Nitric oxide-induced persistent inhibition and nitrosylation of active site cysteine residues of mitochondrial cytochrome-c oxidase in lung endothelial cells

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Zhang, Jianliang, Bilian Jin, Liuzhe Li, Edward R. Block, and Jawaharlal M. Patel. Nitric oxide-induced persistent inhibition and nitrosylation of active site cysteine residues of mitochondrial cytochrome-c oxidase in lung endothelial cells. Am J Physiol Cell Physiol 288: C840–C849, 2005. First published November 23, 2004; doi:10.1152/ajpcell.00325.2004.—Persistent inhibition of cytochrome-c oxidase, a terminal enzyme of the mitochondrial electron transport chain, by excessive nitric oxide (NO) derived from inflammation, polluted air, and tobacco smoke contributes to enhanced oxidant production and programmed cell death or apoptosis of lung cells. We sought to determine whether the long-term exposure of pulmonary artery endothelial cells (PAEC) to pathophysiological concentrations of NO causes persistent inhibition of complex IV through redox modification of its key cysteine residues located in a putative NO-sensitive motif. Prolonged exposure of porcine PAEC to 1 mM NOC-18 increased S-nitrosylation of complex IV S2 by 200%. Site-directed mutagenesis of these two cysteines attenuated NO-increased nitrosylation of complex IV S2 by 200%. Site-directed mutagenesis of these two cysteines of complex IV S2 attenuated NO-induced loss of complex IV activities, suggesting redox regulation of complex IV activity. Sequence analysis of complex IV subunits revealed a novel putative NO-sensitive motif in subunit II (S2). There are only two cysteine residues in porcine complex IV S2, located in the putative motif. Immunoprecipitation and Western blot analysis and “biotin switch” assay demonstrated that exposure of PAEC to 1 mM NOC-18 increased S-nitrosylation of complex IV S2 by 200%. Site-directed mutagenesis of these two cysteines of complex IV S2 attenuated NO-increased nitrosylation of complex IV S2. These results demonstrate for the first time that NO nitrosylates active site cysteines of complex IV, which is associated with persistent inhibition of complex IV. NO inhibition of complex IV via nitrosylation of NO-sensitive cysteine residues can be a novel upstream event in NO-complex IV signaling for NO toxicity in lung endothelial cells.

S-nitrosylation; redox regulation

THE ENDOTHELIUM OF BLOOD VESSELS in the lung is exposed to nitric oxide (NO) when polluted air, tobacco smoke, and therapeutic NO are inhaled and under physiological and pathological conditions, e.g., NO generated by constitutive and inducible isoforms of NO synthase (NOS) in inflammatory cells (2, 31, 32, 34, 37, 67). Despite its physiological importance, excessive NO has cytotoxic effects on vascular endothelial cells (EC), including oxidative injury and programmed cell death or apoptosis in vivo and in cultured cells (4, 21, 24, 28, 44, 45, 54, 61). Loss of EC in vascular beds because of cell death decreases NO production, which impairs endothelium-dependent vessel relaxation and exposes smooth muscle cells, thereby promoting neointimal formation and wall thickening. It is unclear how NO initiates the death process of vascular EC, but mitochondria have been reported to be associated with the signaling of endothelial apoptosis (13, 50). For instance, mitochondrial dysfunction is associated with endothelial apoptosis in vivo and in vitro (40, 50). NO inhibits the mitochondrial transport chain at multiple sites, the most sensitive site of which is mitochondrial cytochrome-c oxidase or complex IV with 50% inhibition at an effective NO concentration of 0.1 μM (15, 48). This short-term exposure to physiological concentrations of NO rapidly inhibits complex IV in a reversible manner that is competitive with oxygen (15, 18). Long-term exposure to pathophysiological concentrations of NO (>1 μM) leads to persistent inhibition of cell respiration due to inhibition of various mitochondrial enzymes including complex IV (16, 52, 60). Peroxynitrite, formed from NO reacting with superoxide, inhibits complex IV in an irreversible manner both in vitro and in vivo (19), indicating that peroxynitrite plays a role in the irreversible inhibition of complex IV. Long-term exposure of murine macrophages to pathophysiological concentrations of NO (~1.5 μM) leads to persistent inhibition of complex IV, which is reported to be reversible throughout the time of exposure (1–12 h) (16), implying a long-lasting but reversible effect of NO on complex IV.

It is not clear how NO causes persistent inhibition of complex IV, but S-nitrosylation of key cysteine residues of complex IV may play a role. This notion is supported by the following observations. First, analysis of complex IV peptide sequences available in the Swiss-Prot database reveals that there are highly conserved cysteine residues across all species (sequences are available in the database) that are reported to be associated with the active center formation (30). Although it is still unclear whether NO can nitrosylate these cysteines to inhibit complex IV activity, NO has been reported to modulate the biological functions of many other intracellular signaling proteins by S-nitrosylation. These include two cysteine transcription factors, NF-κB (22, 23) and activator protein-1 (AP-1) (62). Second, excessive NO may overwhelm the mitochondrial antioxidant systems, e.g., GSH and thioredoxin (Trx), leading to nitrosylation of critical thiols or preventing removal of NO from the already nitrosylated sulfhydryl (SH) moieties (16), which may also contribute to persistent inhibition of complex IV. This inhibition can be long-lasting (compared with NO-heme reactivity) and reversible (compared with...
tyrosine nitration-induced inhibition). Finally, it has been reported that NO inhibition of complex I and II of the mitochondrial electron transport chain in vivo results from S-nitrosylation of critical thiols and ONOO− formation, respectively (9, 49). These observations suggest that critical/active site cysteine residues of complex IV may be unique targets for NO to nitrosylate and may serve as a novel molecular mechanism of NO-induced persistent inhibition of complex IV.

In the present study, pulmonary artery endothelial cells (PAEC) were used as a cell model to determine the effects of the slow-releasing NO donor 2,2'-((hydroxynitrosodihydrazino)-bis-ethanamine (NOC-18) on persistent inhibition and S-nitrosylation of complex IV. Our results demonstrate that long-term exposure of cells to pathophysiological concentrations of NO causes persistent inhibition of complex IV, which is likely due to redox/S-nitrosylation of active site cysteine residues located in a putative NO-sensitive motif in complex IV.

EXPERIMENTAL PROCEDURES

Chemicals. The antibodies against subunit II (S2; S stands for subunit) of complex IV and nitrosothiols were purchased from Molecular Probes (Eugene, OR) and Abcam, respectively. NOC-18 and carboxyl-2-phenyl-4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxyl-PTIO) were purchased from Calbiochem (San Diego, CA). All other chemicals were obtained from Fisher Scientific (Oreland, FL).

Cell culture and NO exposure. Cultured human (Clonetics, San Diego, CA) and porcine (primary cultures) PAEC were used as cell models to determine whether NO-induced persistent inhibition of complex IV is associated with oxidation/nitrosylation of complex IV S2. Porcine PAEC were obtained from the main pulmonary arteries of 6-mo-old pigs from a local slaughterhouse and were propagated in monolayer cultures as described by Zhang et al. (71, 73). Fifth to seventh passage cells in postconfluent monolayers maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) with 4% fetal bovine serum (HyClone Laboratories, Logan, UT) and antibiotics were used for all experiments.

In the present study, NOC-18 and PTIO were used as a NO donor and a NO scavenger, respectively. It has been reported that NO released from 1 mM NOC-18 results in steady-state levels of 1–5 μM NO in medium without any cofactors (Ref. 7, product technique data medium. NO-mediated persistent inhibition of complex IV is spectrally pure, with an average heme-to-protein ratio of 11.9–13.0 nmol heme/μg of protein and a very low 280 nm-to-420 nm absorbance ratio of 1.8.

Measurement of sulfhydryls of complex IV. The effects of NO on free sulfhydryl reactivity were determined by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by the method of Ellman and Gan (25) and Riddles et al. (51) under denaturing conditions. After exposure to NO (NOC-18, 1 mM) or medium only for 0–24 h and then fresh medium for 6 h, PAEC were collected for isolation of mitochondria with a mitochondrial fraction kit (Active Motif, Carlsbad, CA; Refs. 69, 71). Complex IV purified from the mitochondrial fraction was dialyzed against PBS, and protein (5 μM) was desalted with 6 M guanidine HCl and reacted with DTNB (200 μM) in PBS at room temperature for 90 min. The reaction was monitored as an increase in absorbance at 412 nm. The concentration of SH groups was calculated with ε125 = 13,700 M−1·cm−1 (51), and the results are expressed as number of SH groups per complex IV monomer. In some experiments, 50–100 μM carboxyl-PTIO was added 5 min before NO exposure and remained present throughout the 24-h exposure.

Mitochondrial GSH-to-GSSG ratios were isolated from PAEC with or without treatment with 1 mM NOC-18 for 18 h as previously described (69, 71). Mitochondrial GSH-to-GSSG ratios were assessed with the GSH/GSSG Ratio Assay kit (Calbiochem; Refs. 69, 71). Briefly, 10 μl of 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP), a thiol-scavenging reagent to rapidly scavenge GSH, was added to 100 μl of mitochondrial fraction, which was used for GSSG sample preparation. For GSH sample preparation, 50–μl mitochondrial fractions without the presence of M2VP were used. The mixture of sample-blank-standard, chromogen, enzyme, and NADPH (200 μl of each) in a cuvette was examined for the change of absorbance at 412 nm for 3 min with a spectrophotometer. The reaction rate and calibration curves were used to calculate concentrations of GSH and GSSG. GSH-to-GSSG ratios were then calculated.

Adenovirus-mediated allotopic expression of Trx in mitochondria and attenuation of NO inhibition of complex IV. The Trx gene was rescued from pDNA3-3-Trx (70) and cloned into pCMV/myc/mito vector, a vector for allotopic expression, i.e., targeting proteins to mitochondria with a signal sequence encoding a leader peptide to import expressed protein into mitochondria and a myc epitope fused to the COOH terminal for detection of allotropic expression in mitochondria (Invitrogen). The Trx cDNA with additional mitochondrial targeting sequence (coming from the pCMV/myc/mito vector) was rescued and cloned into a transfer vector, pShuttle-CMV, to form pAd-Trx. The same vector containing a green fluorescent protein (GFP) gene, pAd-GFP, and the sham vector, pAd, were made for controls. After sequences were verified by restriction enzyme digestion and sequencing [DNA Sequencing Core, Interdisciplinary Center for Genomic Regulatory Studies, University of California, San Diego] for all experiments.

Measurement of complex IV activity. Mitochondrial protein or purified complex IV was used to measure complex IV activity by following the oxidation of reduced cytochrome c at 550 nm with extinction coefficient ε550 = 27.7 mM−1·cm−1 as previously described (66, 69). The specific activity of complex IV was expressed as micromoles per minute per milligram of protein, and then relative activities (% of activity in control cells) for 4–24 h time points were calculated.

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boiling in the SDS-PAGE gel loading buffer and subjected to Western blot analysis using anti-complex IV S2 antibody to detect biotinylated complex IV S2 (see above).

**Site-directed mutagenesis of complex IV S2.** Cys\(^{196}\) and Cys\(^{200}\) residues of complex S2 were mutated to Ala by changing the Cys codon to Ala codon in the S2 cDNA as described previously (29, 74). The S2 cDNA was modified for nuclear expression, i.e., four TGA codons (for Trp in mitochondria, but for stop in cytosol) were mutated to TGG codons (for Trp in cytosol). The cDNAs with the mutated codons (Cys\(^{196/200}\) to Ala\(^{196/200}\)) were then amplified with the modified cDNA of complex IV S2 as a template and primers 1 and 2/3. Sequences of primers 1 and 2/3 were 5'-GTC GAC ATG GCT TAC CCT TTC CAA CTA GGC TTC-3' and 5'-GCGCCTGTTAACC TGT TAA TAT TGA TGT TGA CCA TTT TTC GAA GTA CTTA TAA TGG GAC AAG TTT AAC AAT GG CAT GAA GCT GTG GTT GTA TCC GCC GAT TAT TGA GGC GCC 3', which contain SalI (in primer 1) and NotI (in primer 2/3) restriction sites (underlined) and the changed codons for Ala\(^{196/200}\) (in primer 2) and Ala\(^{196/200}\) (in primer 3) for Cys\(^{196}\) and Cys\(^{200}\). The mutated cDNA was cloned into pCMV/myc/mito vector in frame between sites for SalI and NotI to form pS2-196 and pS2-200, respectively. The mutations from Cys to Ala codon of pS2-196 and pS2-200 were confirmed by nucleotide sequencing (DNA Sequencing Core, ICBR, University of Florida). The mutated S2 cDNA with additional mitochondrial signaling sequence was cloned into pShuttle-CMV to form pAd-S2-196 or pAd-S2-200. After sequences were verified, the assembled transfer vector was transferred into the Ad genome. Viral particles Ad-S2-196, Ad-S2-200, Ad-GFP, and Ad were produced.

**Allelotopic expression of mutated complex IV S2.** The viruses Ad-S2-196, Ad-S2-200, Ad-GFP, and Ad (5 × 10^6 pfu/ml) were used to infect PAEC with an anti-nitrosocysteine antibody (American Diagnostics) as described previously (69, 71, 73). Relative levels of Trx in the mitochondrial fraction isolated from Ad-Trx-infected cells were elevated two times compared with Ad-Trx-infected cells, indicating an overexpression of Trx in mitochondria. The Ad-Trx- or Ad-GFP-infected cells were exposed to 1 mM NOC-18 or control medium for 18 h and then fresh medium for 6 h. The treated cells were harvested and analyzed for complex IV activity.

**Immunoprecipitation and Western blot analysis of nitrosylated complex IV S2.** Proteins were immunoprecipitated from NO-treated (0–1 mM NOC-18 for 18 h, then fresh medium for 6 h) or control (medium only) PAEC with an anti-nitrosocysteine antibody (4 μg, mouse monoclonal; A. G. Scientific) or 4 μg of isotype-matched control antibody in the dark. The antigen-antibody complexes were isolated with protein A/G agarose beads (Santa Cruz Biotechnology) overnight at 4°C. The beads were then washed five times in high-salt buffer to which 1 mM N-ethylmaleimide was added to block free thiols and thereby prevent artificial S-nitrosylation. Boiled immunoprecipitates in loading buffer with or without DTT were loaded on a 15% SDS-PAGE gel, blotted on nitrocellulose membranes, and hybridized to an anti-complex IV S2 antibody (1:400 dilution, mouse monoclonal; Molecular Probes) and a secondary antibody (anti-mouse IgG conjugated to horseradish peroxidase; Molecular Probes). The protein bands were visualized with enhanced chemiluminescence detection reagent (Amersham) and Bio-Max X-ray film. Band intensities were examined on a densitometer (Fluor-S MultiImager System; Bio-Rad).

**“Biotin switch” assay of nitrosylated complex IV S2.** Biotin switch assay was performed as described previously with slight modifications (33, 41). In brief, control- or NO-exposed PAEC (1 mM NOC-18 for 18 h, then fresh medium for 6 h) were incubated in a nondenaturing lysis solution (in mM: 50 Tris·HCl, pH 7.4, 300 NaCl, 5 EDTA, and 0.1 neocuproine with 1% Triton X-100, aprotinin, and leupeptin). Free thiols were blocked by incubation of the samples in the blocking buffer (in mM: 225 HEPES, pH 7.7, 0.9 EDTA, 0.09 neocuproine, and 20 methylmethanethiosulfonate (MMTS) with 2.5% SDS, 20 min at 50°C). MMTS was removed by protein precipitation with acetone. The pellet was resuspended in HENS buffer (in mM: 250 HEPES, pH 7.7, 1 EDTA, and 0.1 neocuproine with 1% SDS). N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide (biotin-HPDP, 0.4 mM; Amersham Biotech) and sodium ascorbate (1 mM) were added, and the mixture was incubated for 1 h at room temperature in the dark to replace the NO group with biotin on the thiols of cysteine residues. After acetone precipitation, the biotin-labeled proteins were purified with neutravidin-agarose (15 μl/mg protein, Amersham Biotech). Biotinylated proteins were separated from the agrose beads by
residues on complex IV, reactive SH groups of complex IV purified from NO-treated and control PAEC were assessed. Exposure of PAEC to 1 mM NOC-18 for 0–24 h decreased the number of reactive SH groups from 12 to 4 mol/mol complex IV (Fig. 2A), which was attenuated by the NO scavenger PTIO (Fig. 2C). These data suggest that excessive NO causes persistent inhibition of complex IV, likely through modification of cysteine thiols of complex IV.

Excessive NO decreased GSH-to-GSSG ratios in mitochondria. To determine whether excessive NO overwhelms antioxidant systems, e.g., GSH, in mitochondria, levels of mitochondrial GSH and GSSG in PAEC exposed to NO or control medium were assessed. NO decreased GSH-to-GSSG ratios in the mitochondrial fractions of NO-treated cells compared with controls (Fig. 3). The NO scavenger PTIO attenuated the NO-decreased GSH-to-GSSG ratios. Decreased mitochondrial GSH may impair reduction of NO-modified cysteine residues of complex IV. Therefore, NO-induced persistent inhibition of complex IV may be mediated through redox modulation of complex IV.

Overexpression of Trx in mitochondria attenuated NO-induced persistent inhibition of complex IV. Our previous studies showed (70, 72) that NO decreased Trx levels in both mitochondria and cytosol and overexpression of Trx attenuated NO-induced inhibition of a cytosolic enzyme, NOS, in porcine PAEC. To determine whether NO-induced persistent inhibition of complex IV is mediated through redox modulation, PAEC were infected with recombinant Ad harboring a Trx gene with a signal sequence that leads the expressed peptide into mitochondrial (Ad-Trx) or Ad-GFP (sham Ad). Relative levels of Trx protein in mitochondrial and cytosolic fractions of Ad-Trx- or Ad-GFP-infected cells were analyzed by immunoblot techn-
Fig. 3. Excessive NO decreased mitochondrial GSH-to-GSSG ratios. After PAEC were exposed to 1 mM NOC-18 or control medium for 18 h, mitochondrial fractions were isolated from the cells. GSH-to-GSSG ratios in mitochondria isolated from the control and NO-treated cells were assessed with the GSH/GSSG Ratio Assay kit (Calbiochem), following the manufacturer’s instructions. C, control cells; NO, cells exposed to NO; PTIO, cells with PTIO only; NO+PTIO, NO-exposed cells with PTIO. *P < 0.05 vs. control.

Fig. 4. Allotopic expression of thioredoxin (Trx; A) and attenuation of NO inhibition of complex IV (B). PAEC were infected with adenovirus (Ad)-Trx or Ad-green fluorescent protein (GFP) as described in EXPERIMENTAL PROCEDURES. A: mitochondrial and cytosolic lysates (30 μg protein) were subjected to Western blot analysis with anti- Trx antibody to verify overexpression of Trx in mitochondria. Ad-Trx- or Ad-GFP-infected PAEC were exposed to 1 mM NOC-18 (NO) or medium only (C) for 18 h and then incubated in fresh medium for 6 h. Lane 1, Trx level in mitochondria of the Ad-GFP-infected cells; lane 2, Trx level in mitochondria of the Ad-Trx-infected cells; lane 3, Trx level in the cytosol of the Ad-GFP-infected cells; lane 4, Trx level in the cytosol of the Ad-Trx-infected cells. B: treated cells were harvested and analyzed for complex IV activity. Data are means ± SE; n = 3 for each treatment. *P < 0.05 vs. lane 1 in A or control (C) in B.

S-nitrosylation/inhibition of cytochrome-c oxidase by NO

Identification of novel putative NO-sensitive motif in complex IV. Although all NO-accessible cysteine residues of complex IV may be subjected to NO-induced oxidative modification, modification of critical cysteine residues, e.g., active site cysteines and cysteines in NO-sensitive conformation, are most likely to cause the persistent inhibition of complex IV activity. We performed sequence analysis of all subunits of complex IV derived from all species available in the Swiss-Prot database, a comprehensive and nonredundant peptide sequence database, for putative NO-sensitive conformations. Subunits of complex IV can be classified into four groups: group 1 (without cysteines in the peptides), including subunits IVb, VIIa, VIIc, VIIIb, and VIIIc; group 2 (with cysteines but not highly conserved), including subunits I, Va, Vb, Vla, VIIb, and VIIIa; group 3 (highly conserved cysteines but either without the putative NO-sensitive conformation or with conformation not highly conserved), including subunits III, IVa, and Vb; and group 4 (highly conserved cysteine residues in a putative NO-sensitive conformation), including subunit II. There are two highly conserved cysteine residues located in a putative NO-sensitive region Gln-Cys196-Ser-Glu198-Ile-Cys200-Gly of subunit II peptides across all species (sequences are available in the database). The two cysteine residues of subunit II are reported to be associated with the active center formation of complex IV (30). Excessive NO may degrade the copper cluster and nitrosylate the two cysteine residues, preventing recovery of the copper cluster and leading to persistent inhibition of complex IV.

NO-induced S-nitrosylation of complex IV S2. To determine whether exposure of cells to NO causes nitrosylation of cysteine residues on complex IV S2, two approaches were used, namely, immunoprecipitation and Western blot analysis and the biotin switch assay. After exposure of PAEC to 1 mM NOC-18 or control medium for 18 h and then to fresh medium for 6 h, S-nitrosylated proteins were immunoprecipitated from cell lysates with an anti-nitrosocysteine monoclonal antibody. Western blotting analysis of S-nitrosylated complex IV S2 in the immunoprecipitates was carried out with an anti-complex IV S2 antibody (Fig. 5A). Band intensities of nitroso-complex IV S2 were determined and plotted (Fig. 5B). As shown in Fig. 5, A and B, the level of S-nitrosylated complex IV S2 protein in NO-exposed cells is two times higher than that in the control cells.

To verify the NO-induced nitrosylation of complex IV S2, the biotin switch assay was carried out. Free thiols in non-natured lysates of control and NO-treated PAEC were blocked with MMTS. Some lysates were incubated with ascorbate to reduce cysteine residue thiols before the blocking step to serve as a complete negative control. NO groups were removed from nitrosylated cysteine residues with ascorbate, and free thiols of complexes were analyzed. The biotin switch assay was carried out. Free thiols in non-natured lysates of control and NO-treated PAEC were blocked with MMTS. Some lysates were incubated with ascorbate to reduce cysteine residue thiols before the blocking step to serve as a complete negative control. NO groups were removed from nitrosylated cysteine residues with ascorbate, and free thiols of complexes were analyzed.
these cysteine residues were then biotinylated. The biotinylated proteins were isolated and subjected to Western blot analysis with anti-complex IV S2 antibody (Fig. 5, C and D). The relative level of nitrosylated complex IV S2 in the NO-treated lung EC was increased three times compared with that in control cells (Fig. 5, C and D). Pretreatment of cell lysates with ascorbate (lanes C-Vc and NO-Vc, Fig. 5, C and D) diminished bands for biotinylated complex IV S2, verifying the specificity of the biotin switch assay for nitrosylation assessment. Sequence analysis of complex IV S2 demonstrates that there are only two cysteine residues present in porcine complex IV S2 peptide. In addition, the two cysteines of complex IV S2 are involved in the formation of the active center of complex IV. NO-induced S-nitrosylation of one or both of these cysteine residues may prevent recovery of the copper cluster of complex IV, resulting in persistent inhibition of complex IV.

NO-induced S-nitrosylation of complex IV S2 is dose dependent. Proteins were immunoprecipitated from control (medium only) and NO-treated (0.25, 0.5, and 1 mM NOC-18) PAEC with a 6-h recovery incubation and an anti-nitrosocysteine antibody in the dark with 1 mM N-ethylmaleimide. Boiled immunoprecipitates in loading buffer without DTT were loaded on a 15% SDS-PAGE gel, blotted on nitrocellulose membranes, and hybridized to an anti-MCO S2 antibody (A). Band intensities were examined on a densitometer and plotted (B). Data are means ± SD; n = 3. *P < 0.01 vs. control (0 mM NOC-18).

Site-directed mutagenesis and allotopic expression of complex IV S2. To determine whether NO induces nitrosylation of the two cysteine residues of complex IV S2, resulting in persistent inhibition of complex IV activity, Ad harboring mutated complex IV S2 gene, i.e., Cys196 or Cys200 codons replaced by Ala codons, was used to infect PAEC. Ad-GFP (sham Ad) was used as a control. Relative levels of total (endogenous and exogenous) complex IV S2 protein in mitochondrial fractions derived from Ad-S2–196- and Ad-S2–200-

**Fig. 5. NO-induced S-nitrosylation of complex IV S2.** Immunoprecipitation and Western blot analysis and “biotin switch” assay were used. Proteins were immunoprecipitated from NO-treated (1 mM NOC-18 for 18 h, then fresh medium for 6 h) or control (medium only) PAEC with an anti-nitrosocysteine antibody or an isotype-matched control antibody (A). Band intensities were examined on a densitometer and plotted (B). Ig, relative level of nitroso-complex IV S2 in control cell lysates; HC, immunoglobulin heavy chain; LC, light chain. The biotin switch assay was carried out as described in EXPERIMENTAL PROCEDURES. Briefly, nondenaturing cell lysates were subjected to blocking of free thiols, removal of NO groups from the nitrosylated cysteine residues, and biotin labeling of the nitrosylated cysteine residues. Biotin-labeled proteins were isolated and subjected to Western blot analysis with the anti-complex IV S2 antibody (Fig. 5, C and D). Band intensities were examined on a densitometer and plotted (D). Lane C-Vc, relative level of biotin-labeled nitroso-complex IV S2 in control cell lysates pretreated with ascorbate; lane C, relative level of biotin-labeled nitroso-complex IV S2 protein in control cells; lane NO-Vc, relative level of biotin-labeled nitroso-complex IV S2 in NO-exposed cells pretreated with ascorbate; lane NO, relative level of biotin-labeled nitroso-complex IV S2 in NO-exposed cells. Data are means ± SD; n = 3. *P < 0.01 vs. controls.
infected cells were 1.5 times higher than that in the Ad-infected cells (Fig. 7). Relative levels of the myc-tagged complex IV S2 (exogenous) in the mitochondria of Ad-S2–196- or Ad-S2–200-infected cells were higher than those in Ad-infected cells, verifying overexpression of mutated complex IV S2 in mitochondria. The ratios of myc-tagged (exogenous) and total (endogenous and exogenous) complex IV S2 in Ad-S2–196- and Ad-S2–200-infected cells are 50%, suggesting that about half of wild-type complex S2 was replaced by the mutants.

To determine whether removal of the thiol groups from the cysteine residues of complex IV S2 by site-directed mutagenesis prevents NO-induced S2 nitrosylation and leads to persistent inhibition of complex IV, Ad-, Ad-S2–196-, or Ad-S2–200-infected PAEC were exposed to 1 mM NOC-18 for 18 h and then incubated in fresh medium for 6 h. The treated cells were subjected to immunoprecipitation and Western blot analysis and activity assay. Relative levels of nitrosylated complex IV S2 in NO-treated cells with overexpression of S2 mutants were decreased 50% compared with the NO-treated control (sham Ad-infected) (Fig. 8A). If the remaining 50% nitrosylated S2 is due to endogenous expression of wild-type S2, removal of the thiol groups of cysteine residues on S2 eliminates NO-induced nitrosylation of complex IV S2. These results directly support the notion that NO can nitrosylate cysteine residues of complex IV S2. Because these two cysteine residues of S2 are essential for formation of the active center of complex IV, removal of either or both of these thiols is expected to cause loss of its catalytic activity. Complex IV activity in the Ad-S2–196- and Ad-S2–200-infected cells was decreased 52% and 43%, respectively, compared with Ad-infected cells (Fig. 8B). Exposure of Ad-S2–196/200-infected cells to NO decreased further complex IV activity, which is most likely due to nitrosylation or inhibition of endogenously expressed wild-type complex IV S2.

DISCUSSION

Our experimental results demonstrate for the first time that NO-induced persistent inhibition of complex IV is associated with oxidation and S-nitrosylation of cysteine residues located in a novel NO-sensitive motif of complex IV S2. Our present studies indicate that exposure of PAEC to 1 μM NO, equivalent to the low range of NO released under pathophysiological conditions, for 18 h caused persistent inhibition of complex IV, i.e., the catalytic activity did not return to normal after removal of NO for 6 h. This persistent inhibition of complex IV can contribute to NO-induced mitochondrial dysfunction, e.g., increased $K_m$ for $O_2$ and elevation of oxidant leak from the respiratory chain. Low levels of NO (in the nanomolar range) are well known to inhibit complex IV through competitive binding to the active site heme (14, 53). NO-induced persistent inhibition of complex IV is less likely to occur via the NO-heme mechanism because NO competitive inhibition of complex IV is reversed by removal of NO, whereas the persistent inhibition is not. Recently, Cooper and colleagues (19, 53) and other groups reported that NO can induce irreversible inhibition of complex IV via permanent damage due to tyrosine nitration of the enzyme. NO cannot directly nitrate tyrosine residues, but it can react with superoxide anion to form a more powerful oxidant peroxynitrite that is able to nitrate tyrosine

Fig. 8. Removal of thiol groups of complex IV S2 attenuated NO-induced nitrosylation of complex IV S2 and caused loss of complex IV activity. Allotopic expression of mutated complex IV S2 in mitochondria of Ad-S2–196- and Ad-S2–200-infected cells was verified. S2 nitrosylation and complex IV activity in Ad-S2–196/200- or Ad-infected PAEC in the presence or absence of 1 mM NOC-18 for 18 h and a 6-h recovery incubation were then assessed as described in EXPERIMENTAL PROCEDURES. Ad, sham Ad-infected cells; Ad-S2–196, Ad-S2–196-infected cells; Ad-S2–200, Ad-S2–200-infected cells; C, cells without NO treatment; NO, cells treated with 1 mM NOC-18. Data are means ± SE; $n = 4$. *P < 0.05 vs. controls (gene-transferred cells in the absence of NOC-18); **P < 0.05 vs. sham Ad-infected cells.
residues. Because high levels of NO (>1 μM) and superoxide anion released from the respiratory chain are essential for peroxynitrite formation, NO-induced irreversible inhibition of complex IV via a tyrosine nitration mechanism is more likely linked to effects of excessive or sustained NO and/or a late-stage modulation of complex IV under pathophysiological conditions. We found that NO in concentrations of 0.5–2 μM caused persistent inhibition of complex IV. Loss of complex IV activity in NO-exposed cells was correlated with loss of thiol groups from the enzyme. In addition, NO decreased GSH-to-GSSG ratios in mitochondria of PAEC. This suggests that excessive NO may overwhelm mitochondrial antioxidant systems, e.g., mitochondrial GSH and Trx (72). For example, we recently reported (35) that Trx is a direct target for NO-induced thiol modification in porcine PAEC. Diminished defense mechanisms can expose the cysteine residues of complex IV to the NO attack. Studies on overexpression of mitochondrial Trx support the notion that Trx plays a protective role in NO inhibition of complex IV, because high levels of Trx in mitochondria attenuated NO-impaired complex IV. One possible mechanism is reaction of excessive Trx with NO to shield complex IV from NO-induced oxidation/nitrosylation and consequent loss of activity. Nitrosylation and denitrosylation of proteins are currently believed to be a common and critical element in NO-related signaling pathways (17, 26, 40, 58, 63). For instance, NO-induced nitrosylation and persistent inhibition of complex I of the mitochondrial electron transport chain is linked to mitochondrial dysfunction (9, 11, 16, 43). S-nitrosylation of apoptotic factors, e.g., caspases and cytochrome c, by NO modulates cell death (3, 36, 39, 63). NO-mediated nitrosylation of nuclear factors, e.g., NF-κB and AP-1, has been shown to regulate cell proliferation.

Sequence analysis of complex IV peptides revealed a novel putative NO-sensitive motif, Gln-Cys196-Ser-Glu198-Ile-Cys200-Gly, in complex IV S2. Mammalian complex IV consists of at least 15 subunits (30), and cysteine residues in all subunits can be potential targets for NO modification. However, target cysteine residues must be accessible and sensitive to NO and must be essential active site residues of complex IV if modification of the cysteines is to serve as an initiation factor for NO signaling. The two cysteines in complex IV S2 are located in the active center that converts O2 to H2O; hence, they are as accessible to NO as to O2 (1, 55). On the basis of the human Hb structure and accounting for the known acid-base catalyzed Cys β93 nitrosylation and Cys β393 NO-dentitrosylation processes, a putative amino acid sequence, (Lys/Arg/His/Asp/Glu)Cys/(Asp/Glu), has been proposed as the minimum consensus motif for Cys-NO reactivity (5). The most important component of the tri- or tetrapeptide consensus motif has been recognized as the Cys/(Asp/Glu) pair (59). An acidic residue, Glu198, in the putative motif Gln-Cys196-Ser-Glu198-Ile-Cys200-Gly of complex IV S2 may enhance the interaction between the two cysteines and NO, which makes the two cysteines more sensitive to NO, i.e., the two cysteines are more likely modified by NO than other cysteines. NO may react with O2 to form N2O3 in the active site of complex IV, which can immediately attack the nearby cysteine residues of complex IV S2. This selective S-nitrosylation can lead to inhibition of complex IV activity. An autocatalytic mechanism of protein nitrosylation may explain why only particular cysteine residues are targeted within a protein and other cysteine residues are left unmodified (46). These NO-sensitive cysteine residues are essential to bridge two copper atoms to form a Cu center (10, 30). NO-induced oxidation and S-nitrosylation of complex IV S2 may prevent the copper center repair, releasing copper ions. The free copper may catalyze tyrosine nitration of complex IV S2, because Thomas et al. (64) demonstrated that NO-induced protein nitration can be mediated through free heme and metals. Nitration of complex IV can result in its degradation and loss of activity. In addition, the two cysteines in the putative motif are highly conserved across species, which also suggests a vital role for these cysteines in maintaining integrity of complex IV. Similar conformations have been found in the two-cysteine nuclear factors, NF-κB (22, 23) and AP-1 (62). Redox regulation of the two cysteines in these nuclear factors modulates their binding activities.

Our experimental results support the notion that NO oxidizes/nitrosylates the two cysteines in the putative NO-sensitive motif. For instance, exposure of PAEC to NO increased S-nitrosylation of complex IV S2 as determined by two methods, namely immunoprecipitation and Western blot analysis and biotin switch assay, and the effects of NO on nitrosylation were dose dependent. There are only two cysteine residues in the peptide of complex IV S2, which are located in the putative NO-sensitive motif; therefore, NO-induced nitrosylation of complex IV S2 indicates nitrosylation of one or both of the two critical cysteines. Furthermore, removal of thiol groups via replacement of the cysteines by alanines with site-directed mutagenesis diminished NO-induced S2 nitrosylation, verifying the reaction between NO and these key cysteines of complex IV S2. Because the thiol groups of S2 are essential for complex IV activity, removal of the thiols is expected to cause loss of complex IV activity, which was observed in our mutagenesis studies of complex IV S2.

We provide evidence here of a causal relationship between NO-induced persistent inhibition of complex IV and oxidation/S-nitrosylation of cysteine residues located in a putative NO-sensitive motif of complex IV S2 in porcine PAEC. The hypothesis is put forth that NO modulation of complex IV via cysteine oxidation/nitrosylation plays a critical role in NO-induced cytotoxicity of vascular endothelial cells via mitochondrial-mediated pathways. Lung endothelium is exposed to multiple NO sources under pathological conditions, e.g., NO generated by endogenous NOS, NO inhalation therapy, NO in polluted air or smoke, and NO produced by inducible NOS in inflammatory cells (2, 31, 32, 34, 37, 67). Despite its physiological importance, excessive NO has cytotoxic effects on vascular endothelial cells, e.g., oxidative injury and apoptosis (4, 21, 24, 28, 44, 45, 54, 61). Therefore, patients receiving long-term NO inhalation therapy may have impaired endothelial function due to oxidative injury and cell death. Similar loss of lung endothelial function can occur in the event that excessive NO is generated by endogenous processes as a result of diverse pulmonary disorders and/or inflammatory conditions or generated by environmental exposure (20, 24, 27, 37, 38, 57). Therefore, identification of the novel molecular mechanisms underlying NO inhibition, particularly persistent inhibition, of complex IV via oxidation/nitrosylation of critical cysteine residues can help us better understand NO-induced mitochondrial and endothelial dysfunction under pathological conditions.
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