Uncoupling Protein-2 Overexpression Inhibits Mitochondrial Death Pathway in Cardiomyocytes

Yasushi Teshima, Masaharu Akao, Steven P. Jones, Eduardo Marbán

Abstract—Uncoupling proteins (UCPs) are located in the mitochondrial inner membrane and partially dissipate the transmembrane proton electrochemical gradient. UCP2 is expressed in various human and rodent tissues, including the heart, where its functional role is unknown. In the present study, we tested the hypothesis that UCP2 overexpression could protect cardiomyocytes from oxidative stress–induced cell death by reducing reactive oxygen species (ROS) production in mitochondria. Using an adenoviral vector containing human UCP2, we investigated the effects of UCP2 overexpression on the mitochondrial death pathway induced by oxidative stress (100 μmol/L H2O2) in cultured neonatal cardiomyocytes. UCP2 overexpression significantly suppressed markers of cell death, including TUNEL positivity, phosphatidylserine exposure, propidium iodide uptake, and caspase-3 cleavage. Furthermore, UCP2 markedly prevented the catastrophic loss of mitochondrial inner membrane potential induced by H2O2, which is a critical early event in cell death. Ca2+ overload and the production of ROS in mitochondria, both of which contribute to mitochondrial inner membrane potential loss, were dramatically attenuated by UCP2 overexpression. Thus, overexpression of UCP2 attenuates ROS generation and prevents mitochondrial Ca2+ overload, revealing a novel mechanism of cardioprotection. (Circ Res. 2003;93:192-200.)

Key Words: heart • mitochondria • membrane potential • calcium • reactive oxygen species

Uncoupling proteins (UCPs), which are located in the mitochondrial inner membrane, partially dissipate the proton electrochemical gradient.1 The best characterized uncoupling protein, UCP1, is exclusively expressed in brown adipose tissue and is a key molecule in thermogenesis.1 UCP2, another member of the UCP family,2,3 is expressed in various tissues, including the brain, lung, spleen, kidney, liver, adipose tissues, and heart.1–3 Recently, UCP2 was reported to regulate insulin secretion from pancreatic islets by regulating ATP concentration.4–6 Furthermore, UCP2 reportedly contributed to immune response to infection by regulating reactive oxygen species (ROS) production.7 These findings suggest that the physiological role of UCP2 may be organ specific; however, its function in the heart remains unknown.

Mitochondrial ATP-sensitive potassium (mitoKATP) channels have cardioprotective effects against ischemia/reperfusion injury.8,9 The mitoKATP channel opener diazoxide prevents apoptosis induced by oxidative stress in cultured cardiomyocytes.10 Partial depolarization of mitochondrial inner membrane potential (ΔΨm) achieved by the opening of mitoKATP channels and subsequent inhibition of mitochondrial Ca2+ overload have been proposed as potential mechanisms of this effect.11,12 Similarly, low concentrations of mitochondrial uncouplers such as carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) or 2,4-dinitrophenol were reported to have cytoprotective effects. FCCP prevented permeability transition pore (PTP) opening and ROS production induced by staurosporine or chelerythrine in isolated mitochondria from rat liver13,14; 2,4-dinitrophenol reduced infarct size and preserved cardiac function in a rat model of myocardial ischemia/reperfusion injury.15 These results suggest that depolarization of ΔΨm is responsible for cytoprotection. Furthermore, UCP2 overexpression in murine macrophages reduced ROS generation induced by lipopolysaccharide.16

Based on these observations, we hypothesized that UCP2 may have protective effects against oxidative stress in the heart. Using an adenoviral vector, we investigated the effects of UCP2 overexpression on cell injury induced by hydrogen peroxide (H2O2) in cultured cardiomyocytes.

Materials and Methods
All procedures were performed in accordance with the Johns Hopkins University animal care guidelines, which conform to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health.

Primary Culture of Neonatal Rat Cardiac Ventricular Myocytes
Cardiac ventricular myocytes were prepared from 1- to 2-day-old Sprague-Dawley rats and cultured as previously described.10
In Vitro Transduction

Adenovirus (AdEGI-UCP2) that was generated previously was used in the present study. Briefly, adenovirus vectors were generated using Cre-lox recombination of purified V5 viral DNA and shuttle vector pAdEGI-UCP2. This vector coexpressed green fluorescent protein (GFP) and UCP2, driven by the inducible edcsyse promoter. The recombinant vectors were expanded and purified using cesium chloride gradients, yielding concentrations of 1 × 10^10 plaque-forming units per milliliter. Functional expression was inferred from GFP visualization by fluorescence microscopy. The control vector (AdEGI) expressed only GFP, again driven by the edcsyse promoter. Transductions were carried out in culture medium (QBSF; Sigma) for 2 hours at 37°C on day 6 after the isolation of cardiomyocytes. Afterward, the myocytes were washed with virus-free medium and incubated with ponasterone A (Invitrogen) to induce expression from edcsyse-inducible promoter. The experiments were carried out at least 48 hours after transduction.

Immunoblot Analysis

Subcellular fractions of cells were prepared as described before. Primary antibodies for UCP2 and cytochrome c oxidase subunit IV were purchased from Alpha Diagnostic International and Molecular Probes, respectively.

Mitochondrial Oxygen Consumption Rate

For oxygen consumption measurements, cardiomyocytes were trypsinized for detachment from the dishes. After centrifugation, cells were resuspended in DMEM containing 25 mmol/L HEPES. Oxygen consumption was measured using an oxygen sensor (FOXY Fiber Optic Oxygen Sensors; Ocean Optics Inc) at 37°C in a stirred bath. The data for oxygen consumption rate were normalized by the protein concentration in each sample.

TUNEL Staining

TUNEL staining was performed according to the manufacturer’s protocol (Roche) at 16 hours after application of 100 μmol/L H_2O_2. Fluorescein labels incorporated in nucleotide polymers were detected by laser scanning confocal microscopy.

Caspase-3 Activity Assay

Caspase-3 activity was measured as described previously by detection of the cleavage of a colorimetric caspase-3 substrate, N-acetyl-Asp-Glu-Val-Asp-p-nitroaniline, using an assay kit, ApoAlert CPP32 (Clontech), at 8 and 16 hours of 100 μmol/L H_2O_2 stimulation.

Loading of Cells With Fluorescent Indicators

For quantification of cellular viability, cells were double-stained with annexin V and propidium iodide (PI) according to manufacturer’s instructions (Roche). To monitor ΔΨ_m, cells were loaded with 100 mmol/L tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes) at 37°C for 20 minutes. To monitor mitochondrial Ca^2+ levels, cells were loaded with 2 μmol/L rhod-2 AM (Molecular Probes) at 37°C for 30 minutes. We assayed ROS production, such as superoxide anion, hydrogen peroxide, and hydroxyl radical, using chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes). Cells were loaded with 2 μmol/L CM-H_2DCFDA at 37°C for 30 minutes, and the formation of the oxidized derivative, CM-DCF, was monitored.

Confocal Imaging

Cells plated on glass-bottom dishes were loaded with fluorescent probes as described above. After loading with each dye, cells were placed in phenol red-free DMEM supplemented with 25 mmol/L HEPES (pH 7.4). Cells were illuminated (488- and 568-nm lines of a krypton/argon laser), and images were taken by confocal microscopy (UltraVIEW; Perkin-Elmer). Time-lapse confocal imaging was carried out with various intervals between frames, using a ×40 objective lens for DCF and a ×20 objective lens for the other dyes.

Image Analysis

Quantitative image analysis was performed using image analysis software (ImageJ; http://rsb.info.nih.gov/ij/).

FACS Analysis

For FACS analysis of ΔΨ_m, TMRE-loaded cells were harvested by trypsinization at the end of experimental protocols and analyzed using FACS (20 000 cells/sample) (Becton Dickinson). The fluorescence intensity of TMRE was monitored at 582 nm (FL-2 channel). FACS data were analyzed using WinMDI software (http://facs.scripps.edu).

Statistical Analysis

Data are presented as mean±SEM. Multiple comparisons among groups were carried out by one-way ANOVA with Fisher’s least-significant difference as the post hoc test. A level of P<0.05 was accepted as statistically significant.

Results

Adenovirus-Mediated Delivery of UCP2 to Neonatal Cardiomyocytes

To deliver UCP2 to cultured cardiomyocytes, we used a recombinant adenovirus containing the full-length human UCP2 cDNA. Figure 1A shows the results of immunoblot analysis. UCP2 protein level was increased in AdEGI-UCP2 (AdUCP2, 20 multiplicity of infection [moi])-transduced cells compared with AdEGI-transduced cells in the mitochondrial fraction but not in the cytosolic fraction. UCP2 protein level was increased virus volume dependently up to 20 moi, but there was no difference between 20 and 40 moi (n=3). In Figure 1B, cells were stained with TMRE to examine the effect of UCP2 expression on ΔΨ_m under basal conditions. AdUCP2-transduced cells showed partial depolarization of ΔΨ_m (gray area) compared with AdEGI-transduced cells (thick line), verifying functional overexpression of UCP2. This degree of ΔΨ_m dissipation is measurable but very modest compared with the catastrophic depolarization produced by the potent pharmacological uncoupler FCCP (thin line). We analyzed the intensity of fluorescence in the peak of the histogram in each group (n=5). Significant but moderate dissipation of ΔΨ_m was observed in AdUCP2 cells compared with AdEGI. There was no difference between 20 and 40 moi. In Figure 1C, UCP2-transduced cells showed modest but statistically significant increases in oxygen consumption compared with AdEGI cells (n=7, P<0.05). A chemical uncoupler, FCCP (0.5 μmol/L), increased oxygen consumption in both AdEGI and AdUCP2 cells to a similar (presumably maximal) level. However, the magnitude of the FCCP-induced increase was smaller in AdUCP2 compared with AdEGI. This is consistent with the idea that AdUCP2 cells have a higher uncoupled respiration under basal conditions. Conversely, sodium cyanide (5 mmol/L) suppressed the oxygen consumption rate in each group.

Effects of Overexpression of UCP2 on Cell Death

First, we examined the long-term effects of UCP2 overexpression on cell death. We performed TUNEL staining 16 hours after H_2O_2 application. Representative images and quantitative data are shown in Figures 2A and 2B, respectively (n=4 for each group from two independent experiments). In the control group (AdEGI), 15.9±0.6% of the cells...
showed TUNEL-positive nuclei, and exposure to H2O2 (AdEGI/H2O2) increased the number of TUNEL-positive nuclei to 33.7±1.7% (P<0.001). This increase of TUNEL positivity was significantly suppressed in UCP2-transduced cells treated with H2O2 (AdUCP2/H2O2) (19.2±0.5%) (P<0.001 versus AdEGI/H2O2). UCP2 overexpression without H2O2 did not affect TUNEL positivity. Activation of caspase-3 is a critical step in the process of cell death. Figure 2C shows relative caspase-3 activity in each group at 8 and 16 hours after H2O2 application. Caspase-3 activity was significantly increased in AdEGI/H2O2 compared with AdEGI at each time point (P<0.001), and UCP2 overexpression significantly suppressed these increases (P<0.05 and P<0.001 versus AdEGI/H2O2 at 8 hours and 16 hours, respectively) (n=3 for each data point).

Effects of Overexpression of UCP2 on Early Phase of Cell Death
To assess the effects of UCP2 overexpression in the early phase of cell injury, we investigated the alterations of annexin V and PI fluorescence in each group. Time-lapse confocal microscopy at 5-minute intervals began immediately after application of 100 μmol/L H2O2. In AdEGI/H2O2, annexin V fluorescence started to increase ~20 minutes after H2O2 application and gradually increased to a plateau at ~100 minutes (Figures 3A and 3B). UCP2 remarkably suppressed this increase of annexin V fluorescence (Figures 3A and 3B). PI fluorescence was increased after 90 minutes of latency and reached the plateau at ~150 minutes in AdEGI/H2O2 (Figures 3C and 3D). This increase of PI fluorescence was also abrogated by UCP2 overexpression (Figures 3C and 3D). After 150 minutes of scanning, cells were permeabilized with saponin (Sigma) to calculate the percentage of PI-positive cells in each group. In AdEGI/H2O2, 86.3±3.4% of cells were PI-positive at the end of scanning. In contrast, only 7.5±4.9% of cells were PI-positive in AdUCP2/H2O2 (n=3, P<0.001) (Figure 3D, inset). Because annexin V is an indicator of apoptosis and PI of necrosis, these results suggest that UCP2 overexpression reduces both types of cell death.

Effects of UCP2 Overexpression on ΔΨm
Dissipation of ΔΨm is a critical event early in the process of cell death.20 To examine whether preservation of ΔΨm is associated with cardioprotective effects of UCP2, we assessed the change of TMRE fluorescence by H2O2 stimulation in each group using FACS analysis. Incubation with 100...
μmol/L H$_2$O$_2$ for 1 hour decreased TMRE fluorescence and shifted the distribution curve leftward, indicating the depolarization of ΔΨ$_m$ (Figure 4A, top). In AdUCP2 + H$_2$O$_2$, the decrease of TMRE fluorescence was remarkably suppressed, indicating the preservation of ΔΨ$_m$ (Figure 4A, bottom). Summarized data from FACS analysis are shown in Figures 4B and 4C. We evaluated the percentage of cells that exhibited a high level of TMRE fluorescence (defined as >5×10$^{-3}$ in this analysis). UCP2 preserved ΔΨ$_m$ virus volume.
dependently (n=3 for each group) (Figure 4B). Treatment with 1 μmol/L ponasterone A without AdUCP2 transfection had no effect (data not shown). Inhibition of mitoK<sub>ATP</sub> channels by 5-hydroxydecanoate (5HD, 500 μmol/L) or glibenclamide (10 μmol/L) did not cancel ΔΨ<sub>m</sub> preservation by UCP2 overexpression (n=3 for each group) (Figure 4C). Thus, whereas the effects of UCP2 mimic those of mitoK<sub>ATP</sub> channel openers, they are not dependent on mitoK<sub>ATP</sub> channel activation.

**Time-Lapse Analysis of ΔΨ<sub>m</sub> Loss**

To examine time-dependent changes of ΔΨ<sub>m</sub> on a single-cell basis, confocal microscopy was performed using cells loaded with TMRE. Time-lapse scanning began immediately after application of 100 μmol/L H<sub>2</sub>O<sub>2</sub>. At first, we confirmed that TMRE fluorescence did not change during 70 minutes of scanning in AdEGI (Figure 5A). In contrast, cells treated with H<sub>2</sub>O<sub>2</sub> progressively lost red fluorescence intensity, indicating irreversible dissipation of ΔΨ<sub>m</sub> (Figure 5B). TMRE fluorescence was remarkably preserved in AdUCP2/H<sub>2</sub>O<sub>2</sub> (Figure 5C). Twenty cells were randomly selected in each group, and TMRE fluorescence intensity from each cell was plotted in Figure 5D. TMRE fluorescence intensity of individual cells rapidly decreased in AdEGI/H<sub>2</sub>O<sub>2</sub>, but UCP2 overexpression prevented the loss of ΔΨ<sub>m</sub> induced by H<sub>2</sub>O<sub>2</sub> in most cells. The bottom panel in Figure 5D shows the average of TMRE fluorescence intensity from 20 randomly selected cells in each group.

**Effects of UCP2 Overexpression on Mitochondrial Ca<sup>2+</sup> Overload**

Dissipation of ΔΨ<sub>m</sub> is caused by the opening of PTP. Ca<sup>2+</sup> overload in mitochondria is one of the critical triggers of cell death and is known to open the PTP. We examined the effects of UCP2 overexpression on mitochondrial Ca<sup>2+</sup> level using the mitochondrial Ca<sup>2+</sup>-sensitive dye rhod-2. Time-lapse confocal microscopy began after the addition of 100 μmol/L H<sub>2</sub>O<sub>2</sub>. We first confirmed that rhod-2 fluorescence did not change during 70 minutes of scanning in AdEGI (Figure 6A). Rhod-2 fluorescence was rapidly and dramatically augmented in AdEGI/H<sub>2</sub>O<sub>2</sub> at ≈30 minutes after H<sub>2</sub>O<sub>2</sub> application (Figure 6B). UCP2 overexpression almost completely inhibited the Ca<sup>2+</sup> surge observed in AdEGI/H<sub>2</sub>O<sub>2</sub> (Figure 6C). Figure 6D shows the average of rhod-2 fluorescence intensity from 20 randomly selected cells in each group.

**Effects of UCP2 Overexpression on ROS Production**

ROS is another trigger of PTP opening. We investigated the effects of UCP2 overexpression on ROS production using time-lapse confocal microscopy. No changes in fluorescence were observed in AdEGI (Figure 7B, ■). In AdEGI/H<sub>2</sub>O<sub>2</sub>, DCF fluorescence increased immediately after H<sub>2</sub>O<sub>2</sub> application and gradually reached a plateau at ≈40 minutes (Figures 7A and 7B, ●). UCP2 overexpression abrogated the increase in DCF fluorescence (Figures 7A and 7B, ○). Furthermore, the effects of UCP2 overexpression on ROS production were confirmed using an entirely different form of oxidant stress, namely doxorubicin, which exerts cardiotoxicity by increasing ROS production in mitochondria.22,23 Incubation with 50 μmol/L doxorubicin for 3
hours markedly increased DCF fluorescence compared with AdEGI (P<0.001) (Figure 7C), but UCP2 overexpression significantly prevented the increase of fluorescence (P<0.001 versus AdEGI+doxorubicin) (n=6 for each group from two independent experiments) (Figure 7C).

**Discussion**

The major findings in the present study are as follows. First, UCP2 overexpression suppressed markers of cell death such as increased TUNEL positivity, caspase-3 activity, phosphatidylserine exposure, and PI positivity induced by oxidative stress. Second, mitochondrial Ca\(^2\) overload and increased ROS production in mitochondria, and subsequent \(\Delta\Psi_m\) loss induced by oxidative stress, were prevented by UCP2 overexpression in a dose-dependent manner. These results demonstrate that overexpression of UCP2 protects cardiomyocytes against oxidative stress by preserving the integrity of early processes that would normally be recruited as part of the mitochondrial death pathway.

Cardiomyocytes contain abundant mitochondria to maintain cardiac function, which requires a large amount of energy. The plethora of mitochondria can potentially produce ROS that contribute to ischemia/reperfusion injury in the heart.\(^\text{24}\) The mitochondrial respiratory chain generates ROS, especially in state IV respiration, when the electrochemical gradient between the mitochondrial inner membrane is high and the rate of electron transport is limited. In this context, UCP2 should affect ROS production by dissipating the electrochemical gradient. Indeed, the association of UCP2 and ROS production has been described in several reports. GDP, an inhibitor of UCP2, raised mitochondrial membrane potential and increased \(\text{H}_2\text{O}_2\) production in mitochondrial fractions of liver, spleen, and thymus.\(^\text{25}\) Macrophages from mice lacking UCP2 generate more ROS than wild-type mice and had a remarkably increased resistance to *Toxoplasma gondii* infection.\(^\text{7}\) Transfection of UCP2 cDNA in murine macrophage cell line reduced lipopolysaccharide-induced intracellular ROS production,\(^\text{16}\) and the lack of UCP2 in blood cells accelerates atherosclerotic plaque development, probably by increasing ROS production.\(^\text{26}\) This evidence strongly supports the possibility that the principal role of UCP2 is regulation of ROS production in mitochondria, although the precise mechanism of this effect has not been established. Furthermore, a recent report demonstrated that superoxide activated UCPs in various tissues, indicating that UCPs may be recruited endogenously as a compensatory mechanism to counteract oxidative stress.\(^\text{27}\) Consistent with these observations, UCP2 overexpression reduced ROS production in the present study. Exogenous \(\text{H}_2\text{O}_2\)-induced ROS production may not originate only from mitochondrial respiration but also from other reactions, such as the Fenton reaction or interactions with mitochondrial respiratory com-
ponents. Therefore, we cannot be certain that reduced ROS production was caused by increased respiration rate in mitochondria. The present result that UCP2 overexpression suppressed the increase in ROS production by doxorubicin, which is known to increase ROS production in mitochondria,\textsuperscript{22,28,29} indirectly supports our hypothesis. As another possibility, interaction of UCP2 with coenzyme Q, may prevent coenzyme Q from H\textsubscript{2}O\textsubscript{2}-oxidizing action and thereby reduce ROS production.\textsuperscript{30}

This is the first study that shows UCP2 overexpression inhibited mitochondrial Ca\textsuperscript{2+} overload, an important trigger of the mitochondrial death pathway. One possible mechanism is that this effect may simply be attributable to the partial depolarization of ΔΨ\textsubscript{m}, which decreases the driving force for Ca\textsuperscript{2+} uptake into the mitochondrial matrix. Otherwise, inhibition of Ca\textsuperscript{2+} overload may be a secondary effect of reduced ROS production. In fact, ROS was reported to trigger Ca\textsuperscript{2+} increase during hypoxia in pulmonary arterial myocytes.\textsuperscript{31} On the contrary, ROS levels under oxidative stress may depend on extracellular Ca\textsuperscript{2+} concentration.\textsuperscript{32} Therefore, ROS and Ca\textsuperscript{2+} overload may be tightly coupled and there could be a mutual requirement for each to reach its maximal levels.\textsuperscript{33}

PTP is a major player in the cell death pathway. Opening of PTP results in the loss of ΔΨ\textsubscript{m}, massive swelling of mitochondria, rupture of the outer membrane, and release of intermembrane components that induce cell death.\textsuperscript{34} Mitochondrial Ca\textsuperscript{2+} overload and ROS favor PTP opening.\textsuperscript{35,36}
Hence, inhibition of $\Delta \Psi_m$ loss by UCP2 overexpression may be mediated by prevention of $\text{Ca}^{2+}$ overload or ROS production in mitochondria, such that PTP never opens.

Activation of mitoK$_{\text{ATP}}$ channels has cardioprotective effects, and inhibition of mitochondrial $\text{Ca}^{2+}$ overload and subsequent preservation of $\Delta \Psi_m$ are considered the mechanisms of these effects.10–12 We examined the possibility that the cardioprotective effects by UCP2 overexpression observed in the present study may be mediated by activation of mitoK$_{\text{ATP}}$ channels. Neither 5HD nor glibenclamide altered the cardioprotective effects by UCP2 overexpression, indicating that the cardioprotection of UCP2 is not dependent on mitoK$_{\text{ATP}}$ channels. Nevertheless, the fact that UCP2 overexpression mimics the effects of mitoK$_{\text{ATP}}$ channel openers supports the idea that mitoK$_{\text{ATP}}$ channels may be cardioprotective because they are mild uncouplers.11

Although the present findings convincingly demonstrate protective effects of UCP2 overexpression, other models provide discordant results. Namely, a recent report demonstrated that overexpression of UCP2 in HeLa cells prompted cellular oncosis by dramatically decreasing the mitochondrial membrane potential.37 Although we cannot define the reason for the discrepancy, UCP2 overexpression may result in different downstream effects in various cell types. In fact, UCP2 overexpression did not alter the growth and viability of fibroblasts, while causing fatal response in HeLa cells.37 Furthermore, UCP2 negatively regulates insulin secretion in pancreatic $\beta$ cells.5–6 In macrophages, UCP2 contributes to immune function by regulating ROS production.7 A recent study, published while the present article was in revision, demonstrated that transfection of UCP1 conferred cell-protective effects against hypoxia/reoxygenation in H9c2 cells.38 Another study showed that UCP2 expression levels were inversely correlated with caspase-3 activation in neurons.39 Taking these results together, the physiological role of UCP2 may actually be similar in the brain and heart.

Since the discovery of UCP2, several reports have described the regulation of UCP2 mRNA expression in the heart. Thyroid hormone, $\beta$-adrenergic stimulation,40 and fatty acids41 are known as regulators of UCP2 expression. However, the regulation and the functional role of UCP2 in the pathological heart are largely unexplored. In a recent study, UCP2 expression was increased in the hearts of rats with cardiomyopathy induced by aortic regurgitation.42 In contrast, UCP2 expression was decreased in human hearts with idiopathic cardiomyopathy, ischemic cardiomyopathy, and partum cardiomyopathy.43 These studies shed some light on UCP2 expression in the pathological heart, but the functional role is still obscure. Although the results of the present study are restricted to cultured neonatal cardiomyocytes, they suggest that UCP2 may mitigate ischemia-reperfusion injury by reducing cell death. Future studies are necessary to identify the effects of UCP2 overexpression or upregulation on cardiac function in adult heart.

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