Up-Regulation of Uncoupling Protein 2 by Cyanide Is Linked with Cytotoxicity in Mesencephalic Cells

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ABSTRACT

Uncoupling protein 2 (UCP-2) regulates mitochondrial function by increasing proton leak across the inner membrane to dissociate respiration from ATP synthesis and reduce reactive oxygen species generation. A number of studies have shown that UCP-2 expression protects cells from oxidative stress mediated injuries. In the current study, we show UCP-2-mediated reduction in mitochondrial function contributes to the mitochondrial dysfunction and the necrotic death of primary cultured mesencephalic cells (MCs) after exposure to cyanide, a complex IV inhibitor. The necrotic cell death was directly related to the level of mitochondrial dysfunction, as shown by reduction in ATP levels and decreased mitochondrial membrane potential. Treatment with cyanide for 6 h or longer up-regulated UCP-2 expression. Blockade of up-regulation with a transcription or a translational inhibitor reduced the response to cyanide. Knockdown with RNAi or transfection with a UCP-2 dominant-negative interfering mutant reduced the cyanide-induced mitochondrial dysfunction and cell death, showing that constitutive expression of UCP-2 plays a role in the response to cyanide. Overexpression of UCP-2 by transfection with human full-length cDNA potentiated the cyanide toxicity. These findings indicate that UCP-2 can serve as a regulator of mitochondrial-mediated necrotic cell death, in which enhanced expression can increase the vulnerability of primary MCs to injury due to complex IV-mediated inhibition by cyanide.

In Parkinson’s disease (PD), increasing evidence suggests that impairment of mitochondrial function and consequent oxidant stress in select brain regions such as substantia nigra and striatum are important pathophysiologic mechanisms contributing to the loss of dopaminergic neurons in sensitive brain regions and ultimately to the decline of motor coordination and functioning in patients. Uncoupling proteins (UCPs) are a family of inner mitochondrial membrane proteins that regulate mitochondrial respiration, ATP, and reactive oxygen species (ROS) production by catalyzing an inner membrane protein leak (Chavin et al., 1999). In the nervous system, at least three UCPs are expressed, including the widely expressed UCP-2, neuron-specific UCP-4, and UCP-5 (brain mitochondrial carrier protein-1) (Richard et al., 1998). Even mild uncoupling of the electron transport chain by proton leak through a UCP markedly reduces the generation of ROS by decreasing mitochondrial membrane potential (ΔΨm) (Negre-Salvayre et al., 1997; Miwa and Brand, 2003; Teshima et al., 2003). Moderate to strong uncoupling markedly reduces ATP levels in cells and induces nonapoptotic cell death (Mills et al., 2002; Li et al., 2005). Taken together, these findings suggest that mitochondrial uncoupling in neurons may impact the susceptibility of particular brain regions to degeneration in conditions such as PD.

UCP-2 can function as a death or survival factor, dependent on the initiation stimulus and death pathway executed (Duval et al., 2002). Hypoxic stress induces expression of UCP-2, which may be an adaptive mechanism to oxidative stress (Pecqueur et al., 2001). UCP-2 may provide cytoprotection by acting as a negative regulator of oxidative phosphorylation to decrease ROS production (Sullivan et al., 2003). Also, UCP-2 may be neuroprotective by modulating cellular redox signaling or inducing mild mitochondrial uncoupling that prevents mitochondrial release of proapoptotic proteins (Mattiasson et al., 2003).

Other studies have associated a high level of UCP-2 expression and activity with necrotic death. Overexpression of UCP-2 in HeLa cells decreased ΔΨm, NADH, and ATP levels.
and subsequent cell death was not prevented by caspase inhibitors (Mills et al., 2002). Uchino et al. (2004) demonstrated that liver allografts with steatosis up-regulated UCP-2, which enhanced the vulnerability of hepatocytes to necrosis. In cortical cells, transfection with UCP-2 switched cyanide-induced apoptosis to necrosis (Li et al., 2005). This was a specific action mediated by UCP-2 since RNA interference and a dominant-negative mutant blocked the necrotic response.

Induction of UCP-2 expression may be a double-edged sword; increased action would reduce efficiency of ATP synthesis and reduce ROS generation to decrease execution of apoptosis (Diehl and Hoek, 1999). On the other hand, this initially protective response may progress to catastrophic disruption of cellular metabolism, leading to marked ATP depletion, mitochondrial dysfunction, and necrotic death. It is possible that up-regulation of UCP-2 may enhance the cytotoxic response to a mitochondrial toxin such as cyanide. Cyanide is a rapid-acting neurotoxicant that stimulates ROS generation to produce cell injury and death (Jones et al., 2003). Cyanide inhibits cytochrome oxidase to block complex IV in the mitochondrial respiratory chain and thereby enhances ROS generation at complex III (Chen et al., 2003). In mesencephalic cells (MCs), cyanide-induced necrosis is associated with excess oxidative stress and rapid breakdown of mitochondrial function, characterized by onset of mitochondrial membrane permeability transition (MPT) and ATP depletion (Prabhakaran et al., 2002). The necrosis is inhibited by preventing changes in mitochondrial function, indicating that mitochondrial dysfunction is a primary initiator of the cell death. Mitochondrial actions that lead to necrosis include MPT, respiratory inhibition, uncoupling of oxidative phosphorylation, and excess ROS generation (Castilho et al., 1999; Li et al., 2005).

Because in PD dopaminergic neuronal structures such as the substantia nigra in the mesencephalic tegmentum are selectively damaged, we used primary cultures of rat dopaminergic MCs as a model to test the hypothesis that activation of UCP-2 may contribute to the robust sensitivity of mesencephalic neurons to mitochondrial respiratory inhibition by cyanide. It is proposed that expression and activation of UCP-2 excessively uncouples mitochondrial oxidative phosphorylation, and inhibition of cytochrome oxidase by cyanide leads to a catastrophic drop of cellular ATP and subsequent execution of necrosis. In the present study, it was observed that exposure of MCs to cyanide increased UCP-2 expression, which in turn enhanced the sensitivity of cells to cyanide.

Materials and Methods

Cell Culture of Primary Rat Mesencephalic Cells. Ventral mesencephalon tissue was removed from fetal Sprague-Dawley rats (15–17 days gestation). Briefly, the tissue was placed in Hank’s balanced salt solution, and the cells were dissociated with the addition of 0.025% trypsin at 37°C for 15 min. Trypsin digestion was stopped by adding 100 μl of trypsin inhibitor and 100 μl of DNase I. Dissociated cells were centrifuged at 1000g for 10 min and suspended in DMEM supplemented with 22 mM glucose, 2 mM glutamine, 3.7 g/l bicarbonate, 10% fetal bovine serum, 1% horse serum, and 1% penicillin/streptomycin (5000 U/ml). Cells were plated at a density of 5 × 10^4 cells/cm² in culture flasks or in six-well culture plates precoated with 0.1% poly-l-lysine and maintained in DMEM under controlled incubation conditions (37°C, 5% CO₂ and 95% air). On day 6, 10 μM cytochrome arabinofuranoside was added to the medium for 24 h to suppress proliferation of astrocytes. DMEM was changed every 48 to 72 h until cells reached confluency (12–16 days). At the time of experimentation, the percentage of astrocytes in the culture was approximately 30% as determined by immunostaining of the astrocyte-specific marker glial fibrillary acidic protein and 70% neurons as determined by immunostaining of the neuron-specific marker microtubule associated protein-2.

Transient Transfections. The UCP-2 cDNA or mutant of UCP-2 (UCP-2ΔD212N) was subcloned into the expression vector pCDNA3.1 as previously described (Mills et al., 2002). Transient transfections of UCP-2, UCP-2ΔD212N plasmids, and siRNA were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described by Li et al. (2005). Lipofectamine diluted in Opti-MEM was applied to the plasmids or dsRNA and incubated for 45 min. To each microtiter plate well containing cells, 1 μg of plasmid or 0.2 μg of 21-bp dsRNA (siRNA) containing Lipofectamine was applied in a final volume of 1 ml. The medium was changed to regular MC culture medium 5 h after transfection, and cells were treated with cyanide after 24 h of transfection. The full-length UCP-2 cDNA was cotransfected with the nuclear-localizing green fluorescent protein construct p-CMV-GFPnuc (Invitrogen), which allowed for visualization of cells undergoing transfection. The dominant-interfering mutant was a site-directed mutant of UCP-2 created by substitution of position 212 (Asp→Asn) and was subcloned in the expression vector pCDNA3.1 (Mills et al., 2002).

siRNA Preparation and Transient Transfection. siRNA corresponding to the UCP-2 reporter gene was synthesized by Ambion (Austin, TX) with 5’ phosphate, 3’ hydroxyl, and two base overhangs on each strand. The gene-specific sequences were used for UCP-2 interference: sense 5’-GAACGGGACACCUUUAGAGtt-3’ and antisense 5’-CUCUAAAGGUGUCGUUCGtt-3’. annealing for duplex siRNA formation was performed as described by the manufacturer.

Evaluation of Apoptosis (Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Staining). After transfection, cells were treated with KCN (400 μM for 24 h) and/or pretreated with 1 μM cyclosporin A for 30 min before cyanide. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed on paraformaldehyde (4% in phosphate-buffered saline)-fixed cells using the Apoptag in situ apoptosis detection kit (Oncor, Gaithersburg, MD). Briefly, cells were preincubated in equilibration buffer containing 0.1 M potassium cacodylate (pH 7.2), 2 mM CaCl₂, and 0.2 mM dithiothreitol for 10 min at room temperature and then incubated in TUNEL reaction mixture containing 200 nM potassium cacodylate (pH 7.2), 4 mM MgCl₂, 2 mM 2-mercaptoethanol, 30 μM biotin-16-dUTP, and 300 U/ml terminal deoxynucleotidyl transferase in a humidified chamber at 37°C for 1 h. After incubating in stop/wash buffer for 10 min, the elongated digoxigenin-labeled DNA fragments were visualized using anti-digoxigenin peroxidase antibody solution followed by staining with 3,3’-diaminobenzidine/H₂O₂ (0.2 mg/ml diaminobenzidine tetrachloride and 0.005% H₂O₂ in PBS, pH 7.4). Cells were then counterstained with hematoxylin. The selectivity of the assay is based on the presence of 3-OH DNA fragment ends in apoptotic cells.

Quantitation of Necrotic Cell Death. Necrosis was quantitated using two DNA fluorescent dyes, SYTO-13 and propidium iodide (PI) (Prabhakaran et al., 2004). Both dyes bind DNA, but only SYTO-13 is membrane permeable. Thus, SYTO-13 stains normal cells with a green fluorescence, whereas only cells with disrupted plasma membranes stain red with propidium iodide. The percentage of cells stained positive for PI was determined as an estimate of necrosis.

Western Blot Analysis. UCP-2 expression was assessed with Western blots. After various treatments or transient transfection, cells were washed with ice-cold PBS and harvested by centrifugation at 500g for 5 min. Cell pellets were lysed in a buffer containing 220 mM mannitol, 68 mM sucrose, 20 mM HEPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 1 mM EDTA, 2 mM MgCl₂, 1 mM dithiothreitol, 0.1% nonidet P-40, and a protease inhibitor cocktail. Cell lysates were then boiled in -20°C and analyzed by Western blot using antibodies against UCP-2.
Triton X-100, and protease inhibitors on ice for 15 min. After centrifugation, supernatants were taken as whole-cell protein extraction. The protein content in the extractions was determined by the Bradford assay (Bio-Rad, Hercules, CA). The samples containing 30 μg of protein were boiled in Laemmli buffer for 5 min and subjected to electrophoresis in 12% SDS-polycrylamide gel, followed by transfer to a nitrocellulose membrane. After blocking with phosphate-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20, the membrane was exposed to the primary UCP-2 antibody or β-actin antibody for 3 h at room temperature on a shaker. The UCP-2 antibody was a rabbit anti-mouse polyclonal antibody (1:2000) directed toward the C-terminal domain of UCP-2 (Alpha Diagnostic International, Inc., San Antonio, TX). Antibody specificity was determined by using a 14- amino acid UCP-2 blocking peptide according to the manufacturer’s protocol. Antibodies were detected with a fluorescent-linked anti-mouse IgG (second antibody) conjugated to horse- radish peroxidase using enhanced chemiluminescence. Densitometric analysis was performed using Scion Image software (Scion Corporation, Frederick, MD).

Reverse Transcriptase-PCR Analysis. Total RNA was isolated from mesencephalic cells using the RNaseasy Mini Kit (QIAGEN, Valencia, CA) and was reverse transcribed into cDNA, and this was used as template for the PCR. RNA samples (1 μg) with oligo(dT) were denatured at 75°C for 5 min followed by addition of 1× reverse transcriptase buffer, 4 μl of deoxynucleoside triphosphate mix, 1 μl of RNase inhibitor, and 1 μl of reverse transcriptase for a total of 20 μl (Retroscript Kit; Ambion). After gently mixing, samples were incubated at 42°C for 1 h, and the reaction was stopped by heating to 92°C for 10 min. PCR amplimer pairs for analysis of UCP-2 cDNA were 5’-CGA CAG TGC TCT GGT ATC TCC-3’ (sense) and 5’-ACA TCA ACG GGG GAG GCA ATG-3’ (antisense). β-Actin cDNA amplimer pairs were 5’-GTG GGC CGC TCT AGG CACCAA-3’ (sense) and 5’-CTC TTT GAT GTC ACG CAC GAT-3’ (antisense). β-Actin mRNA was assessed to control for the amount and integrity of RNA in each sample. Each PCR was performed on a 1-μl cDNA sample using Tag DNA polymerase (Promega, Madison, WI) in a total volume of 25 μl in a PerkinElmer Life and Analytical Sciences (Boston, MA) 2400 DNA thermal cycler. Each cycle consisted of a denaturation step (94°C for 30 s), an annealing step (45 s), and a primer extension step (72°C for 1 min). Annealing temperature and cycle number for UCP-2 were 54°C and 30 cycles and for β-actin were 63°C and 23 cycles. PCR products were separated by electrophoresis on 12% agarose gel and detected by ethidium bromide staining using a UV transilluminator.

Measurement of Cellular ATP. Cellular ATP content was determined using a bioluminescence assay according to the manufacturer’s instructions (Sigma Chemical, St. Louis, MO). Cells (2 × 10⁵) were treated with cyanide, and 24 h later, cells were washed in PBS, lysed in 0.5% Triton X-100, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and incubated for 10 min on ice. After removal of cell debris by centrifugation (10,000g; 15 min, 4°C), the ATP content was measured by the luciferin/luciferase method (Los et al., 2002).

Measurement of Mitochondrial Membrane Potential. The ΔΨm was monitored with rhodamine 123 (R123) as previously described (Prabhakaran et al., 2002). After treatment, cells were loaded with 10 μM R123 and incubated at 37°C for 30 min in the dark. Uptake of R123 into mitochondria is a direct reflection of its permeability: increases in R123 fluorescence reflect a lowering of ΔΨm. Loaded cells were washed twice with Krebs-Ringer buffer, and changes in R123 fluorescence were monitored using a fluorescence plate reader at 498-nm excitation and 525-nm emission.

Statistics. Data were expressed as mean ± S.E.M., and statistical significance was assessed by one-way analysis of variance, followed by the Tukey-Kramer multiple range test. Differences were considered significant at P < 0.05.

Results

Cyanide-Induced Necrosis Is Associated with Cellular Energy Depletion. Cyanide-induced necrotic death in MCs (Fig. 1A) is consistent with our previous report (Prabhakaran et al., 2002). A concentration-related necrotic death was observed following 24 h of exposure to KCN, with 400 μM being the threshold concentration required to produce cytotoxicity. This correlates with a decrease of cellular ATP (Fig. 1B). It was concluded that ATP depletion below a critical level is likely an initiation signal for cell death.

Cyanide-Induced Up-Regulation of UCP-2 and Cell Death. Cells treated with KCN (400 μM or higher concentrations) for 20 h showed elevated cellular UCP-2 above constitutive levels (Fig. 2A). Up-regulation of UCP-2 was rapid; increased expression was detected within 6 h of KCN exposure and reached a maximal level within 12 to 24 h (Fig. 2B). As shown in Fig. 2C, KCN exposure induced a time-dependent increase of UCP-2 mRNA, parallel to increased UCP-2 expression. It was concluded that exposure to cyanide enhanced expression of UCP-2 in MCs. To determine whether up-regulation of UCP-2 was transcriptionally regulated, cells were pretreated with a protein synthesis inhibitor (cycloheximide) or a transcriptional inhibitor (actinomycin D) before exposure to KCN. Cycloheximide attenuated induction of UCP-2 protein expression,

![Fig. 1](https://example.com/Fig1.png)
whereas actinomycin D produced an even greater block of UCP-2 induction (Fig. 3A). Interestingly, these treatments produced parallel reductions in the level of KCN-induced cell death (Fig. 3B). It was concluded that KCN increased UCP-2 expression through a transcriptionally regulated process and the enhanced expression was linked with increased cell death.

Altered UCP-2 Expression Modulates Cyanide-Induced Necrosis. To elucidate its role in cyanide-induced cell death, UCP-2 levels were enhanced by transfection with a UCP-2 expression plasmid or knocked down by RNA interference. Western analysis of the constructs showed that the UCP-2+/+ plasmid increased cellular expression, whereas siRNA decreased constitutive levels (Fig. 4A). Overexpression of UCP-2 did not significantly increase the level of cell death above that observed in control cells but did markedly enhance necrosis produced by KCN (Fig. 4B). Interestingly, RNAi knockdown significantly reduced the necrotic response to KCN. Changes in ATP levels 24 h after KCN treatment paralleled the cell death; transfection with UCP-2 potentiated the cyanide-induced reduction of cellular ATP, whereas RNAi blocked the reduction of ATP (Fig. 4C). It was concluded that enhanced expression of UCP-2 potentiates the
necrotic response to cyanide, and knockdown of constitutive expression reduces the response to KCN. Changes in cellular energetics paralleled the cell death.

Mitochondrial Dysfunction Is Involved in UCP-2-Mediated Cell Death. MPT is involved in execution of both necrosis and apoptosis (Crompton, 1999). Necrotic cell death is characterized by a rapid opening of the MPT pore, which is inhibited by cyclosporin A to block cell death. To determine whether MPT was involved in UCP-2-mediated cytotoxicity, UCP-2 expression was increased by transfection; then, opening of mitochondrial membrane pore was blocked by CsA (Fig. 5). Cyanide lowered the $\Delta \psi_m$, and in UCP-2-overexpressed cells, an additional lowering of $\Delta \psi_m$ was observed, correlating with the level of cell death. Note that an increase in R123 fluorescence indicates a reduction of $\Delta \psi_m$. CsA blocked the ability of UCP-2 to enhance both cyanide-induced mitochondrial depolarization and necrotic death. These observations show that $\Delta \psi_m$ collapse and the cell death are the consequences of MPT pore opening. It is concluded that KCN in the presence of enhanced expression of UCP-2 induces MPT by inducing a greater reduction in $\Delta \psi_m$; thus, mitochondrial dysfunction plays an important initiation role in the necrotic cell death.

UCP-2 Mutant Protects from Cyanide-Induced Necrosis. Since previous studies show that UCP-2D212N is a functional dominant-interfering antagonist to UCP-2 (Mills et al., 2002), experiments were conducted to verify the in-

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Fig. 4. Effect of transfection with UCP-2 cDNA or siRNA on cell responses to cyanide. All studies were conducted 24 h after transfection with a UCP-2 expression plasmid or UCP-2 siRNA (knockdown). A, effect of transfection on cellular UCP-2 expression. Twenty-four hours after transfection, cells were treated with KCN (400 $\mu$M) for 20 h, and then cell lysates were subjected to SDS-PAGE and immunoblotted. Densitometric analysis is the relative density of three or more assays. B, 24 h after KCN treatment (400 $\mu$M), PI-positive cells were counted, and percentage of necrotic cells was determined. Data represents mean ± S.E.M. *, significantly different from control; #, significantly different from KCN group; $P < 0.05$. C, effect of UCP-2 cDNA or siRNA transfection on ATP levels 24 h after KCN treatment. ATP levels are expressed as percent control (nontreated cells), and each value is the mean of six or more experiments ± S.E.M. *, significantly different from control and UCP$^+$ group; #, significantly different from KCN group, $P < 0.05$.

Fig. 5. Blockade of mitochondrial membrane permeability pore transition blocks cyanide-induced necrosis. A, 24 h after transfection with human UCP-2 cDNA, cells were treated with CsA for 30 min and then with KCN (400 $\mu$M) for 2 h. Cells were then loaded with rhodamine 123 and $\Delta \psi_m$ monitored by change in fluorescence of R123-loaded cells. B, cells were transfected with human UCP-2 cDNA for 24 h and then pretreated with CsA before addition of KCN. Twenty-four hours later, the level of necrosis was determined. Data represent mean ± S.E.M. of six or more experiments. *, significantly different from control; #, significantly different from KCN treatment group; +, significantly different from UCP$^+$-KCN treatment group; $P < 0.05$. 

volvement of constitutive UCP-2 in cyanide-induced necrosis and mitochondrial dysfunction. Expression of the UCP-2 mutant reduced necrotic death (Fig. 6A). KCN-induced cell death was not completely blocked by the mutant, thus confirming that cyanide's action is not mediated by the protein but rather enhanced. Transfection with the mutant significantly attenuated KCN-induced changes in $\Delta\Psi_m$ and ATP levels, thus showing that UCP-2 contributes to KCN-induced mitochondrial dysfunction (Fig. 6, B and C). Thus, constitutive expression of UCP-2 contributes to the cytotoxic action of cyanide by enhancing mitochondrial dysfunction.

Discussion

Cyanide produced a necrotic death of MCs that was partly dependent on the level of UCP-2. Continuous exposure to cyanide for 6 h or longer up-regulated UCP-2 expression, which in turn enhanced cellular sensitivity, whereas knockdown with RNAi reduced the necrosis and preserved $\Delta\Psi_m$ and cellular ATP. A dominant-interfering mutant also reduced the response to cyanide, showing that constitutive expression of UCP-2 plays a role in the cyanide response. Overexpression of UCP-2 by transfection with human UCP-2 cDNA enhanced cyanide-induced mitochondrial dysfunction, leading to a drop of ATP levels and $\Delta\Psi_m$, followed by necrosis. It was concluded that the level of UCP-2 expression in MCs influences the cytotoxicity by modulating cyanide-induced mitochondrial dysfunction.

Cyanide is a potent neurotoxin that inhibits cytochrome oxidase in mitochondrial complex IV to block oxidative metabolism and stimulate ROS generation upstream at complex III (Jones et al., 2003). In mice treated with cyanide, two distinct modes of cell death were noted in specific brain regions, with apoptosis in cortex and necrotic death in basal ganglia (Mills et al., 1999). In cultured primary neuronal cells, cyanide also produced apoptosis in cortical cells and necrosis in MCs, confirming that the response was dependent on cell type (Prabhakaran et al., 2002). These modes of cell death share common initiation stimuli, but divergent intracellular cascades are activated to produce either apoptosis or necrosis (Shou et al., 2003; Prabhakaran et al., 2004). Cells undergoing necrosis display a more intense level of oxidative stress and experience a rapid breakdown of mitochondrial function characterized by onset of MPT and ATP depletion (Prabhakaran et al., 2002). On the other hand, we have shown that primary cultured cortical cells exposed to low concentrations of cyanide undergo primarily an apoptotic death (Li et al., 2005). Overexpression of UCP-2 in these cells switched the response to necrosis, which was related to a greater level of mitochondrial dysfunction. The present study shows that in MCs, cyanide produces primarily a necrotic death that is enhanced by up-regulation of UCP-2 expression above constitutive levels.

Under constitutive conditions, MC and cortical cells respond to cyanide with different modes of cell death. It is possible that the selective vulnerability of brain regions to cyanide may be related in part to differences in regional expression and activation of UCP-2 (Mills et al., 1999). It should be pointed out that in these studies, mixed cultures of cells composed of neurons and astrocytes were used, and the contribution of astrocytic cells to the necrotic response is not known. However, we have observed that astrocytic cells are resistant to cyanide (J. L. Borowitz, L. Li and K. Prabhakaran, unpublished data), and there may be a difference among the sensitivity of the various neuronal populations, i.e., dopaminergic versus other types of neurons. By defining the susceptible cell population in the primary cultures, a more definitive link could be established between UCP-2 expression and specific cell populations.

There is considerable evidence to suggest mitochondrial dysfunction may underlie the pathophysiologic changes in vulnerable brain regions occurring during neurodegenerative conditions such as Alzheimer's disease and PD. Inhibitors of mitochondrial function such as cyanide or MPTP produce similar neuroanatomic changes and consequent motor dysfunction in animal models to those observed in patients with PD. Furthermore, mutations in mitochondrial complex I and increased mitochondrial oxidant stress are associated with substantia nigra lesions in PD patients (for review, see Beal,
edly decreased
This enhanced mitochondrial dysfunction was evident in inhibition of complex IV could lead to rapid ATP depletion.

Inhibiting ATP synthesis (Boss et al., 1998).

Depolarize the inner mitochondrial membrane, resulting in increased mitochondrial dysfunction. UCP-2 is known to increase ATP synthesis (Boss et al., 1998).

Efficient mitochondrial ATP synthesis coupled with partial inhibition of complex IV could lead to rapid ATP depletion. This enhanced mitochondrial dysfunction was evident in cells transfected with UCP-2. In these cells, cyanide markedly decreased ΔΨm, leading to opening of the MPT pore and extensive energy failure; the result was increased execution of cellular necrosis.

To confirm involvement of UCP-2 in cyanide-induced cell death, cellular levels of the protein were modified. Knockdown of UCP-2 by RNAi markedly reduced necrosis, whereas overexpression enhanced cell death. To determine the role of constitutive expression in the response, cells were transfected with a dominant-interfering mutant, which antagonizes UCP-2 activity (Mills et al., 2002). The mutant reduced cyanide-related changes in ΔΨm and ATP levels and subsequent cell death. Previous studies have established that the mutant functions as a dominant-negative antagonist to endogenous UCP-2 and antagonizes UCP-2 and hypoxia-induced death. Based on these observations, it was concluded that mitochondrial sensitivity to cyanide is related in part to the level of UCP-2 expression.

Cyanide-induced up-regulation of UCP-2 expression may be mediated through oxidative stress. In neurons, cyanide induces a rapid burst of ROS, accompanied by a high level of nitric oxide formation by nitric-oxide synthase (Gunasekar et al., 1996; Prabhakaran et al., 2002). Furthermore, UCP-2 expression is known to be rapidly induced under high oxidative stress, possibly through translational regulation of the UCP-2 mRNA (Pecqueur et al., 2001; de Bilbao et al., 2004). Also, Nakatani et al. (2002) showed that in hepatocytes, up-regulation of UCP-2 was prevented by cycloheximide pretreatment. Following up-regulation of expression, mitochondrial UCP-2 could then be activated by cyanide-induced generation of ROS (Echtay et al., 2002). In isolated mitochondria, KCN generates superoxide in the mitochondrial matrix to activate UCP-2, which then increases uncoupling and progressively reduces proton conductance. It is likely the cyanide-induced oxidative stress leads to both up-regulation and then activation of UCP-2 in mitochondria.

Studies have indicated that UCP-2 up-regulation is important in cell death. Sriram et al. (2002) showed that two neurotoxins (methamphetamine and kainic acid) increased UCP-2 expression in mouse brain and, in turn, proposed that abnormal expression of UCP-2 may cause excessive energy depletion that can produce marked mitochondrial dysfunction and necrosis. Moreover, Diehl and Hoek (1999) proposed that UCP-2 up-regulation may place the cell in a situation in which a decrease in substrate availability or an increase in ATP requirements may have lethal consequences.

Other studies have considered involvement of UCP-2 in necrosis. Overexpression or activation of UCP-2 leads to a rapid fall in ΔΨm and reduction of both mitochondrial NADH and ATP levels to selectively produce necrosis (Mills et al., 2002). Expression of a dominant-interfering mutant of UCP-2 confers resistance to necrosis, but not apoptosis (Mills et al., 2002). Also, overexpression of the antiapoptotic Bcl-2 does not influence UCP-2-mediated cell death. Bechmann et al. (2002) observed that UCP-2-overexpressing transgenic mice exhibited a lower level of neuronal apoptosis following brain lesions but did not report the level of necrosis. Furthermore, Mattson and Liu (2003) proposed that uncoupling proteins are involved in regulating mitochondria-mediated cell death. In parallel studies, we have observed that overexpression of UCP-2 in cortical cells switches the death mode from apoptosis to necrosis in response to cyanide exposure (Li et al., 2005).

As opposed to necrosis, numerous studies have shown that UCP-2 modulates apoptotic cell death. Overexpression of human UCP-2 in transgenic mice protected against stroke and traumatic brain injury (Mattiaison et al., 2003) and also against dopamine cell loss in a mouse model of Parkinson’s disease (Andrews et al., 2005). In a seizure model, transgenic mice overexpressing UCP-2 displayed a lower level of hippocampal lesions characterized by degenerating or apoptotic pyramidal cells in the CA1 region (Diano et al., 2003). Brains of transgenic mice expressing human UCP-2 show decreased oxidative stress, elevated ATP, and an increased number of mitochondria, suggesting that UCP-2 provides protection from oxidative stress. In cultured cortical cells, UCP-2 inhibits caspase activation and protects against oxygen/glucose deprivation-induced death (Mattiaison et al., 2003). Apoptosis is an energy-consuming process (Liu et al., 1996) and may not occur in the presence of moderate cellular ATP depletion (Leist et al., 1997; Zamzami et al., 1997). Additionally, UCP-2 may inhibit apoptosis either by limiting ATP synthesis or by decreasing pH of the intermembranous space that would decrease the opening of pores in the outer mitochondrial membrane to permit efflux of apopgetic factors into the cytosol for activation of the caspase cascade. Although increased UCP-2 activity has the potential to constrain apoptosis, it could become maladaptive, thus promoting necrosis if neuronal energy requirements increase.

Results of the present study involving cyanide and UCP-2 may have pathological significance. Takuma et al. (2005) linked cyanide-induced mitochondrial dysfunction with Alzheimer’s disease. Transgenic mice overexpressing both amyloid-β peptide and mitochondrial alcohol dehydrogenase show enhanced ROS production in brain cells, leading to cell death. The dehydrogenase enzyme binds to amyloid-β peptide to generate oxidative species. Cyanide (but not mitochondrial complex I inhibitors) further enhances the level of oxidative stress in these transgenic mice through inhibition of complex IV. This report is of special interest since cyanide is known to be endogenously generated in brain mitochondria (Gunasekar et al., 2004).

Inhibition of oxidative phosphorylation by cyanide is associated with collapse of ΔΨm and ATP depletion that terminates in necrotic MC death. When UCP-2-mediated mitochondrial uncoupling is increased, the mitochondrial dysfunction produced by cyanide is enhanced, thus potentiating cell death. It is concluded that the level of UCP-2
expression can serve as a regulator of mitochondria-mediated necrotic cell death in which enhanced expression and subsequent activation of UCP-2 can increase the vulnerability of neurons to neurotoxin-induced mitochondrial dysfunction.

References


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