh medium at 37°C for 1 h. Fluorescence was measured using a fluorescence plate reader (Tecan, Switzerland) with excitation at 485 nm and emission filter at 535 nm.

Detection of phosphorylated Akt and eNOS

Western blotting analysis was performed to investigate a possible mechanism of crocetin's action on Akt and eNOS phosphorylation. HUVECs were plated onto a six-well plate and exposed to different intervention conditions as described above. Then cells were harvested in lysis buffer containing 1% Triton X-100, Pefabloc (1 mM), aprotinin (10 μg/mL), pepstatin (1 μg/mL), leupeptin (10 μg/mL), NaF (10 mM), and Na_3VO_4 (1 mM). Protein determination was performed by Bradford assay (Bio-Rad, Richmond, CA, U.S.A.). Equal amounts of protein (40 μg) for each sample were migrated in acryl amide gels and blotted onto nitrocellulose filters. Blotted filters were probed with primary antibodies for the total of phosphorylated Akt and phosphorylated eNOS (Cell Signaling Technology, Beverly, CA, U.S.A.). After incubation with peroxidase-

conjugated anti-rabbit immunoglobulin G, specific reactions were revealed by the enhanced chemiluminescence method. Membranes were stripped by incubation in Re-Blot 1X antibody stripping solution (Chemicon International, Temecula, CA, U.S.A.) and reprobed for the respective total protein kinase content or β-actin to verify loading evenness. Densitometric values of band intensity were analyzed by Image Quant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Determination of NO production

NO production in the culture medium using a spectrophotometric method based on the Griess reaction (Green *et al.*, 1982). Briefly, culture medium (100 μL) was mixed with an equal volume of modified Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenedi amine dihydrochloride, and 2% phosphoric acid). After 10 min of incubation at room temperature, the concentration of the resultant chromophore was spectrophotometrically measured at 540 nm with a spectrophotometer (SpectraMax Plus384, Molecular Device, Sunnyvale, CA, U.S.A.). The nitrite concentrations in the samples were calculated from freshly prepared nitrite standard curves made from sodium nitrite with the same culture medium.

Statistical analysis

Results were presented as mean±SE for three or more independent experiments. Statistical significance among multiple groups was analyzed by one-way ANOVA followed by student-Newman-Keuls test for comparison of several groups in SPSS11.5 software package. $P<0.05$ was considered statistically significant.

RESULTS

Effect of crocetin on high glucose-induced apoptosis of HUVECs

In this study we used the DNA fragmentation staining with Hoechst 33,258 and cell death detection ELISA method to confirm the apoptosis in HUVECs induced by high glucose (33 mM) in accordance with previous study (Ho *et al.*, 1999; Ho *et al.*, 2006; Ho *et al.*, 2007).

Morphologically, nuclear condensation and chromatin fragmentation is one important and specific morphological change. Therefore, we used Hoechst 33,258 to show nuclear chromatin in HUVECs, and observed cell morphology with phase contrast microscopy. As showed in Fig. 2, nuclei of HUVECs in normal control group (glucose 5.5 mM) stained light with Hoechst. In contrast, when endothelial cells were exposed to high glucose for 48 h, nuclei in HUVECs showed chromatin condensed, and multiple chromatin fragments were seen in some of the cells, indicating the characteristic of apoptotic nuclei. And apoptosis was not evident until 36 h of high glucose treatment. Pretreatment of crocetin made the chromatin not even bright and few chromatin fragments were seen as compared to high glucose treatment, which showed that crocetin inhibited high glucose-induced apoptosis.

Quantitatively, we used the ELISA to determine high glucose-induced apoptosis in $HUVEC_S$ and inhibitory effect of crocetin (as illustrated in Fig. 3 and Fig. 4). High glucose-induced apoptosis markedly increased at 36 h and 48 h (vs. HG at 0 h or at 24 h, P <0.01), and high glucose induced apoptosis in the time-dependent manner of apoptosis (apoptosis at 48 h compared with at 36 h, P<0.05). Results also showed that crocetin (1.0 μM) pronouncedly inhibited high glucose induced-apoptosis compared with the high glucose treatment(vs. HG at 36h, P < 0.05;vs HG at 48 h, P < 0.01). Furthermore, the antiapoptotic effect of crocetin increased with the increase of concentration of crocetin (crocein 1.0 μM vs. crocetin 0.1 μM, n=5, P<0.05), indicating its dose-dependent manner of antiapoptosis of crocetin on high glucose-induced cell apoptosis. Crocetin (0.01 μM) showed no significant difference compared with high glucose group.

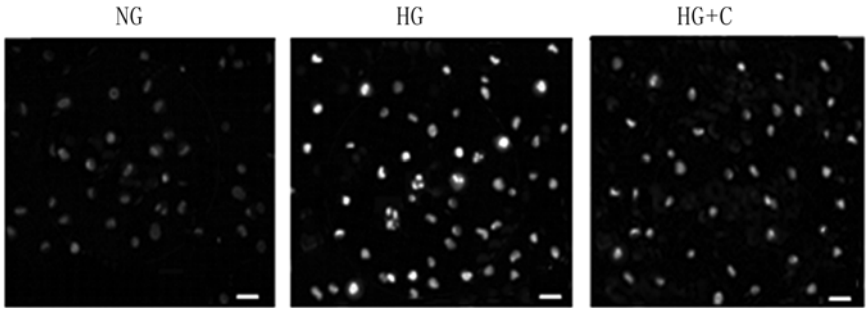

Fig. 2. High glucose induces apoptosis (stained with Hoechst) in HUVECs (phase-contrast micrographs). Compared with normal group, exposure to high glucose (33 mM) for 48 h indued more HUVECs apoptosis, showing condensed and brightly stained chromatin and some fragments of apoptotic nuclei. Pretreatment of crocetin (1.0 μM), HUVECs were lightly stained with Hoechst, rare cells with condensed and brightly stained chromatin or fragments of apoptotic nuclei. scale bars=30 μm.

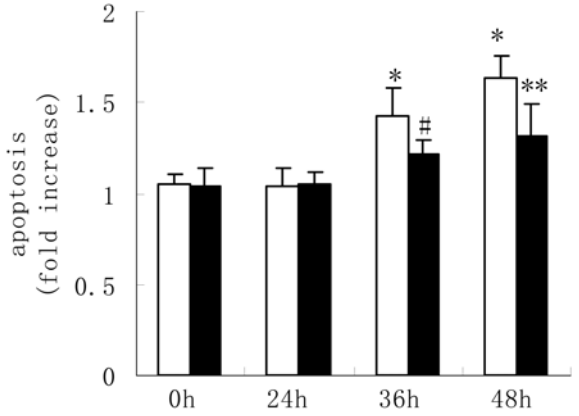

Fig. 3. High glucose-induced apoptosis in HUVECs and inhibitory effect of crocetin on apoptosis determined by ELISAs. $HUVEC_S$ were incubated with high glucose (33 mM, open bar) for 24 h to 48 h with or without pretreatment of crocetin (1.0 uM, solid bar). Data were expressed as mean±SE (n=5). High glucose significantly increased apoptosis at 36 h and 48 h compared with at 0 h and 24 h (*p<0.01). Crocetin time-dependently decreased high glucose-induced apoptosis (**P<0.05, HG+C at 36h vs. HG+C at 48 h).

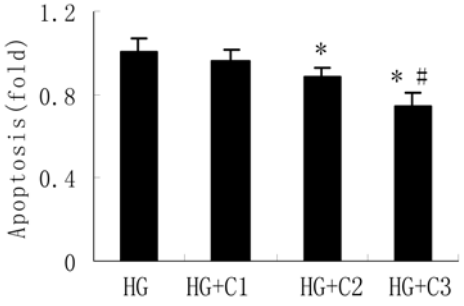

Fig. 4. Crocetin dose-dependently decreased apoptosis determined by ELISA. Data were expressed as mean±SE (n=5). $HUVEC_S$ were pretreated with different concentration of crocetin (C1, 0.01 μm; C2, 0.1 uM; C3, 1.0 Um), then incubated with high glucose (33 mM) for 48 h. Compared with HG, crocetin (0.01 uM) showed no marked inhibition on apoptosis (P>0.05). However, crocetin (0.1 uM), and crocetin (1.0 uM) pronouncedly attenuated the apoptosis (#P<0.01), and in a dose-dependent manner (#P<0.01, crocetin (1.0 uM) vs. (0.1uM) crocetin).

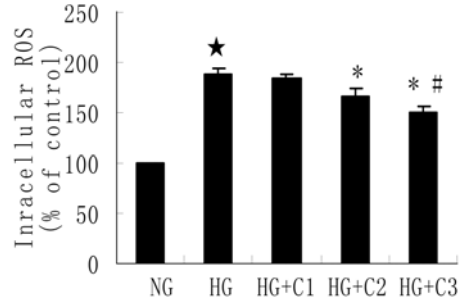

Fig. 5. The inhibitory effect of crocetin on intracellular ROS. Data were presented as means±SE (n=5). High glucose (33 mM) increased ROS production in HUVECs (HG vs. NG, ★P<0.01). However, crocetin (C2, 0.1 μM; C3, 1.0 μM) markedly reduced ROS generation compared with high glucose (*P<0.01), and in a dose-dependent manner (HG+C3 vs. HG+C2, #P<0.01). Crocetin (C1, 0.01 μM¨)showed no significant inhibitory effect on ROS production (#P>0.05, HG+C1 vs.).

Effect of crocetin on intracellular ROS

As shown in Fig. 5, the generation of intracellular ROS in $HUVEC_S$ increased significantly after treatment with 33 mM glucose compared with normal glucose (5 mM). However, pretreatment with crocetin (0.1 μM, 1.0 μM) reduced intracellular ROS from 188% to 166% or 150%, respectively (P<0.01). There was no statistically significant between high glucose group and high glucose plus crocetin (0.01 μM) group (p>0.05).

Effect of crocetin on activationn of Akt and eNOS

To explore the possible mechanism for anti-apoptotic effect of crocetin, we investigated the effect of crocetin on Akt and eNOS phosphorylation in HUVECs. As shown in Fig. 6A and B, high glucose showed no affection on Akt expression; however, it significantly reduced the phosphorylation of Akt (HG vs. NG, P<0.01). Preincubation of HUVECs with crocetin resulted in a significant increase in the phosphorylation of Akt compared with high glucose (P<0.05). The PI3K inhibitor LY294002 pronouncedly at-

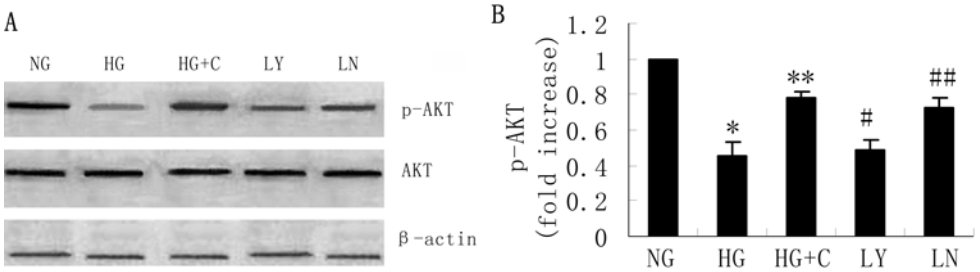

Fig. 6. Expression of Akt and P-Akt in cultured $HUVEC_S$ determined by western blot (**A**). Data of quantitative densitometry were presented as mean±SE (n=5) (**B**). Before crocetin (1.0 μM) was added, $HUVEC_S$ were pretreated with the inhibitors LY (10 μM) or L-NAME (100 μM) for 30 min, and then $HUVEC_S$ were incubated with high glucose (33 mM). Akt expressions were statistically insignificant among different group (data not shown). However, High glucose decreased p-Akt expression in $HUVEC_S$ compared with NG (5.5 mM) (*P<0.01). On the contrary, crocetin markedly increased p-Akt expression (HG+C vs. HG, **P<0.01); LY reduced the increase of p-Akt by crocetin (LY vs. HG+C, #P<0.01); LN had no influence on the increase of p-Akt by crocetin (LN vs. HG+C, ##P>0.05).

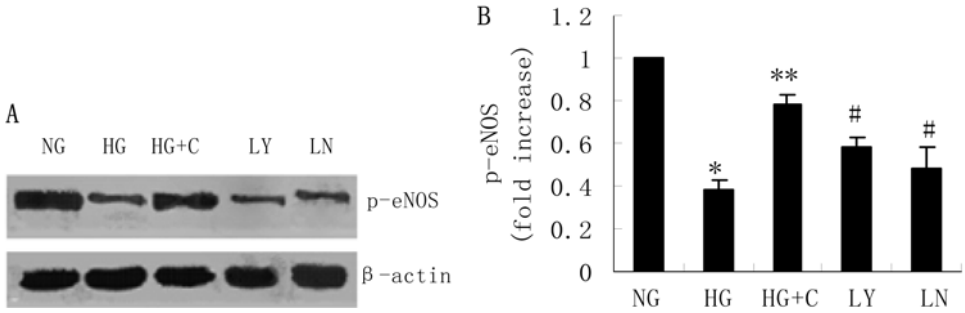

Fig. 7. Expression of p-eNOS (western blot, **A**) in $HUVEC_S$ exposed to different treatments as described in Fig. 6. Data obtained from quantitative densitometry were expressed as mean±SE (n=5) (**B**). Compared with NG (5.5 mM), High glucose (33 mM) decreased p-eNOS production in $HUVEC_S$ (*P<0.01). Crocetin (1.0 μM) markedly increased P-eNOS production compared with HG (**P < 0.01). LY (10 μM) and LN (100 μM) both reduced the increase of p-eNOS production by crocetin (LY vs. HG+C, P<0.01; LN vs. HG+C, P<0.01).

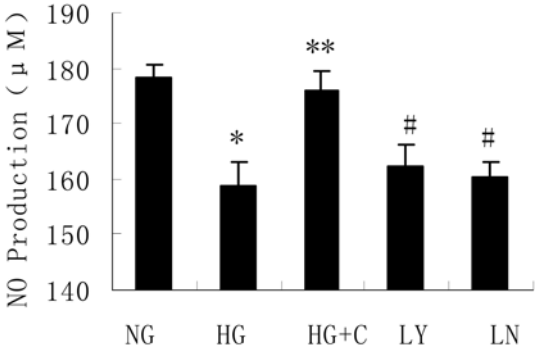

Fig. 8. Total NO concentration in $HUVEC_S$ culture medium exposed to different treatments as described in Fig. 6. Results are expressed as mean±SE (n = 5). Compared with normal glucose (5.5 mM), High glucose (33 mM) markedly inhibited the NO production in the culture medium (*P<0.01). Crocetin (1 μM) significantly increased the NO concentration in medium (HG+C vs. HG, **P<0.01). LY (10 μM) and LN (100 μM) both reduced the increase of NO by crocetin (LY or LN vs. HG+C, #P<0.01).

tenuated the effects of crocetin on phosphorylation of Akt, making the p-Akt decrease (LY vs. HG+C, P<0.01); LN, the NOS inhibitor, showed no influence on the increase of p-Akt by crocetin (LN vs. HG+C P>0.05). Similarly, crocetin reversed the inhibition of high glucose-induced eNOS phosphorylation as shown in Fig. 6A and B. Preincubation of endothelial cells with L-NAME or LY294002 abolished the increase of phosphorylation of eNOS caused by crocetin.

Effects of crocetin on NO content in culture medium

As illustrated in Fig. 8, high glucose (33 Mm) significantly decreased NO production compared with normal glucose (P<0.01). In contrast, preincubation of HUVECs with crocetin (1 μM) protected against the high glucose-induced depletion of NO level (HG+C vs. HG, P<0.01). L-NAME and LY294002 inhibited the increase of NO production caused by crocetin (LY or LN vs. HG+C, P<0.01).

DISCUSSION

Recent studies have suggested crocetin attenuated apoptosis of vascular endothelial cells induced by ox-LDL or AGEs, which is related to the increase of eNOS and /or NO concentration caused by crocetin (He *et al.*, 2005; Tang *et al.*, 2005; Zheng *et al.*, 2005; Xiang *et al.*,2006). However, there is no report on whether crocetin inhibits high glucose-induced apoptosis in human umbilical endothelial cells. In present study, for the first time we confirmed crocetin has strong antioxidant activity and exposure of cultured HUVEC to high glucose resulted in an increased apoptosis confirmed by cell death ELISA, and DNA staining by Hoechst. Furthermore, we found crocetin significantly inhibited high glucose-induced $HUVEC_S$ apoptosis, which may exert its protection£¨at least partly, in a PI3K-Akt-activated eNOS-derived NO-dependent manner.

It has been established that under diabetes, hyperglycemia causes the autoxidation of glucose, glycation of proteins, and the activation of polyol metabolism. These changes accelerate generation of ROS and increase in oxidative chemical modification of lipids, DNA, and proteins in various tissues (Robertson *et al.*, 2003; Bhat and Zhang, 1999). The ROS production has been demonstrated to cause endothelial dysfunction and apoptosis, which may play an important role in the development of diabetic vascular complications (Giuglano *et al.*, 1996; Wang *et al.*, 1998; Basta *et al.*, 2004; Yokozawa *et al.*, 2007). These observations indicate that antioxidant may be useful in the prevention of endothelial cell injury induced by ROS. Our results showed that high glucose (33 mM) significantly increased the intracellular ROS level, which was correlated with subsequent cell apoptosis. However, the addition of crocetin (0.1 μM, 1.0 μM), in a dose-dependent manner, inhibited high glucose-induced ROS production and signi-

ficantly prevented high glucose-induced $HUVEC_S$ apoptosis. Therefore, these results indicate that crocetin has a strong ROS-scavenging activity, and the antiapoptotic mechanisms of crocetin may be, at least partly, through its antioxidant activity.

Akt was recognized as a major downstream target of PI3K (Burgering *et al.*, 1995), only through the phosphorylation at both Thr 308 and Ser 473, the Akt was activated. Akt activation has been reported to promote HUVEC survival (Fulton *et al.*, 1999). Our study found pretreatment of cells with crocetin partly offset decrease of Akt phosphorylation mediated by high glucose. Pretreatment with LY294002 significantly attenuated the antiapoptotic effect of crocetin, leading to an increased number of apoptotic cells. Taken together, at least partly through PI3K/Akt, crocetin exert its anti-apoptotic effect on endothelial cells.

By inducing eNOS activity, the PI3K/Akt/eNOS pathway can provide an enhanced survival signal for the cells (Datt *et al.*, 1999; Kim *et al.*, 2000). It is found that NO donor, sodium nitroprusside, can prevent high glucose-mediated endothelial cell apoptosis in vitro (Ho *et al.*,1999). Moreover, hyperglycemic impairment of NO production by endothelial cells has been implicated in increased cardiovascular disease risk caused by diabetes (Ho *et al.*, 1999). In this study, we found that crocetin markedly protected endothelial cells from high glucose-induced apoptosis via PI3K/Akt pathway. Furthermore, our results showed crocetin inhibited down-regulation of eNOS phosphorylation expression caused by high glucose, resulting in an increase of NO production, whereas eNOS inhibitors L-NAME significantly reduced the increase of NO production caused by crocetin. Moreover, PI3K inhibitor LY294002 decreased the up-regulation effect of crocetin on eNOS phosphorylation and NO production. All these results indicated that PI3K/Akt pathway is involved in eNOS activation.

In conclusion, the present study shows that, despite its precise anti-apoptotic mechanism has not yet been thoroughly elucidated, crocetin is capable of inhibiting high glucose-induced apoptosis in HUVECs. Moreover, this study indicates crocetin ameliorates the apoptosis, at least in part, via PI3K/Akt/eNOS pathway, and crocetin may exert beneficial effects in preventing diabetes-associated cardiovascular complications.

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