Chronic Treatment with Azide in Situ Leads to an Irreversible Loss of Cytochrome c Oxidase Activity via Holoenzyme Dissociation*

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Chronic treatment of cultured cells with very low levels of azide (I50 < 10 μM) leads to slow (t1/2 = 6 h), irreversible loss of cytochrome c oxidase (COX) activity. Azide-mediated COX losses were not accompanied by inhibition of other mitochondrial enzymes and were not dependent upon electron flux through oxidative phosphorylation. Although azide treatment also reduced activity (but not content) of both CuZn superoxide dismutase and catalase, a spectrum of pro-oxidants (and anti-oxidants) failed to mimic (or prevent) azide effects, arguing that losses in COX activity were not due to resultant compromises in free radical scavenging. Loss of COX activity was not attributable to reduced rates of mitochondrial protein synthesis or declines in either COX subunit mRNA or protein levels (COX I, II, IV). Co-incubation experiments using copper (CuCl2, Cu-His) and copper chelators (neocuproine, bathocuproine) indicated that azide effects were not mediated by interactions with either CuA or CuB. In contrast, difference spectroscopy and high performance liquid chromatography analyses demonstrated azide-induced losses in cytochrome aa3 content although not to the same extent as catalytic activity. Differential azide effects on COX content relative to COX activity were confirmed using a refined inhibition time course in combination with blue native electrophoresis, and established that holoenzyme dissociation occurs subsequent to losses in catalytic activity. Collectively, these data suggest that COX deficiency can arise through enhanced holoenzyme dissociation, possibly through interactions with the structure or coordination of its heme moieties.

Most ATP required by eukaryotic cells under resting conditions is generated aerobically by mitochondrial oxidative phosphorylation (OXPHOS). Maintenance of appropriate stoichiometry of OXPHOS complexes is critical not only to ensure efficient flux of electrons through the ETC but also to minimize the potentially cytotoxic impact of mitochondrially derived ROS (1, 2). Declines in the content of individual OXPHOS complexes frequently accompany cardiovascular and neurological diseases, as well as aging (3, 4).

The origins and consequences of the loss of a given OXPHOS complex are frequently studied in cell lines established from patients with metabolic deficiency (5–7). However, the pathophysiological consequences of complex deficiencies have also been studied using specific inhibitors to chronically impair catalytic activity (2, 8). Sodium azide is commonly used in vitro as a rapid and reversible inhibitor of COX, the terminal enzyme of the ETC that catalyzes the transfer of electrons from reduced cytochrome c to molecular oxygen (9–11). The ability of azide to rapidly bind to the bimolecular center, thereby inhibiting COX activity, has led to its frequent use in acute studies, including those designed to address the role of mitochondrial oxygen sensing in signal transduction (12, 13). We previously reported that chronic treatment of cultured myocytes with micromolar levels of azide results in the irreversible inhibition of COX activity (14). This mode of inhibition clearly differs from the classic mechanism of azide-mediated COX inhibition in that it is of higher affinity (0.01–0.1 μM) and irreversible in nature. The mechanism(s) by which azide mediates these effects is unknown. Moreover, its potential relevance to COX deficiency observed in a more physiologically relevant context has not been evaluated despite previous reports of selective COX losses leading to Alzheimer’s disease-like symptoms in rats that had been chronically infused with sodium azide (15, 16).

Several plausible mechanisms exist that could account for the azide-mediated, irreversible inhibition of COX activity. First, the capacity of azide to act as a chelator of first order transition series metals could affect COX activity by either terminal binding to or stripping of one or both copper centers from highly conserved domains within mitochondrially encoded subunits I and II (CuA on COX II, CuB on COX I; Refs. 11 and 17). Second, because copper metabolism is critical to the maturation and assembly of individual COX subunits into a functional holoenzyme complex (18–20), azide may impair either its delivery or insertion into the complex. Third, azide may act as a suicide metabolite to inhibit COX activity during enzyme

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1 The abbreviations used are: OXPHOS, oxidative phosphorylation; ASMC, aortic smooth muscle cells; COX, cytochrome c oxidase; CS, citrate synthase; Cu-His, copper histidine; ETC, electron transport chain; HEK293, human embryonic kidney 293 cells; HS, horse serum; O2O−, peroxynitrite; O2−, superoxide anion; PBS, phosphate-buffered saline; PC12, pheochromocytoma 12 cells; ROS, reactive oxygen species; HPLC, high performance liquid chromatography; SOD, superoxide dismutase; SSC, standard saline citrate; DCF, 2,7-dichlorofluorescin.

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turnover in a manner analogous to azide-mediated loss of catalase activity (21). Accordingly, it has recently been shown that the COX-mediated conversion of azide to the azidyl radical leads to its irreversible inactivation in vitro, although this mechanism requires very high concentrations of both azide and $\text{H}_2\text{O}_2$ (22). Fourth, chronic azide effects on COX activity may arise indirectly from elevated bulk phase production of ROS since azide is also a potent inhibitor of SOD (23) and catalase (21, 24) activities.

In the present study, we systematically addressed these possibilities to clarify the mechanism(s) by which azide irreversibly inhibits COX activity. Collectively, our studies suggest that azide treatment results in a loss of catalytic activity by accelerating the rate of holoenzyme dissociation, possibly through interactions with the structure or coordination of heme moieties.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—All culture media, sera, and antibiotics were purchased from Invitrogen. C2C12 cells were grown in proliferation medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum. At 70% confluency, myoblasts were switched to differentiation medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 1% HS, 10% FBS (H11003), and 1% w/v hyaluronic acid (S1). The medium was changed every 2 days. Mitochondrial extracts in the presence of 5 mM KCN to inhibit any non-native COX activity (EC 1.15.1.1) were assayed at 550 nm using the aerobic xanthine/xanthine oxidase (EC 1.11.1.6) (27) were measured from whole cell extracts as previously described. COX I and IV antibodies were resuspended in 1% w/v Triton X-100. Samples were centrifuged for 20 min at 14,000 $\times$ g. The resultant supernatant (S1) was diluted to 1:2,000. COX I and IV antibodies were reconstituted in Tris-buffered saline containing 0.05% SDS, 1% w/v Triton X-100, and 0.15 mM NaCl and 0.015 mM sodium citrate, 0.1% SDS, and 10 mM EDTA. Membranes were washed twice at room temperature for 15 min with 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 0.1% SDS and twice at 50 °C with 0.1× SSC, 0.1% SDS. Blots were visualized by phosphorimaging, and relative signal strength was quantified using ImageQuant software (Molecular Dynamics). Differences in loading across lanes were normalized using a probe for $\alpha$-tubulin mRNA.

**Electrophoresis and Immunoblotting**—Plates were rinsed and harvested in ice-cold PBS. Pelleted cells were flash-frozen in liquid nitrogen and stored at −80 °C for at least 24 h. Thawed cells pellets were resuspended in ice-cold lysis buffer (250 mM sucrose, 20 mM HEPES (pH 7.4), 1% w/v deoxycholic acid, 1% sodium deoxycholate, 1 mM ethylenediaminetetraacetic acid (EDTA) supplemented with first-dimension gels and second dimension electrophoresis (36) were as previously described. NADH ubiquinone oxidoreductase and COX holoenzyme levels from first-dimension immunoblots were quantified using an enhanced chemiluminescence detection system (Amersham Biosciences, Inc.) according to the manufacturers’ specifications.

**Fluorescence Microscopy**—Subconfluent myoblasts were grown in a 12% SDS-PAGE gel (31), and electroblotted (Bio-Rad, Mini-structural and functional effects of Azide on COX

**RNA Isolation and Northern Analysis**—Total RNA was purified from guanidinium thiocyanate extracts using a standard acid phenol protocol (38). Denatured RNA (10 µg) was fractionated using a 1% agarose-formaldehyde gel system. A cDNA was used for the muscle-specific isoform of COX-IV. Single-stranded cDNA was reversetranscribed using GES at 60 °C with 5’-ctgacgtctgtgtgct-3’ and 5’-gattgagctggattggtg-3’ using standard PCR conditions, cloned into pCR 2.1 (Invitrogen), and sequenced. All other probes for mtDNA- and nuclear-encoded mRNA species were obtained as previously described (16, 25).

**Pulse Labeling Experiments**—To pulse label mitochondrial translation products (39), myotubes grown in 60-mm plates were washed twice with sterile PBS and incubated for 30 min in pre-warmed, methionine-free Dulbecco’s modified Eagle’s medium supplemented with 2% diazylated HS. After an additional 5-min incubation in the presence of 100 µCi of [35S]methionine, cells were washed with 400 µCi of Expres-35S (Man- denviron, Inc.) according to the manufacturers’ specifications. Samples (10 µg) were sonicated on ice and fractionated on a 12–20% gradient gel at 7 mA for 14–16 h. The gel was then fixed for 1 h and dried for autoradiography.

**Statistical Analyses**—Significant differences ($p < 0.05$) between control and treated groups for all measured parameters were detected.
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Fig. 1. Chronic treatment of cultured cells with azide results in loss of COX activity, bioenergetic deficit, and altered cellular capacity for ROS metabolism. A, whole cell respiration (■) and lactate production (●) in day-6 serum-starved C2C12 myotubes treated with 50 μM azide for 6 days (mean ± S.E., n = 6). B, titration of COX activity in HEK293, ASM, PC12, and C2C12 cells treated with increasing concentrations of azide (0–500 μM) for 24 h. Rates (μmol/min·mg−1) in controls were 16.6 (ASM), 12.1 (HEK293), 212.2 (PC12), and 105.3 (C2C12). C, activities of bioenergetic enzymes assayed from isolated mitochondria. D, antioxidant enzymes measured from whole cell extracts of day-6 serum-starved C2C12 myotubes treated with 100 μM azide for 24 h (mean ± S.E., n = 3). All rates are expressed relative to controls. The asterisk denotes a significant difference (p < 0.05) between control and azide-treated cells.

using one-way analysis of variance and identified post hoc using the Tukey-Kramer honestly significantly different.

RESULTS

Chronic Azide Treatment Results in an Irreversible Loss of COX Activity, a Concomitant Decline in Whole Cell Respiration, and a Resultant Bioenergetic Stress—Chronic treatment of C2C12 myotubes with micromolar amounts of azide caused a time-dependent loss of COX activity (data not shown; Ref. 14), a resultant decline in whole cell respiration, and a concomitant increase in the rate of intracellular lactate accumulation (Fig. 1A). The azide effect on COX activity was also observed in three other cell backgrounds (HEK293, PC12, and ASM), with a 24-h treatment with 100 μM azide, resulting in the inhibition of 65–90% of total activity (Fig. 1B).

Loss of catalytic activity was irreversible, both in situ and in vitro. C2C12 myotubes pulsed for 6 h with 100 μM azide continued to lose COX activity 24 h after the removal of azide from the culture media (6 versus 24 h, 49 versus 33% of control). Azide-treated C2C12 cell extracts dialyzed for 24 h (50 mM Tris (pH 8.0), 4 °C) in the presence or absence of reductant (β-mercaptoethanol, dithiothreitol) also failed to recover catalytic activity (data not shown).

To determine whether azide required access to the binuclear center to irreversibly inhibit COX activity in situ, C2C12 myotubes were co-incubated with equimolar amounts of azide and cyanide (10 μM each). Despite a much higher relative affinity of cyanide (K_i 1 versus 64 μM; Refs. 10 and 41) and its ability to competitively displace bound azide from the binuclear center in vitro (42), it neither attenuated nor abrogated azide-mediated COX inhibition (expressed as % of control: azide, 51.1; KCN, 102.5; azide + KCN, 47.1). Collectively, these data argue against azide exerting its in situ effects on COX activity via classically described ligand interactions with the binuclear center (see Refs. 9 and 10).

Chronic Azide Effects on Mitochondrial Enzymes Are Specific to COX and Do Not Require a Functionally Intact Respiratory Chain—The irreversible loss of catalytic activity could not be mimicked by dialyzing extracts of untreated C2C12 myotubes, rat skeletal muscle, or purified bovine COX against 0–100 μM azide (24 h in 50 mM Tris (pH 8.0), 4 °C; data not shown). Given that azide-induced losses in catalytic activity only occurred in situ, we investigated the potential requirement of an intact ETC to the observed azide effects (see Ref. 42). Previous reports that azide is a potent inhibitor of the ATPase activity of complex V (43, 44) also prompted us to evaluate its effects on the activity of a spectrum of other mitochondrial enzymes. Although azide treatment resulted in a significant loss of COX activity, it had no effect on a Krebs cycle enzyme (CS), individual complexes of the ETC, and the ATPase component of complex V (Fig. 1C). Incubation of C2C12 cells for 24 h with specific inhibitors of complexes I (2–200 nM rotenone) and III (2 μg/ml antimycin A, 0.1–10 μM myxothiazol) in the presence or absence of azide neither caused a specific COX loss nor abrogated azide-mediated COX losses (data not shown). Although 24-h incubations with oligomycin (2.4 μg/ml), a complex V inhibitor, enhanced the specific loss of COX activity mediated by azide (Table 1), marked inhibition of 35S incorporation into mitochondrial translation products (36.2% of control; data not shown) suggested that the oligomycin effect was attributable to reduced availability of mitochondrial encoded COX subunits required for holoenzyme assembly.

Chronic Azide Effects on COX Activity Are Independent of Changes in Bulk Phase ROS Production—Although other bioenergetic enzymes were unaffected by azide treatment, marked inhibition of CuZn SOD (12.0 ± 3.6% of control) and catalase (30.8 ± 1.3% of control) was also observed (Fig. 1D). This suggested the possibility that altered cellular capacity to metabolize ROS may contribute to the loss of COX activity. However, myoblasts treated for 24 h with 100 μM azide showed no increase in DCF fluorescence, an indicator of H_2O_2 production, despite 80% losses in COX activity (Figs. 2, A and B). Moreover, azide had no effect on catalase mRNA levels, a sensitive indicator of oxidative stress (data not shown).

To further confirm that azide effects on COX were not mediated by altered ROS production, C2C12 myotubes were treated for 24 h with a spectrum of pro-oxidants. Treatment with the O_2^- generator paraquat (45) and the catalase inhibitor aminotriazole (46) alone and in combination did not result in a loss of COX activity (Figs. 2, C and D). A complementary approach, in which myotubes were incubated with a series of antioxidants, also failed to prevent azide-induced COX losses. Treatments included supplementation with sodium selenite (47), which induced a 4-fold increase in glutathione peroxidase (GPx) content (Fig. 2C), and chemical antioxidants N-acetyl cysteine (H_2O_2), Tiron (O_2^-), carnosine (O^-), and mannitol (hydroxyl radical) (each 0.1–1 mM) (n = 3, data not shown).
C2C12 cells were differentiated for 6 days under serum-starved conditions and treated for 24 h with either 10 μM azide or 2.4 μg/ml oligomycin alone or in combination. COX, NADH ubiquinone oxidoreductase (NDH), catalase, and total SOD activities were assayed at 37 °C from solubilized cell extracts as outlined under “Experimental Procedures.” All rates are expressed relative to controls (n = 5).

| Treatment                        | Enzyme activity |
|---------------------------------|-----------------|
|                                 | COX             | NDH             | Catalase        | Total SOD       |
| 10 μM azide                     | 53.2 ± 4.6<sup>a</sup> | 112.3 ± 6.9    | 74.4 ± 4.8<sup>a</sup> | 63.5 ± 4.8<sup>a</sup> |
| 2.4 μg/ml oligomycin            | 91.5 ± 1.8      | 128.7 ± 4.2<sup>a</sup> | 123.7 ± 4.9<sup>a</sup> | 91.5 ± 8.2 |
| 10 μM azide + 2.4 μg/ml oligomycin | 26.9 ± 5.1<sup>a</sup> | 147.9 ± 20.4<sup>a</sup> | 107.8 ± 7.3<sup>a</sup> | 38.2 ± 3.8<sup>a</sup> |

<sup>a</sup> Denotes a significant difference (p < 0.05) between control and treated cells.

<sup>b</sup> Denotes a significant difference (p < 0.05) between cells treated with azide alone versus azide plus oligomycin.

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**Table I. Azide and oligomycin effects on enzyme activity**

**Fig. 2. Azide effects on COX activity are not dependent on altered bulk phase ROS production.** A, DCF fluorescence in control (C) and azide-treated (Az, 100 μM, 24 h) myoblasts; B, panel A, expressed as a percentage of H<sub>2</sub>O<sub>2</sub>-stimulated maximal fluorescence (n = 5). C, activities of glutathione peroxidase (GPx), total cellular SOD, catalase; D, COX in day 6 serum-starved C2C12 myocytes grown either in minimal or sodium selenite (50 nM)-supplemented media and treated with azide (100 μM), aminotriazole (ATA, 20 μM), paraquat (PQ, 50 μM), or aminotriazole plus paraquat for 24 h (mean ± S.E., n = 3). All rates are expressed relative to sodium selenite-supplemented controls. Parallel treatments in particular had pronounced effects on cellular ROS production, as indicated by elevated catalase mRNA levels (100% increase after 1 day) and increased total SOD and catalase activities (200 and 50% increase, respectively, after 3 days; data not shown). Chronic treatment with higher doses of paraquat resulted in loss of viability and cell death (data not shown). The asterisk denotes a significant difference (p < 0.05) between cells grown in minimal versus sodium selenite-supplemented media. The single dagger denotes a significant difference (p < 0.05) between azide–treated and control cells. The double dagger denotes a significant difference (p < 0.05) between azide- and ATA-treated cells.

NO is a well known, reversible inhibitor of COX (48, 49). One of its metabolic byproducts, ONOO⁻, may also be catabolized by COX, a process that leads to irreversible catalytic inactivation of the enzyme (50). We therefore considered the potential contribution of altered NO and reactive NO species metabolism to the azide effects. No effects comparable with azide-dependent losses in COX were seen with the NO and ONOO⁻ generators S-nitroso-N-acetylpenicillamine (SNAP) and 3-morphilinosydnonimine (SIN-1; 0.01–3 μM), the nitric-oxide synthase inhibitor Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME; 0.1–1 μM), or the soluble guanylyl cyclase inhibitor 8-bromo-cGMP (5–500 μM) (data not shown).

**Chronic Azide Treatment Does Not Affect the Levels of Mitochondrially and Nuclear-encoded COX mRNAs and Proteins**—Northern and western analyses were conducted to evaluate whether the loss in COX activity was accompanied by changes in mRNA and/or protein levels of individual COX subunits. Levels of two mitochondrially encoded (COX I and II) and two nuclear-encoded subunit (COX IV and V1α) mRNAs were unchanged after 1, 3, or 6 days of treatment with 50 μM azide (Fig. 3A; n = 5). Lack of a general protection of COX II mRNA in response to azide-mediated COX inhibition (see Ref. 14) was further confirmed by measuring COX II/COX I mRNA ratios from azide titrations of ASMC, HEK293, and PC12 cells (data not shown). Protein levels of COX I, II, and IV subunits were also comparable in azide-treated and control cells after either 1 or 3 days of treatment with 100 μM azide (Fig. 3B). Similarly, levels of the ND1 subunit of complex I and of cytochrome c, the electron donor to complex IV, were unaffected by azide treatment.

To address whether azide inhibited the synthesis of individual subunits required for complex assembly without altering the levels of pre-existing ETC enzymes, the rate of mitochondrial protein synthesis was measured in control and azide-treated cells. Azide had no effect on the rate of mitochondrial protein synthesis, either acutely (added with label) or chronically (added 24 h before pulse-chase) (data not shown). Parallel treatment of myocytes with azide and thiamphenicol, a specific inhibitor of mitochondrial translation (51), revealed that both agents caused significant losses of COX activity without affecting the activities of complex I and CS (Fig. 4A). However, despite a 65% depression in the rate of mitochondrial protein synthesis relative to controls (Fig. 4B), the thiamphenicol-induced loss of COX activity was significantly slower than that observed with azide.

**Chronic Azide Effects on Catalytic Activity Do Not Involve Copper Chelation**—Irreversible losses of COX activity could also conceivably arise as a result of damage to either its metal centers or surrounding protein moieties. The ability of azide to act as a monodentate chelator of first transition series metals by binding to open coordination sites raised the possibility that it may exert its effects on COX activity by either chelation or stripping of copper centers within the holoenzyme. To evaluate the potential importance of ligand interactions with the copper centers in contributing to azide-induced COX losses, C2C12 cells were incubated with 10 μM azide in the presence or absence of copper (CuCl<sub>2</sub>, Cu-His) and copper chelators (neocuproine, BCS) (Fig. 5A). Azide effects on COX activity were unperturbed when it was added in combination with a 10-fold molar excess of CuCl<sub>2</sub> or Cu-His. Treatment with either neocuproine or bathocuproine disulfonic acid failed to mimic the
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**FIG. 3.** Azide effects on catalytic activity do not involve reduced steady-state mRNA or protein levels of individual COX subunits. A, representative autoradiograms of COX I, II, IV, and VIa mRNA levels as a function of azide treatment (50 μM) over a 6-day period (n = 5). B, representative immunoblots for bioenergetic (COX I, II, and IV; ND1; cytochrome c) and ROS-scavenging (catalase, CuZnSOD and MnSOD) proteins as a function of treatment with azide (0, 10, 100 μM) for 3 days. Porin and actin were used as loading controls for bioenergetic and ROS-scavenging enzymes, respectively.

The primary finding of the present study is that chronic exposure results in irreversible losses in COX activity by 24 h (Fig. 7A). Immunoblots of second-dimension gels of 24 h control and azide-treated samples demonstrated that a disproportionate amount of the total pool of available COX I and II subunits in azide-treated cells were present as unassembled, full-length polypeptides (Fig. 7B). Collectively, these data argue that azide treatment leads to an initial loss of catalytic activity that ultimately results in an accelerated rate of holoenzyme dissociation, an event that may be triggered by irreversible damage to either the structure or coordination of its heme moieties.

**DISCUSSION**

The present study characterized the mechanism by which chronic azide treatment mediates irreversible losses in COX activity. Although azide has traditionally been used in vitro as a rapid and reversible inhibitor of COX activity (9, 10), it has been shown that chronic exposure results in “selective” and irreversible COX losses both in situ (14) and in vivo (15, 16). However, the mechanistic basis of these two distinct effects on COX had not been previously investigated. Because selective COX losses and Alzheimer’s disease-like symptoms have been observed in rats chronically infused with azide (15, 52), this model may be clinically relevant to the pathophysiology of COX deficiency.

The primary finding of the present study is that chronic azide treatment causes the irreversible loss of COX activity by...
were treated with 0 or 100 μM H9262 and azide plus copper chelators. A significant difference (p < 0.05) between control and treated cells. The double dagger denotes a significant difference (p < 0.05) between cells treated with azide alone and azide plus copper chelators. B-D, day 6 serum-starved myotubes were treated with 0 or 100 μM azide for 24 h. B, difference spectra (air-oxidized minus dithionite-reduced) were generated from solubilized mitochondrial extracts to quantify cytochrome contents in both samples (control versus azide, n = 2: aa₃, 40.3 versus 14.7 nm; b, 268.2 versus 267.9 nm; c, 252.8 versus 274.7 nm). C and D, reverse phase HPLC profiles of protoheme (first peak) and heme A (third peak) content in control (C) and azide-treated (D) cells respectively (n = 3). The integrated area under the curve is shown in parentheses with heme A content normalized to protoheme to account for different amounts of starting protein (control versus azide, n = 3: 0.498 ± 0.111 versus 0.167 ± 0.021).

The loss of COX activity was greater than the decline in COX content, as assessed by three independent indices (spectroscopy (aa₃)), reverse phase HPLC (total heme A), and blue native PAGE (heme oxygenases). Such differential losses of COX activity and cytochrome aa₃ content also accompany COX deficiency in a number of disease models (Alzheimer’s disease, Ref. 53; Menkes’ syndrome, Ref. 54). The ability of azide to induce COX deficiency in the absence of any apparent defects in the expression of mitochrondrially encoded and nuclear-encoded COX subunits represents another important finding and further emphasizes the potential shortcomings associated with using subunit profiles as a diagnostic tool to assess the molecular basis of COX deficiency (55).

Results from in situ and in vitro manipulations strongly suggest that chronic azide effects are not mediated by classic, high affinity interactions with the binuclear center (10). Although the Kᵢ for this site is within the range of doses used in the present study (64 versus 0–100 μM), the binding of azide to the binuclear center is readily reversible in vitro. In contrast, extensive in vitro dialysis and in vivo washout of azide-treated cells failed to reverse in situ losses in COX activity. Co-incubation with cyanide was also unable to abrogate the observed azide effects despite its higher affinity for the binuclear center (40) and its ability to rapidly displace bound azide (41).

Our in vitro findings with purified COX, tissue homogenates, and cell culture extracts further extend the argument that azide-induced COX losses only occur in situ. The inhibition of COX activity in both muscle (C2C12, Sol 8, ASMC) and non-muscle (HEK293, PC12) cell backgrounds eliminates the possible requirement of either a muscle-specific factor or the expression of muscle-specific COX isoforms in order for azide to exert its effects. Although one possible in vivo influence common to all tissues is redox cycling of COX inhibition of other OXPHOS complexes to alter the rate of electron flow to COX had no effects on the efficacy of azide. The minor losses in COX activity seen with oligomycin treatment in both control and azide-treated cells appear to arise through indirect inhibition of mitochondrial protein synthesis rather than to direct effects on COX.

COX has previously been shown to catalyze the one-electron

FIG. 5. Azide-mediated loss of catalytic activity results in a lesser decline in aa₃ content that is independent of copper chelation. A, day 6 serum-starved myotubes were treated for 24 h with azide (Az, 10 μM) in the presence or absence of 100 μM copper (CuCl₂, Cu-His) and copper chelators (neocuproine (NEO), bathocuproine disulfonic acid (BCS)). C, control. COX activity was measured in solubilized cell extracts, and rates were expressed relative to controls (mean ± S.E., n = 3). The asterisk denotes a significant difference (p < 0.05) between control and treated cells. The double dagger denotes a significant difference (p < 0.05) between cells treated with azide alone and azide plus copper chelators. B-D, day 6 serum-starved myotubes were treated with 0 or 100 μM azide for 24 h. B, difference spectra (air-oxidized minus dithionite-reduced) were generated from solubilized mitochondrial extracts to quantify cytochrome contents in both samples (control versus azide, n = 2: aa₃, 40.3 versus 14.7 nm; b, 268.2 versus 267.9 nm; c, 252.8 versus 274.7 nm). C and D, reverse phase HPLC profiles of protoheme (first peak) and heme A (third peak) content in control (C) and azide-treated (D) cells respectively (n = 3). The integrated area under the curve is shown in parentheses with heme A content normalized to protoheme to account for different amounts of starting protein (control versus azide, n = 3: 0.498 ± 0.111 versus 0.167 ± 0.021).

FIG. 6. Azide-mediated losses in catalytic activity are more pronounced than declines in holoenzyme content. Proliferating myoblasts were treated with 0 or 100 μM azide (Az) for 0, 6, 12, and 24 h. A, control. Mitoplasts were isolated from digitonin-permeabilized cells at a detergent to protein ratio of 1.2:1, solubilized (1.5 mg/ml) in lysis buffer (750 mM 6-aminoacaproic acid, 50 mM Bistris (pH 7.0), and 1.5% laurylmaltoside) and electrophoresed under native conditions as described under “Experimental Procedures.” A, in-gel assays (35) for COX and NADH ubiquinone oxidoreductase (NDH) activity after electrophoresis in the first dimension (n = 3). Complimentary measurements were also made spectrophotometrically using whole cell extracts (n = 5). B, first-dimension native gels were transferred using semi-dry immunoblotting (36) and incubated overnight at 4°C with either anti-Cox1p (1:1,000) or anti-39-kDa (1:2,000) monoclonal antibodies (Molecular Probes). COX and NADH ubiquinone oxidoreductase holoenzyme levels were quantitated using an enhanced chemiluminescence detection system (Amersham Biosciences, Inc.) according to the manufacturer’s specifications (n = 3). C, summary of azide-mediated declines in COX catalytic activity (open bar) and COX content (solid bar) over the course of a 24-h treatment. Significant (p < 0.05) stepwise reductions in COX catalytic activity were observed at each time point and are not presented on the graph for the sake of clarity. The asterisk denotes a significant difference (p < 0.05) in COX holoenzyme content between azide-treated cells relative to t = 0 control. The single dagger denotes a significant difference (p < 0.05) in COX holoenzyme content between azide-treated cells. The double dagger denotes a significant difference (p < 0.05) between residual COX catalytic activity and COX holoenzyme content in azide-treated cells at a given time point.
oxidation of azide to the azidyl radical, a reaction that results in its irreversible inactivation in vitro (22). The analogous reaction with cyanide leads to loss of COX activity by cyanyl radical attack of the non-metal protein matrix intrinsic to the holoenzyme (57). Although azidyl radical attack could in principle lead to COX damage and dissociation in our model, this seems improbable for at least two reasons. First, in vitro catalysis of azide to the azidyl radical requires very high levels of exogenous H$_2$O$_2$ (1 mM) and azide (10 mM). In contrast, manipulation of cellular capacity either to produce (i.e. aminotriazole-mediated catalase inhibition, ETC inhibitors) or scavenge (e.g. -acetylcysteine, sodium selenite-induced increases in glutathione peroxidase content) H$_2$O$_2$ did not alter the azide effects on COX. Second, unlike cyanyl-inhibited COX, which showed no alteration in difference spectra (57), spectroscopically detectable cytochrome a$_3$ content was reduced as a result of azide treatment.

Several lines of evidence also argue against the involvement of bulk phase ROS production in azide-mediated COX losses despite the additional inhibition of catalase and CuZn SOD activities. First, extensive experimental manipulations that resulted in significant changes in the ability to either produce or quench a suite of free radicals (H$_2$O$_2$, hydroxyl radical, NO, ONOO$^-$, O$_2^-$) had no effect on COX activity. Second, inhibition of complexes I and III, the two major intramitochondrial sites of O$_2^-$ production (58), neither contributed to nor abrogated azide-mediated losses of COX activity. And third, the activity of aconitate, a mitochondrial hemoprotein that is highly sensitive to oxidative stress (59, 60), was the same in control and azide-treated cells (data not shown).

Reduced COX content does not appear to be due to its impaired synthesis since azide had no effect on levels of COX mRNA transcripts (I, II, IV, V1a), COX subunits (I, II, IV), mtDNA copy number (14), or the rate of mitochondrial protein synthesis. Although potential azide effects on the expression of the 10 remaining subunits were not assessed, the azide phenotype differs dramatically from those observed in model systems in which either the proteolytic or chaperone function of mitochondrial proteases has been modified (61, 62) or in which the function of nuclear-encoded proteins critical to complex assembly has been lost (5, 64). Therefore it seems unlikely that defects in COX biogenesis contributed to the azide-induced loss of holoenzyme content. Lack of an equivalent temporal loss of COX activity in thiamphenicol-treated cells provides further support that azide-mediated COX losses occur at a much greater rate than can be accounted for simply by the kinetic inhibition of processes relating to complex biogenesis.

The inability of either copper to abrogate or copper chelators to mimic the azide effects further suggests that the loss of catalytic activity was independent of azide interactions with the copper centers within COX. Although previous in vitro studies have described alterations in the kinetic and spectroscopic properties of COX in response to incubation with bathocuproine disulfonic acid, substantially higher doses were required to obtain an effect (0.05–1 μM; Refs. 56 and 63). In contrast, comprehensive spectroscopic, HPLC, and native electrophoretic analyses all argue that structural damage either at or near the heme groups caused an initial loss of catalytic activity that ultimately led to a reduction in COX content through holoenzyme dissociation. The ability of azide to mediate the differential loss of catalytic activity and holoenzyme content and to induce COX deficiency without altering the expression of mitochondrially encoded and nuclear-encoded COX subunits has important implications for diagnostic tools used to assess the molecular basis of COX deficiency.

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