Interaction of Cyanide and Nitric Oxide with Cytochrome c Oxidase: Implications for Acute Cyanide Toxicity

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Acute cyanide toxicity is attributed to inhibition of cytochrome c oxidase (CcOX), the oxygen-reducing component of mitochondrial electron transport; however, the mitochondrial action of cyanide is complex and not completely understood. State-3 oxygen consumption and CcOX activity were studied in rat N27 mesencephalic cells to examine the functional interaction of cyanide and nitric oxide (NO). KCN produced a concentration-dependent inhibition of cellular respiration. Cyanide's median inhibitory concentration (IC50) of oxygen consumption (13.2 ± 1.8μM) was higher than the CcOX IC50 (7.2 ± 0.1μM). Based on respiratory threshold analysis, 60% inhibition of CcOX was necessary before oxygen consumption was decreased. Addition of high levels of exogenous NO (100μM S-nitroso-N-acetyl-DL-penicillamine) attenuated cyanide inhibition of both respiration and CcOX. On the other hand, when endogenous NO generation (penicillamine) attenuated cyanide inhibition of both respiration and CcOX activity were studied in rat N27 mesencephalic cells to examine the functional interaction of cyanide and nitric oxide (NO). KCN produced a concentration-dependent inhibition of cellular respiration. Cyanide's median inhibitory concentration (IC50) of oxygen consumption (13.2 ± 1.8μM) was higher than the CcOX IC50 (7.2 ± 0.1μM). Based on respiratory threshold analysis, 60% inhibition of CcOX was necessary before oxygen consumption was decreased. Addition of high levels of exogenous NO (100μM S-nitroso-N-acetyl-DL-penicillamine) attenuated cyanide inhibition of both respiration and CcOX. On the other hand, when endogenous NO generation (penicillamine) attenuated cyanide inhibition of both respiration and CcOX (Way, 1984). As a result of mitochondrial electron transport inhibition by cyanide, excess reactive oxygen species (ROS) are generated at complexes I and III, producing intense oxidative stress that contributes to cellular dysfunction (Chen et al., 2003; Gunasekar et al., 1998; Jones et al., 2000).

Cyanide binding to CcOX is complex and dependent on the enzyme’s redox state (Nicholls and Soulimane, 2004). Under normal cellular conditions, CcOX reduces O2 to H2O at the enzyme’s heme a3-CuB binuclear center. This reaction produces a proton gradient across the mitochondrial inner membrane to generate the mitochondrial membrane potential (∆Ψm) that drives ATP synthesis at complex V. The binuclear center can exist in reduced, oxidized, or partially reduced states (Nicholls and Soulimane, 2004). When the binuclear center is fully reduced with iron in the ferrous (Fe2+) state and copper as cuprous (Cu+), oxygen binds (KD = 1μM) and then complex IV transfers electrons from cytochrome c to molecular oxygen. Although oxygen only binds to the fully reduced binuclear center, cyanide can interact with all three states. Cyanide binds tightly to the fully oxidized state (Fe3+/Cu2+) (KD = 10M−1 s−1, KD = 1μM), whereas its binding to the fully reduced binuclear center is weaker (KD = 1.3 × 105M−1 s−1, KD = 500μM). Cyanide has the highest affinity (KD = 2 × 106M−1 s−1, KD = 20nM) for the partially reduced state (Fe2+/Cu2+) (Antonini et al., 1971; Jones et al., 1984).

Nitric oxide (NO) also binds to the CcOX binuclear center, and under physiological conditions, NO is thought to be an endogenous modulator of oxidative phosphorylation (Cooper and Giulivi, 2007). In mitochondria, NO is produced by a constitutively expressed mitochondrial nitric oxide synthase (mtNOS) (Ghafoorifar and Richter, 1997). Mitochondrial generated NO binds to the reduced heme a3 (KD = 1 × 108M−1 s−1) to inhibit CcOX (Stubauer et al., 1998). Inhibition is competitive with O2 and increases with decreasing oxygen tensions (Mason et al., 2006). Although NO binds to heme a3 (Fe2+) with high affinity (KD = 0.1nM), it can also bind to oxidized CuB (KD = 28nM), where it is metabolized to nitrite. Reversal of NO-Fe3+ binding is thought to be linked to metabolism of NO to nitrite (NO2−) on Cu2+ (Giuffre et al., 2000; Sarti et al., 2003).

Cyanide is a potent toxicant that produces a rapid onset, histotoxic anoxia by inhibiting mitochondrial oxidative phosphorylation (Way, 1984). Cyanide binds to the cytochrome c oxidase (CcOX) heme a3-CuB binuclear center to inhibit both cellular oxygen utilization and ATP production (Way, 1984). Due to CcOX inhibition, cyanide initiates a catastrophic cascade of reactions leading to neurological and myocardial dysfunction and death if the inhibition is not reversed immediately (Way, 1984). As a result of mitochondrial electron transport inhibition by cyanide, excess reactive oxygen species (ROS) are generated at complexes I and III, producing intense oxidative stress that contributes to cellular dysfunction (Chen et al., 2003; Gunasekar et al., 1998; Jones et al., 2000).

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NO and cyanide appear to compete for binding at the CcOX binuclear center since excess cyanide antagonizes NO-heme a3 binding. Stubauer et al. (1998) found that in the presence of cyanide, NO (μM) binding was 1000 times slower (seconds vs. milliseconds) than without cyanide. Furthermore, the dissociation rate for NO (K_app = 0.3μM NO per minute) was 10 times slower in the presence of cyanide (K_app = 3.2μM NO per minute). Interestingly, high NO (micromolar) concentrations effectively antagonize cyanide by displacing cyanide from the binding site. Pearce et al. (2003) showed that when cyanide and NO (in 10-fold excess of cyanide) are both present, they do not inhibit CcOX additively; instead, NO decreases cyanide-mediated inhibition. It was proposed that when both molecules are present, cyanide is displaced from the binuclear center by NO and NO is converted to nitrite (NO2−). After NO dissociates from CcOX binding or is converted to nitrite (NO2−), cyanide can then bind the heme a3 to inhibit CcOX. It was concluded that the cyanide antitodal activity of nitrites may be explained in part by generation of NO from nitrite anion to interact with cyanide binding at the CcOX binuclear center.

In the current study, cyanide-mediated inhibition of cellular aerobic respiration and CcOX were examined in the presence or absence of NO in rat N27 mesencephalic cells. The objective was to study the effect of increasing or decreasing intracellular NO on cyanide-mediated inhibition of CcOX and oxygen utilization at toxicologically relevant concentrations. It was concluded that the interaction between NO and cyanide influences both cyanide-mediated toxicity and reversal of CcOX inhibition.

**MATERIALS AND METHODS**

**Materials.** RPMI 1640, penicillin/streptomycin, and fetal bovine serum were purchased from Gibco (Grand Island, NY). Digitonin, succinate, 2,5'-adenosine diphosphate (ADP), S-nitroso-N-acetyl-ε-penicillamine (SNAP), Nω-monomethyl-l-arginine ester (l-NAME), potassium ferricyanide (K3Fe(CN)6), bovine heart cytochrome c, dithiothreitol (DTT), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), and superoxide dismutase (SOD) were purchased from Sigma Chemical Company (St Louis, MO). 4-Amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) and MitoTracker Red chloromethyl-X-rosamine (CMX Ros) were obtained from Invitrogen (Eugene, OR). (±/-)-(E)-Methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide (NOR-1) was purchased from EMD Chemicals, Inc. (La Jolla, CA).

**N27 cell cultures.** 1RBAN27 (N27) cells were provided by Dr. A.G. Kanthasamy (Iowa State University, Ames, IA). All experiments were performed using the immortalized, mesencephalic cell line N27 derived from rat brain. This 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) and has been used as a cell model of cyanide-induced cytotoxicity (Zhang et al., 2007). Cells were cultured on 100 × 20-mm plates at an initial density of 5 × 104 cells/ml. Over a 3- to 4-day period, cells were grown to confluency (1 × 106 cells/ml) in RPMI media (pH 7.2) containing 2 g/l NaHCO3, 10% fetal bovine serum, and penicillin (100 U/ml)/streptomycin (100 μg/ml) at 37°C in a 5% CO2, 95% air environment. Cells were passaged at least two times before use.

**Cellular oxygen consumption.** Oxygen consumption was measured polarographically (Kim-Han et al., 2001; Mattiazzi et al., 2004). Confluent N27 cells were rinsed with phosphate-buffered saline (PBS), harvested using trypsin-EDTA, and then resuspended in PBS prior to a 5-min centrifugation at 1000 rpm. The remaining pellet was resuspended in 600 μl oxygenated buffer (20mM HEPES, pH 7.1, 250mM sucrose, 10mM MgCl2, 2mM KH2PO4, 4mM rotenone, and 15mM succinate) containing 0.01% digitonin. A Clark-type oxygen electrode was used to measure cellular respiration polarographically (Digital Model 10 Controller, Rank Brothers, Ltd, Cambridge, UK). For each experiment, the solution of permeabilized cells was placed into the electrode’s reaction chamber. Oxygen consumption was monitored and recorded using the Pico Technology software program (Pico Technology, Ltd, Cambridge, UK). To initiate state-3 respiration, 500μM ADP was added to the cell suspension. Resulting oxygen uptake (in the absence of KCN or any additional compounds) was referred to as control respiration. When the voltage generated by the chamber’s oxygen (VO2) reached 0.65–0.70 V (~70% atmospheric oxygen remaining, 30% consumed by cells), KCN was added to inhibit state-3 respiration. For coaddition of cyanide and NO, cells were briefly (2–3 min) incubated with an NO donor (SNAP) prior to initiating state-3 respiration and adding KCN. Finally, for NOS inhibition, cells were pretreated with l-NAME (100μM) for 1 h at 37°C, 5% CO2, and 95% air prior to experimentation.

To determine oxygen consumption, slopes of the oxygen utilization plots were calculated. The slope, or state-3 respiration rate resulting from a given treatment, was expressed as change in voltage due to O2 (ΔVO2)/change in time (Δt min), or ΔVO2/Δt min. The voltage and time values were obtained directly from the automated Pico Technology data chart.

**Quantitation of mitochondrial NO: fluorescence microscopy and laser scanning cytometry.** N27 cells were prepared according to a protocol by Dedkova et al. (2004). Cells were grown overnight on 24- or 96-well (black, clear-bottom) plates to obtain cultures that were ~60% confluent. Upon experimentation, the cell media was replaced with Krebs-Ringer bicarbonate solution (12mM KH2PO4, 1mM CaCl2, 125mM NaCl, 5mM KCl, 25mM HEPES, 6mM glucose, 5mM NaHCO3, 1.2mM MgSO4, pH 7.4) containing 7μM DAF-FM DA. After 30 min DAF-FM DA treatment, 200μM MitoTracker Red CMX Ros was added and cells were incubated for an additional 10 min. Cells then were rinsed with Krebs-Ringer bicarbonate buffer for 15 min at 37°C, prior to addition of KCN (5–60μM), l-NAME (100μM), or SNAP (100μM) for 15 min. For in situ visualization of mitochondrial NO, N27 cells (grown on 24-well plates) were imaged with a Nikon Eclipse TE200 inverted fluorescence microscope (Nikon, Melville, NY) equipped with blue (420–495 nm) and green excitation (520–560 nm) filters for visualization of DAF-FM DA and MitoTracker Red CMX Ros, respectively. Images (~400) were captured using the SPOT Advanced software system (Diagnostic Instruments, Sterling Heights, MI), Confocal Assistant 4.02 (courtesy of Purdue University Cytometry Laboratories) was used to merge the images.

For quantitative analyses of mitochondrial NO levels, cells were imaged using the CompuCyte iCyts Research Imaging Cytometer (CompuCyte, Cambridge, MA), a laser scanning cytometer (LSC) attached to an inverted fluorescence microscope. Stained and treated cells (plated on black, clear-bottom, 96-well plates) were identified by mitochondrial fluorescence, using a 633-nm laser for MitoTracker Red excitation. Within the identified mitochondria, the NO levels were measured using a 488-nm laser for DAF-FM excitation. Using the iCyts Data Integration software (CompuCyte), NO intensities within individual sets of mitochondria (up to 50 per 5-s scan) were quantified in terms of integral fluorescence or the mean sum of fluorescing DAF-FM pixels per well.

**CeCoX activity.** After 30-min pretreatments with l-NAME, SNAP, NOR-1, KCN, DTT, PTIO, and/or SOD, confluent cultures (1 × 106 cells/ml) were rinsed and scraped from tissue culture plates prior to resuspending in PBS, harvesting, and centrifugation (as described above in respiratory studies). Cells were further resuspended in 300 μl Krebs-Ringer bicarbonate buffer. Homogenization was performed for 30 s using a motorized pestle at low speed. Total protein in the homogenate was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) based on the Bradford
method. Protein concentrations were normalized so that the final CcOX reaction mixture contained 120 µg/ml homogenate protein.

CcOX activity was determined by monitoring the decrease in reduced cytochrome c absorbance over time (Wharton and Tzagoloff, 1967). Cytochrome c was reduced according to the method of Isom and Way (1984). Briefly, 2% palladium asbestos was added to a 600nM solution of cytochrome c in 10mM KH2PO4 (pH 7.0). The solution was then bubbled with N2 for 5 min, H2 for 1 h, and N2 for 10 min prior to gravity filtration. The 600 µl reaction mixture was composed of 3mM KH2PO4, 100nM reduced cytochrome c, and 120 µg/ml cell homogenate protein in a plastic cuvette. Each reaction cuvette was placed in a 37°C water-jacketed spectrophotometer (Hitachi U-3000, Hitachi, Ltd, Tokyo, Japan) and absorbance of reduced cytochrome c at 550 nm monitored every 5 s for 1 min. To obtain a standard or blank, the cuvette was oxidized for 1 min using 3mM potassium ferricyanide (in 100mM KH2PO4, pH 7.0).

The rate of reduced cytochrome c oxidation (or CcOX activity) was determined by calculating the slope of the log (Absred.cyt c – Absox cyt. c) versus time (min). Rates were expressed as micromole of reduced cytochrome c oxidized per minute (Wharton and Tzagoloff, 1967).

CcOX kinetic analysis. Kinetics studies were performed using the same procedure as for the CcOX assays, except reduced cytochrome c concentrations were varied over the range of 100–300nM, and CcOX enzyme activity was determined in the presence of KCN and NO. Lineweaver-Burk plots were constructed by plotting the inverse of CcOX activity (micromole of reduced cytochrome c oxidized per minute) against inverse of reduced cytochrome c concentration (nanomolar). Vmax and Km values were determined using GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

Statistical analysis. Statistical analyses were performed using GraphPad Prism. Data were evaluated using a one-way ANOVA followed by a Tukey’s multiple comparisons test. Differences relative to control values were considered statistically significant when p < 0.05.

Respiratory threshold analysis was conducted as described by Mazat et al. (1997). The threshold was defined as the percent CcOX inhibition that decreased oxygen consumption below 100%. The respiratory threshold curve was established from the tabulated data of the best fit curve (r² = 0.99) of KCN inhibition of cellular oxygen consumption versus CcOX inhibition in the presence of KCN.

RESULTS

Cyanide Inhibition of Cellular Oxygen Consumption

It is well established that cyanide inhibits cellular oxygen consumption (Mazat et al., 1997). In N27 cells, KCN produced a concentration-dependent inhibition of state-3 respiration over the 2–60µM concentration range (Fig. 1A). The IC50 for inhibition of oxygen consumption was estimated at 13.2 ± 1.8µM KCN. No changes in cell count or morphology were observed after 1 h treatment with either 5µM or 60µM KCN concentrations, thus eliminating a reduction of viable cells as a cause of the lower oxygen consumption (Fig. 1B).

Interaction of Cyanide and NO with Cellular Oxygen Consumption

To examine the interaction between NO and cyanide, oxygen consumption was studied in the presence of exogenous NO generated from an NO donor. SNAP was selected as the NO donor since it produces a concentration-related generation of NO that inhibits cellular respiration (Xie et al., 1998). To confirm that SNAP’s action was due to NO generation, NO levels resulting from the 100µM SNAP treatment were estimated using the Griess nitrite assay (data not shown). Also, cells were loaded with the NO-sensitive fluorescence indicator, DAF-FM, followed by addition of SNAP to the cells. Intracellular NO levels increased rapidly over a 5-min period and peak levels (1–7µM NO) were observed within 10–15 min (data not shown). SNAP (100µM) decreased state-3 respiration by 36% (Fig. 2A). The NO donors 3-morpholinosydnonimine and sodium nitroprusside produced parallel responses (data not shown). Under these conditions, KCN (20µM) inhibited oxygen consumption by 56%. Addition of SNAP to KCN did not increase the inhibition, rather KCN (20µM) + SNAP (100µM) reduced state-3 respiration by 44% of control, which was not significantly different from KCN alone. Cotreatment with SNAP (NO) changed the shape of the cyanide inhibition curve (Fig. 2B). At KCN concentrations less than −10µM, treatment with exogenous NO produced an additive inhibition of cellular oxygen consumption, whereas at higher KCN concentrations (20–60µM), exogenous NO attenuated cyanide inhibition.

Cyanide-Mediated Respiratory Inhibition and NO Synthase

Our previous work has shown that cyanide elevates endogenous NO levels in a variety of primary and cultured cells (Gunasekar et al., 1996; Prabhakaran et al., 2006). Therefore, the effect of endogenously generated NO on cyanide respiratory inhibition was examined by inhibiting NOS activity with L-NMMA (100µM), a potent inhibitor of the
four NOS isoforms. L-NMMA alone elevated the level of oxygen consumption, reflecting removal of the inhibition produced by endogenous NO generated by mtNOS (Fig. 3A). When KCN (20 μM) was added to cells pretreated with L-NMMA, state-3 respiration was not significantly inhibited compared to control, whereas this concentration of KCN alone inhibited cellular respiration by 56%. L-NMMA shifted the cyanide concentration-response curve upward and to the right and the cyanide IC_{50} was increased nearly 4.5-fold, from 13.2 ± 1.8 to 59.6 ± 0.9 μM (Fig. 3B). Since L-NMMA is a selective NOS inhibitor that has no direct effect on cellular respiration, the change in sensitivity to cyanide was most likely due to depletion of endogenous NO in mitochondria. Similar results were observed with the pan-NOS inhibitor N^ω-monomethyl-l-arginine methyl ester (l-NAME) (data not shown).

**Effect of Cyanide on Mitochondrial NO Generation**

To determine if the respiratory effects of the treatments can be attributed to altered NO levels, mitochondrial NO levels were determined by fluorescence microscopy and LSC.

Subcellular NO distribution was visualized by fluorescence microscopy by use of the NO-sensitive fluorescence dye DAF-FM and mitochondria were visualized with MitoTracker Red (Fig. 4A). Under control conditions, both total cellular and mitochondrial NO levels fluctuated from cell to cell. Treatment of cells with SNAP (100 μM) or L-NMMA (100 μM) altered cellular NO levels (Fig. 4B). In SNAP-treated cells, the green DAF-FM fluorescence due to NO binding was prevalent throughout the cytosol, suggesting that SNAP increased total cellular NO levels. Mitochondria of SNAP-treated cells appeared as punctate regions of yellow fluorescence, showing that NO accumulated in mitochondria. On the other hand, L-NMMA decreased NO in both cytosol and mitochondria, as indicated by a sparse cytosolic green fluorescence and orange-red mitochondrial fluorescence. After cyanide treatment, mitochondria were bright yellow at 5 and 20 μM KCN, indicating markedly elevated levels of NO. Interestingly, 60 μM KCN decreased NO levels in both cytosol and mitochondria. LSC analysis showed that 5 μM KCN increased mitochondrial NO levels, which progressively decreased as the KCN concentration increased over the 20–60 μM range (Fig. 4C). Since Mitotracker Red is a potentiometric dye, it is possible that changes in ΔΨ_{m} could alter its mitochondrial localization. To eliminate this possibility, ΔΨ_{m} was monitored by...
the rhodamine-123 method following cyanide. Under these conditions, cyanide (5–60μM) did not increase Δψm (data not shown). These observations confirm that SNAP and l-NMMA alter cellular NO levels and that low KCN concentrations (<10μM) increase mitochondrial NO generation, whereas higher concentrations inhibit mtNOS activity to reduce cellular NO.

**Cyanide Inhibition of CcOX and Respiratory Threshold**

To correlate cyanide inhibition of cellular respiration with CcOX activity, cellular CcOX activity was analyzed in cell suspensions. Cyanide (5–100μM) inhibited CcOX activity in a concentration-dependent manner with an IC50 of 7.2 ± 0.1μM (Fig. 5A). A number of reports indicate that cellular CcOX levels are functionally in excess, and respiratory inhibition by cyanide may not be stoichiometrically coupled to CcOX inhibition (Davey et al., 1998; Pettersen and Cohen, 1993). A 60–70% inhibition of CcOX is necessary before a reduction of cellular respiration is observed (Davey et al., 1998; Mazat et al., 1997). Respiratory threshold analysis correlating cyanide-mediated inhibition of cellular oxygen consumption (data from Fig. 1A expressed as percent control) with CcOX activity showed that in N27 cells, ~60% inhibition of CcOX was necessary before a decrease of cellular respiration was observed (Fig. 5B).

**CcOX Inhibition in Presence of Exogenous NO**

To correlate NO-induced changes in cyanide respiratory inhibition with CcOX inhibition, enzymatic assays were performed in the presence and absence of exogenous NO. SNAP (100μM) alone decreased CcOX activity by 71%...
However, when cells were treated with KCN (20μM) in the presence of SNAP, CcOX activity was elevated above that observed with KCN or SNAP. NO attenuated cyanide-mediated inhibition of CcOX at KCN concentrations as high as 100μM (Fig. 6B). The inhibition of CcOX by KCN/SNAP cotreatment paralleled inhibition of cellular oxygen consumption (Fig. 2B) in which the effect of low concentrations of KCN (<10μM) was increased by NO, whereas at higher KCN concentrations (>10μM), the inhibitory effect of KCN inhibition was reduced. To confirm that the SNAP action was NO mediated, NOR-1 was used to generate high intracellular levels of NO. NOR-1 rapidly releases NO ($t_{1/2}=1.7$ min) and has no known actions other than NO generation. NOR-1 inhibited CcOX and antagonized KCN’s inhibition, similar to that observed with SNAP (Fig. 6C).

Since SNAP is known to mediate S-nitrosylation of respiratory proteins (Dahm et al., 2006), the involvement of S-nitrosylation in the SNAP-mediated antagonism of cyanide was examined. To inhibit S-nitrosylation of cysteine residues, cells were pretreated with the thiol-reducing agent DTT as described by Arnelle and Stamler (1995). In the presence of DDT (100μM), the effect on SNAP’s interaction with cyanide was slightly reduced (Fig. 6D). On the other hand, PTIO (200μM), an NO scavenger, blocked the SNAP action. To determine the involvement of peroxynitrite in the SNAP-mediated actions, cells were pretreated with SOD (100 U/ml) that blocks the generation of peroxynitrite by scavenging superoxide. In the presence of SOD, the interaction of SNAP with cyanide was not altered. It is concluded that the interaction of SNAP with cyanide-mediated inhibition of CcOX is attributed to NO generation, and S-nitrosylation and peroxynitrite play minimal roles in the response.

**CcOX Kinetic Analysis of the Cyanide and NO Interaction**

In a previous analysis of NO and cyanide interaction using purified CcOX and excess reduced cytochrome c (substrate), NO altered cyanide inhibition of CcOX by increasing the $V_{\text{max}}$ for cyanide inhibition (Pearce et al., 2003). Based on this observation, CcOX kinetics was evaluated using nanomolar excess of reduced cytochrome c. KCN (20μM) decreased the CcOX $V_{\text{max}}$ to below control levels (homogenates treated with reduced cytochrome c alone; 0.80 ± 0.7 μmol/min for control vs. 0.43 ± 0.02 μmol/min for KCN) (Fig. 7A and Table 1). The inhibition was noncompetitive with respect to reduced cytochrome c since the $K_m$ (202.5nM for control vs. 200.6nM for KCN) was unaltered. When KCN was combined with SNAP, the $V_{\text{max}}$ increased above control ($V_{\text{max}} = 1.41 ± 0.1 \mu\text{mol/min for SNAP + KCN vs. } 0.80 ± 0.7 \mu\text{mol/min for control}$) (Fig. 7B). However, the $K_m$ (192.2nM for SNAP + KCN vs. 202.5nM for control) did not change. It was concluded that SNAP alone inhibited CcOX, whereas addition of SNAP to KCN-treated CcOX produced an antagonism of the KCN-mediated inhibition.
Inhibition of CcOX in the KCN

Inhibition of CcOX in the presence of exogenous NO. N27 cells were pretreated with KCN (20 μM) and/or SNAP (100 μM) for 30 min at 37°C prior to analysis of CcOX activity. The rates of CcOX activity for cell homogenates were determined using nanomolar concentrations of reduced cytochrome c. (A) CcOX activity of cells treated with KCN or SNAP alone. (B) CcOX activity of cells treated with KCN + SNAP. The data are displayed as Lineweaver-Burk plots and are the means ± standard errors for three or more experiments.

Kinetic Analysis of KCN Inhibition in Absence of NO

To determine whether endogenously generated NO directly influences the cyanide-mediated inhibition of CcOX, cells were pretreated with L-NMMA (100 μM), followed by KCN (20 μM). L-NMMA pretreatment used to block generation of endogenous NO attenuated KCN-mediated CcOX inhibition (Fig. 8A). Inhibition of CcOX in the KCN + L-NMMA group was not significantly different from control. In the presence of L-NMMA, KCN’s inhibition of CcOX was 14 times less potent than in cells treated with KCN alone (IC50 = 102 ± 10 μM vs. 7.2 ± 0.1 μM) (Fig. 8B). The effect of L-NMMA on CcOX was similar to that observed on cellular oxygen consumption with a shift of the response curve to the right (Fig. 4B).

L-NMMA stimulated CcOX activity at KCN concentrations below 100 μM, possibly due to removal of endogenous NO-mediated modulation of cellular respiration. Kinetic analysis of cyanide inhibition of CcOX in the presence of L-NMMA is shown in Figure 9. L-NMMA reversed KCN-mediated inhibition of CcOX, increasing the Vmax above control (Vmax = 1.41 ± 0.5 μmol/min for KCN + L-NMMA vs. 0.80 ± 0.7 μmol/min for control) (Table 1). Interestingly, L-NMMA alone decreased the Km for reduced cytochrome c (202.5 nM for control vs. 123 nM for L-NMMA), thus showing the effect of endogenously generated NO on control CcOX activity. The respiratory threshold of CcOX, as determined by cyanide inhibition in the presence of L-NMMA, was calculated at ~60% (data not shown), the same as that observed in the absence of L-NMMA (Fig. 5B).

It was concluded that the respiratory threshold is due to excess CcOX and not modification of respiration by endogenous NO.

Table 1

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<thead>
<tr>
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<th>Vmax (μmol reduced cytochrome c oxidized/min)</th>
<th>Km (μM reduced cytochrome c)</th>
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<tr>
<td>Control</td>
<td>0.80 ± 0.7</td>
<td>202.5</td>
</tr>
<tr>
<td>KCN</td>
<td>0.43 ± 0.02</td>
<td>200.6</td>
</tr>
<tr>
<td>SNAP</td>
<td>0.61 ± 0.16</td>
<td>192.2</td>
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<tr>
<td>SNAP + KCN</td>
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<tr>
<td>L-NMMA</td>
<td>0.88 ± 0.61</td>
<td>123.3</td>
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<tr>
<td>L-NMMA + KCN</td>
<td>1.41 ± 0.49</td>
<td>201.3</td>
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Note. Each value represents the mean ± standard error of three or more experiments.

Discussion

Both cyanide and NO are classic inhibitors of CcOX that interact with the enzyme’s catalytic binuclear center to inhibit oxygen binding, thus producing an altered Δψm and reduced oxidative phosphorylation (Way, 1984). The complex interaction of cyanide and NO observed in this study is due to differences in their mode of inhibition of CcOX. Modification of cyanide’s CcOX inhibition and cellular oxygen consumption by NO has important toxicological implications. Cyanide non-competitively inhibited CcOX with respect to cytochrome-c-mediated reduction of the enzyme. Micromolar levels of exogenous NO also markedly inhibited CcOX in N27 cells. However, exogenous NO (100 μM SNAP) reversed cyanide inhibition of CcOX, as reflected by an increased Vmax of cyanide-treated CcOX above control. These results are similar to those observed by Pearce et al. (2003) using purified CcOX in which NO + cyanide did not produce an additive inhibition of CcOX.

Cyanide is a complex toxicant that produces multiple actions in intact biological systems, including inhibition of oxidative metabolism (CcOX inhibition), inhibition of the antioxidant defense, and alteration of critical cellular ion homeostasis (Gunasekar et al., 1996). The objective of the present study was to extend the observations of Pearce et al. (2003) that were conducted with purified enzyme at millimolar concentrations of cyanide to a toxicological cell model. The response to cyanide in a cell model is the sum of all actions, but it is recognized that the primary toxicological action is related to inhibition of CcOX. With a cell model it is difficult to make accurate inferences about the molecular interaction of cyanide with the CcOX binuclear
center. However, the enzyme kinetics of cyanide binding and inhibition of CcOX in purified enzyme systems have been extensively reported and can be used as a basis to explain the compound’s toxicity in cells from the target organs.

In the present study, it was observed that low levels of endogenously generated NO (nanomolar range) appeared to enhance the cyanide inhibition as shown in the studies with the NOS inhibitor L-NMMA. Treatment with L-NMMA alone increased oxygen consumption, indicating that endogenous NO (nanomolar range) generation normally limits oxidative metabolism. Also when 20µM cyanide was added in the presence of L-NMMA (blocked NO generation), the oxygen consumption returned to control levels and the concentration-response relationship of cyanide inhibition was shifted to the right. It was concluded that low levels of endogenous NO enhanced cyanide inhibition of respiration, as shown by L-NMMA reversal of the inhibition. Low physiological levels (nanomolar range) of NO enhance cyanide inhibition of CcOX, whereas high levels of exogenous NO (micromolar range) antagonize the inhibition.

Role of Endogenously Generated NO and mtNOS

Under normal physiological conditions, endogenous NO can produce up to ~50% inhibition of cellular respiration by binding competitively (with respect to oxygen) to Fe$_{a3}^{2+}$ and noncompetitively to Cu$_{b}^{2+}$ (Mason et al., 2006). The non-competitive inhibition at Cu$_{b}^{2+}$ occurs under low electron flux, slow CcOX turnover, and low oxygen concentrations (< 30µM) (Cooper, 2002). It has been proposed that the function of endogenously generated NO is to regulate mitochondrial oxygen consumption and ∆ψm through reversible inhibition of CcOX (Ghafourifar and Cadenas, 2005). Physiological NO directly inhibits CcOX to reduce oxygen consumption and indirectly activates mitochondrial signaling by altering the mitochondrial redox state and ROS production (Cooper and Giulivi, 2007). As observed in this study, physiological levels of NO appear to enhance mitochondrial dysfunction produced by mitochondrial toxicants such as cyanide.

Increased generation of mitochondrial NO observed following low cyanide exposure (5µM) is likely due to cyanide-mediated activation of mtNOS. Several studies have shown that cyanide can stimulate NOS to increase synthesis of NO. Gunasekar et al. (1996) showed that cyanide stimulates NOS and increases ROS production by 50% in cerebellar granule cells. Cyanide also increases levels of the NO metabolite, nitrite, in mesencephalic cells (Prabhakaran et al., 2006). Moreover, cyanide upregulates inducible NOS (iNOS) expression in a time- and concentration-dependent manner. It is likely
that NOS, and more specifically mtNOS, participates in cyanide inhibition of mitochondrial function by facilitating CcOX inhibition.

Cyanide most likely activates mtNOS by increasing mitochondrial Ca\textsuperscript{2+} influx. mtNOS is a constitutive NOS activated by the Ca\textsuperscript{2+}-calmodulin complex to convert L-arg to NO (Mason et al., 2005). Cyanide elevates free cytosolic Ca\textsuperscript{2+} by three independent processes: (1) through IP\textsubscript{3}-gated channels, (2) by increasing NMDA receptor activity, and (3) by release from intracellular Ca\textsuperscript{2+} stores (Mills et al., 1999). It is likely that the rapid elevation of free cytosolic Ca\textsuperscript{2+} enhances mitochondrial Ca\textsuperscript{2+} influx, followed by activation of mtNOS to generate NO, which then interacts with CcOX. On the other hand, sustained elevation of mitochondrial Ca\textsuperscript{2+} can eventually downregulate mtNOS activity, which would decrease mitochondrial NO levels, as observed at cyanide concentrations greater than 20\textmu M.

Interaction of Cyanide and NO with CcOX

To explain the observed CcOX kinetics and respiratory interaction between cyanide and NO, a simplified model of competitive inhibition is inadequate (Antonini et al., 1971; Sarti et al., 2003). Binding of cyanide and NO to the CcOX binuclear center is complex and dependent on enzyme catalytic turnover, flux rate, and binuclear center redox status. At the enzyme active site, cyanide and NO have different chemistry and different on and off rates that need to be considered in explaining their interaction (Hill et al., 1983). NO and cyanide bind to both the reduced heme a\textsubscript{3} (Fe\textsuperscript{2+}) or oxidized Cu\textsubscript{B}\textsuperscript{2+}, and in turn, the redox changes of the binuclear center during the catalytic cycle influence binding and the pattern of inhibition (Sarti et al., 2003; van Buuren et al., 1972). NO inhibits CcOX by a combination of competitive binding with oxygen at heme a\textsubscript{3} (Fe\textsuperscript{2+}) and noncompetitive binding with oxygen at cupric Cu\textsubscript{B}\textsuperscript{2+} (Mason et al., 2006). NO binds rapidly to the cupric (Cu\textsuperscript{2+}) site to reduce it to the cuprous state (Cu\textsuperscript{+}) and produces nitrite (NO\textsubscript{2} -), which is subsequently released from the binuclear center. At low endogenous levels, NO binds predominately to oxidized Cu\textsubscript{B}\textsuperscript{2+} to produce an additive inhibition with cyanide that binds preferentially to reduced heme a\textsubscript{3}, as observed in this study. At high NO concentrations (exogenous micromolar levels), the reduced CcOX state begins to accumulate (Mason et al., 2006). As the fraction of CcOX in the reduced state increases, NO at micromolar concentrations competes with cyanide for binding to the reduced binuclear binding site to partly antagonize cyanide inhibition. This is consistent with observations of Pearce et al. (2003), who proposed that NO displaces cyanide from reduced heme a\textsubscript{3} (Fe\textsuperscript{2+}) and then NO dissociates from the heme a\textsubscript{3} to bind Cu\textsubscript{B}\textsuperscript{2+} and then is oxidized to NO\textsubscript{2} -.

It is also important to consider that inhibition by cyanide and NO are in competition with oxygen at the reduced heme a\textsubscript{3} site. In low oxygen tension present in brain mitochondria (< 15\textmu M), inhibition by cyanide and NO would be enhanced. This partly explains why cyanide is such a rapid acting and potent intoxicant. Since this study was conducted at atmospheric oxygen levels (~210\textmu M), the effect of cyanide and NO on cell respiration and CcOX is most likely underestimated as compared to their actions and interaction in vivo (Cooper, 2002).

Role of Excess CcOX

Respiratory threshold analysis showed that 60% of the cellular CcOX has to be inhibited before decreased mitochondrial respiration becomes apparent. Interestingly, a respiratory threshold analysis of cells treated with cyanide in the presence of L-NMMA produced the same threshold value, 60%, showing that the CcOX respiratory threshold is not the result of endogenous NO binding, but rather an excess quantity of CcOX. This observation is further supported by Pettersen and Cohen (1993), who found in murine brain mitochondria after administration of lethal doses of KCN, that only 50% of COX activity was inhibited. Other reports suggest that excess CcOX prevents severe respiratory inhibition, as in the case of cyanide toxicity (Davey et al., 1998).

It is interesting to note that cyanide is not cytotoxic to N27 cells at concentrations as high as 400\textmu M (Zhang et al., 2007), whereas primary cultured neurons undergo significant cell death at this concentration (Prabhakaran et al., 2006). The IC\textsubscript{50} for inhibition of oxygen consumption in N27 cells was ~13\textmu M as compared to < 1\textmu M for primary neurons (Davey et al., 1998), and the respiratory threshold for both cell types is ~60%. It is concluded that N27 cells are more resistant to cyanide than primary neurons and that this may be explained by a higher level of anaerobic metabolism (glycolysis) and less dependence on mitochondrial aerobic respiration in the immortalized cells (N27). However, it appears that the resistance of N27 to cyanide may be attributed in part to mitochondrial regulation since overexpression of uncoupling protein-2 in N27 cells leads to an enhanced cyanide cytotoxicity, similar to that observed in primary cells (Zhang et al., 2007).

Toxicological Implications

Many neurological disorders, including multiple sclerosis and Parkinson’s disease, are characterized by substantial nitration of complexes I and IV. In these diseases, complexes I and IV are the primary sites of nitration, resulting in inhibition of oxidative phosphorylation (Qi et al., 2006). Interestingly, acute cyanide toxicity has been linked to a delayed neuropathy resembling Parkinson’s disease (Rosenberg et al., 1989). The resulting pathology may be due in part to cyanide-mediated nitration of complexes I and IV through upregulation of mtNOS. In a study by Broderick et al. (2006), the NO and cyanide scavenger cobinamide was found to rescue cellular respiration and growth after cyanide treatment in animal cell models, providing further support that acute cyanide toxicity is enhanced by mtNOS generation of NO. It should be noted that NO generation undergoes a greater level of regulation in
humans as compared to rodents (Kroncke et al., 1998). Thus, caution should be taken when extrapolating NO-mediated pathology from rodent models to humans.

Implications for Antidotal Treatment of Acute Cyanide Intoxication

Current results provide valuable insight into cyanide-mediated inhibition of CcOX and explains the cyanide antidotal action of nitrite. Based on the present study, it appears that the anti-cyanide action of nitrite is due only in part to its ability to generate blood methemoglobin, a cyanide scavenger (Way, 1984). Pearce et al. (2003) proposed that nitrite (NO$_2^-$) provides an exogenous source of NO, which would displace cyanide from CcOX to subsequently undergo metabolism by rhodanese or scavenging by methemoglobin. We have provided in vivo evidence that the cyanide antidotal activity of nitrite may be related in part to NO generation (Sun et al., 1995). The antagonism is attributed to nitrite-mediated oxidation of hemoglobin (Fe$^{2+}$(a$_3$)) to methemoglobin (Fe$^{3+}$(a$_3$)), a cyanide scavenger (Bhattacharya, 2000). However, NO generated from nitrite may play a role in the antagonism of cyanide. Isosorbide dinitrate (ISDN), an NO generator, produced only a slight increase in methemoglobin levels, but more than doubled cyanide’s LD$_{50}$ in mice (Sun et al., 1995). The antagonism exceeded the cyanide scavenging capacity of the low methemoglobin levels. Furthermore, pretreatment with the methemoglobin-reducing agent, methylene blue, blocked methemoglobin generation but did not block ISDN’s ability to antagonize cyanide. It was concluded that ISDN’s cyanide antidotal activity was due in part to NO generation.

The present study also provides a basis for the antidotal action of hyperbaric oxygen. Since cyanide is competitive with oxygen for binding to reduced heme a$_3$, increased oxygen tension would facilitate displacement of cyanide to reactivate the enzyme. Thus, cyanide would in theory inhibit cellular respiration less effectively as O$_2$ tension increases. Secondly, in the presence of the nitrite antidote, oxygen would alter the complex binding kinetics of both cyanide and NO, leading to reactivation of CcOX. It was observed that in the presence of micromolar NO, the inhibited CcOX was partially restored. When mitochondrial electron flux rate and NO/O$_2$ ratio are high, NO-Fe$_{a3}^{2+}$ binding is elevated (Mason et al., 2006; Sarti et al., 2003). NO may also bind to Fe$_{a3}^{3+}$ to prevent cyanide binding (Pearce et al., 2003). Although excess NO, like cyanide, inhibits cellular respiration, oxygen would decrease the NO/O$_2$ ratio, promoting NO-Cu$_B^{2+}$ binding and NO metabolism. The result would be increased CcOX activity.

CONCLUSION

In summary, low endogenous nanomolar levels of NO enhance inhibition of CcOX by cyanide, whereas exogenous micromolar levels of NO antagonize cyanide. Cyanide increases endogenous NO levels, possibly by upregulating mtNOS activity. In turn, this excess NO contributes to cyanide inhibition of CcOX, which is competitive with oxygen at the enzyme’s heme a$_3$ catalytic site. On the other hand, high exogenous NO levels reverse cyanide inhibition of CcOX. These observations explain why cyanide is a rapid, potent intoxicant at the low oxygen tensions within mitochondria and provide mechanistic information on the antidotal action of the nitrite ion.

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REFERENCES


