The Mitochondrial Uncoupling Protein-2 Promotes Chemoresistance in Cancer Cells

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Abstract

Cancer cells acquire drug resistance as a result of selection pressure dictated by unfavorable microenvironments. This survival process is facilitated through efficient control of oxidative stress originating from mitochondria that typically initiates programmed cell death. We show this critical adaptive response in cancer cells to be linked to uncoupling protein-2 (UCP2), a mitochondrial suppressor of reactive oxygen species (ROS). UCP2 is present in drug-resistant lines of various cancer cells and in human colon cancer. Overexpression of UCP2 in HCT116 human colon cancer cells inhibits ROS accumulation and apoptosis after exposure to chemotherapeutic agents. Tumor xenografts of UCP2-overexpressing HCT116 cells retain growth in nude mice receiving chemotherapy. Augmented cancer cell survival is accompanied by altered NH2-terminal phosphorylation of the pivotal tumor suppressor p53 and induction of the glycolytic phenotype (Warburg effect). These findings link UCP2 with molecular mechanisms of chemoresistance. Targeting UCP2 may be considered a novel treatment strategy for cancer.

Introduction

Cancers are often exposed to adverse conditions such as nutrient limitation, ischemia, hypoxia, host defense mechanisms, and anticancer therapy. Cancer cells typically respond to these stimuli by increased abundance of reactive oxygen species (ROS) resulting in oxidative stress (1). In this complex interplay, ROS promote further genomic instability and stimulate signaling pathways of cellular growth and proliferation. Paradoxically, ROS may also initiate cell death pathways, if present in excessive amounts (2, 3). The ability of cancer cells to regulate ROS levels greatly contributes to autonomous growth, evasion of apoptosis, and other hallmarks of adaptation associated with chemoresistance (4, 5). A better understanding of how oxidative stress is controlled by cancer cells is therefore essential to identifying new molecular targets for the treatment of cancer.

Mitochondria are the primary source of metabolically derived ROS (6). Substrate oxidation by mitochondrial respiration generates a proton gradient across the mitochondrial inner membrane that establishes the chemical potential (Δψm). The energy contained within Δψm can be either used for ATP synthesis (oxidative phosphorylation) or dissipated as heat that is mediated via proton leak in a process termed uncoupling (7). Elevated Δψm levels impede rapid flow of electrons along the respiratory chain, facilitating escape of more electrons and formation of superoxide, the primary mitochondrial ROS (6). Because proton leak decreases Δψm and the rate of superoxide production, mitochondrial uncoupling is a principal mechanism in the regulation of oxidative stress (8, 9). Accordingly, uncoupling protein-2 (UCP2), a widely distributed member of the anion carrier protein superfamily located in the mitochondrial inner membrane, is the major regulator of mitochondrial ROS (9, 10).

UCP2 expression correlates with neoplastic changes in human colon cancer (11), and drug-resistant sublines of various cancer cells also exhibit increased levels of UCP2, lower Δψm, and reduced susceptibility to oxidative damage (12). Overexpression of UCP2 in HepG2 human hepatoma cells lowers intracellular ROS levels and attenuates apoptosis induced by various challenges (13). Thus, whereas UCP2 is a marker of chemoresistance, expression of this mitochondrial protein may facilitate cancer cell adaptation to oxidative stress. However, the precise molecular mechanisms by which increased UCP2 expression may promote cancer cell survival are not known and have been examined here.

Materials and Methods

Cell lines. Human colon cancer cell lines HCT116, HT29, DLD1, and CaCo2 were obtained from American Type Culture Collection. p53−/− HCT116 cells and their wild-type isogenic cell line were a generous gift of Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). Cells were cultured in McCoy's modified medium (HCT116 and HT29), RPMI 1640 (DLD1), or Eagle's MEM (CaCo2), all supplemented with 10% fetal bovine serum (20% in case of CaCo2), 2 mmol/L L-glutamine, and 1% penicillin-streptomycin. Cells were kept in a humidified incubator at 37°C, 5% CO2.

Plasmids and cell transfection. For UCP2 overexpression experiments, human spleen total RNA (Ambion) was reverse transcribed and full-length human UCP2 cDNA was amplified by PCR with sequence-specific primers [forward, 5′-TACAGTCCATGTTGGTTC-3′; reverse, 5′-GGAAGGACACACTCT-3′, containing restriction sites for KpnI and HindIII, respectively (underlined)]. The double-digested cDNA was inserted into pcDNA 3.1/Zeocin− using the rapid DNA ligation kit (Roche). Successful ligation of the full-length hUCP2 was confirmed by sequencing (W.M. Keck Facility, Yale University). The same plasmid was used to generate a standard curve in the real-time PCR assay. The full-length human TATA-box binding protein (TBP) was cloned by a similar technique (forward primer, 5′-AGAACAAGGCCGCGCA-3′; reverse primer, 5′-TACGCTTGTCTCTTGTAATTC-3′) and subsequently inserted into the pCR 2.1 vector. HCT116 cells (5 × 104 per reaction) were transfected by nucleofection (Amza Biosystems) using 2 μg plasmid following the manufacturer's instructions. The UCP2-overexpressing HCT116 stable cell line was generated by using 10 μg/mL zeocin (Invitrogen) in the culture medium for several passages, and colonies raised from a single cell were analyzed for UCP2 expression by Western blotting.

Chemicals and UV irradiation. Camptothecin (CPT), doxorubicin-hydrochloride, etoposide, carbonyl cyanide-4-trifluoro-methoxyphenylhydrazone (FCCP), oligomycin, N-acetyl-L-cysteine, MG132 (Z-Leu-Leu-Leu-Ala),...
and routine chemicals were ordered from Sigma unless otherwise specified. Camptothecin stock solution (2.5 mmol/L) was prepared in DMSO. Doxorubicin stock solution (2 mg/ml) was prepared in water. Etoposide stock solution (30 mmol/L) was prepared in methanol. FCCP (40 mmol/L) was dissolved in ethanol and kept at –20°C until usage. Irinotecan (CPT-11; Pfizer) was dissolved in physiologic saline (20 mg/ml). Cells were exposed to UV irradiation essentially as described elsewhere (14). Briefly, cells were washed thrice with PBS, which was then removed, and the plates were irradiated for 15 min with 40 J/m² intensity on ice using FB-UVX1-1000 cross-linker (Fisher), followed by overnight incubation at 37°C. Cell death was assessed by cell cycle analysis.

Real-time PCR. The PARIS kit (Ambion) was used to isolate total cellular RNA and protein from the samples following the manufacturer's instructions. The total RNA was reverse transcribed using First Strand cDNA Synthesis Kit (Roche), and 5-ng cDNA was amplified with sequence-specific primers using iCycler iQ Multi-Color Real-time PCR Detection System (Bio-Rad). Serial dilutions of the human UCP2 and TBP plasmids were used to create standard curves. Thermal cycling conditions involved 45 cycles, with denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, using 0.4 μmol/L of intron-spanning primers (UCP2, forward 5’-CTCTGAAGGAAACACCAT-3’ and reverse 5’-CCCAAGGCGAAGTGAATG-3’; TBP, forward 5’-CAGGACCGG- CACGTATT-3’ and reverse 5’-TTTTCTCTGCGCACTGAC-3’). Samples were run in triplicates, normalized using their TBP mRNA content as endogenous reference, and data were expressed in arbitrary units as relative abundance of UCP2 mRNA over TBP mRNA.

Mitochondrial isolation and fractionation. Mitochondria were isolated using standard protocol (15). Purified mitochondria were either homogenized in cell disruption buffer (PARIS Kit, Ambion) and snap-frozen for further use in immunoblot analysis or further fractionated using the previously described digitonin/alkaline treatment (16). Briefly, 100 μL of purified mitochondria were dissolved in 500 μL of digitonin solution (1.2 mg/mL). After incubation on ice for 25 min, the suspension was centrifuged at 10,000 × g for 10 min to generate mitoplasts, which consisted of the mitochondrial inner membranes and the matrix. The supernatant contained the intermembrane space fraction and outer membrane. For alkaline treatment, mitoplastic pellets were washed and resuspended in freshly prepared 0.1 mol/L sodium carbonate (pH 11.5) and subsequently incubated at 0°C for 30 min. The membrane fraction was recovered by centrifugation at 100,000 × g for 30 min at 4°C; the supernatant represented the soluble fraction of the mitochondria. Mitoplasts and mitochondrial membranes were reconstituted in cell disruption buffer.

Antibodies and immunoblot analysis. Cell lysates were prepared in cell disruption buffer (PARIS Kit, Ambion) supplemented with protease inhibitors (Roche). For the detection of phosphoproteins, we used the disruption buffer. Antibodies and immunoblot analysis. Cell lysates were prepared in cell disruption buffer (PARIS Kit, Ambion) supplemented with protease inhibitors (Roche). For the detection of phosphoproteins, we used the disruption buffer.

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Measurement of mitochondrial membrane potential. Mitochondrial membrane potential (ΔΨm) was measured qualitatively using the lipophilic fluorescent probe 5,5,6,6-tetramethyl-1,1’3,3’-tetraethylbenzimidazolyl-carboxyrmthylene iodide (JC-1, Sigma). Cells were cultured in 96-well plates, washed with PBS, and incubated with 6 μmol/L JC-1 for 30 min at 37°C. Cells were then washed with Tris-buffered saline and JC-1 fluorescence was immediately measured in a SpectraMax MS spectrophotometer (Molecular Devices). The ratio of red (530 nm) to green (590 nm) fluorescence of JC-1 was calculated for each well. To control experimental conditions, FCCP (50 μmol/L) and oligomycin (10 μmol/L) were used to dissipate and increase ΔΨm respectively. Each condition was reproduced in at least six wells for each experiment.

Measurement of whole-cell oxygen consumption. Cells were harvested and resuspended in medium containing 125 mmol/L NaCl, 5.2 mmol/L KCl, 1 mmol/L Na2PO4, 0.5 mmol/L CaCl2, 10 mmol/L dextrose, and 10 mmol/L HEPES. Batches of 5 × 10⁶ cells were placed in the chamber of a Digital Model 10 polarography apparatus equipped with a Clark-type oxygen electrode (Rank Brothers), and oxygen consumption was measured for up to 15 min until the medium was depleted of oxygen according to the manufacturer's instructions. Initial oxygen content was calculated to be 0.20625 mmol/L based on temperature, altitude, and osmolarity of cell medium. Electrode potentials were recorded on a computer via an interface system using Pico Log Recorder (Pico Technology). The rate of oxygen consumption was calculated for each run, and each condition was repeated at least in triplicate.

Biochemical assays. Cellular ATP content was measured with ATPlite kit (Perkin-Elmer). Lactate levels in cell culture supernatants were measured by Lactate Assay Kit (BioVision). Both ATP and lactate levels were normalized to viable cell numbers.

DNA fragmentation assay. DNA fragmentation was assessed by the accelerated apoptotic DNA ladderizing protocol (17) with slight modifications. Cells were homogenized in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 1% NP40, 20 mmol/L EDTA], pelleted at 16,000 × g (5 min, 4°C), and the supernatant was subjected to one round of phenol/chloroform/isooamyl alcohol (25:24:1, pH 7.4, 0.5 mL) extraction. Apoptotic DNA fragments were precipitated from the liquid phase by adding 50 mL of 3 mol/L sodium acetate (pH 5.2), 1 mL of nuclease free glycerogen (Roche), and 0.6 mL of isopropanol. After incubation on ice for 5 min, precipitated nucleic acids were pelleted by centrifugation at 12,900 × g (10 min, 4°C). After washing with 70% ethanol, the pellet was reconstituted in Tris-EDTA buffer and DNA concentrations were measured by spectrophotometry. Equal amounts of DNA were digested with RsaseOne (Promega). After digestion, 5λ Orange G dye was added to each sample and the apoptotic DNA fragments were resolved by 1.8% Tris-acetate-EDTA agarose gel electrophoresis.

Annexin flow cytometry. To assess apoptosis by the appearance of Annexin V on the cell surface, cells were washed with PBS, harvested using 0.25% trypsin (Sigma), and centrifuged at 500 × g for 5 min. After repeated washing, cells were resuspended in Annexin binding buffer and stained with the Vybrant Apoptosis Assay Kit #3 (Invitrogen) according to the manufacturer's instructions. Briefly, the cells were stained with 5 μL of Annexin V conjugated with FITC (component A) and 1 mL of 100 μg/mL propidium iodide (component B). Following incubation for 30 min in darkness at room temperature, the cells were immediately analyzed in a FACSort flow cytometer (Becton Dickinson). Annexin binding and propidium iodide internalization were quantified using FL1 and FL3 channels, respectively. A minimum of 10,000 events were collected for each condition. All experiments were reproduced at least in triplicate using three independent experiments.

Measurement of intracellular ROS. Cellular ROS generation was assayed by using 2’,7’-dichlorodihydrofluorescin diacetate (DCF; Invitrogen). Cells were washed with PBS, harvested using 0.25% trypsin, centrifuged at 500 × g for 5 min, and resuspended in PBS. Cells were incubated with 10 μmol/L DCF for 10 min at room temperature. Because DCF is unstable in
solution, a fresh 10 mmol/L stock was prepared for each experiment. After staining, cells were treated with various agents and promptly analyzed by flow cytometry using a FACSort flow cytometer (Becton Dickinson). A minimum of 50,000 events were collected for each condition and all experiments were reproduced at least in triplicate.

**Tumor cell xenotransplantation.** HCT116 cells stably expressing UCP2 (clone ZUT) and empty vector controls (clone ZEU12) were s.c. injected into the lower flanks of NCr nude mice (Taconic Farms) at a dose of 3 x 10^6 viable tumor cells. Mouse tumor growth was measured with digital caliper and calculated by using the formula of a rotational ellipsoid: V = π/6 x A x B^2, where V is volume, A is the longest tumor axis, and B is the perpendicular shorter tumor axis. In vivo chemotherapy with irinotecan hydrochloride (CPT-11, Pfizer) was started after 2 wk once xenografts reached an average volume of at least 100 mm^3. CPT-11 was administered at a dose of 25 mg/kg ip. every third day for 2 wk. All animal experiments were done in accordance to the institutional guidelines of Lifespan Animal Welfare Committee of Rhode Island Hospital.

**Immunohistochemistry.** Tumor xenografts were removed and fixed overnight in 4% paraformaldehyde dissolved in PBS at 4°C, then dehydrated, embedded in paraffin, and cut into 4-μm thickness. Tissue slides were stained with C-20 goat polyclonal anti-UCP2 antibody (1:100; Santa Cruz), followed by biotinylated secondary horse anti-goat antibody (1:500; Vector), and visualized with peroxidase (Vector).

**Statistical analysis.** Data are presented as mean ± SE and analyzed with unpaired Student's t test or ANOVA when multiple comparisons were made. Differences with calculated P < 0.05 were regarded as significant.

**Results and Discussion**

UCP2 overexpression protects cancer cells from apoptosis and oxidative stress. To examine the functional importance of UCP2 in cancer cells, we have overexpressed the plasmid-encoded cDNA of human UCP2 in HCT116, a human colon cancer cell line with low endogenous UCP2 levels (Fig. 1A and B). Recombinant UCP2 was synthesized at high levels and targeted successfully to the mitochondrial inner membrane of HCT116 cells (Fig. 1C). Consistent with increased uncoupling (9), UCP2-overexpressing HCT116 cells displayed diminished baseline ΔΨm (Fig. 1D, left) and increased oxygen consumption (Fig. 1D, middle), whereas their intracellular ATP levels remained unchanged (Fig. 1D, right). Because UCP2 has no apparent effect on net proton conductance unless activated by superoxide or ROS-derived alkenals (9), these findings affirm that baseline oxidant levels are sufficiently high to activate plasmid-encoded UCP2 in HCT116 cells.

To determine whether UCP2 overexpression can block apoptosis induced by chemotherapeutic drugs, we treated HCT116 cells with CPT, a topoisomerase I inhibitor with derivatives widely used in the clinical management of colon cancer (18). Such agents cause DNA strand breaks and initiate a series of events that may contribute to increased oxidative stress, culminating in cell death (18). Apoptosis induced by CPT was markedly diminished in UCP2-overexpressing HCT116 cells as determined by Annexin V flow cytometry (Fig. 2A, left) and DNA ladder gel electrophoresis (Fig. 2A, top right). Furthermore, UCP2 overexpression in HCT116 cells resulted in decreased cleavage and activation of the key apoptosis effector caspase-3, increased abundance of the antiapoptotic Bcl-XL protein, and less expression of PUMA-α protein, and less expression of PUMA-α (Fig. 2A, bottom right), an essential proapoptotic protein targeted by the tumor suppressor p53 (19, 20). These data further indicate that plasmid-encoded UCP2 confers resistance to CPT-induced apoptosis. Apoptosis was similarly diminished in UCP2-overexpressing HCT116 cells.

**Figure 1.** Overexpression of UCP2 in cancer cells. A, cell line selection for overexpression experiments. Endogenous UCP2 mRNA levels in various human colon cancer cell lines determined by quantitative real-time PCR and expressed as relative ratios over the mRNA of TATA-box binding protein shown in arbitrary units. Bars, SE. B, immunoblot analysis of UCP2 in the mitochondrial fraction of HCT116 cells transfected with various amounts of HUCP2-pcDNA3.1(Zero) plasmid containing the full-length human ucp2 cDNA (pUCP2) or empty vector (EV) indicates dose-dependent expression of plasmid-encoded UCP2, whereas endogenous UCP2 protein in these cells is essentially nondetectable. Subunit IV of cytochrome c oxidase (COX IV) served as loading control. C, plasmid-encoded UCP2 is properly targeted to the mitochondrial inner membrane. Mitochondria of HCT116 cells were isolated and subfractionated 48 h after transfection with 2 μg of plasmid. Immunoblotting indicates the presence of plasmid-encoded UCP2 in the inner membrane fraction (identified by cytochrome c oxidase subunit IV), but not in the intermembrane space (identified by cytochrome c). OM, mitochondrial outer membrane. D, functional analysis of plasmid-encoded UCP2. Left, mitochondrial membrane potential (ΔΨm) of HCT116 cells in response to UCP2 overexpression assessed by red to green JC-1 fluorescence ratios and shown in percentages relative to nontransfected cells (no DNA); bars, SE. Experimental controls to abolish or elevate ΔΨm included the chemical uncoupler FCCP (10 μmol/L) and the ATP synthase inhibitor oligomycin (10 μmol/L), respectively. Middle, oxygen consumption (pmol/min/cell ± SE) measured by polarography using a Clark-type oxygen-sensitive electrode. Right, intracellular ATP content (pmol/10^6 cells ± SE) measured by luciferin-luciferase assay. * P < 0.05, pUCP2 versus empty vector.
HCT116 cells treated with two topoisomerase II inhibitors, etoposide and doxorubicin, showing that the protective effect of UCP2 is not limited to CPT and topoisomerase I blockade (Fig. 2B, top). Moreover, UCP2 overexpression resulted in a 30% decrease of apoptosis induced by exposure of HCT116 cells to UV radiation (40 J/m² for 15 minutes), indicating the ability of UCP2 to rescue cancer cells from various types of cytotoxic injury (not shown).

Chemical uncoupling simulates the effect of UCP2 overexpression in cancer cells. Pretreatment with low doses of the chemical uncoupler FCCP also protected HCT116 cells from apoptosis induced by topoisomerase inhibitors (Fig. 3A). Because FCCP is a pure protonophore, these findings support the role of proton leak in the antiapoptotic effect of UCP2. The protective effect of uncoupling was further shown by lower intracellular ROS levels in HCT116 cells exposed to chemotherapeutic agents (Fig. 3B, top). DNA fragmentation was assessed by accelerated DNA ladder gel electrophoresis. Marker, DNA molecular weight control. Right bottom, immunoblot analysis of proapoptotic (full caspase-3, cleaved caspase-3, and PUMA-α) and antipapoptotic proteins (Bcl-XL). B, effect of UCP2 overexpression on cellular responses to various cytotoxic drugs. Top, columns, mean rates of apoptosis expressed as the percentage of total cell number assessed by Annexin V flow cytometry; bars, SE. Bottom, intracellular ROS levels assessed by DCF flow cytometry. Columns, mean DCF fluorescence expressed as the percentage of levels measured in nontransfected, untreated cells at baseline, and following treatment for 30 min; bars, SE. *, P < 0.05, pUCP2 versus empty vector. All treatments were initiated 24 h after transfection.

UCP2 promotes in vivo chemoresistance in cancer cells. Next, we analyzed the in vivo effects of UCP2 on drug resistance of colon cancer cells. We generated s.c. xenografts in NCr nu/nu mice by using wild-type p53 HCT116 cells that stably overexpress UCP2 (clone ZU7) compared with empty vector controls (clone ZE12; Fig. 4A). Triaxial measurements indicated no difference between spontaneous growth rates of xenografts containing cells overexpressing UCP2 and cells transfected with empty vector (Fig. 4B). Tumor growth markedly regressed in response to treatment with the topoisomerase I inhibitor CPT-11 in mice that received xenografts of ZE12 cells. In contrast, UCP2-overexpressing ZU7 cells were much more resistant to CPT-11. These in vivo observations provide further evidence that increased levels of UCP2 augment chemoresistance in colon cancer cells.

p53-dependent protection of cancer cells from apoptosis by UCP2. To investigate the link between diminished intracellular ROS levels and cell death rates in UCP2-overexpressing HCT116 cells treated with cytotoxic agents, we next examined the role of the pivotal tumor suppressor p53 in this process. HCT116 cells possess wild-type p53, which is a plausible target of UCP2 for several reasons. As shown above, UCP2 overexpression results in reduced expression of PUMA-α, an important proapoptotic effector of p53. Furthermore, as recently proposed, ROS provide a major stimulus to p53 stabilization and subsequent induction of apoptosis by a
feed-forward regulatory loop (21). Consistent with this concept, decreases of mitochondrial ROS levels by chemical inhibitors of oxidative phosphorylation abrogate p53-dependent apoptosis in human T lymphocytes and MOLT-3 leukemia cells expressing wild-type p53 (22). To determine if inhibition of apoptosis by increased mitochondrial uncoupling depends on the presence of p53, we overexpressed UCP2 in p53<sup>-/-</sup> HCT116 cells. Cell cycle analysis in p53<sup>-/-</sup> HCT116 cells transfected with empty vector and treated with the protonophore FCCP 30 min before the addition of CPT for 24 h. Apoptosis was assessed by Annexin V staining (left), DNA ladder formation (right top), caspase-3 cleavage (right middle), and disappearance of Bcl-X<sub>L</sub> (right bottom). For additional details, please see Fig. 1. Note the increased number of cells staining for both Annexin V and propidium iodide in response to 5 μmol/L FCCP (and at higher doses; data not shown) in the top right quadrants, indicating concomitant increase in necrotic cell death. Results are each from at least two independent experiments. B, columns, mean intracellular ROS levels (expressed as the percentage of levels measured by DCF in untreated cells) at baseline and in response to treatment with 2.5 μmol/L CPT for 30 min; bars, SE. ROS levels in cells treated with the antioxidant N-acetylcysteine (NAC; 2.5 mmol/L) are shown for comparison. C, Intracellular ATP content (pmol/10<sup>3</sup> cells ± SE) measured by luciferin-luciferase assay shows dose-dependent decrease following treatment with FCCP, but FCCP has no further effect on markedly decreased ATP levels in cells exposed to 2.5 μg CPT for 24 h. *, P < 0.05, between cells with or without treatment with CPT; †, P < 0.05, between cells with or without treatment with FCCP.

Figure 3. Uncoupling mimics the effect of UCP2 in cancer cells. A, at the doses indicated, HCT116 cells were treated with the protonophore FCCP 30 min before the addition of CPT for 24 h. Apoptosis was assessed by Annexin V staining (left), DNA ladder formation (right top), caspase-3 cleavage (right middle), and disappearance of Bcl-X<sub>L</sub> (right bottom). For additional details, please see Fig. 1. Note the increased number of cells staining for both Annexin V and propidium iodide in response to 5 μmol/L FCCP (and at higher doses; data not shown) in the top right quadrants, indicating concomitant increase in necrotic cell death. Results are each from at least two independent experiments. B, columns, mean intracellular ROS levels (expressed as the percentage of levels measured by DCF in untreated cells) at baseline and in response to treatment with 2.5 μmol/L CPT for 30 min; bars, SE. ROS levels in cells treated with the antioxidant N-acetylcysteine (NAC; 2.5 mmol/L) are shown for comparison. C, Intracellular ATP content (pmol/10<sup>3</sup> cells ± SE) measured by luciferin-luciferase assay shows dose-dependent decrease following treatment with FCCP, but FCCP has no further effect on markedly decreased ATP levels in cells exposed to 2.5 μg CPT for 24 h. *, P < 0.05, between cells with or without treatment with CPT; †, P < 0.05, between cells with or without treatment with FCCP.

Figure 4. UCP2 promotes in vivo drug resistance in cancer cells. A, detection of UCP2 expression by immunoblot analysis (top) and immunohistochemistry (bottom) in s.c. xenografts of HCT116 cells stably expressing UCP2 (ZU7; right) or empty vector controls (ZE12; left) inoculated at a dose of 3 x 10<sup>6</sup> into both flanks of 4- to 6-wk-old male NCr nu/nu mice (n = 8). ZE12 cells with scattered and faintly positive staining reflect endogenous UCP2 expression. β-Actin served as loading control. Magnification, ×400. B, growth of HCT116 cancer cell xenografts monitored by triaxial measurements. Two weeks after inoculation of HCT116 cells, mice were treated with CPT-11 at a dose of 25 mg/kg i.p. every 3 d (arrows). Controls received saline injection. *, P < 0.05, ZU7 versus ZE12 following CPT-11 treatment.
with CPT showed lack of G1-S arrest and lower rates of cell death ($P < 0.0001$, versus wild-type), both consistent with augmented chemoresistance in the absence of functional p53 (Fig. 5A).

However, diminished susceptibility to CPT in p53$^{-/-}$ HCT116 cells was not further altered by UCP2 overexpression ($P > 0.05$ (not significant), versus wild-type), suggesting that cytoprotection by mitochondrial uncoupling is not additive with the absence of wild-type p53 (Fig. 5A). Notably, UCP2 overexpression did not alter CPT-induced activation of the G1-S checkpoint in p53$^{+/+}$ HCT116 cells (Fig. 5A), suggesting that plasmid-encoded UCP2 interferes with p53-mediated apoptosis but has no effect on p53-mediated cell cycle arrest under these conditions.

**UCP2 interferes with posttranslational modification of p53 in cancer cells.** A well-recognized mechanism of p53 activation involves phosphorylation of its NH$_2$-terminal domain in response to upstream stress signals (23). Rapid phosphorylation of Ser$_{15}$ is considered a "priming event" in response to genotoxic stresses (24). This is followed by modifications that involve a number of NH$_2$-terminal p53 residues directly or indirectly responsive to oxidative stress, including Ser$_{33}$ and Ser$_{46}$ by the stress-activated protein kinase p38 (25, 26), Ser$_{20}$ and Ser$_{46}$ by protein kinase C$\gamma$ (27, 28), and Thr$_{81}$ by c-jun NH$_2$-terminal kinase (29). To explore links between diminished ROS levels and p53-mediated apoptosis in HCT116 cells, we tested the effect of plasmid-encoded UCP2 on NH$_2$-terminal p53 phosphorylation. We noted that phosphorylation of p53 induced by 2.5 $\mu$mol/L CPT in UCP2-overexpressing HCT116 cells was markedly diminished at the designated Ser$_{15}$, Ser$_{33}$, and Ser$_{46}$ residues (Fig. 5B). Of note, total p53 abundance remained essentially unchanged, indicating no apparent effect of UCP2 overexpression on p53 accumulation in response to CPT. These data suggest that UCP2 inhibits apoptosis of HCT116 cells by interfering with the ROS-mediated phosphorylation of p53 within the transactivation domain. Further studies will be necessary to identify UCP2-specific patterns for posttranslational modification of p53 and the signaling mediators and other molecular partners involved in this process.

**UCP2 overexpression promotes the glycolytic phenotype in cancer cells.** p53 seems to be involved in the regulation of energy metabolism, potentially via interactions with UCP2. As recently reported, p53 stimulates mitochondrial oxygen consumption by inducing the expression of SCO2, a subunit of the cytochrome $c$ oxidase complex that is embedded in the respiratory chain, revealing a further novel mechanism for tumor suppression (30). Furthermore, the product of another p53-inducible gene,
TP53-induced glycolysis and apoptosis regulator (TIGAR), lowers the intracellular levels of fructose-2,6-bisphosphate, a key substrate in glycolysis (31). Thus, p53 may compromise the Warburg effect, a metabolic hallmark of many cancer cells (32) by increasing oxidative phosphorylation, inhibiting glycolysis, and preserving the balance between these two differing ATP-generating pathways. Indeed, there is decreased oxygen consumption and increased lactate production in p53-deficient cells (30). In line with these observations, we found that HCT116 cells that stably overexpress UCP2 produce progressively more lactate compared with empty vector–transfected control cells in culture (Fig. 5C). Moreover, treatment of UCP2-overexpressing HCT116 cells with the glucose analogue 2-deoxyglucose, a potent inhibitor of glycolysis, results in suppression of cell growth, consistent with increasing dependence on glycolytic ATP production (Fig. 5D).

Conclusions

Our findings indicate that UCP2 modulates the cellular adaptive response of cancer cells. Increased expression of UCP2 may provide a marker of chemoresistance in p53-mutant cell lines (Fig. 1A) and in the setting of neoplasia (11) of the human colon. We also show that UCP2 seems to have an active role in promoting cancer cell survival that is linked to mitochondrial suppression of ROS production. Moreover, the antiapoptotic effects of UCP2 via ROS involve modulation of the p53 pathway, a pivotal tumor suppression mechanism. Finally, this interaction also affects the balance of cellular energy production because UCP2 overexpression preferentially induces the glycolytic phenotype in cancer cells. Altogether, the data identify UCP2 as a potential molecular target of novel treatment strategies in cancer.

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References

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