Thioredoxin overexpression prevents NO-induced reduction of NO synthase activity in lung endothelial cells

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Zhang, Jianliang, Yong D. Li, Jawaharlal M. Patel, and Edward R. Block. Thioredoxin overexpression prevents NO-induced reduction of NO synthase activity in lung endothelial cells. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L288–L293, 1998.—We recently reported that nitric oxide (NO) induces posttranscriptional modulation of lung endothelial cell NO synthase (ecNOS) that results in loss of activity. The loss of activity can be reversed by the redox regulatory proteins thioredoxin (Thx)/thioredoxin reductase (Thx-R). The present study was designed to examine whether diminished expression of endogenous Thx and Thx-R may account for regulation of ecNOS activity in NO-exposed cells and whether overexpression of Thx can prevent NO-induced reduction of ecNOS activity in cultured porcine pulmonary artery endothelial cells (PAEC). Exposure to 8.5 ppm NO gas for 24 h resulted in an 80% decrease of Thx and a 27% decrease of Thx-R mRNA expression. Similarly, NO exposure caused 30 and 50% reductions in Thx and Thx-R protein mass, respectively. This NO-induced decrease in the expression of Thx-R mRNA and protein was accompanied by a significant (P < 0.05) decrease in the catalytic activity of Thx-R but not of glutaredoxin or the cellular levels of reduced glutathione and oxidized glutathione. Overexpression of Thx gene in PAEC was achieved by transient transfection of these cells with pcDNA 3.1 vector inserted in sense or antisense (native) orientation in a human Thx cDNA. Thx mRNA and protein contents in transfected cells were four- and threefold higher, respectively, than those in native PAEC. Exposure of native cells to 10 µM NO solution for 30 min resulted in a significant (P < 0.01) loss of ecNOS activity, whereas ecNOS activity was comparable in Thx-overexpressed cells with or without NO exposure. These results demonstrate that NO exposure results in diminished expression of Thx and Thx-R in PAEC. Endogenous levels of Thx are critical to restoring the NO-induced loss of ecNOS activity because overexpression of Thx prevented the NO-induced loss of ecNOS catalytic activity. These results also demonstrate that NO modulation of ecNOS and Thx proteins is regulated by a physiologically relevant redox mechanism.

Nitric oxide (NO), an endogenous product of lung endothelial cells as well as an exogenous gas used in inhalation therapy, plays a critical role in the physiology and pathophysiology of the lungs of animals and humans (2, 4, 8). Vascular endothelial cells generate NO from metabolism of L-arginine via an oxidative catabolic reaction mediated by the constitutively expressed isoform of NO synthase (ecNOS; see Ref. 19). Despite its role in the regulation of pulmonary vascular function, excessive production of endogenous NO or prolonged inhalation of exogenous NO gas has been shown to be toxic to a number of cells, including vascular endothelial cells (7, 20, 27, 30, 31, 33, 35). For example, in vivo and in vitro studies have demonstrated that NO causes inhibition of protein synthesis and enzyme activities, including ecNOS activity, S-nitrosylation of proteins, and altered vasoreactivity (3, 24, 29, 31, 33, 35, 37). S-nitrosylation of active-site cysteine residues and disulfide formation have been suggested to be responsible for the modulation of N-methyl-D-aspartate receptor function, for tissue plasminogen activation, and for the activities of type I adenylate cyclase, glyceraldehyde-3-phosphate dehydrogenase, protein kinase C, and ecNOS (11, 14, 22, 26, 33, 37). S-nitrosothiols have also been reported to accelerate disulfide formation and to play a role in physiological functions (3, 37). We have recently reported that exposure of intact pulmonary artery endothelial cells (PAEC), isolated total membranes, plasma membranes, or purified ecNOS to NO significantly decreases catalytic activity of ecNOS and that disulfide-reducing chemicals, e.g., dithiothreitol or 2-mercaptoethanol, or enzymatic reduction of disulfide by thioredoxin (Thx)/thioredoxin reductase (Thx-R), but not by glutaredoxin, restore NO-induced loss of ecNOS activity (33). This suggests that Thx/Thx-R may play a critical role in the regulation of ecNOS activity under oxidative stress.

The role of intracellular thiol-disulfide-reducing systems such as Thx (consisting of Thx, Thx-R, and NADPH) and the thioredoxin-glutaredoxin systems such as glutathione (GSH), GSH reductase, glutaredoxin, and NADPH in regulating thiol-disulfide exchange and catalytic activity of proteins is well established (15, 16, 34). The Thx/Thx-R system is a general intramolecular disulfide oxidoreductase that catalyzes NADPH-dependent reduction of intramolecular disulfides in a variety of proteins (16, 22). Thx is an 11- to 12-kDa protein with a highly conserved consensus sequence (Trp-Cys-Gly-Pro-Cys-Lys) that represents the active site of this protein (17, 18). The two half-cysteines at the active site of oxidized Thx are reduced by an NADPH-dependent reaction catalyzed by Thx-R (15, 34). Thus the redox protein Thx is the physiological substrate for Thx-R and is known to regulate a variety of redox-sensitive enzymes, including enzymes responsible for DNA synthesis and gene expression (1, 13, 25, 34). The objective of the present study was to examine whether diminished expression of endogenous Thx accounts for the inability of PAEC to protect against NO-induced inhibition of ecNOS in PAEC and, if so, to determine whether overexpression of Thx prevents NO-induced loss of ecNOS activity in these cells.

NITRIC OXIDE

NITRIC OXIDE (NO), an endogenous product of lung endothelial cells as well as an exogenous gas used in inhalation therapy, plays a critical role in the physiology and pathophysiology of the lungs of animals and humans (2, 4, 8). Vascular endothelial cells generate NO from metabolism of L-arginine via an oxidative catabolic reaction mediated by the constitutively expressed isoform of NO synthase (ecNOS; see Ref. 19). Despite its role in the regulation of pulmonary vascular function, excessive production of endogenous NO or prolonged inhalation of exogenous NO gas has been shown to be toxic to a number of cells, including vascular endothelial cells (7, 20, 27, 30, 31, 33, 35). For example, in vivo and in vitro studies have demonstrated that NO causes inhibition of protein synthesis and enzyme activities, including ecNOS activity, S-nitrosylation of proteins, and altered vasoreactivity (3, 24, 29, 31, 33, 35, 37). S-nitrosylation of active-site cysteine residues and disulfide formation have been suggested to be responsible for the modulation of N-methyl-D-aspartate receptor function, for tissue plasminogen activation, and for the activities of type I adenylate cyclase, glyceraldehyde-3-phosphate dehydrogenase, protein kinase C, and ecNOS (11, 14, 22, 26, 33, 37). S-nitrosothiols have also been reported to accelerate disulfide formation and to play a role in physiological functions (3, 37). We have recently reported that exposure of intact pulmonary artery endothelial cells (PAEC), isolated total membranes, plasma membranes, or purified ecNOS to NO significantly decreases catalytic activity of ecNOS and that disulfide-reducing chemicals, e.g., dithiothreitol or 2-mercaptoethanol, or enzymatic reduction of disulfide by thioredoxin (Thx)/thioredoxin reductase (Thx-R), but not by glutaredoxin, restore NO-induced loss of ecNOS activity (33). This suggests that Thx/Thx-R may play a critical role in the regulation of ecNOS activity under oxidative stress.

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EXPERIMENTAL PROCEDURES

Chemicals. Calmodulin, NADPH, GSH, GSH reductase, oxidized glutathione (GSSG), leupeptin, bovine insulin, EDTA, EGTA, and phenylmethylsulfonyl fluoride were purchased from Sigma (St. Louis, MO). Tetrahydrodiproterin was purchased from B. Schirck's laboratory (Jena, Switzerland); Dowex AG1-X8 (hydroxide form, 200–400 mesh), AG50W X8 (sodium form, 200–400 mesh), and l-arginine were purchased from Bio-Rad (Richmond, CA). l-[3H]arginine was obtained from NEN (Boston, MA). Escherichia coli and human Thx and Thx-R were purchased from American Diagnostics (Greenwich, CT). A PolyATtract mRNA isolation system was purchased from Promega (Madison, WI). Digoxigenin-labeled dUTP and Genius labeling and detection kits were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). All other chemicals were obtained from Fisher Scientific (Orlando, FL). NO gas (>99% purity) was obtained from Air Liquid (Morrisville, PA). Premixed NO (8.5 ppm) gas in nitrogen containing 5% CO2 was obtained from Air Products (J. acksonville, FL).

Tissue culture. Endothelial cells were obtained from the main pulmonary artery of 6- to 7-mo-old pigs and propagated in monolayer cultures as described by Patel et al. (33). Fifth- to seventh-passage PAEC in postconfluent monolayers maintained in RPMI 1640 (Life Technologies, Grand Island, NY) with 4% fetal bovine serum (HyClone Laboratories, Logan, UT) and antibiotics were used for all experiments.

Cloning and expression of Thx in PAEC. Human Thx cDNA (kindly provided by Dr. Garth Powis, Arizona Cancer Center, Tucson, AZ) was cloned into a mammalian expression vector pCDNA-3 (Invitrogen, San Diego, CA) in sense orientation to form pThx-S and in antisense orientation to form pThx-A. Porcine PAEC (60% confluent) were transiently transfected with pThx-S and pThx-A using Transfectam reagent (Promega) as described by the manufacturer. Cells in 100-mm dishes were incubated in serum-free RPMI 1640 medium containing 15 µg of Transfectam and 5 µg of DNA at 37°C for 5 h. After incubation, the cells were washed and reincubated in RPMI 1640 containing 10% fetal bovine serum at 37°C for 24 h as previously described (41). The cells containing pThx-S and pThx-A plasmid were selected by adding 500 µg/ml of Zeocin (Invitrogen) in complete medium and were used for characterization by Northern blot and slot blot analyses for expression of Thx mRNA and protein contents, respectively, as described below.

NO exposure. Confluent native PAEC monolayers and/or PAEC-expressing Thx-S and Thx-A were exposed to a continuous flow of 8.5 ppm NO gas at 95% N2-5% CO2 at 37°C for 24 h or to 10 µM NO solution prepared in O2-free Hanks' balanced salt solution (HBSS) at room temperature for 30 min as previously described (33). Controls for NO gas and NO solution were exposed to 95% N2-5% CO2 or to HBSS alone, respectively, under identical conditions (33). Exposure to NO gas for 24 h under these conditions did not alter the pH (7.4) of the culture medium. Total nitrite (after reduction of nitrate), which represents the stable products of NO/NO2 in the medium, was determined as previously described (21). Nitrite content of the medium from 24-h NO gas-exposed cells was 16.3 ± 4.2 µM (n = 8), whereas the nitrite content of the control medium before and after the 24-h exposure was not detectable. In addition, exposure of cells to 14 ppm of NO2 for 24 h or 2.5 mM each of sodium nitrite or nitrate for 4 h had no effect on the catalytic activity of eNOS (33). After exposure, cells were washed and used for the following measurements: cellular GSH and GSSG contents, catalytic activities of partially purified glutaredoxin and Thx-R, total membrane fraction eNOS, RNA isolation, Northern and Western blot analyses of Thx and Thx-R, and slot blot analysis of Thx. Determinations of GSH and GSSG content were carried out using GSH reductase and 2-vinylpyridine as described by Griffith (12) and modified by Bhat et al. (5). Protein was quantified by the method of Lowry et al. (23).

Measurement of eNOS activity. Total membrane fraction eNOS activity was measured by monitoring the formation of l-[3H]citrulline from l-[3H]arginine as previously described by Patel et al. (33). The specific activity of eNOS is expressed as picomoles l-citrulline per minute per milligram protein.

Purification and measurement of glutaredoxin activity. Control and NO-exposed PAEC glutaredoxin were purified using a method described previously by Gan and Wells (10). The catalytic activity of this purified preparation was measured using hydroxyethyl disulfide as previously reported (32). One unit of glutaredoxin activity was defined as 1 µmol NADPH oxidized/min under our assay conditions.

Measurement of Thx-R activity. Thx-R activity was measured using partially purified Thx-R from control and NO-exposed PAEC as previously described (28). The catalytic activity of Thx-R was measured by monitoring the change in absorbance at 340 nm due to oxidation of NADPH by Thx-R in the presence of Thx and insulin (28). Briefly, 200 µl of 100 mM potassium phosphate buffer containing 1 mM EDTA and 0.2 mg/ml bovine serum albumin were mixed with 10 µl of NADPH (20 mM), 30 µl of bovine insulin (80 µM), 10 µl of Thx (3 mM), and 50 µl of Thx-R sample (final volume = 300 µl). One unit of Thx-R activity was defined as 1 µmol NADPH oxidized/min at room temperature under our experimental conditions.

Western blot analysis of Thx and Thx-R. Cell lysate proteins (20 µg) from control and NO-exposed cells were separated using 7.5% SDS-PAGE and were electrophoretically transferred from gel to polyvinylidene difluoride membranes as described previously (41). To separate nonspecific protein binding, the membranes were incubated in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20 (TBST) containing 3% blot-qualified bovine serum albumin for 1 h. Subsequently, the membranes were incubated in TBST containing a 1:1,000 diluted mouse anti-Thx monoclonal antibody (kindly provided by Dr. Reen Wu, University of California, Davis, CA) or a 1:2000 diluted human anti-Thx-R monoclonal antibody (kindly provided by Dr. Garth Powis, Arizona Cancer Center) with agitation for 1 h at room temperature. After several washes with TBST, the membranes were incubated with anti-mouse IgG horseradish peroxidase-linked whole antibody (Amersham Life Sciences, Arlington Heights, IL) diluted 1:5,000 in TBST with agitation for 1 h. The membranes were then washed, and the immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham). Quantification of Thx and Thx-R was performed by scanning laser densitometry as previously described (21).

RNA isolation and Northern analysis. Total mRNA was extracted directly from control and NO-exposed cells as well as from Thx-overexpressed cells with the PolyATtract mRNA isolation system (Promega) according to the manufacturer's instructions. The glyoxal-denatured total mRNA (~10 µg/sample) and digoxigenin-labeled RNA molecular-weight marker II (1.6–7.4 kb; Boehringer Mannheim) were fractionated on a 0.8% (wt/vol) agarose gel before blotting onto nylon membranes (Zeta-Probe GT; Bio-Rad). Membranes were prehybridized in DIG-Easy-Hyb solution (Boehringer Mann-
The human Thx and Thx-R cDNAs (394 and 3.7 kb, respectively) were labeled with digoxigenin-dUTP by the random-primer method (9). The labeled probe (1 µg) was denatured by boiling for 5 min and added to 50 ml of DIG-Easy-Hyb solution. Hybridization was carried out at 43°C for 16 h. After hybridization, the blot was washed two times in 2× SSC (10× SSC = 1.5 M NaCl and 0.15 M sodium citrate)-0.1% SDS at room temperature and with 1× SSC-0.1% SDS at 65°C for 25 min. The hybridized probes were immunodetected with anti-digoxigenin-alkaline phosphatase and the chemiluminescence substrate CSPD (Genius Labeling and Detection Kits; Boehringer), as described by the manufacturer, and exposed to Kodak ZAR-5 film for 30–60 min. To document the amount of RNA loaded, the same blot was stripped two times in 0.1× SSC-0.1% SDS at 65°C for 1 h. The 18S complementary oligonucleotide (ACG GTA TCT GAT CGT CTT CGA ACC; see Ref. 40) was labeled at its 3′-end with terminal transferase by synthesis of a DNA tail of 40–50 nucleotide length with several incorporated digoxigenin-labeled dUTPs (Boehringer) as described previously (36) using a Genius labeling and detection kit. Membranes were hybridized at 50°C overnight and washed two times at 65°C in 1× SSC-0.1% SDS for 25 min. The ECL method was used to detect the hybrids under the conditions described above.

Quantification of Thx and Thx-R mRNA was performed using a laser densitometer (21). The levels of Thx and Thx-R mRNAs were standardized to the 18S mRNA contents.

Slot blot analysis of Thx protein. To confirm overexpression of Thx in PAEC, cell lysate protein (0.4, 4, and 40 µg) from Thx cDNA transfected cells in sense (pThx-S) and antisense (pThx-A) orientation were loaded onto a 9×12-cm nitrocellulose membrane (Bio-Rad). The blot was incubated in blocking solution (0.2% nonfat milk in Tris-buffered saline, 20 mM Tris-HCl, pH 7.5, and 500 mM NaCl) and then hybridized with monoclonal anti-Thx antibody, and the immunoreactive bands were visualized by the ECL detection system as described above.

Statistical analysis. Statistical significance for the effects of NO exposure on GSH and GSSG contents, on the catalytic activities of ecNOS, glutaredoxin, and Thx-R, and on Thx and Thx-R mRNA and protein expression was determined by analysis of variance and Student’s t-test (38).

RESULTS

Effect of NO on GSH and oxidized GSH contents and ecNOS, glutaredoxin, and Thx-R activities in PAEC. As shown in Table 1, exposure to 8.5 ppm NO gas for 24 h or 10 µM NO solution for 30 min did not alter GSH and oxidized GSH (GSSG) contents or the catalytic activity of glutaredoxin in PAEC. However, there were significant decreases in ecNOS and Thx-R activities in cells exposed to NO gas (P < 0.05 for both) or NO solution (P < 0.05 for ecNOS and P < 0.01 for Thx-R) compared with controls. In addition, inhibition of ecNOS and Thx-R activities in cells exposed to NO solution was almost twofold greater than that in cells exposed to NO gas.

Effect of NO on Thx- and Thx-R-specific mRNA and protein expression. As shown in a representative autoradiograph, exposure to 8.5 ppm NO gas for 24 h resulted in decreased expression of both Thx (Fig. 1A) and Thx-R (Fig. 1C) mRNAs. Densitometric analysis revealed that exposure to NO significantly diminished expression of Thx (Fig. 1B) and Thx-R (Fig. 1D) mRNAs (P < 0.001 for Thx and P < 0.05 for Thx-R) in PAEC. Similarly, exposure of PAEC to 8.5 ppm NO gas for 24 h significantly decreased the protein mass of Thx (Fig. 2, A and B) and Thx-R (Fig. 2, C and D; P < 0.05 for both). The loss of Thx mRNA expression was much greater than the loss of Thx-R mRNA expression in NO-exposed cells, whereas the loss of Thx and Thx-R protein expression in NO-exposed cells was comparable.

Overexpression of Thx in PAEC. Because NO exposure diminished Thx expression and the cellular level of Thx is critical for the regulation of redox-sensitive proteins, we focused on the role of Thx expression in NO-induced loss of ecNOS activity. As shown in Fig. 3, the transfection of human Thx cDNA resulted in a fourfold increase of expression of Thx mRNA compared with control PAEC. Similarly, Thx protein mass, as measured by slot blot analysis (Fig. 4), showed comparable increased expression.

Effect of NO on ecNOS activity in PAEC overexpressing Thx gene. To determine whether overexpression of Thx prevents NO-induced inhibition of ecNOS activity (sense) and Thx-A (antisense) cDNA transfected cells were exposed to oxygen-free water with and without 10 µM NO. Exposure of Thx-A PAEC to 10 µM NO resulted in a 70% (P < 0.05, n = 8) loss of ecNOS activity compared with the respective control (not shown). In contrast, as shown in Fig. 5, ecNOS activities in Thx-S-overexpressed cells exposed to control conditions were comparable to those in overexpressed cells exposed to NO solution.

DISCUSSION

We have recently reported that NO-induced posttranslational regulation of the catalytic activity of ecNOS...
is associated with the formation of intramolecular disulfide bonds within the ecNOS protein because exogenous addition of the disulfide-reducing enzyme Thx/Thx-R was able to restore ecNOS activity in PAEC (33). The results of the present study demonstrate for the first time that exposure of PAEC to NO gas reduces the expression of Thx and Thx-R mRNA and protein as well as the catalytic activity of Thx-R without altering GSH and GSSG contents or the catalytic activity of glutaredoxin. Overexpression of Thx protected against the NO-mediated loss of catalytic activity of ecNOS in PAEC, suggesting that the diminished expression of Thx in NO-treated cells allows for the NO-induced inhibition of ecNOS activity in these cells. These results also suggest that the redox regulatory protein Thx plays a critical role in maintaining the catalytic activity of ecNOS.

Thiol-disulfide oxidoreductases such as Thx and Thx-R are well recognized for their role in the cellular defense against oxidative stress in general and for reduction of intramolecular disulfides in a variety of enzymes, transport proteins, and receptors (15, 16, 31, 33, 34). Exposure to physiologically relevant concentrations of NO is associated with the loss of selective protein function by at least two distinct mechanisms. First, NO can interact with allosteric thiols in the protein, resulting in intramolecular disulfide formation and altered protein function. We have previously reported that NO can inhibit ecNOS activity in porcine PAEC by this posttranscriptional mechanism (31–33). Second, NO can also regulate protein function by...
acting at the transcriptional level. The NO-induced loss of Thx and Thx-R mRNA and protein expression observed in this study may represent an example of this second mechanism. Diminished activity of Thx-R can occur posttranslationally by direct action of NO with active-site cysteines in this protein. Similarly, interaction with allosteric thiols of proteins that regulate transcriptional and/or translational processes may indirectly reduce expression of Thx and Thx-R mRNA and protein contents. Although NO is known to S-nitrosylate a number of proteins, cellular GSH and GSSG contents were not altered by the concentrations of NO used in our study. This is consistent with previous observations that NO does not directly react with GSH under physiological conditions (39) and that the loss of cellular GSH content requires an excess of 50 µM NO (6). The precise mechanism responsible for the decreased expression of Thx and Thx-R expression in PAEC exposed to NO is currently being examined.

In summary, we have shown that exposure to NO can diminish expression of a critical redox regulatory enzyme system, Thx and Thx-R, and that increased expression of Thx can prevent NO-induced loss of redox-sensitive enzymes such as ecNOS. The implications of these observations are significant for a number of other redox-sensitive proteins critical for cellular processes such as DNA synthesis and gene expression.

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