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Cyanide-induced Death of Dopaminergic Cells is Mediated by Uncoupling Protein-2 Up-regulation and Reduced Bcl-2 Expression

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Abstract

Cyanide is a potent inhibitor of mitochondrial oxidative metabolism and produces mitochondria-mediated death of dopaminergic neurons and sublethal intoxications are associated with a Parkinson-like syndrome. Cyanide toxicity is enhanced when mitochondrial uncoupling is stimulated following up-regulation of uncoupling protein-2 (UCP-2). In this study, the role of a pro-survival protein, Bcl-2, in cyanide-mediated cell death was determined in a rat dopaminergic immortalized mesencephalic cell line (N27 cells). Following pharmacological up-regulation of UCP-2 by treatment with Wy14,643, cyanide reduced cellular Bcl-2 expression by increasing proteasomal degradation of the protein. The increased turnover of Bcl-2 was mediated by an increase of oxidative stress following UCP-2 up-regulation. The oxidative stress involved depletion of mitochondrial glutathione (mtGSH) and increased H₂O₂ generation. Repletion of mtGSH by loading cells with glutathione ethyl ester reduced H₂O₂ generation and in turn blocked the cyanide-induced decrease of Bcl-2. To determine if UCP-2 mediated the response, RNAi knock down was conducted. The RNAi decreased cyanide-induced depletion of mtGSH, reduced H₂O₂ accumulation, and inhibited down-regulation of Bcl-2, thus blocking cell death. To confirm the role of Bcl-2 down-regulation in the cell death, it was shown that overexpression of Bcl-2 by cDNA transfection attenuated the enhancement of cyanide toxicity after UCP-2 up-regulation. It was concluded that UCP-2 up-regulation sensitizes cells to cyanide by increasing cellular oxidative stress, leading to an increase of Bcl-2 degradation. Then the reduced Bcl-2 levels sensitize the cells to cyanide-mediated cell death.

Keywords

Bcl-2; Cyanide; Mitochondrial glutathione; Uncoupling protein-2

Introduction

Cyanide is a potent neurotoxicant that produces a rapid onset of toxicity and death within minutes. In sub-lethal toxicity, lesions of the central nervous system may develop which may manifest as a Parkinson-like syndrome (Utti et al., 1985; Sarkaya et al., 2006). In these individuals, select damage to the basal ganglia is apparent, with dopaminergic pathways

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displaying the highest sensitivity (Rosenberg et al., 1989; Mills et al., 1999; Jones et al., 2003). We recently reported that mice exposed to cyanide over 9 days had selective loss of dopaminergic neurons in the substantia nigra mid-brain area (Zhang et al., 2009). Since cyanide inhibits cytochrome oxidase to disrupt mitochondrial function, degeneration of dopaminergic neurons likely involves mitochondrial-mediated death pathways (Leavesley et al., 2008).

Up-regulation of uncoupling protein-2 (UCP-2), an anion carrier expressed in the inner mitochondrial membrane, has been associated with several models of brain injury and neurodegeneration in which the level of expression appears to regulate the level of cell injury (Mizuno et al., 2000; Sullivan et al., 2003). A low level UCP-2 expression stimulates leakage of protons across the mitochondrial inner membrane, thereby reducing the mitochondrial membrane potential ($\Delta\Psi_m$) and decreasing generation of reactive oxygen species (ROS). This action protects cells from oxidative stress (Mattiasson et al., 2003). On the other hand, excess mitochondrial uncoupling, which occurs with UCP-2 over-expression, sensitizes cells to cytotoxic agents, possibly by lowering cellular ATP levels (Li et al., 2005, 2006; Prabhakaran et al., 2005). We have shown that up-regulation of UCP-2 can enhance cyanide toxicity (Li et al., 2005). Pharmacological up-regulation of UCP-2 by Wy14,643 in primary cortical cells can switch cyanide-induced apoptosis to necrotic death (Li et al., 2006) and the level of UCP-2 expression appears to serve as a regulator of mitochondrial mediated necrotic cell death (Prabhakaran et al., 2005). These observations have significant toxicological implications in that functional changes in mitochondrial regulation, such as that mediated by UCP-2, may influence the level of vulnerability to injury, particularly to target organs that are highly dependent on oxidative phosphorylation.

Recently, it has been shown that UCP-2 up-regulation may contribute to cell death by altering expression of Bcl-2 proteins, such as increasing levels of the pro-death protein BNIP3 (Bcl-2/adenovirus E1B 19kDa-interacting protein 3) (Bodyak et al., 2007) and decreasing levels of the anti-apoptotic protein Bcl-2 (Sun et al., 2004). Bcl-2 is reduced in a number of conditions associated with cell apoptosis, including lipopolysaccharide (LPS)-mediated death of endothelial cells (Blocksom et al., 2004) and neuronal death following cerebral ischemia (Sasaki et al., 2006). In these cell death models, UCP-2 also undergoes up-regulation, but the effect of up-regulating UCP-2 on Bcl-2 expression and the subsequent execution of neuronal cell death is not known.

In the case of cyanide, increased expression of Bcl-2 protects neural cells from cyanide-induced mitochondrial dysfunction and loss of viability, suggesting a protective role of Bcl-2 in cyanide-induced cell death (Myers et al., 1995). In the present study, we demonstrate in an immortalized mesencephalic cell line that UCP-2 up-regulation by treatment with Wy14,643 is linked with down-regulation of Bcl-2 expression, which in turn contributes to enhanced cyanide-mediated mitochondrial dysfunction and cell death.

Materials and methods

Materials

4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid (Wy14,643) was purchased from BioMol (Plymouth Meeting, PA) and Sytox green and 10-acetyl-3,7-dihydroxyphenoxazine (Amplex red) from Invitrogen (Carlsbad, CA). Pre-stained SDS-PAGE standards and the Bio-Rad protein assay kit were from Bio-Rad (Hercules, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Wy14,643 was dissolved in DMSO and the final concentration of DMSO did not exceed 0.1% (v/v). Other chemicals were dissolved in cell culture medium.

Cell culture

Rat immortalized mesencephalic IRB₃AN₂₇ neuronal cells (N27 cells) display features of dopaminergic neurons (Adams et al., 1996). N27 cell are tyrosine-hydroxylase-positive and express a functional dopamine transporter. This rat dopaminergic cell line was selected for the study since it is a well-characterized cell model used for evaluation of neurotoxic mechanisms (Kaul et al., 2005). The cells have been used as a cell culture model of Parkinson disease and to study chemical-induced toxicity in dopaminergic cells. Cells were plated at a density of 1×10^4 cells/cm² on poly-L-lysine coated 6 or 24-well plates and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37°C in an atmosphere of 5% CO₂ and 95% air.

UCP-2 was up-regulated by pretreatment with Wy14,643 (100 µM). We have previously characterized the up-regulation of UCP-2 by Wy14,643 in N27 cells (Zhang et al., 2007b). Wy14,643 produces a rapid upregulation of UCP-2 over a 6–12 hour period.

Quantitation of cytotoxicity

Cell death was determined by use of Sytox green, as previously described with minor modifications (de la Monte et al., 2001). Sytox green is a membrane-impermeant fluorescence dye and excluded from viable cells with an intact plasma membrane. The dye enters only necrotic or late apoptotic cells and intercalates with DNA to produce a green fluorescence (Kitazawa et al., 2004). After treatment, cell cultures were incubated with 1 µM Sytox green for 30 min (37°C) and then medium was removed and cells were washed with phosphate buffered saline (PBS). Cells were examined by fluorescence microscopy in which the number of cells in the microscopic field exhibiting green fluorescence (dead cells) was counted. Cell death was expressed as percentage of dead cells in the treatment group compared to control (untreated cells). Also, cell death was determined in cell suspensions in 24 well microtiter plates by measuring fluorescence at excitation 485 nm and emission 535 nm with a fluorescence microplate reader. The relative level of cell death was expressed as percent increase of fluorescence above control cell fluorescence.

Cellular peroxide generation

Cellular H₂O₂ was determined using Amplex red as previously described with minor modification (Mohanty et al., 1997). In the presence of peroxidase, Amplex red reacts with H₂O₂ in a 1:1 stoichiometry to produce the fluorescent-red oxidation product resorufin. Briefly, pretreated cells were incubated with 50 µM Amplex red reagent and 0.1 U/ml horseradish peroxidase in Krebs–Ringer phosphate (145 mM NaCl, 5.7 mM Na₂HPO₄, 4.86 mM KCl, 0.54 mM CaCl₂, 1.22 mM MgSO₄, 5.5 mM glucose, pH 7.35) at room temperature for 30 min. Fluorescence was monitored with a microplate reader at excitation wavelength of 540 nm and emission wavelength of 590 nm. Relative cellular H₂O₂ levels were directly proportional to fluorescence intensity. Treatment group data were expressed as percent of fluorescence generated in control cells under identical incubation conditions.

Glutathione analysis

Cytosolic and mitochondrial subcellular fractions were isolated as described by Muyderman et al (2004) with slight modification. Cells were harvested by trypsinization (0.05% trypsin and 0.02% EDTA) and washed twice in PBS. The cells were resuspended in isolation medium (10mM Tris, 1 mM EDTA, 0.32 M sucrose, pH 7.4) containing 0.2mg/ml digitonin on ice for 10 min and centrifuged (15,000 g) for 5 min. The supernatant was used as the cytosolic fraction. The pellet was washed twice with isolation medium by centrifugation. The final pellet was resuspended in the same medium for subsequent studies. Fractionation purity was confirmed by assessing the presence of cytochrome oxidase for mitochondria and α -tubulin for the cytosol.

Glutathione was determined by the 5,5-dithiobis-2-nitrobenzoate-oxidized glutathione (GSSG) reductase recycling assay, in which the rate of 2-nitro-5-thiobenzoic acid (TBN) formation is proportional to total GSSG and reduced glutathione (GSH) concentrations (Eyer and Podhradsky, 1986). The cell lysate was centrifuged for 5 min at $10,000 \times g$, and aliquots of the supernatant removed and neutralized with buffer (143 mM NaH_2PO_4 , 6.3 mM EDTA, pH 7.4). The reaction mixture, containing 5,5'-dithiobis-2-nitrobenzoic acid (1 mM) and NADPH (0.34 mM), was added to samples and the reaction was started by adding 8.5 IU/ml glutathione reductase (Vandeputte et al., 1994). Total glutathione (GSH+GSSG) levels were determined by measuring the increase in absorbance at 415 nm.

Real-time PCR

Total RNA was isolated from cells with the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed to cDNA. The forward and reverse primers for target genes were obtained from Integrated DNA Technologies (Coralville, IA). The primers are as follows: Bcl-2: forward, 5'-TCG TCA CCC CAC CCT TAC AT - 3', and reverse, 5'-AGT GCC TGT CTG GGA TGC A - 3'; β -actin: forward, 5'-TCC TCC TGA GCG CAA GTA CTC T - 3', and reverse, 5'-GCT CAG TAA CAG TCC GCC TAG AA - 3'. The ABsolute QPCR SYBR green Mix kit (ABgene, Rochester, New York) was used for RT-PCR analysis. Amplification was carried out in the Mx3000P RT-PCR System (Stratagene, La Jolla, CA) for 15 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 1 min at 60 °C and 30 s at 72 °C. The relative differences in gene expression between groups were expressed using cycle time (Ct) values. The Ct values of the interested genes were first normalized with that of β -actin in the same sample, and then the relative differences between control and treatment groups were calculated and expressed as relative increases, with the control as 100%.

Western blot analysis

After various treatments, cells were washed with ice-cold PBS and harvested by centrifugation at $500 \times g$ 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 MgCl_2 , 1 mM dithiothreitol, 0.1% 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). Samples containing 30 μg of protein were boiled in Laemmli buffer for 5 min and then subjected to electrophoresis in 12% (UCP-2, Bcl-2) or 10% (ubiquitin) SDS-polyacrylamide gel, followed by transfer to a polyvinylidene difluoride membrane. After blocking with Tris-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20, the membrane was exposed to primary antibodies to Bcl-2, ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA), UCP-2 (Alpha Diagnostic International, Inc., San Antonio, TX) or β -actin (Sigma Chemical Co., St. Louis, MO) for 3 h at room temperature. The fluorescein signal was then amplified by a secondary antibody with an anti-fluorescein alkaline phosphatase conjugate, followed by fluorescent ECF substrate according to the ECF Western Blotting KitTM (Amersham, Piscataway, NJ). Densitometric analysis was performed using Scion Image software (Scion Corporation, Frederick, MD). Data were normalized to the internal control (β -actin) and then expressed as relative density of each band compared with the respective vehicle control band. For each study, Western blot analysis was conducted two to three times and representative blots are shown.

Transient transfection and RNA interference

The full-length human UCP-2 cDNA (UCP-2⁺) was subcloned into the expression vector pcDNA3.1 as previously described (Mills *et al.*, 2002). The full-length human Bcl-2 cDNA (Bcl-2⁺) was subcloned into the expression vector LTR-SV neo vector (a kind gift from Dr

David M. Hockenbery, University of Washington). Small interfering RNA corresponding to the UCP-2 gene was designed as recommended (Elbashir et al., 2001) and synthesized by Ambion, Inc. (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'-CUCUAAAGGUGUCCCGUUCtt-3'; annealing for duplex siRNA formation was performed as described by the manufacturer. In N27 cells, this siRNA sequence effectively knocks UCP-2 expression down 24 h after transfection (Zhang et al., 2007b). The Silencer Negative Control siRNA, which does not target rat, mouse, or humans genes, was used as a negative control (Ambion, Austin, TX). Transient transfections of UCP-2, Bcl-2 plasmids and UCP-2 siRNA were performed with Lipofectamine 2000™ (Invitrogen, Carlsbad, CA) as previously described (Zhang et al., 2007b).

Statistics

Data were expressed as mean $\pm$ S.E.M., and statistical significance was assessed by one-way ANOVA. The Tukey-Kramer multiple range test was used for post hoc comparisons. Differences were considered significant at $P < 0.05$.

Results

UCP-2 up-regulation enhances cyanide toxicity and reduces Bcl-2 expression

In N27 cells, cyanide produced a low level cytotoxicity as determined by Sytox green staining (Fig. 1A,B). Visual inspection (counting green fluorescence cells) showed less than 5% cell death was induced by KCN (400 μ M) (Fig. 1A). On the other hand, Wy14,643 (100 μ M) alone induced a slight increase in cell death which was approximately 12% of the cells. When cell death was expressed as an increased of Sytox fluorescence, a similar level of death was observed (Fig. 1B). Pretreatment with Wy14,643 enhanced cyanide toxicity, consistent with our previous report that showed Wy14,643 + KCN produced >38% cell death (Zhang et al., 2007b). It was concluded that wildtype N27 cells are resistant to KCN and that pretreatment with Wy14,643 significantly increased the sensitivity of the cells to cyanide.

We previously established that Wy14,643 rapidly up-regulates UCP-2 expression (Zhang et al., 2007b). To determine whether the level of UCP-2 is linked with changes of Bcl-2 expression, UCP-2 was up-regulated by treatment with Wy14,643 and the subsequent expression level of Bcl-2 examined. Wy14,643 induced a concentration- and time-dependent increase of UCP-2 expression that was accompanied by down-regulation of Bcl-2 (Fig. 2A,B). Decreased Bcl-2 expression was initiated within 12 h and continued to decrease over 18 h. Bcl-2 down-regulation paralleled the increase of UCP-2 expression. The down-regulation of Bcl-2 was significantly enhanced when cells were treated with cyanide (Fig. 3A). To confirm that UCP-2 up-regulation produced changes in Bcl-2 expression, cells were transiently transfected with UCP-2 plasmid to force UCP-2 overexpression. In UCP-2⁺ cells, Bcl-2 expression was reduced by 25% as compared to wild type cells, thus showing elevation of UCP-2 above constitutive expression produces Bcl-2 down-regulation (Fig. 3B).

UCP-2 up-regulation increases proteasomal degradation of Bcl-2

Bcl-2 expression is tightly regulated at both transcriptional and post-transcriptional levels (Breitschopf et al., 2000). To determine whether UCP-2 up-regulation alters Bcl-2 expression, Bcl-2 mRNA levels were analyzed by real-time PCR. UCP-2 up-regulation did not affect Bcl-2 mRNA levels, either in the presence or absence of cyanide, thus it appeared in this model that post-transcriptional modification regulated Bcl-2 expression (Fig. 4A). Since Bcl-2 undergoes proteasomal degradation (Chanvorachote et al., 2006), lactacystin, a specific inhibitor, was used to inhibit proteasome metabolism. Lactacystin increased whole cell ubiquitinated protein levels as noted on Western blot analysis using an anti-ubiquitin antibody (Fig 4B.).

Accumulation of ubiquitinated proteins indicated lactacystin blocked the proteasomal degradation pathway. Pretreatment with lactacystin reversed UCP-2-mediated down-regulation of Bcl-2 (Fig. 4C) and it was concluded that Bcl-2 was post-transcriptionally down-regulated by increased proteasomal degradation.

Increased H₂O₂ generation and Bcl-2 down-regulation

H₂O₂ can stimulate proteasomal degradation of Bcl-2 (Wang et al., 2008). To determine if excess generation of H₂O₂ was involved in UCP-2-mediated down-regulation of Bcl-2, H₂O₂ levels were measured in intact cells. H₂O₂ generation increased slightly in UCP-2 up-regulated cells (Wy14,643 treatment group) and significantly enhanced by cyanide + Wy14,643 (Fig. 5A). To specifically determine if increased H₂O₂ generation mediated the Bcl-2 down-regulation, H₂O₂ was scavenged with catalase. Western blotting showed that down-regulation of Bcl-2 was blocked by catalase, thus showing a strong association of H₂O₂ generation with Bcl-2 down-regulation (Fig. 5B).

H₂O₂ generation and mitochondrial GSH depletion

Since mtGSH plays an important protective role against H₂O₂-mediated oxidative damage in mitochondria (Sen et al., 2000), the levels of mtGSH and H₂O₂ were measured after UCP-2 up-regulation. A marked decrease of mtGSH was induced by cyanide in UCP-2 up-regulated cells (Wy14,643 group) (Fig. 6A). Repletion of cellular GSH by loading with glutathione ethyl ester (GSH-EE) reversed the UCP-2 mediated enhancement of mtGSH depletion, significantly reduced levels of H₂O₂ generation and blocked down-regulation of Bcl-2 (Fig. 6A,B,C). It was concluded that oxidative stress was enhanced by mtGSH depletion and was an initiator of Bcl-2 down-regulation.

UCP-2 Knockdown attenuates oxidative stress and Bcl-2 down-regulation

To conclusively establish the role of UCP-2 up-regulation in reducing cellular levels of Bcl-2, UCP-2 was knocked down by RNA interference (RNAi) and then subsequent changes in mtGSH, H₂O₂ accumulation, and Bcl-2 expression determined. We have previously shown in N27 cells that this UCP-2 RNAi effectively knocks UCP-2 expression down (Zhang et al., 2007b). UCP-2 knockdown significantly reduced cyanide-mediated depletion of mtGSH and the increased generation of H₂O₂ (Fig. 7A,B). In control studies, treatment with UCP-2 siRNA alone did not significantly alter mtGSH or H₂O₂ generation. Wy14,643 alone did not alter mtGSH levels, but significantly increased H₂O₂ generation as we previously reported (Zhang et al., 2007b). On the other hand, the combined treatment with KCN + Wy14,643 produced a marked level of H₂O₂ generation. UCP-2 knockdown blocked the cyanide-mediated decrease of Bcl-2 expression and cell death (Fig. 8A,B). It should be noted in control studies that UCP-2 knockdown did not alter Bcl-2 levels. However, Wy14,643 alone reduced Bcl-2 levels and produced a low level cell death, but when combined with KCN, a marked level of cell death was observed. We have previously reported the potentiation of cyanide-induced cell death by Wy14,643 (Zhang et al., 2007b). It was concluded that UCP-2 up-regulation increases the level of oxidative stress produced by cyanide, which in turn initiates down-regulation of Bcl-2.

Increased Bcl-2 expression attenuates cyanide toxicity

To determine if changes of Bcl-2 expression can alter cyanide-induced toxicity, cells were transiently transfected with Bcl-2 cDNA and the effect on cyanide-induced cell death determined. Under the transfection conditions, Bcl-2 levels increase over 200% of control wildtype cells (Fig. 9A). The forced over-expression of Bcl-2 (Bcl-2⁺) attenuated the cell death produced by up-regulation of UCP-2 (Wy14,643 treatment group) and importantly, produced a 60% reduction of cell death by cyanide in UCP-2 up-regulated cells, as determined by both counting the number of death cells (green fluorescence) in a microscopic field or by measuring

fluorescence (Fig. 9B,C). It was concluded that the level of Bcl-2 expression modulates sensitivity of the cells to cyanide and up-regulation of UCP-2 reduces Bcl-2 levels and enhances sensitivity to cyanide.

Discussion

Cyanide-induced cell death was enhanced in a dopaminergic cell model by UCP-2 up-regulation. The action of UCP-2 was attributed to reduced expression of Bcl-2, an anti-apoptotic protein. In cells undergoing up-regulation of UCP-2, cyanide induced excessive oxidative stress as a result of mtGSH depletion and increased production of H_2O_2 . The oxidative stress increased proteasomal degradation of Bcl-2, thereby increasing susceptibility to cell death. This mechanism is supported by the observation that RNAi knockdown of UCP-2 blocked cyanide-mediated reduction of mtGSH and inhibited Bcl-2 degradation. Over-expression of Bcl-2 protected against the UCP-2 enhancement of cyanide toxicity, thus providing strong evidence that Bcl-2 down-regulation contributes to the cell death.

Cyanide is a rapid acting toxicant that produces death within minutes of exposure to lethal levels (Way, 1984). Cyanide inhibits cytochrome c oxidase to block complex IV in the mitochondrial respiratory chain to produce histotoxic hypoxia in which cells cannot utilize oxygen via oxidative phosphorylation (Leavesley et al., 2008). The result is rapid reduction of cellular ATP, leading to a catastrophic loss of homeostasis. In organs dependent on aerobic respiration, including brain and heart, dysfunction ensues resulting in death. In sublethal toxicity, a post-intoxication sequelae may manifest in which individuals develop a Parkinson-like syndrome characterized by selective degeneration of dopaminergic pathways in basal ganglia (Uitti et al., 1985; Rosenberg et al., 1989; Sarkaya et al., 2006). The mechanism underlying the neurodegeneration is complex and involves activation of specific mitochondria-mediated cell death pathways, similar to that activated by cellular hypoxia (Zhang et al., 2007a). In this study, UCP-2 expression and activation modulated the sensitive of the cell model to cyanide, thus showing that regulators of mitochondrial function can modulate cyanide-induced dysfunction. Thus, conditions that alter UCP-2 activity in mitochondria can influence the effects of cyanide on neuronal cells.

UCP-2 resides in the inner mitochondrial membrane where it regulates mitochondrial oxidative respiration by catalyzing a proton leak across the inner mitochondrial membrane. The proton leak reduces the $\Delta\Psi_m$, the driving force for ATP synthesis (Brand, 1990). UCP-2 increases susceptibility of cells to mitochondrial active compounds, including cyanide (Prabhakaran et al., 2005; Zhang et al., 2007b). The mechanism by which UCP-2 increases cell death produced by mitochondrial toxins appears to be related to UCP-2-mediated reduction of cellular ATP and $\Delta\Psi_m$ (Krauss et al., 2005). Recently, it was proposed that UCP-2 can function as a Ca^{2+} transporter to regulate mitochondrial Ca^{2+} influx and total Ca^{2+} load (Trenker et al., 2007). UCP-2 up-regulation may induce a mitochondrial Ca^{2+} overload, which then can induce mitochondrial dysfunction by activating mitochondrial transition pore opening. Alternatively, UCP-2 may modulate cell death by altering function of the Bcl-2 protein family. For instance, UCP-2 over-expression up-regulates BNIP-3, a BH3-only cell death protein, which is activated in myocardial ischemic injury and cyanide-induced neuronal degeneration (Bodyak et al., 2007; Zhang et al., 2007a). In this study, it was shown that Bcl-2 down-regulation contributed to the enhancement of cyanide toxicity in cells expressing high levels of UCP-2. It was concluded that decreased Bcl-2 levels and reduced ATP generation contributed to mitochondrial dysfunction that manifested as increased susceptibility to cytotoxicity.

Bcl-2 is constitutively expressed and localized to the outer mitochondrial membrane where it attenuates cell death signals to promote cell survival (Kroemer, et al., 1998). Bcl-2 exerts an anti-apoptotic effect by inhibiting mitochondrial outer membrane permeabilization to suppress

release of cytochrome c into the cytosol (Gross et al., 1999). Bcl-2 may also inhibit necrotic-like cell death by blocking opening of the mitochondrial permeability transition pore to maintain cellular ATP levels within survival limits (Garland and Halestrap, 1997). Forced over-expression of Bcl-2 can block cell death produced by a variety of stimuli, including cyanide (Tsujimoto et al., 1997). In this study it was observed that over-expression of Bcl-2 blocked enhancement of cyanide toxicity by UCP-2 up-regulation. It appears that the cell death is due in part to reduced Bcl-2 levels and transfection with Bcl-2 cDNA increased Bcl-2 expression which then enabled the cells to maintain survival.

Bcl-2 expression is regulated at both transcriptional and post-transcriptional levels (Breitschopf et al., 2000). Transcriptional regulation controls expression, as reflected by mRNA levels, whereas post-translational modifications, including dephosphorylation and ubiquitination, are essential for stability and function of the protein under various pathologic conditions (Haendeler et al., 1996; Dimmeler et al., 1999). In this study, cyanide markedly reduced Bcl-2 levels in UCP-2 up-regulated cells. Since levels of Bcl-2 mRNA were not altered as compared to constitutive expression, it appeared that post-transcriptional events were involved in the down-regulation. Proteasome inhibition blocked Bcl-2 down-regulation, therefore increased proteasomal degradation likely mediated the reduction in protein levels. Bcl-2 degradation is stimulated by oxidative stress, including mitochondrial generation of H_2O_2 (Li et al., 2004; Wang et al., 2008). Peroxides promote Bcl-2 proteasomal metabolism by inducing dephosphorylation and ubiquitination (Breitschopf et al., 2000). In cells undergoing UCP-2 up-regulation, cyanide increased H_2O_2 generation. The increased oxidative stress then mediated Bcl-2 degradation since pretreatment with catalase, a H_2O_2 scavenger, blocked the down-regulation of Bcl-2.

In mitochondria, GSH is crucial for maintaining redox homeostasis and protection against H_2O_2 (Sen et al., 2000). mtGSH depletion results in H_2O_2 accumulation to increase cellular oxidative damage (Sims et al., 2004). Decreased mtGSH levels have been associated with a reduction of Bcl-2 expression and increased apoptosis (Celli et al., 1998). UCP-2 up-regulation enhanced cyanide-mediated depletion of mtGSH, thus increasing cellular accumulation of H_2O_2 and subsequently stimulating Bcl-2 degradation. Pretreatment with GSH-EE restored mtGSH levels and blocked Bcl-2 down-regulation, thus indirectly showing mtGSH depletion contributed to the oxidative stress and reduction of Bcl-2 expression.

The decrease of cellular GSH following exposure to cyanide is likely due in part to reduced cellular ATP resulting from inhibition of cytochrome c oxidase (Mithofer et al., 1992; Prabhakaran et al., 2006). Additionally, inhibition of mitochondrial oxidative phosphorylation stimulates ROS production, leading to reduced mtGSH. In this study, UCP-2 up-regulation enhanced cyanide depletion of mtGSH. UCP-2 knockdown significantly reduced the mtGSH depletion, thus indicating a role of UCP-2 in mtGSH depletion (de Bilbao et al., 2004). It is reasonable to link UCP-2 up-regulation with a reduction of mtGSH, since both cellular GSH synthesis and mitochondrial uptake from the cytosolic pool require ATP. Reduced levels of ATP resulting from UCP-2 up-regulation may compromise cellular GSH synthesis and in turn mitochondrial uptake of GSH is reduced. Importantly, in the presence of cyanide, ATP synthesis was further reduced, thus leading to a marked depletion of mtGSH.

In the cell model used in this study, the increased sensitivity to cyanide was due to reduced expression of Bcl-2, an anti-apoptotic protein. By reducing Bcl-2 levels, the sensitivity of the cells to cyanide is enhanced, leading to increased cytotoxicity. It would be interesting to determine if this mechanism of cell death is specific for dopaminergic cells. We have recently shown that cyanide induces activation of BNIP3, a BH3 only Bcl-2 protein, to produce selective dopaminergic cell death in both *in vivo* and *in vitro* models (Zhang et al., 2009). These observations may provide an explanation of the underlying mechanism of increased sensitivity

of dopaminergic cells to cyanide and explain in part why central dopaminergic pathways are predisposed to cyanide-induced degeneration (Mills et al., 1999, Rosenberg et al, 1989). A variety of environmental and chemical agents can enhance expression of UCP-2 via the PPAR α pathway and changes in constitutive expression of UCP-2 in select brain areas may explain their vulnerability to injury by mitochondrial active compounds, similar to that observed with cyanide (Li et al., 2006; Takeuchi et al., 2006).

UCP-2 is a target gene of PPAR α (Nakatani et al., 2002) and UCP-2 expression can be up-regulated in N27 cells and primary neurons by Wy14,643, a high affinity, selective PPAR α agonist (Li et al., 2006; Zhang et al., 2007b). Pharmacologic up-regulation of UCP-2 was dependent on PPAR α activation. However, it should be pointed out that Wy14,643 can produce actions independent of PPAR α , including low level generation of ROS, GSH depletion and a reduction of Bcl-2 (Strakova et al., 2005; Martinasso et al., 2007). In turn, it can not be ruled out that in the Wy14,643 treatment groups these actions contributed to cell death. Nevertheless, the knockdown studies provided strong evidence that UCP-2 up-regulation was the primary pathway that enhanced cyanide toxicity. It is interesting to note that PPARs agonists have been employed in clinical trials of several neurodegenerative conditions, including Parkinson's disease, amyotrophic lateral sclerosis and Alzheimer's disease (Heneka and Landreth, 2007). PPAR γ agonists show promise in suppressing the CNS inflammation associated with these neurodegenerative conditions, whereas PPAR α agonists have produced more variable responses associated with inhibition of microglial proinflammatory responses (Heneka and Landreth, 2007; Ramanan et al., 2008). In light of safety concerns involving use of PPAR agonists, it is important to note that the present study shows that activation of PPAR α can lead to enhanced toxicity of a potent mitochondrial toxicant (Rubenstruck et al., 2007).

In summary, it is concluded that Bcl-2 down-regulation is involved in UCP-2 enhancement of cyanide toxicity. UCP-2 up-regulation produces depletion of mtGSH and accumulation of H₂O₂, thus stimulating proteasomal degradation of Bcl-2. Reduced cellular levels of Bcl-2 then enhance the cytotoxicity produced by cyanide.

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Fig. 1. Effect of UCP-2 up-regulation on cyanide-induced cell death. Cells were treated with Wy14,643 (100 μ M) for 5 h to up-regulate UCP-2, followed by incubation with KCN (400 μ M) for 24 h. Cell death was determined with Sytox green. **(A)** Representative photomicrographs of Sytox green stained cells: a. Control; b. KCN; c. Wy14,643; d. KCN + Wy14,643. Scale bar = 100 μ m and magnification was 200 $\times$. **(B)** Level of cell death, as reflected by Sytox green fluorescence intensity, was expressed as percent increase of control cell fluorescence. *Significantly different from control group; #Significantly different from Wy14,643 group, $P < 0.05$.

Fig. 2. Effect of UCP-2 regulation on Bcl-2 expression. **(A)** Cells were treated with Wy14,643 (25–200 μM) for 12 h, and then UCP-2 and Bcl-2 expression determined by Western blot analysis. **(B)** Time course of UCP-2 and Bcl-2 expression after treatment with Wy14,643 (100 μM) for 6–18 h. β-Actin was used as an equal-loading control. Results are representative of three separate experiments. Densitometric analysis shows relative Bcl-2 expression levels (mean ± SEM). *Significantly different from control group; #significantly different from 12 h treatment group, $P < 0.05$.

Fig. 3.

Effect of cyanide on Bcl-2 expression after UCP-2 up-regulation or over-expression. **(A)** UCP-2 was up-regulated by Wy14,643 (100 μ M) treatment for 5 h, followed by KCN (400 μ M) for 12 h. Cellular Bcl-2 expression was determined by Western blotting. Control cells were incubated with 0.1% (v/v) DMSO. **(B)** Cells were transiently transfected with UCP-2⁺ expression plasmid for 24 h, followed by KCN for 12 h. Bcl-2 expression was determined by Western blotting and β -actin was used as an equal-loading control. Control cells were treated with Lipofectamine 2000 and empty vector. Densitometric analysis shows relative Bcl-2 expression levels (mean $\pm$ SEM). *Significantly different from control group; #Significantly different from same treatment without KCN, $P < 0.05$.

Fig. 4.

Effect of cyanide on Bcl-2 mRNA levels and proteasomal metabolism following UCP-2 up-regulation. **(A)** Bcl-2 mRNA expression was determined by real-time PCR analysis. **(B)** Western analysis of whole cell ubiquitinated proteins was determined by blotting with anti-ubiquitin antibody. Cells were pretreated with lactacystin (5 μ M) for 1 h, followed by Wy14,643 and then by KCN for 12 h. Lactacystin increased accumulation of ubiquitinated proteins as indicated by increased staining of protein smears as compared to control group. **(C)** Expression of Bcl-2 following lactacystin inhibition of proteasomal metabolism. β -Actin was used as an equal-loading control. Densitometric analysis shows relative Bcl-2 expression levels (mean $\pm$ SEM). Control cells were incubated with 0.1% (v/v) DMSO. *Significantly

different from control group; [#]Significantly different from Wy14,643 group; [&]Significantly different from same treatment without lactacystin, $P < 0.05$.

Fig. 5. Cyanide-mediated changes in intracellular hydrogen peroxide generation and Bcl-2 expression. **(A)** Cellular levels of H₂O₂ after treatment with Wy14,643 (100 μ M) for 5 h, followed by KCN (400 μ M) for 12 h. H₂O₂ was detected by the Amplex red method and each value is expressed as percent control. **(B)** Effect of H₂O₂ scavenging by catalase on Bcl-2 down-regulation. Cells were pretreated with catalase (200 U/ml) for 1 h, followed by Wy14,643 and then KCN. Bcl-2 expression was determined by Western blotting and densitometric analysis shows relative protein levels (mean $\pm$ SEM). β -actin was used as an equal-loading control. Control cells were incubated with 0.1% (v/v) DMSO. Each value is the mean of three experiments $\pm$ SEM. *Significantly different from control group; #Significantly different from the Wy14,643 group; &Significantly different from same treatment without catalase, $P < 0.05$.

Fig. 6.

Effect of mtGSH repletion on H₂O₂ accumulation and Bcl-2 down-regulation. Cells were pre-loaded with GSH-EE (2 mM) for 1 h and then treated with Wy14,643 (100 μ M) for 5 h followed by KCN (400 μ M) for 12 h. **(A)** Cytosolic and mitochondrial fractions were isolated and glutathione levels determined. **(B)** Effect of GSH-EE on H₂O₂ generation. H₂O₂ was detected by the Amplex red method and is expressed as percent control. **(C)** Effect of GSH-EE on Bcl-2 expression. Bcl-2 expression was determined by Western blotting and β -actin was used as an equal-loading control. Densitometric analysis shows relative Bcl-2 expression levels (mean $\pm$ SEM). Control cells were incubated with 0.1% (v/v) DMSO. Each value is the mean of three

experiments $\pm$ SEM. *Significantly different from control group; #Significantly different from the Wy14,643 group; &Significantly different from same treatment without GSH-EE, $P < 0.05$.

Fig. 7.

Effect of UCP-2 knockdown on mtGSH levels and H₂O₂ generation. Cells were transfected with UCP-2 RNAi and 24 h later treated with Wy14,643 (100 μ M) for 5 h, followed by KCN (400 μ M) for 12 h. **(A)** Effect of UCP-2 RNAi on mtGSH levels. Control cells were treated with Silencer Negative Control siRNA (open bar) or with UCP-2 siRNA (shaded bar). **(B)** Effect of UCP-2 RNAi on H₂O₂ generation. H₂O₂ generation is expressed as percent control. Control wildtype N27 cells were transfected with Silencer Negative Control siRNA. Each value is the mean of three experiments $\pm$ SEM. *Significantly different from control group; #Significantly different from the Wy14,643 group; &Significantly different from treatment without UCP-2 RNAi, $P < 0.05$.

Fig. 8.

Effect of UCP-2 knockdown on Bcl-2 expression and cyanide toxicity. Cells were transfected with UCP-2 RNAi and 24 h later treated with Wy14,643 (100 μ M) for 5 h, followed by KCN (400 μ M) for 12 h. **(A)** Bcl-2 levels as detected by Western blotting. β -Actin was used as an equal-loading control. Densitometric analysis shows relative Bcl-2 expression levels (mean $\pm$ SEM). Control wildtype N27 cells did not receive any treatment. **(B)** Cyanide-induced cell death was determined by Sytox green fluorescence intensity. Control cells were treated with Silencer Negative Control siRNA (open bar) or with UCP-2 siRNA (shaded bar). *Significantly different from control group; #Significantly different from the Wy14,643 group; &Significantly different from treatment without UCP-2 RNAi, $P < 0.05$.

Fig. 9.

Effect of Bcl-2 over-expression on cyanide toxicity following UCP-2 up-regulation. 24 h after transfection with a Bcl-2⁺ expression plasmid, cells were treated with Wy14,643 (100 μ M) for 5 h, followed by KCN (400 μ M) for 24 h. (A) Cells were transfected with Bcl-2⁺ plasmid for 24 h and then Bcl-2 expression determined by Western blot analysis. (B) Representative photomicrographs of Sytox green stained cells. Magnification was 200 $\times$ and scale bar = 100 μ m a. Control; b. KCN; c. Wy14,643; d. KCN + Wy14,643; e. Bcl-2⁺ alone; f. Bcl-2⁺ + KCN; g. Bcl-2⁺ + Wy14,643; h. Bcl-2⁺ + KCN + Wy14,643. (C) Level of Cell death was reflected by Sytox green fluorescence intensity. Cell death is expressed as percent increase of control cell fluorescence. Control cells were treated with Lipofectamine 2000 without plasmid. Each

value is the mean of three experiments $\pm$ SEM. *Significantly different from control; #Significantly different from Wy14,643 group; &Significantly different from the same treatment without Bcl-2⁺, $P < 0.05$.