Increased activity of mitochondrial uncoupling protein 2 improves stress resistance in cultured endothelial cells exposed in vitro to high glucose levels

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Koziel A, Sobieraj I, Jarmuszkiewicz W. Increased activity of mitochondrial uncoupling protein 2 improves stress resistance in cultured endothelial cells exposed in vitro to high glucose levels. Am J Physiol Heart Circ Physiol 309: H147–H156, 2015. First published April 24, 2015; doi:10.1152/ajpheart.00759.2014.—The endothelium is relatively independent of the mitochondrial energy supply, but mitochondria-derived ROS may play an important role in the development of many cardiovascular diseases. Energy-dissipating uncoupling proteins (UCPs) mediate free fatty acid-activated, purine nucleotide-inhibited proton conductance (uncoupling) in the inner mitochondrial membrane. We have described a functional characteristic and an antioxidative role for UCP2 in endothelial cells and isolated mitochondria and how this function is altered by long-term growth in high concentrations of glucose. Human umbilical vein endothelial cells (EA.hy926 line) were grown in media with either high (25 mM) or normal (5.5 mM) glucose concentrations. Under nonphosphorylating and phosphorylating conditions, UCP activity was significantly higher in mitochondria isolated from high-glucose-treated cells. More pronounced control of the respiratory rate, membrane potential, and ROS by UCP2 was observed in these mitochondria. A greater UCP2-mediated decrease in ROS generation indicates an improved antioxidative role for UCP2 under high glucose conditions. Mitochondrial and nonmitochondrial ROS generations were significantly higher in high-glucose-treated cells independent of UCP2 expression. UCP2 gene silencing led to elevated mitochondrial ROS formation and ICAM1 expression, especially in high-glucose-cultured cells. UCP2 influenced endothelial cell viability and resistance to oxidative stress. Endothelial cells exposed to high glucose concentrations were significantly more resistant to peroxide. In these cells, the increased activity of UCP2 led to improved stress resistance and protection against acute oxidative stress. Our results indicate that endothelial UCP2 may function as a sensor and negative regulator of mitochondrial ROS production in response to hyperglycemia.

mitochondria; endothelium; uncoupling protein; antioxidative activity; bioenergetics; high glucose levels

MITOCHONDRIAL OXIDATIVE PATHWAYS are not used much in endothelial cells, because the synthesis of ATP in these cells occurs mainly via a glycolytic pathway. However, isolated endothelial mitochondria are efficient and highly coupled (14). Moreover, several recent observations have suggested that endothelial mitochondria not only contribute to ATP generation but are also centrally involved in maintaining the fine regulatory balance between mitochondrial Ca\(^{2+}\) concentrations, ROS production, and nitric oxide production (4, 5, 11, 13, 14). Endothelial mitochondria may also function as sensors of alterations in the local environment of circulating blood and contribute to the survival of endothelial cells under oxidative stress, and mitochondrial ROS in endothelial cells are important signaling molecules (28). Endothelial mitochondria may play an important role in the development of many cardiovascular diseases (1, 5, 9, 22). It has been proposed that the production of mitochondrial ROS in response to chronic hyperglycemia might be the key initiator for several mechanisms by which hyperglycemia damages cells.

Many mitochondrial functions that impact the cell’s response to the environment are linked to the electron transport chain and the resultant mitochondrial membrane potential (\(\Delta \Psi\)). Control of \(\Delta \Psi\) involves a proton leak through the inner mitochondrial membrane that is largely regulated by uncoupling proteins (UCPs) belonging to the mitochondrial carrier superfamily (21). UCPs mediate free fatty acid-activated, purine nucleotide-inhibited proton conductance, are involved in the control of cellular energy balance, and help to prevent the production of ROS by mitochondria. In endothelial cells, the upregulation of mitochondrial UCP2 by AMP-activated protein kinase attenuates oxidative stress in diabetes (27). Increased UCP2 expression is common in highly proliferative cell types, including cancer cells (20). In the absence of UCP2, endothelial growth stimulation provokes mitochondrial network fragmentation and premature senescence via a mechanism involving superoxide-mediated p53 activation.

Many questions must be addressed with respect to understanding the physiological role that UCP2 plays in endothelial mitochondria and the contribution of endothelial UCP2 to vascular function and disease. The present work presents the functional characteristics of UCP2 in isolated endothelial mitochondria and endothelial cells and how this function is altered by long-term growth in glucose concentrations that are well above the physiological range. To address this issue, mitochondrial UCP2 functions were compared in endothelial cells cultured in media with either high (25 mM) or normal (5.5 mM) glucose concentrations. Here, we report that high glucose exposure of endothelial cells involves the upregulation of mitochondrial UCP2, which reduces \(\Delta \Psi\) and limits the production of mitochondrial ROS that promote increased stress resistance.

MATERIALS AND METHODS

Cell culture and cell fraction preparation. The stable human endothelial cell line EA.hy926 was originally derived from a human umbilical vein (8). Cells were grown in DMEM supplemented with 10% FBS, 1% l-glutamine, 2% hypoxanthine-aminopterin-thymidine, and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO\(_2\) at 37°C. EA.hy926 cells were cultured for 5–6 days in DMEM with either 5.5 or 25 mM d-glucose (representing normal and high glucose conditions, respectively). In some experiments, the culture medium with 5.5 mM d-glucose plus 19.5 mM l-glucose was used as an osmolarity control. During cell culture, the medium was changed every 3 days. EA.hy926 cells were cultured in 140-mm dishes until
they reached ~90–100% confluence. Cells used in this study were between passages 5 and 12.

EA.hy926 cell cultures for both normal and high glucose cultures were harvested with trypsin-EDTA, rinsed twice with PBS (with 10% and 5% FBS, respectively), and centrifuged at 1,200 g for 10 min. Subsequently, cells were washed in cold PBS medium and then centrifuged once again. The final cell pellet was resuspended in the same medium. Protein content was determined using the Bradford method (Bio-Rad).

SDS-PAGE and immunoblot analysis. For immunoblot analysis, cellular fractions were isolated in the presence of protease inhibitors (Sigma). The spectra Multicolor Broad Range Protein Ladder (Fermentas) was used as a molecular weight marker. RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, and 50 mM Tris; pH 8.0) was used to lyse cells. Proteins were separated on 12% or 8% SDS-polyacrylamide gels and then electrotransferred to nitrocellulose membranes. The following primary antibodies were used: mouse monoclonal anti-β-actin (42 kDa, CP01, Calbiochem), purified goat polyclonal anti-UCP2 (35 kDa, sc-6525, Santa Cruz Biotechnology), rabbit polyclonal anti-UCP3 (34 kDa, ab3477, Abcam), and mouse monoclonal anti-ICAM1 (89 kDa, ab53013, Abcam). The appropriate horseradish peroxidase-conjugated secondary antibodies were used. Expression levels of β-actin were used as a loading normalization control. Protein bands were visualized using the Amersham ECL system and digitally quantified using the GeneTools 4.03 software package.

Mitochondria preparation. Mitochondria were isolated from EA.hy926 cells according to a very efficient isolation procedure that produces highly active and well-coupled mitochondria (14). After being harvested and washed in PBS, EA.hy926 cells were resuspended in PREP medium (0.25 M sucrose, 1.5 mM EDTA, 1.5 mM EGTA, 0.2% BSA, and 15 mM Tris-HCl; pH 7.2) at a ratio of 3 ml medium/1 g cells. Cells were then homogenized by 10 passes with a tight Dounce homogeniser, and homogenates were subsequently centrifuged at 1,200 g for 10 min. Pellets were resuspended, and cells were once again homogenized (8–10 passes) and centrifuged to collect the mitochondria remaining in the pellet. Supernatants were combined and then centrifuged at 1,200 g for 10 min, and the resultant supernatants were then centrifuged at 12,000 g for 10 min. Mitochondrial pellets were washed with PREPII medium containing 0.25 M sucrose and 15 mM Tris-HCl (pH 7.2) and then centrifuged at 12,000 g for 10 min. All steps were performed at 4°C. The final mitochondrial pellet was resuspended in medium containing 0.25 M sucrose and 15 mM Tris-HCl (pH 7.2) and centrifuged at 12,000 g for 10 min. The yields of the isolated mitochondria were equal to 4.3 ± 0.5 and 3.3 ± 0.3 mg mitochondrial protein/g cells (means ± SD, n = 25) for cells grown in normal glucose and high glucose conditions, respectively.

Mitochondrial O2 consumption and ΔΨ measurements. O2 uptake was determined polarographically using an O2 electrode (Rank Bros., Cambridge, UK) in 2.8 ml standard incubation medium, which consisted of 150 mM sucrose, 2.5 mM KH2PO4, 1 mM MgCl2, 20 mM Tris-HCl (pH 7.2), and 0.1% BSA with 2 mg mitochondrial protein at 37°C. ΔΨ was measured simultaneously with O2 uptake using a tetraphenylphosphonium (TPP+)-specific electrode. The TPP+-specific electrode was calibrated by four sequential TPP+ additions (0.4, 0.8, and 1.6 μM). For baseline correction, after each run, 0.5 μM FCCP was added to release TPP+. For calculation of the ΔΨ value, the ratio of O2 uptake values in the presence of 0.05 mM ATP to activate succinate dehydrogenase. Succinate (5 mM) plus rotenone (2 μM) was used as a respiratory substrate. Resting, nonphosphorylating respiratory rate measurements were performed in the presence of 1.8 μM carboxyatractyloside (to inhibit ATP/ADP antiporter activity) and 0.5 μg/ml oligomycin (to inhibit ATP synthase). Phosphorylating respiration was measured using 150 μM ADP (pulse) or 0.4 mM ADP. Uncoupled respiration was measured using up to 0.5 μM FCCP. O2 uptake values are presented as nanomoles O2 per minute per milligram of protein. Values of ΔΨ were given in millivolts. To induce UCP2 activity, linoleic acid (up to 32 μM) was used. To inhibit UCP2 activity, 4 mM GTP was applied. Proton leak measurements were performed with 5 mM succinate (plus 2 μM rotenone) as an oxidizable substrate. The response of proton conductance to its driving force (proton leak kinetics) can be expressed as the relationship between the O2 uptake rate and ΔΨ (flux-force relationship) when varying ΔΨ by titration with respiratory chain inhibitors. To decrease the rate of the Q-reducing pathway, succinate dehydrogenase was titrated with cyanide (up to 20 μM).

 Determination of superoxide anion formation. ROS production was detected by a nitroblue tetrazolium (NBT) assay with EA.hy926 cells and isolated mitochondria. NBT (yellow water soluble) was reduced by superoxide to formazan-NBT (dark blue insoluble). Detached cells (0.2 mg protein in 1 ml DMEM with 5.5 or 25 mM glucose) were incubated for 1 h (37°C) with 0.2% NBT under agitation in the presence or absence of 10 μM diphenylene iodonium (DPI; a NADPH oxidase inhibitor). Cells were centrifuged (1,200 g for 10 min at 4°C), the supernatant was removed, and formazan-NBT release was determined spectrophotometrically at 560 nm (UV 1602, Shimadzu spectrophotometer). In isolated endothelial mitochondria, the level of superoxide anion release was determined spectrophotometrically at 560 nm (UV 1602, Shimadzu) by measuring the rate of NBT (0.07 mg/ml) reduction at 37°C. Mitochondria (0.2 mg) were incubated in 0.7 ml of the standard incubation medium (see above) in the presence of succinate (5 mM) plus rotenone (2 μM) as a respiratory substrate. Under nonphosphorylating conditions, measurements were performed in the presence 1.8 μM carboxyatractyloside and 0.5 μg/ml oligomycin. Measurements were performed in the absence or presence of SOD (20 units, Sigma-
The differences were considered to be statistically significant at comparisons was used to identify significant differences; in particular, duplicate. ANOVA followed by a post hoc Tukey’s test for pairwise from at least seven to eight independent cellular or mitochondrial
larity, we compared the effect of treatment with 19.5 mM D-glucose) conditions exhibited similar UCP3 expression 
but not UCP1, could be detected in normal glucose-treated cells. This osmolarity control did not significantly change the expression of UCP2 protein, suggesting that the observed upregulation of UCP2 is specific for metabolized 25 mM D-glucose. Moreover, it seems that UCP2 is the only UCP involved in high glucose-induced modifications in EA.hy926 endothelial cells. Therefore, we could attribute the high glucose-induced changes in UCP activity to UCP2.

To determine UCP2 function in endothelial mitochondria, we evaluated the activation of UCPs by free fatty acids (linoleic acid) and their inhibition by purine nucleotides (GTP) in isolated EA.hy926 mitochondria. Figure 1C shows examples of O2 consumption and ∆Ψ measurements for nonphosphorylating mitochondria respiring with succinate as a reducing substrate. In nonphosphorylating mitochondria isolated from cells grown in high glucose conditions, the respiratory rate and ∆Ψ were observed to be more sensitive to 16 μM linoleic acid (i.e., a greater stimulation of respiratory rate and a greater decrease in ∆Ψ) and the subsequent addition of 4 mM GTP (i.e., a more pronounced recoupling effect), indicating a higher level of UCP activity (mainly UCP2 activity) compared with that of control mitochondria isolated from cells grown in normal glucose conditions. Moreover, a proton leak kinetics experiment obtained with cyanide titration (Fig. 1D) indicated that for given linoleic acid (16 μM) and GTP (4 mM) concentrations, the linoleic acid-induced, GTP-inhibited, UCP-mediated proton leak at the same ∆Ψ (154 mV) was twofold greater in mitochondria from high glucose-treated cells.

Under phosphorylating conditions, a direct consequence of UCP activity is a decrease in the amount of ATP synthesized per O2 consumed, i.e., the yield of mitochondrial oxidative phosphorylation. As shown in Fig. 1E, in both types of endothelial mitochondria, linoleic acid-induced GTP-inhibited UCP activity was able to divert energy from oxidative phosphorylation, indicating an energy-dissipating function during phosphorylating respiration. The increased activity of UCP2 in phosphorylating mitochondria from high glucose-treated cells led to a significantly greater reduction in the oxidative phosphorylation yield (ADP/O ratio) and the respiratory control ratio (Fig. 1E). For given linoleic acid (8 or 16 μM) and GTP (4 mM) concentrations, the linoleic acid–induced, GTP-reversed drops in the ADP/O ratio and respiratory control ratio were approximately twofold greater in mitochondria from high glucose-treated cells. Thus, a greater contribution of UCP2 activity to phosphorylating respiration was observed in these mitochondria compared with mitochondria from normal glucose-treated cells.

The voltage dependence of the electron flux (Fig. 1F) shows that the linoleic acid–induced GTP-inhibited respiration (for 16 μM linoleic acid and 4 mM GTP) only occurs due to UCP-mediated proton recycling, as it corresponds to a pure protonophoretic effect of linoleic acid that is not distinguishable from the effect of a well-known protonophore, FCCP. Indeed, for both types of mitochondria, couples of respiratory rate and ∆Ψ measurements in resting (state 4) respiration with increasing concentrations of FCCP or in the presence of 16 μM linoleic acid (with or without 4 mM GTP), as well as in phosphorylating state 3 respiration, constituted a single flow-force relationship. Thus, a modulation of the force (∆Ψ), either by phosphorylation potential, linoleic acid, or protonophore,
led to the same modification of the flow (respiratory rate). The results shown in Fig. 1F indicate that linoleic acid (at 16 μM concentration) did not interact with the respiratory chain of endothelial mitochondria and that the growth of endothelial cells under high glucose conditions did not disturb the voltage dependence of the electron flux in the respiratory chain of these mitochondria.

UCP2 control of respiratory rate, ΔΨ, and ROS in mitochondria from high glucose-treated endothelial cells is more pronounced. In EA.hy926 mitochondria from normal glucose- and high glucose-treated cells, respiration rates and ΔΨ during the oxidation of succinate were similar in the absence of linoleic acid (Figs. 1A and 2, A and B). We determined the maximal linoleic acid concentration required to obtain a simultaneous increase in O$_2$ consumption and a decrease in ΔΨ for nonphosphorylating endothelial mitochondria from both studied types of cells (Fig. 2). To exclude UCP activation by endogenous free fatty acids, all measurements were performed in the presence of defatted BSA. To exclude uncoupling mediated by an adenine nucleotide carrier, all measurements were performed in the presence of carboxyatractyloside. Figure 2, A and B, shows the influence of four different concentrations of linoleic acid (8, 16, 24, and 32 μM) on the respiratory rate (Fig. 2A) and ΔΨ (Fig. 2B). In mitochondria from normal glucose-treated cells, the maximal linoleic acid-induced uncoupling effect was observed with 16 μM linoleic acid, which increased the respiration rate by ~55% (Fig. 2A). In mitochondria from high glucose-treated cells, the maximal linoleic acid-induced uncoupling effect was observed with 24 μM linoleic acid, with which the respiration rate increased significantly, by ~160%. These increases in respiration were accompanied by a decrease in ΔΨ of ~11 and 23 mV for mitochondria from normal glucose- and high glucose-treated cells, respectively (Fig. 2B). These results confirm a much greater linoleic acid-induced uncoupling in mitochondria from high glucose-treated cells at any linoleic acid concentration up to 16 μM. At 24 μM linoleic acid, there was decrease (in mitochondria from normal glucose-treated cells) and no further increase (in mitochondria from high glucose-treated cells) in O$_2$ consumption compared with that at 16 μM linoleic acid. At 32 μM linoleic acid, an inhibition of respiration was also revealed in mitochondria from high glucose-treated cells. Thus, an inhibition of the respiratory chain occurred at a higher linoleic acid concentration in mitochondria from cells exposed to high glucose, indicating less sensitivity to the inhibitory excess of fatty acid.

The determination of superoxide anion formation showed that in the absence of linoleic acid, ROS production was similar in both types of mitochondria respiring with succinate (Fig. 2C). With increasing linoleic acid concentrations up to 16 or 24 μM, superoxide generation was gradually reduced in mitochondria from normal glucose- and high glucose-treated cells, respectively. However, for a given linoleic acid concentration (up to 16 μM), the linoleic acid-induced attenuation of ROS formation was significantly stronger in mitochondria from high glucose-treated cells. In mitochondria from normal glucose- and high glucose-treated cells, concentrations of linoleic acid higher than 16 and 24 μM, respectively, caused an increase in superoxide formation compared with that which occurred in the presence of lower fatty acid concentrations. These observations indicate an inhibition of the mitochondrial
respiratory chain that occurred when concentrations of fatty acid were too high. However, in the case of mitochondria from high glucose-treated cells, less sensitivity to the inhibitory excess of fatty acid was observed.

Compared with nonphosphorylating conditions in both types of endothelial mitochondria, the production of superoxide (Fig. 3B) and H$_2$O$_2$ (Fig. 3C) was considerably reduced by $\sim$30–50% under phosphorylating (in the presence of ADP) and uncoupled (in the presence of FCCP) conditions. In the presence of GTP, superoxide formation during uncoupled respiration did not change (Fig. 3B). While noninduced or linoleic acid-induced changes in mitochondrial function, including ROS production, are sensitive to purine nucleotides, they can be considered to indicate UCP activation. Effects of UCP2 activation (by linoleic acid) or inhibition (by GTP) on ROS formation were studied in nonphosphorylating mitochondria in the presence of oxidative phosphorylation inhibitors (Fig. 3, A and C). In the presence of oligomycin and carboxyatractyside, the highest level of ROS formation was observed. In both types of mitochondria, the addition of GTP, which blocks linoleic acid-induced UCP2 activity, increased superoxide formation in a concentration-dependent manner (Fig. 3A). The inhibitory effect of GTP on inducible UCP2 activity and thereby on UCP2 antioxidant activity was much greater in mitochondria from high glucose-treated cells. In the presence of 4 mM GTP, superoxide formation was restored to the level that was detected before fatty acid addition. Similar effects of linoleic acid (i.e., decrease in ROS formation) and GTP (i.e., reincrease in ROS formation), which were more pronounced in high glucose-treated cells, were observed when the H$_2$O$_2$ production rate was measured under nonphosphorylating conditions (state 4) and phosphorylating conditions in the presence of oxidative phosphorylation inhibitors (state 3, in the presence of 0.4 mM ADP; state U, in the presence of 0.6 mM FCCP and in the presence or absence of 4 mM GTP) conditions. A and B: superoxide formation relative to control nG mitochondria in state 4 in the absence of CATR and Oligo (100%; first bar in Fig. 3B). A–C: results obtained from 7–8 independent mitochondrial isolations ($n$), in which each determination was performed at least in duplicate, are presented as means ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

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production rate was measured in nonphosphorylating EA.hy926 mitochondria (Fig. 3C). Thus, for the first time, it has been shown that linoleic acid-induced GTP-inhibited UCP2 activity lowers mitochondrial ROS production in isolated endothelial mitochondria and that this ROS-decreasing protective function of UCP2 is much more pronounced in mitochondria from endothelial cells grown under high glucose conditions.

In high glucose-treated cells, lack of UCP2 induces increased inflammation and ROS production. To elucidate a physiological role for UCP2 in endothelial cells, we compared EA.hy926 cells cultured under normal glucose (normal UCP2 level) and high glucose (UCP2 upregulation) conditions (with control siRNA) with cells that had been treated with UCP2 siRNA to knockdown UCP2 (no UCP2). In normal glucose- and high glucose-tolerant cells, the efficiency of knockdown was confirmed by a lack of UCP2 detection in UCP2-null cells (Fig. 4). UCP3 expression did not change in both types of endothelial cells independent of UCP2 silencing. Immunodetection of ICAM1 indicated an increased level of the inflammation marker in high glucose-treated cells compared with normal glucose-treated cells that was independent of the presence of UCP2. In both types of cells, the absence of UCP2 increased ICAM1 expression, although the increase was much greater in high glucose-treated cells.

Compared with UCP2-expressing cells cultured in normal glucose conditions, the exposure of UCP2-expressing cells to high glucose concentrations caused a significant increase in total (Fig. 5A) and mitochondrial (Fig. 5A and B) superoxide generation. Mitochondrial superoxide generation was measured in endothelial cells either as DPI-insensitive NBT reduction (Fig. 5A) or MitoSOX oxidation (Fig. 5B). Therefore, in EA.hy926 cells, high glucose-induced ROS appear to be produced by the enzyme NADPH oxidase and from mitochondrial sources (including DPI-insensitive sources). To confirm that the high glucose-induced enhancement of superoxide formation was not due to the high osmolarity, we compared the effect of treatment with 19.5 mM L-glucose plus 5.5 mM D-glucose, providing an equivalent osmolarity as 25 mM D-glucose (Fig. 5B, right). This osmolarity control did not significantly change...
superoxide production, suggesting that the observed high glucose-induced enhancement of superoxide formation is specific for metabolized 25 mM d-glucose.

In normal glucose- and high glucose-treated cells, absence of UCP2 caused a significant increase in total and mitochondrial superoxide formation (Fig. 5, A and B). However, the enhancement of superoxide formation in UCP2-deficient cells was much more pronounced in high glucose-treated cells than in normal glucose-treated cells, indicating an increased need for the antioxidative activity of UCP2 under high glucose conditions.

**UCP2** influenced endothelial cell viability and resistance to oxidative stress; under high glucose conditions, stress resistance was improved. Despite increased ICAM1 expression (Fig. 4) and pronounced ROS generation (Fig. 5), endothelial cells grown in high glucose conditions revealed a similar viability compared with cells grown in normal glucose conditions (Fig. 6). Moreover, UCP2 silencing led to a similar (~12%) decrease in cell viability in both types of cells (Fig. 6, no H$_2$O$_2$ treatment). To verify whether the lack of UCP2 and/or high glucose conditions had an impact on endothelial cell resistance to stress, normal glucose- and high glucose-treated cells were incubated with peroxide, and their survival was evaluated relative to that of control cells without H$_2$O$_2$ treatment. Figure 6 shows that peroxide significantly impaired cell viability in normal glucose- and high glucose-treated cells (by ~45% and 26% in UCP2-expressing cells and by ~52% and 40% in UCP2 knocked down cells, respectively). Interestingly, high glucose-treated cells were significantly less sensitive to peroxide than normal glucose-treated cells independent of UCP2 expression. UCP2 silencing led to slightly more impaired stress resistance to peroxide in high glucose-treated cells compared with normal glucose-treated cells (~26% reduction and 19% reduction versus high glucose- and normal glucose-treated UCP2-expressing cells, respectively; Fig. 6). Thus, in endothelial cells exposed to high glucose concentrations, increased expression of UCP2 led to improved stress resistance.

Exposure of cells to H$_2$O$_2$ induces not only cell necrosis but also cell apoptosis. However, besides necrotic cells, only parts of apoptotic cells (with plasma membrane damage) were detected in the present trypan blue exclusion assay (Fig. 6). Moreover, the method cannot distinguish between necrotic and apoptotic cells. Therefore, further studies are needed to assess more accurately effects of high glucose and UCP2 knockdown on H$_2$O$_2$-induced apoptosis of endothelial cells.

**DISCUSSION**

We investigated the effect of UCP2 on mitochondrial function in mitochondria isolated from endothelial cells exposed to high glucose levels. The first characteristic we noted was that in isolated endothelial mitochondria, high glucose conditions induced an increase in free fatty acid-induced GTP-inhibited UCP2 activity under both nonphosphorylating and phosphorylating conditions. In nonphosphorylating mitochondria from high glucose-treated endothelial cells, the increased UCP2 activity had a bigger impact on the mitochondrial respiratory rate, ΔΨ, and ROS generation. UCP2-mediated uncoupling modulates the proton leak across the mitochondrial inner membrane. The antioxidative activity of endothelial UCP2 was dependent on its effect on mitochondrial ΔΨ. The high glucose-induced UCP2-mediated uncoupling caused an increased antioxidative efficiency (increased ROS attenuation) in mitochondria from high glucose-exposed cells. However, the high glucose-induced increase in the activity of UCP2 led to a greater reduction in the oxidative phosphorylation yield. These findings indicate that under high glucose-induced oxidative stress, the maintenance of ATP synthesis efficiency is not as important as the attenuation of mitochondrial ROS production, especially given that endothelial mitochondria are not particularly dependent on oxidative phosphorylation. Our results confirm the implication that UCP2 may serve as a sensor and negative regulator of mitochondrial ROS production in endothelial cells with elevated glucose levels (2, 7, 15, 21, 23).

In endothelial mitochondria from both normal glucose- and high glucose-treated cells, an excess of linoleic acid inhibited the respiratory chain, with no further activation of mitochondrial uncoupling through UCP2 (Fig. 2). It has been recently shown that high glucose exposure induces a shift in endothelial aerobic metabolism from carbohydrate oxidation toward the oxidation of lipids and amino acids (14). Therefore, in hyperglycemic endothelial cells, the increased oxidation of fatty acids by mitochondria may protect the mitochondrial respiratory chain against the inhibitory effects of excess levels of fatty acids. On the other hand, in endothelial mitochondria with high glucose-induced increased levels of UCP2, lower levels of fatty acids may still activate UCP2-mediated uncoupling to a larger extent than in mitochondria from normal glucose-exposed cells. Our results indicate that the mitochondrial respiratory chain in high glucose-treated cells is less sensitive to free fatty acid inhibition due to UCP2 upregulation.

The exposure of endothelial cells to high glucose levels leads to increased intracellular and mitochondrial ROS production and therefore produces excessive oxidative stress (6, 10, 17, 18, 23, 27). Under our experimental conditions, the in-
creased oxidative stress in EA.hy926 cells grown under chronic high glucose conditions was revealed by significantly higher intracellular and mitochondrial ROS generation and the upregulation of UCP2 as a mitochondrial antioxidative system protein. Increased expression levels of UCP2 in response to high glucose have previously been observed in other endothelial cell lines (such as human umbilical vein endothelial cells, bovine retinal capillary endothelial cells, and human microvascular endothelial cells) (2, 7, 19, 29) and endothelial cells isolated from animal models of diabetes (e.g., diabetic mice) (24, 27). In EA.hy926 endothelial cells, high glucose-induced ROS appear to be produced by the enzyme NADPH oxidase and by mitochondrial sources (Fig. 5). In general, mitochondrial ROS generation is associated with an increased reduction of respiratory chain components that may be caused by the increased oxidation of mitochondrial fuels and/or the impairment of the QH2-oxidizing pathway. Our results indicate that under high glucose-induced oxidative stress conditions, UCP2 activity may attenuate mitochondrial ROS production by lessening the reduction level of mitochondrial respiratory chain complexes. A greater enhancement in superoxide formation was observed in UCP2-deficient EA.hy926 cells exposed to high glucose concentrations compared with UCP2-deficient control cells, indicating an increased need for antioxidative UCP2 activity under high glucose conditions. Our experiments show that endothelial UCP2 participates in the control of mitochondria-derived ROS.

We showed that UCP2 influenced cell viability in both high glucose- and normal glucose-treated EA.hy926 cells (Fig. 6). There was a significant difference in the viability of UCP2-expressing and also UCP2-knocked down cells after H2O2 treatment between normal glucose and high glucose conditions (Fig. 6), although UCP2 siRNA reduced UCP2 protein to similar very low levels in both types of cells (Fig. 4). UCP2 silencing only slightly more impaired stress resistance in high glucose-treated cells compared with normal glucose-treated cells. The findings indicate the presence of additional UCP2-independent mechanisms of endothelial cell injury induced by high glucose conditions. They could involve high glucose-induced UCP2-independent mitochondrial changes and nonmitochondrial alternations that influence endothelial cell viability. However, high glucose-treated cells were significantly more resistant to peroxide independent of UCP2 expression. Thus, in endothelial cells exposed to high glucose concentrations, the increased activity of energy-dissipating protein, i.e., UCP2, and thereby increased antioxidant defense led to improved stress resistance and protection against acute oxidative stress. These results indicate that in endothelial cells, hyperglycemia could be involved in the hormetic induction (3) of antioxidant defenses and stress resistance. Moreover, inhibition of high glucose-induced apoptosis by lentivirus-mediated UCP2 overexpression has been observed in human umbilical vein endothelial cells (12). Thus, our data highlight and support the importance of mitochondrial UCP2-mediated uncoupling in endothelial stress resistance.

Several studies have shown that in response to hyperglycemia, endothelial UCP2 may function as a sensor and negative regulator of mitochondrial ROS overproduction, which is an initiating cause in the pathogenesis of diabetic complications, such as diabetic retinopathy, hypertension, or atherosclerosis. In diabetic mice, AMP-activated protein kinase activation increases UCP-2 expression, resulting in the inhibition of both superoxide and prostacyclin synthase nitration and thus attenuation of oxidative stress (27). In diet-induced obese mice, UCP2 protects endothelial function through increasing nitrite oxide bioavailability secondary to the inhibition of ROS formation (25). Similarly, in diabetic ob/ob mice, a vascular benefit is likely to result from the upregulation of UCP2 expression, which reduces oxidative stress and increases the level of nitric oxide (24). For example, this effect accounts for the endothelium-dependent relaxation observed in capsaicin-treated db/db mice. Thus, our data showing a greater UCP2-mediated decrease in ROS generation, an improved antioxidative role for UCP2, and improved resistance to oxidant stress-induced cell death in association with increased UCP2 expression and activity in endothelial cells exposed to high glucose levels support studies on diabetes-induced vascular disease. The increase in UCP2 expression in endothelial cells may aid in preventing the development and progression of vascular dysfunction. For instance, UCP2 could be a useful target in treating atherosclerosis or hypertension-related vascular events (15, 16). However, many questions must be addressed with respect to understanding the physiological role that UCP2 plays in endothelial mitochondria and its contribution to vascular function and disease.

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