Inhibition of high glucose-induced apoptosis by uncoupling protein 2 in human umbilical vein endothelial cells

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Received October 30, 2013; Accepted February 21, 2014

DOI: 10.3892/ijmm.2014.1676

Abstract. Studies have shown that an overproduction of mitochondrial reactive oxygen species (ROS) is an initiating cause in the pathogenesis of diabetic complications. However, uncoupling protein 2 (UCP2) can protect retinal vascular endothelial cells from damage by inhibiting the overproduction of mitochondrial ROS, although the protective mechanism involved is not completely clear. This study aimed to assess the effect and mechanism of UCP2 on the apoptosis of human umbilical vein endothelial cells (HUVECs). HUVECs were cultured in normal glucose (NG, 5.5 mmol/l) or high glucose (HG, 30 mmol/l) medium in the presence or absence of UCP2+/– lentiviral transfection. Lentivirus-mediated UCP2 overexpression inhibited the apoptosis of HUVECs induced by HG. Treatment with HG resulted in the upregulation of caspase-3 and cytochrome c and the downregulation of Bcl-2 in vitro. Furthermore, compared with the NG group, the rate of apoptosis was significantly increased in the HG group. On day two post-infection, NG cells showed significantly greater HUVEC cell proliferation than HG cells. Notably, UCP2 overexpression inhibited these processes. Taken together, these results suggest that UCP2 promotes cell proliferation and inhibits HG-induced apoptosis in HUVECs via the Bcl-2 up- and downregulation of caspase-3 and cytochrome c in vitro. This may provide experimental evidence for the application of UCP2 as a new protective factor for diabetic complications, such as diabetic retinopathy.

Introduction

Diabetic retinopathy (DR) is a major cause of blindness in developed countries, and oxidative stress caused by hyperglycemia is one of the most common causes of diabetic microangiopathy (1). Currently, the specific cause of diabetic microangiopathy is not completely understood. Recently, a unifying hypothesis has been suggested whereby the production of mitochondrial reactive oxygen species (ROS) in response to chronic hyperglycemia may be the key initiator of four pathogenic pathways: the polyol pathway, increased formation of advanced glycation end-products, activation of protein kinase C, and the hexosamine pathway (1-3). These studies have emphasized the important role of increased mitochondrial ROS production in diabetes complications, including retinopathy. Therefore, mitochondrial ROS may serve as an important target for DR treatment. We previously found (4) that uncoupling protein 2 (UCP2) has a negative regulatory role in ROS generation; however, the exact mechanism remains to be determined.

UCPs are mitochondrial transporters present in the inner membrane of mitochondria. They belong to the family of anion mitochondrial carriers. Five different UCPs have already been identified: UCP1-4 and UCP5, or brain mitochondrial carrier protein 1 (BMCP1). These proteins are expressed in different tissues and play different roles in cellular metabolism. Brain-specific UCP4 and UCP5 have been suggested to play a role in apoptosis in the brain (5,6). Immunocytochemistry of UCP expression in endothelial cells and pericytes showed that UCP1 and UCP2 expression was positive in both types of cells, while UCP3 was negatively expressed (7). UCP2 was initially described by Fleury et al (8). It shares 59% identity to UCP1 and is expressed most abundantly in mitochondria from the spleen, certain regions of the brain, the pancreas, lungs, stomach, intestine, white adipose tissue, thymocytes, kidney, cardiomyocytes, macrophages and mast cells, but not in muscle, heart, liver, or brown adipose tissue (8-18). Expression of UCP2 has been shown to result in a decrease in mitochondrial superoxide (19-24). UCP2 has been shown to have a protective effect in the brain, preventing acute damage produced by ischemia (25-28); in the circulatory system, preventing atherosclerotic plaques (29); and in cardiomyocytes, where its overexpression is anti-apoptotic (30). Findings of previous studies have shown that UCP2 plays a role in macrophages and pancreatic β cells by affecting the killing capacity and glucose-induced insulin secretion, respectively (13,19,22,31,32).

Considering that the overexpression of UCP2 can inhibit vascular damage in DR and induce pathological changes associated with vascular injury and nerve-tissue degeneration, we aimed to determine whether UCP2 was able to reduce...
high glucose-induced endothelial cell apoptosis and protect blood vessels from damage. In this study, we investigated whether UCP2 inhibits high glucose-induced apoptosis of human umbilical vein endothelial cells (HUVECs) to provide experimental evidence for the application of UCP2 as a new protective factor of DR.

Materials and methods

Construction and identification of the UCP2 expression plasmid. The primers targeting the human UCP2 gene were synthesized based on a cDNA library (GenePharma, Shanghai, China), and the sequences based as follows: UCP2 forward, 5'-GGGATACCAAGGCACGTCATTACG-3' and reverse, 5'-AGCACAGTGACAATTGGCATTCAGG-3'; hACTB forward, 5'-CCCTGGCAACCACGAC-3' and reverse, 5'-GCCGATCCACAGGATGTC-3'.

Each of these fragments was amplified, digested with AgeI and then ligated into the pcG-FC-F3FLAG vector for 15 min at 23 and 42°C. The recombiant was transformed into competent Escherichia coli cells treated with calcium chloride and incubated for 16 h at 37°C.

PCR conditions were as follows: 94°C for 5 min, 30 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 2 min and then 72°C for 10 min. Positive clones, as confirmed by PCR, were chosen for sequencing. The plasmids with the correct sequence were transfected into 293T cells, and expression of the protein was observed under a fluorescence microscope (Nikon, Tokyo, Japan). Western blotting was performed to detect the expression of the protein. The virus titer was determined by quantitative PCR (qPCR).

Cell culture and lentivirus transduction. HUVECs were obtained from the ScienCell Research Laboratories (San Diego, CA, USA). The cells were cultured in Endothelial Cell Medium (ECM; ScienCell Research Laboratories, Carlsbad, CA, USA) with 5% (v/v) fetal bovine serum (FBS) at 37°C in 5% (v/v) CO₂ and 95% humidity. When they reached confluence, the cells were maintained in 1% (v/v) fetal calf serum and exposed to normal glucose (NG, 5.5 mmol/l) or high glucose (HG, 30 mmol/l) for 3-7 days, during which the medium was changed every 2 days. When the HUVECs were ~50% confluent in fresh serum-free medium, they were transiently transfected with control lentivirus or UCP2-overexpression lentivirus at a multiplicity of infection (MOI) of 100. The cells were cultured in ECM with 5% (v/v) FBS after infection for 4 h and then selected using 200 µm/ml puromycin. The stable overexpressing lines were established when >90% of the transfected cells were found to strongly express GFP under a fluorescent microscope. The HUVECs were divided into four groups: NG, HG, high glucose + the lentiviral-negative vector control group (NC), and high glucose + UCP2-overexpressed group (UCP2).

RNA isolation and RT-qPCR. Total RNA was isolated from HUVECs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA extract (2 µl) was reverse-transcribed into cDNA in a total reaction volume of 20 µl using a RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Burlington, ON, Canada). qPCR was performed using IQ Supermix (Bio-Rad, Hercules, CA, USA), with 20 µl reaction mixtures containing 1 µl cDNA, 8 µl sterilized water, 10 µl SYBR-Green real-time PCR Master Mix (Takara Bio Inc., Shiga, Japan), and 1 µl of primer. The UCP2, cytochrome c, caspase-3 and Bcl-2 amplification signals were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and evaluated using the equation: fold-change = 2^ΔΔct. The primer sequences used in this study were: hUCP2 forward, 5'-GGCTGAGGATGTGCGAG-3' and reverse, 5'-CAGAATGAGTTGCAAGGAG-3'; cytochrome c forward 5'-TTTATATTAGATGTGCCCATGGC-3' and reverse, 5'-CTCCAGATGATGCCTTGGT-3'; caspase-3 forward, 5'-CAGCCGCAAATAAAGACAAAAAG-3' and reverse, 5'-CCCGCTTACAAATAGCCACC-3'; Bcl-2 forward, 5'-TACCTGAAACCGCACCCTG-3' and reverse, 5'-GCGCTACAGTTCCAAACAGG-3'; hGAPDH forward, 5'-GGGTGTAAGACCATGAGAATGTG-3' and reverse, 5'-GATGCCATGACTGGTGCAT-3'.

Protein extraction and western blot analysis. HUVECs were washed three times with ice-cold phosphate-buffered saline (PBS, 4°C, pH 7.4) for 5 min at room temperature and prepared using a protein extraction kit and a protease inhibitor kit (Pierce, Rockford, IL, USA). The supernatant was collected and the protein content of each lysate was determined using a BCA Protein Assay kit (Tianlai, Shanghai, China) was used to measure apoptosis in NG, HG, NC and UCP2 cells. Cells (1x10⁵) were plated in a volume of 50 µl using a 96-well plate. After 48 h, the cells were trypsinized, and the cells and culture medium were collected. Following centrifugation at 1,000 x g for 10 min, the supernatant was discarded. The cells were resuspended in 1 ml PBS, and transferred to an Eppendorf tube. Annexin-V FITC was added and mixed on ice and left in the dark for 15 min. Cell apoptosis was detected by flow cytometry. Experiments were performed in triplicate.

Flow cytometry. According to the manufacturer's instructions, the Annexin-V FITC apoptosis detection kit (Beyotime, Shanghai, China) was used to measure apoptosis in NG, HG, NC and UCP2 cells. Cells (1x10⁵) were plated in a volume of 1 ml into each well of a 6-well plate. After various incubation periods, cells were trypsinized, and the cells and culture medium were collected. Following centrifugation at 1,000 x g for 10 min, the supernatant was discarded. The cells were resuspended in 1 ml PBS, and transferred to an Eppendorf tube. Annexin-V FITC was added and mixed on ice and left in the dark for 15 min. Cell apoptosis was detected by flow cytometry. Experiments were performed in triplicate.

Immunofluorescence and confocal microscopy. Cells cultured on glass coverslips were fixed for 15 min at 4°C in PBS containing 4% (w/v) paraformaldehyde. The fixative was removed, and cells were either permeabilized with 0.5% (v/v) Triton X-100 in PBS for 6-7 min or were not permeabilized.
Cells were then blocked with 4% (w/v) bovine serum albumin in PBS for 30 min at room temperature. The non-permeabilized and permeabilized cells were treated with Bcl-2, cytochrome c, caspase-3 monoclonal antibody (1:100, 1:300 and 1:300, respectively) and/or rabbit polyclonal antibody for different organelle markers (2-5 µg/ml) overnight at 4°C. After removing the unbound primary antibodies and washing them five times with PBS containing 0.5% (v/v) Triton X-100 (PBST), the cells were incubated with Oregon Green-conjugated (excitation/emission wavelength 496/524 nm) or Rhodamine Red-conjugated (excitation/emission wavelength, 570/590 nm) anti-rabbit secondary antibodies for 40 min at room temperature. The cells were washed and then stained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) nuclear dye for 10 min at 37°C. After the cells were washed five times with PBST, coverslips were mounted onto glass slides and sealed with quenching agent and glycerol. Confocal images were acquired using an Olympus Fluoview 1000 microscope (Olympus, Center Valley, PA, USA). Experiments were performed in triplicate.

Statistical analysis. SPSS 17.0 was used to analyze experimental data. Experimental findings were presented as the mean ± standard deviation. One-way ANOVA followed by the Student-Newman-Keuls test was used to compare the effect of treatment on the various parameters. Non-parametric data were analyzed using Chi-square test or the Fisher’s exact method. P<0.05 was considered to indicate statistical significance.

Results

Identification of recombinant plasmid Plenti6.3/V5 DEST. The positive clones were identified after PCR amplification, and the size of the PCR product was 911 bp. The detected sequence was identical to the known UCP2 sequence in GenBank (Gene ID NM 003355.2). Western blotting revealed a 32 kDa band in cell extracts, which was in accordance with the expected size of the UCP2-Flag protein (32 kDa). These results indicated that the UCP2 recombinant plasmid was successfully expressed in HUVECs and suggested that these cells were successfully transduced with lentivirus.

Overexpression of UCP2 by stable transfection of Plenti6.3/V5 DEST. Total mRNA and protein extracts were prepared from untransfected controls (NG and HG), lentiviral-negative vector controls (NC) and UCP2-transfected (UCP2) HUVECs. The UCP2 mRNA and protein expression levels in these cells were determined by RT-qPCR and western blot assays, respectively. RT-qPCR demonstrated that the mRNA levels of UCP2 protein expression in the transfectants containing Plenti6.3/V5 DEST were increased 3-fold when compared with the untreated control HUVECs, which was consistent with the increase of UCP2 protein expression (Fig. 1). There was no significant difference between the cells transfected with the control lentiviral-negative vector and the untransfected cells (p=0.05). These results indicated that the stable transfection of Plenti6.3/V5 DEST upregulated UCP2 expression in HUVECs.

UCP2 promoted HUVEC cell proliferation at HG concentrations. The CCK8 assay (Fig. 2) revealed a gradual increase in HUVEC cell proliferation from day one to six in the four cell lines post-infection. On day one post-infection, there was no statistically significant difference in the proliferation of HUVECs among the four cell lines by ANOVA (p=0.05). On day two post-infection, NG cells showed significantly greater HUVEC cell proliferation than the HG and NC cells (p<0.05), and UCP2 cells showed significantly greater HUVEC cell proliferation as compared to NC cells (p<0.05). However, there was no statistically significant difference in HUVEC cell proliferation between the HG and NC cells (p>0.05). The difference between cell lines over the six successive days was statistically significant by ANOVA.

UCP2 attenuated high glucose-induced apoptosis in HUVECs. With Annexin V FITC labeling, cell apoptosis was analyzed using flow cytometry on day three post-infection. The apoptotic rate in the NG group was 9.31% (Fig. 3A). This apoptotic rate was significantly different when compared with the HG, NC or UCP2 group (p<0.05), with the apoptotic rates being 28.73, 25.25 and 13.5%, respectively (Fig. 3A). There was no significant difference in the apoptotic rate between the HG and NC groups (p>0.05) (Fig. 3).

Differing effects of UCP2 on apoptosis-related protein expression. We investigated whether the overexpression of UCP2 induced by Plenti6.3/V5 DEST affected apoptosis-associated gene expression, such as caspase-3, cytochrome c and Bcl-2.
HG-, NC- and UCP2-transfected cells were determined by RT-qPCR and western blotting, respectively. The expression trend of the three proteins was found to be consistent with the flow cytometric results. When compared with the NG group, caspase-3 and cytochrome c expression in the HG or NC group was significantly greater than that in the NG group (p<0.05), while the anti-apoptotic Bcl-2 expression in the HG or NC group was significantly lower than that in the NG group (p<0.05). The protein levels of caspase-3, cytochrome c and Bcl-2 showed no significant changes in the HG and NC groups (p>0.05). Caspase-3 and cytochrome c expression in the UCP2 group was significantly lower than that in the NG group (p<0.05), while the anti-apoptotic Bcl-2 expression in the UCP2 group was significantly greater than that in the NG group (p<0.05) (Fig. 4A-D). We also analyzed the levels of the three proteins in the conditioning media by RT-qPCR. The results were consistent with our western blot analysis (Fig. 4E-G).

Localization of apoptosis-related proteins in HUVECs. HUVECs were either labeled against the primary antibodies for caspase-3, cytochrome c or Bcl-2 and nuclei were labeled with blue fluorescent DAPI. Lentivirus-transfected cells were labeled with green fluorescent EGFP. Laser confocal scanning microscopy was employed to observe alterations in the localization of the three proteins. The localized region appeared yellow or orange-yellow. All three proteins were distributed throughout HUVECs, with the fluorescent density in the cytoplasmic area being relatively strong and aggregating in the nuclear periphery. Fluorescence in the nucleus was not well distributed and clustered in aggregates in the plasmosome. When compared with the NG group, caspase-3 and cytochrome c density in the HG or NC group was significantly stronger than that in the NG group, while the anti-apoptotic Bcl-2 density in the HG or NC group was significantly weaker than that in the NG group. The protein densities of caspase-3, cytochrome c and Bcl-2 showed no significant changes in the HG and NC groups. Caspase-3 and cytochrome c densities in the UCP2 group were significantly weaker than those in the NC group (p<0.05) while the anti-apoptotic Bcl-2 density in the UCP2 group was significantly stronger than that in the NC group (Fig. 5). The density trend of the three proteins was found to be consistent with western blotting and RT-qPCR.

Discussion

In comparison to hUCP1, hUCP2 was found to have a greater effect on mitochondrial membrane potential when expressed in yeast. It was found to have properties consistent with a role in diabetes and obesity. Sayeed et al. (33) found that UCP2 silencing in poorly differentiated breast tumor cells rapidly led to the induction of apoptosis and cell differentiation. However, whether UCP2 plays a role in apoptosis induced by HG remains unclear. In this study, we found that UCP2 promoted HUVEC proliferation and attenuated high glucose-induced apoptosis. Beltramo et al. (34) found that apoptosis of endothelial cells and pericytes increased in the presence of high levels of glucose after 3 days of culture. In previous studies (35-37) it has been reported that cultured retinal pericytes exposed to high levels of glucose (25-30 mM) for a period of 27 days show a higher rate of apoptosis than cells grown at 5.5 mM glucose. Moreover, Cui et al. (7) found that cells cultured in HG for 24-25 (23 mM) days and 45-46 (30 mM) days, resulted in an
increased rate of apoptosis in pericytes and endothelial cells when compared with cells exposed to low glucose concentrations (5 mM). There were no significant differences between the rates of apoptosis in the 23 and 30 mM glucose groups. Our results were consistent with these studies. Compared with the NG group, the apoptotic rate was significantly increased in the HG and NC groups, while the apoptotic rate in the UCP2 group was significantly decreased. There was no significant difference in the rate of apoptosis between the HG and NC groups. Thus, we concluded that UCP2 attenuates high glucose-induced apoptosis in HUVECs.

Evidence suggests that UCP2 is related to cell proliferation. Elorza et al. (38) found that UCP2 deficiency results in a significant decrease in cell proliferation at the erythropoietin-dependent phase of erythropoiesis. By contrast, Pecqueur et al. (39) found that UCP2−/− cells exhibit enhanced proliferation associated with a metabolic switch from fatty acid oxidation to glucose metabolism. At present, the results are controversial. Therefore, to solve this problem, we tested UCP2+/+ HUVEC cell proliferation following HG treatment. Our results were consistent with those of Pecqueur et al. (39). On day one post-infection, there was no statistically significant difference in the proliferation of HUVECs among the four cell lines. On day two post-infection, NG cells showed significantly greater HUVEC cell proliferation than the HG and NC cells, and UCP2 cells showed significantly greater HUVEC cell proliferation than the NC cells. However, there was no statistically significant difference in HUVEC cell proliferation between the HG and NC cells. Thus, UCP2 promoted HUVEC proliferation.

Apoptosis is a programmed cell death process that serves to remove abnormally proliferative cells, and UCP2 has been previously associated with apoptotic and proliferative activity in poorly differentiated breast tumor cells. However, various
cell types and rat species have been shown to use different mechanisms of UCP2 to influence both apoptotic and proliferative activity. For example, Sayeed et al (33) showed that the negative regulation of UCP2 by TGFβ signaling rapidly leads to the induction of apoptosis and cell differentiation. In addition, proliferation signaling pathways such as Rb/E2F, c-Myc, and Ras are characterized by their ability to activate nuclear transcription factors located in the cytosol (40). However, Elorza et al (38) showed that UCP2 modulates cell proliferation through the MAPK/ERK pathway during erythropoiesis. Furthermore, Pecqueur et al (39) demonstrated that UCP2 controls proliferation by promoting fatty acid oxidation and limiting glycolysis-derived pyruvate utilization.

To investigate the effect of UCP2 on cell apoptosis and proliferation, we focused on the expression of the apoptosis-related proteins caspase-3, cytochrome c and Bcl-2, which determine cell fate at the mitochondrial level. Specifically, western blotting and RT-qPCR were used to detect expression levels of the three proteins. Compared with the NG group, caspase-3 and cytochrome c expression in the HG or NC group was significantly increased, while the anti-apoptotic Bcl-2 expression in the HG or NC group was significantly decreased. Moreover, protein levels of caspase-3 and cytochrome c expression in the UCP2 group were significantly lower than those in the lentiviral-negative vector control group (P<0.05), while the anti-apoptotic Bcl-2 density in the UCP2 group was significantly stronger than that in the lentiviral-negative vector control group (A-C).

In conclusion, to the best of our knowledge, these are the first results showing that UCP2 promotes cell proliferation and inhibits HG-induced apoptosis in HUVECs via the Bcl-2 up- and downregulation of caspase-3 and cytochrome c.

In conclusion, to the best of our knowledge, these are the first results showing that UCP2 promotes cell proliferation and inhibits HG-induced apoptosis in HUVECs via the Bcl-2 up- and downregulation of caspase-3 and cytochrome c in vitro. Future efforts should focus on whether these effects are also present in vivo.
Acknowledgements

This study was supported by grants from the Research Fund for the National Nature Science Funding of China (nos. 30930097, 81273424 and 81170862), Major National Science and Technology projects during the 12th Five-Year Plan (2011ZX09302-007-02).

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