Cell-Surface Protein Disulfide Isomerase Is Required for Transnitrosation of Metallothionein by S-Nitroso-Albumin in Intact Rat Pulmonary Vascular Endothelial Cells

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S-nitrosation of the metal binding protein, metallothionein (MT) appears to be a critical link in affecting endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS)-derived nitric oxide (NO)-induced changes in cytoplasmic and nuclear labile zinc, respectively. Although low molecular weight S-nitrosothiols also appear to affect this signaling system, less is known about the ability of extracellular protein nitrosothiols to transnitrosate MT. Accordingly, we synthesized fluorescently labeled S-nitroso-albumin (SNO-albumin, a major protein S-nitrosothiol in plasma) and determined, *via* confocal microscopy in fixed tissue, that it is transported into cultured rat pulmonary vascular endothelial cells in a temperature sensitive fashion. The cells were transfected with an expression vector that encodes human MT-IIa cDNA sandwiched between enhanced cyan (donor) and yellow (acceptor) fluorescent proteins (FRET-MT) that can detect conformational changes in MT through fluorescence resonance energy transfer (FRET). SNO-albumin and the membrane-permeant low molecular weight S-nitroso-L-cysteine ethyl ester (L-SNCEE) caused a conformational change in FRET-MT as ascertained by full spectral laser scanning confocal microscopy in live rat pulmonary vascular endothelial cells, a result which is consistent with transnitrosation of the reporter molecule. Transnitrosation of FRET-MT by SNO-albumin, but not L-SNCEE, was sensitive to antisense oligonucleotide–mediated inhibition of the expression of cell surface protein disulfide isomerase (csPDI). These results extend the original observations of Ramachandran *et al.* (Ramachandran N, Root P, Jiang XM, Hogg PJ, Mutus B. Proc Natl Acad Sci U S A 98: 9539–9544, 2001) and suggest that csPDI-mediated denitrosation helps to regulate the ability of the major plasma NO carrier (SNO-albumin) to transnitrosate endothelial cell molecular targets (e.g. MT). Exp Biol Med 231:1507–1515, 2006

Key words: metallothionein; fluorescence resonance energy transfer; S-nitroso-albumin; transnitrosation; cell surface protein disulfide isomerase; pulmonary endothelium

Introduction

S-nitrosation of the metal binding protein, metallothionein (MT) appears to be a critical link in affecting nitric oxide (NO)-induced changes in the cytoplasmic (1) and nuclear (2) labile zinc pool. *In vitro* Raman spectroscopy and fluorometric assay studies of exposure of authentic MT to NO gas (3) or low molecular weight nitrosothiols (4), respectively, revealed S-nitrosation of MT. The predicted unfolding of MT after S-nitrosation has led us (5) and others (6) to devise genetic and chemical fluorescence resonance energy transfer (FRET)-based strategies, respectively, to follow these reactions in intact cells.

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Collectively, the latter two studies provide indirect evidence that gaseous NO, endothelial nitric oxide synthase (eNOS)-derived NO, and small molecular weight nitrosothiols (S-nitroso-glutathione, -cysteine, or -acetylpenicillamine) all resulted in S-nitrosation of MT and metal release from MT (1, 5–8). Nonetheless, the effect of protein S-nitrosothiols on MT has not been investigated. Protein S-nitrosothiols such as S-nitroso-albumin (SNO-albumin) may act as significant extracellular NO reservoirs in health and disease (9, 10). As with low molecular weight nitrosothiols, the capacity of extracellular protein nitrosothiols to participate in intracellular transnitrosation is affected by a number of factors (11). It has been suggested that cell surface protein disulfide isomerase (csPDI) acts as a critical cell surface cysteine reductase that affects the activity of SNO-albumin (12). Accordingly, we examined the effect of genetically silencing csPDI in cultured rat pulmonary artery and microvascular endothelial cells (RPAEC and RPMEC, respectively) on the transnitrosation of MT (using a FRET-based MT reporter molecule, FRET-MT) by SNO-albumin and S-nitroso-L-cysteine ethyl ester (L-SNCEE), a membrane permeant, low molecular weight nitrosothiol (13). Our results show that exposure of both RPAEC and RPMEC to SNO-albumin or L-SNCEE results in transnitrosation of FRET-MT, but only SNO-albumin–mediated transnitrosation is sensitive to inhibition of csPDI by antisense oligonucleotides. This suggests that extracellular csPDI-mediated denitrosation of SNO-albumin, the major plasma NO carrier, regulates its ability to transnitrosate endothelial cell molecular targets (e.g. metallothionein). By removing NO from SNO-albumin at the plasma membrane, csPDI may minimize promiscuous transnitrosation of intracellular targets.

Materials and Methods

Endothelial Cell Cultures. RPAEC were cultured as previously described (14) and grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). RPMEC (Vascular Endothelial Cell Company, VEC Technologies, Rensselaer, NY) were grown in flasks, chamber slides, or Petri dishes precoated with sterile 0.1% gelatin in phosphate buffered saline (PBS) and maintained in MCDB-131 complete medium (Vascular Endothelial Cell Company) containing DMEM supplemented with 5% FBS, endothelial cell growth factor, heparin, 50 U/ml penicillin, and 50 μg/ml streptomycin in humidified 5% CO_2, 95% room air at 37°C. Both RPAEC and RPMEC were characterized by their uptake of acetylated low density lipoprotein (LDL), von Willebrand factor (vWF), and expression of platelet/endothelial cell adhesion molecule (PECAM).

Drugs and Reagents. All drugs and reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise. The RNA sequence for PDI (rat PDI cDNA) was obtained from the National Center for Biotechnology Information protein database library (NM_012998). Bases 124–146 of the coding sequence were used to design the antisense oligodeoxynucleotide (ODN) sequence. The antisense ODN (5′-GCTCTTCTTCAGCAC-CAGGACGT-3′) to rat PDI mRNA was synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Alexa Fluor 488–conjugated goat anti-mouse IgG antibody and Alexa Fluor 488 bovine serum albumin conjugate were purchased from Molecular Probes (Eugene, OR). The monoclonal anti-mouse PDI IgG antibody was from Stressgen Biotech, Inc. (San Diego, CA). FRET-MT (1) and L-SNCEE (8) were synthesized as previously described.

Preparation of SNO-Albumin-Cy3. We first labeled human albumin (fatty acid–free) with fluorescent Cy3 monofunctional N-hydroxysuccinimide (NHS) ester at a molar dye to protein ratio of 40:1 under alkaline conditions (pH 7.5–8.5). Microcon 30,000 cutoff centrifuge filter tubes (Millipore, Billerica, MA) were used for recovering the albumin-Cy3 complex. We then generated reduced Cy3-albumin-SH by reaction with equimolar amounts of dithiothreitol (DTT) and diethylenetriaminepentaacetic acid (DTPA). SNO-albumin-Cy3 was then prepared through a transnitrosation reaction with S-nitroso-glutathione (GSNO). GSNO was prepared in a reaction of acidified sodium nitrite ($NaNO_2$) with glutathione (GSH). Typically, 100 m*M* GSH was mixed with 100 m*M* $NaNO_2$ in 20 m*M* hydrochloric acid (HCl) at room temperature. The final dye to protein ratio was determined by measuring the absorbance of Cy3 at 552 nm and calculating the fluorophore concentration using the appropriate extinction coefficient (molar extinction coefficients of 150,000 $M^{-1}cm^{-1}$ at 552 nm for the Cy3 dye and 170,000 $M^{-1}cm^{-1}$ at 280 nm for the protein).

The content of S-nitrosothiols was determined fluorometrically using 4,5-diaminofluorescein (DAF-2) as a specific probe for NO (15). A typical sample contained a mixture of albumin-rich fraction 2 of plasma (50 μl), 1 μl DAF-2 (844 μ*M*), and 150 μl PBS, pH 7.4. To release NO from S-nitrosothiols, samples were irradiated with UV light (Oriel UV light source, model 66002; Pittsburgh, PA) using a cutoff filter (Balzers, Pittsburgh, PA) of >330 nm for 10 min (80 $\mu W/cm^2$). Prior experiments showed that under these conditions all NO is released from human serum albumin (16). Because proteins have been reported to interfere with fluorescence-based assays of S-nitrosothiols (17), after irradiation, samples (200 μl) were heated at 80°C for 5 min and then mixed with chloroform (50 μl) and centrifuged at 10,000 *g* for 5 min to remove proteins; the resulting supernatant was used for measurements. Fluorescence intensity of DAF-2 was measured at by excitation at 495 nm and emission at 515 nm (slits 5.0 nm and 5.0 nm, respectively) using a Shimadzu RF-5301 spectrofluorometer (Kyoto, Japan). A solution of GSNO was used for calibration of the DAF-2 assay as described (15). The concentration of GSNO was determined photometrically using a molar extinction coefficient of 900 $M^{-1}cm^{-1}$ at 336 nm (18).

Cellular PDI Determination by Fluorescence Immunoassay and Western Blot Assay. RPAEC and RPMEC were passaged into Lab-Tek II 8-well chamber slides (Invitrogen, Grand Island, NY) into 10% FBS-DMEM and cultured for 24 hrs to 50% confluence. Cells were transfected with PDI and 0.3 μ*M* or 1.2 μ*M* antisense ODN in serum-free Opti-MEM (Invitrogen) and cultured 3 hrs at 37°C. Media was then removed and replaced with normal culture medium (10% FBS-DMEM). Transfected cells were incubated for another 24 hrs at 37°C, washed with PBS (pH 7.4), and fixed with 2% paraformaldehyde for 20 mins at room temperature. Cells were then incubated for 60 mins at room temperature with a blocking buffer containing 1x PBS, 0.1% Triton X-100, and 10% goat serum. Cells were incubated with the primary antibody (anti-mouse monoclonal PDI IgG antibody diluted 1:200) for 60 mins at room temperature. Cells were then washed three times with PBS that contained 0.05% Tween-20 (PBS-T) for 30 mins, incubated with the secondary antibody (Alexa Fluor 488–labeled goat anti-mouse IgG antibody, used at 1:1000) for 60 mins with light protection, and subsequently washed three times with PBS-T for 30 mins. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; 1 μg/ml) for 30 secs and washed three times with PBS. The cells on the slides were sealed with Fluoromount-Gand (Fisher Scientific, Pittsburgh, PA) examined by fluorescence microscopy at ×60 magnification. Data were analyzed with MetaMorph Imaging Series 5.0 software. Total cellular PDI was estimated by Western blot using the method described by Jiang *et al.* (19).

FRET Detection (8). RPAEC and RPMEC were bathed in Hanks balanced salt solution (Gibco/BRL, Invitrogen) containing 100 μ*M* EDTA to chelate trace amounts of metal ions in the buffer and prevent extracellular decomposition of SNO-albumin, and imaged at 37°C using a thermocontrolled stage insert (Harvard Apparatus Inc., Holliston, MA). Images were obtained with a ×40 oil immersion lens at 512 × 512 pixels using the confocal-based Zeiss spectral imaging system (LSM510 META; Carl Zeiss, Jena, Germany). Cyan fluorescent protein was excited at 458 nm (HFT 458 filter). Resolved fluorescence spectra at each pixel were detected by an array of eight spectrally-separate photomultiplier tube elements within the META detection head and recorded on a voxel-by-voxel basis during scanning to generate a set of images, each corresponding to the fluorescence wavelength resolved at 10 nm intervals. Color separation of cyan and yellow fluorescent protein emission spectra was determined from the resolved image using a linear unmixing algorithm based on reference spectra obtained in cells expressing only cyan or yellow fluorescent protein. Images were collected at baseline and at intervals up to 15 mins after addition of either SNO-albumin or L-SNCEE. In separate control experiments, FRET was confirmed by acceptor photobleaching (data not shown).

Results

Uptake of SNO-Albumin by RPAEC. Since human serum albumin has only one cysteine (available for nitrosation), synthesis of fluorescently-labeled SNO-albumin required a strategy to attach the fluorophore at a non-cysteine site. We ultimately targeted lysine residues and used Cy3 conjugation to facilitate multicolor fluorescence detection with Alexa Fluor 488–conjugated albumin. In Figure 1, we note that SNO-albumin-Cy3 (red vesicles, middle panel) was taken up in a pattern similar to that of Alexa Fluor 488–albumin (green vesicles, left panel). Indeed, overlay of the two images shows a considerable degree of colocalization (yellow vesicles, right panel). The image shown was taken after RPAEC were incubated with a mixture of Alexa Fluor 488–albumin (50 μg/ml) and SNO-albumin-Cy3 (50 μg/ml) in the presence of bovine serum albumin (5 mg/ml) for 15 mins at 37°C and then fixed in 2% paraformaldehyde. In Figure 2, we note that uptake of SNO-albumin-Cy3 is temperature sensitive (as is the case for Alexa Fluor 488–albumin; data not shown). In particular, as in Figure 1, the fluorophore demonstrated a punctate distribution in the cytoplasm (e.g. outside the nuclear signal of DAPI; blue) of RPAEC at 37°C (Fig. 2, right panel) that was not apparent at 4°C. We used scanning laser confocal and multiphoton microscopy to provide further evidence that the fluorophore was taken up into RPAEC at 37°C (data not shown). Collectively, these approaches suggest that SNO-albumin is taken up by cells in a process similar to that of native albumin; however, the fluorescence assays used do not allow us to conclude that NO remains with SNO-albumin during our experiment.

csPDI and Transnitrosation of FRET-MT in Live Rat Pulmonary Endothelial Cells. RPAEC were exposed to antisense ODN against rat csPDI for approximately 24 hrs. Immunofluorescence of fixed (2% paraformaldehyde) cells revealed a dose dependent silencing of csPDI expression with both 0.3 μ*M* and 1.2 μ*M* oligonucleotides (Fig. 3). A separate group of RPAEC were treated in a similar fashion, lysed, and proteins analyzed by Western blot analysis (Fig. 4, left panel). Relative expression of PDI (normalized to actin) decreased by almost a factor of 10 with the higher concentration of antisense ODN (figure 4, right panel). Accordingly, we then performed experiments on the ability of nitrosothiols to transnitrosate FRET-MT in live RPAEC and RPMEC treated with the higher dose of PDI antisense ODN. Both RPAEC and RPMEC were infected, as previously described (5), with an adenovirus (multiplicity of infection [MOI] of 50:1) that contains cDNA for a fluorescent chimeric protein comprising the 6 kDa MT sandwiched between enhanced cyan and yellow fluorescent proteins (FRET-MT). We recently described (8) the application of a laser scanning confocal microscope capable of acquiring full spectral reports and have used this system to contrast spectra after application of SNO-albumin or L-SNCEE

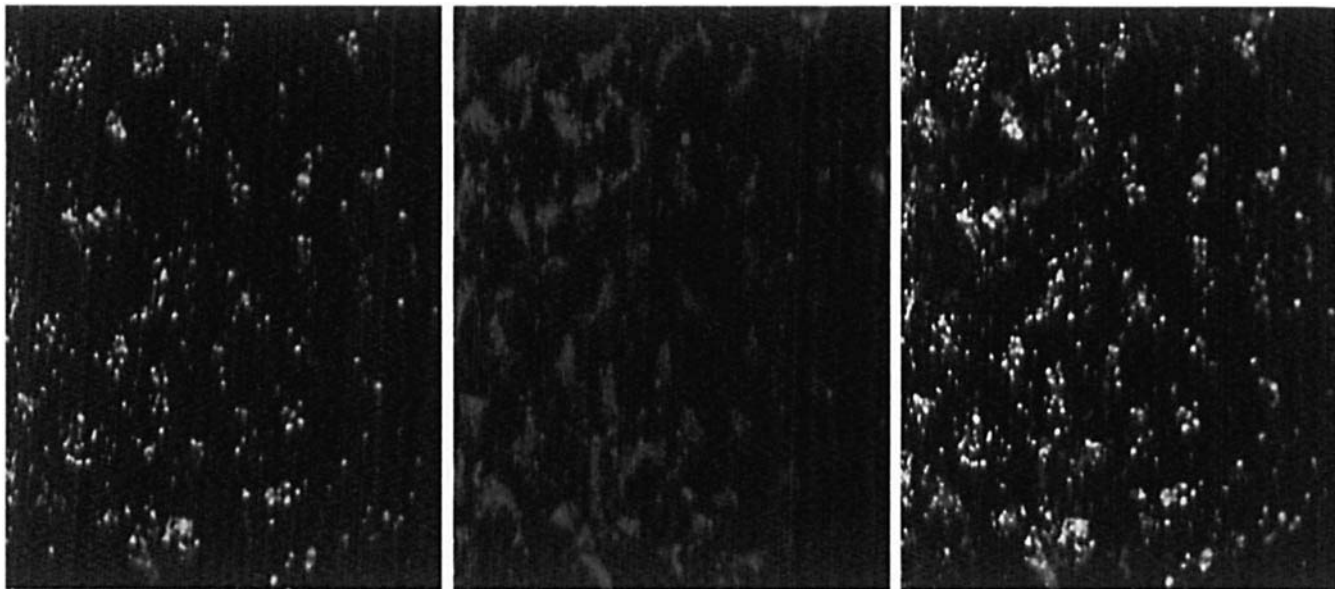

Figure 1. Co-uptake of Alexa 488–albumin and SNO-Albumin-Cy3 by RPAEC. Fluorescent images of Alexa 488–albumin (green vesicles, left panel), SNO-albumin-Cy3 (red vesicles, middle panel), and an overlay of their colocalization (yellow vesicles, right panel) are shown. This image was obtained by confocal microscopy after cells were incubated with a mixture of Alexa 488–albumin (50 μg/ml) and SNO-albumin-Cy3 (50 μg/ml) in the presence of bovine serum albumin (5 mg/ml) for 15 mins at 37°C and then fixed in 2% paraformaldehyde.

(with or without antisense arrest of csPDI). Figure 5 shows representative spectral reports for a single RPAEC before and after application of SNO-albumin and L-SNCEE. SNO-albumin caused a decrease in the acceptor signal (yellow, approximately 525 nm) and an unquenching of the donor (cyan, approximately 485 nm) in cells not treated with antisense ODN (Fig. 5A). This response was not observed in RPAEC treated with 1.2 μ*M* antisense ODN to csPDI (Fig. 5B). L-SNCEE produced a change in FRET-MT similar to SNO-albumin under control conditions (Fig. 5C). Unlike SNO-albumin, however, L-SNCEE–induced changes in FRET-MT were unaffected in RPAEC treated with antisense ODN to csPDI (Fig. 5D). Similar effects were noted in RPMEC (Fig. 6). These

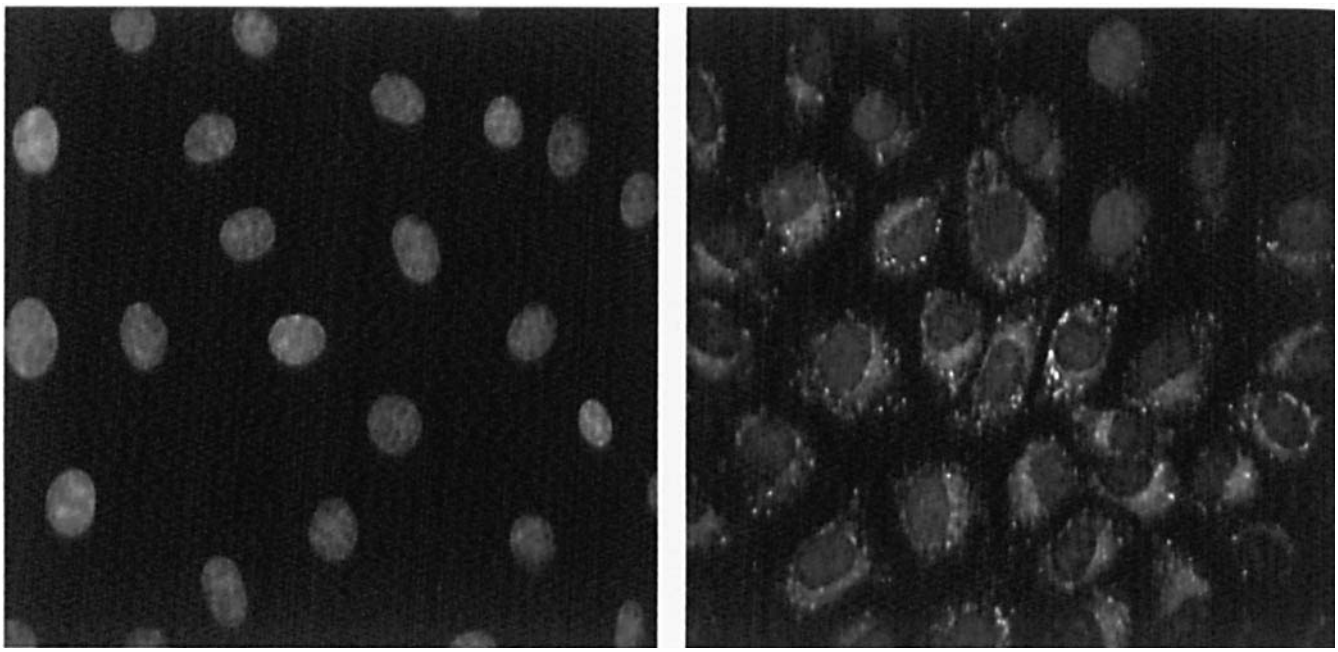

Figure 2. Temperature-dependent uptake of SNO-albumin-Cy3 by RPAEC. The effect of temperature on uptake of SNO-albumin-Cy3 by RPAEC is shown. Uptake of SNO-albumin-Cy3 (50 μg/ml) was performed at 4°C or 37°C for 15 mins and then fixed in 2% paraformaldehyde. The absence of vesicle fluorescence (left panel) confirms the lack of SNO-albumin-Cy3 uptake at 4°C. The right panel shows SNO-albumin-Cy3 uptake at 37°C. Cell nuclei were stained with DAPI.

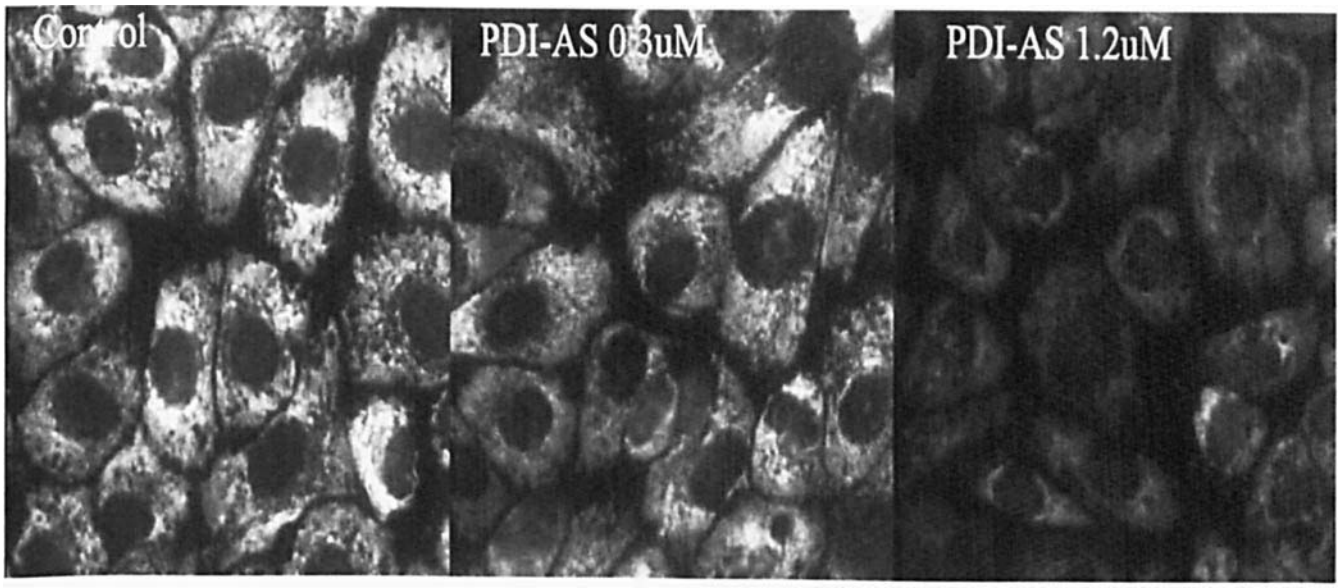

Figure 3. Suppression of csPDI expression with antisense ODN in RPAEC. Cells were transfected with 0.3 μ*M* or 1.2 μ*M* antisense ODN, or without ODN as a control, in serum-free Opti-MEM for 3 hrs and, after another 24 hrs incubation in 10% FBS-DMEM, fixed with 2% paraformaldehyde. Cells were treated with primary antibody (anti-mouse monoclonal PDI IgG, diluted 1:200) and secondary antibody (Alexa Fluor 488–labeled goat anti-mouse IgG, diluted 1:1000). Slides were examined by fluorescence microscopy at ×60 magnification.

experiments were repeated in multiple cells from two separate subcultures of RPMEC, and we observed that SNO-albumin caused a 16 ± 3% decrease in FRET-MT that was completely reverted (2 ± 2% decrease in FRET-MT) in the presence of antisense ODN to csPDI; L-SNCEE caused a 30 ± 1% decrease in FRET-MT compared with the control, and still caused a 21 ± 8% decrease in the presence of antisense ODN to PDI.

Discussion

In addition to the classical paracrine/autocrine function of locally produced NO, it is now apparent that NO transnitrosates a number of components in plasma and some of these reaction products may serve to prolong and redirect the action of NO in an endocrine fashion (10). Furthermore, in addition to the well known soluble guanylyl cyclase/cGMP–mediated effects of NO, considerable attention has

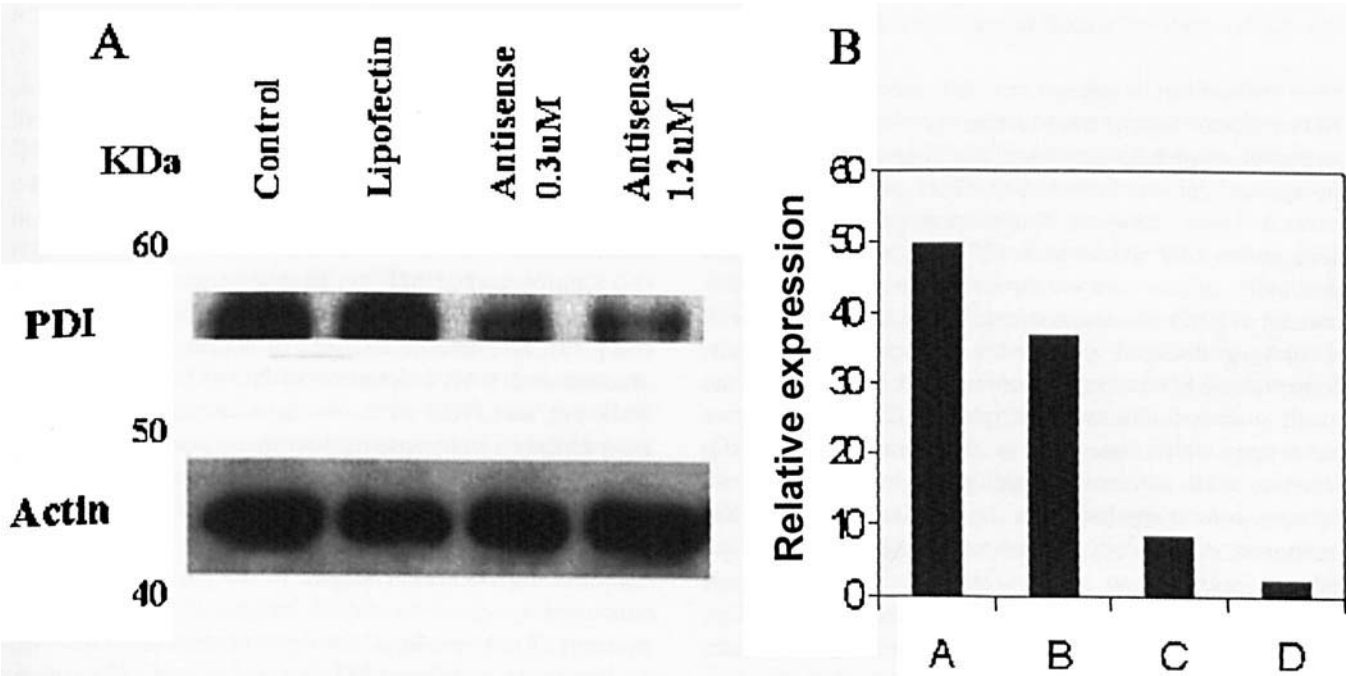

Figure 4. Western blot analysis of total cellular PDI protein levels in RPAEC transfected with antisense ODN. (A) Lane 1, control cells. Lane 2, cells treated with transfection reagent (Lipofectin; Invitrogen, Carlsbad, CA) without ODN. Lanes 3 and 4, cells transfected with 0.3 μ*M* and 1.2 μ*M* antisense ODN, respectively. (B) The amount of cellular PDI after normalization with actin.

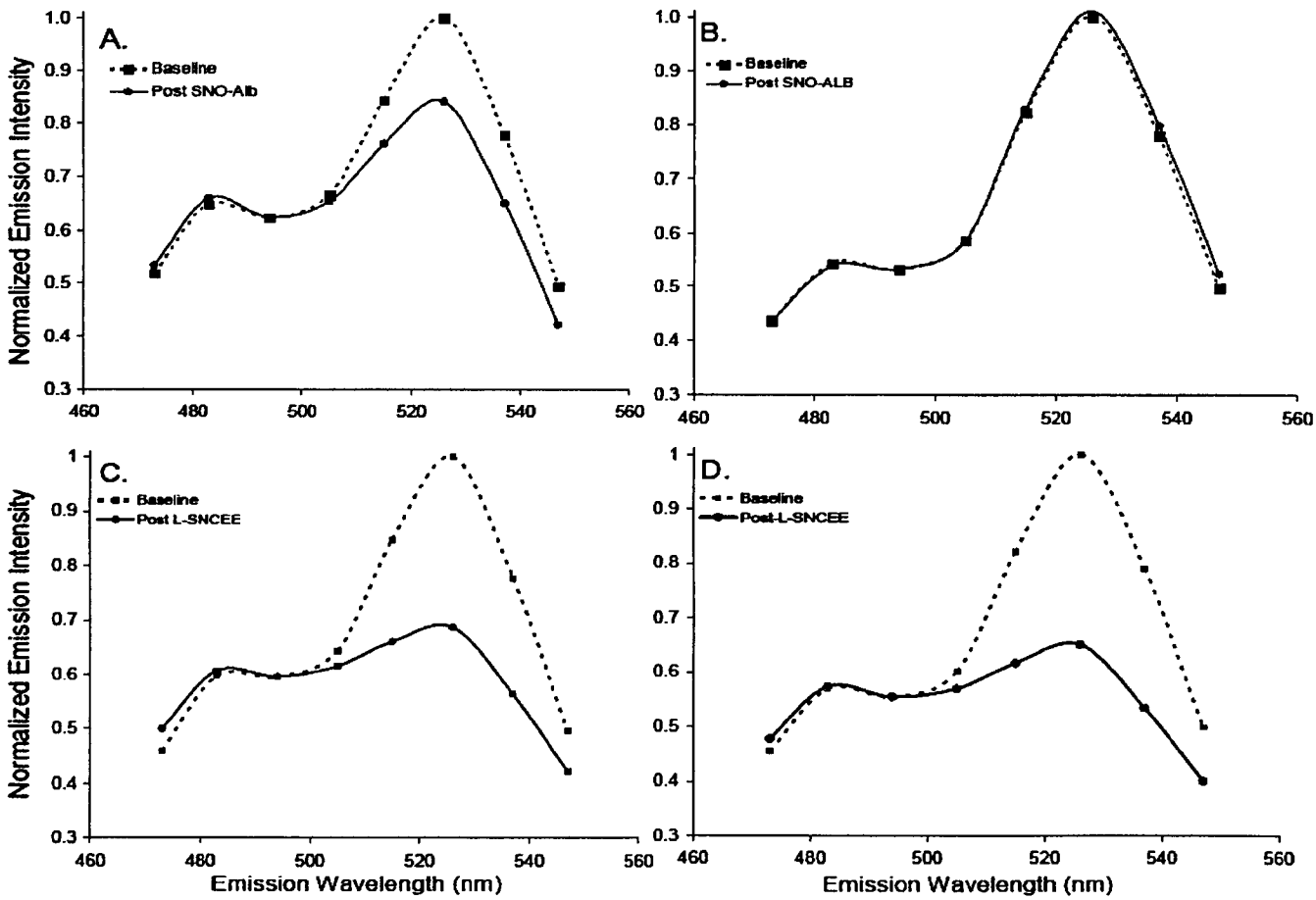

Figure 5. Spectral FRET-MT changes after exposure of RPAEC to S-nitrosothiols in real time. RPAEC were infected with an adenovirus (MOI: 50:1) that carries cDNA that encodes a chimeric fluorescent protein consisting of the 6 kDa heavy metal binding protein, MT, sandwiched between enhanced cyan and yellow fluorescent proteins (FRET-MT). Full spectral reports of FRET-MT were recorded by laser scanning confocal microscopy. (A) The effect of SNO-albumin in control cells. (B) The effect of SNO-albumin in cells treated with 1.2 μM antisense ODN to PDI. (C) The effect of L-SNCEE in control cells. (D) The effect of L-SNCEE in cells treated with 1.2 μM antisense ODN to PDI.

been focused on the process of S-nitrosation and its role in NO-mediated signal transduction (20). Candidate NO-interacting molecules in blood that may account for NO storage and delivery include nitrite (21), iron-nitrosyls (22), nitrated lipids (23) and S-nitrosated proteins including hemoglobin (24) and albumin (25). As the specificity and sensitivity of our assays improve, the absolute concentrations of SNO-albumin continue to decrease in plasma of normal or diseased patients or experimental animals. Nonetheless, SNO-albumin continues to appear to be the major protein S-nitrosothiol in plasma (25). As such, we were particularly interested in the interaction of SNO-albumin with pulmonary vascular endothelium and its potential role in regulating the S-nitrosation of intracellular molecules. Accordingly, we report that SNO-albumin-Cy3 behaves similarly to Alexa 488–albumin in that both fluorophores are taken up in a temperature-sensitive (Fig. 2), vesicle-like pattern (Fig. 1) into rat pulmonary vascular endothelial cells. Nonetheless, it appears that the biologically-important fraction of NO in SNO-albumin that is responsible for S-transnitrosation of MT in intact RPAEC and RPMEC is denitrosated by csPDI (Figs. 5 and 6). In this regard, our findings confirm observations by Ramachandran *et al.* (12), in which they used quenching of intracellular N-dansylhomocysteine as a reporter for nitrosation to reveal a transnitrosation role for csPDI in human umbilical vein endothelium.

S-nitrosation of MT has been described *in vitro* with various structural approaches including Raman spectroscopy (3), fluorometric analyses of copper-mediated denitrosation and DAF-2 detection of NO (4), cadmium (Cd) NMR (7), and FRET (26). We have described an indirect assay based on a chimeric reporter that encodes human MT-IIa sandwiched between donor (cyan) and acceptor (yellow) enhanced fluorescent proteins that is transfected into pulmonary endothelial cells (5). Changes in MT conformation are detected as changes in FRET efficiency, as monitored by narrow bandwidth imaging (5) or full spectral reporting (8). As predicted from our original study (27) on the interaction of NO and MT, S-nitrosation of MT requires oxygen (28), is suppressed by reduced GSH (29), and is stereoselective for small molecular weight S-nitrosothiols

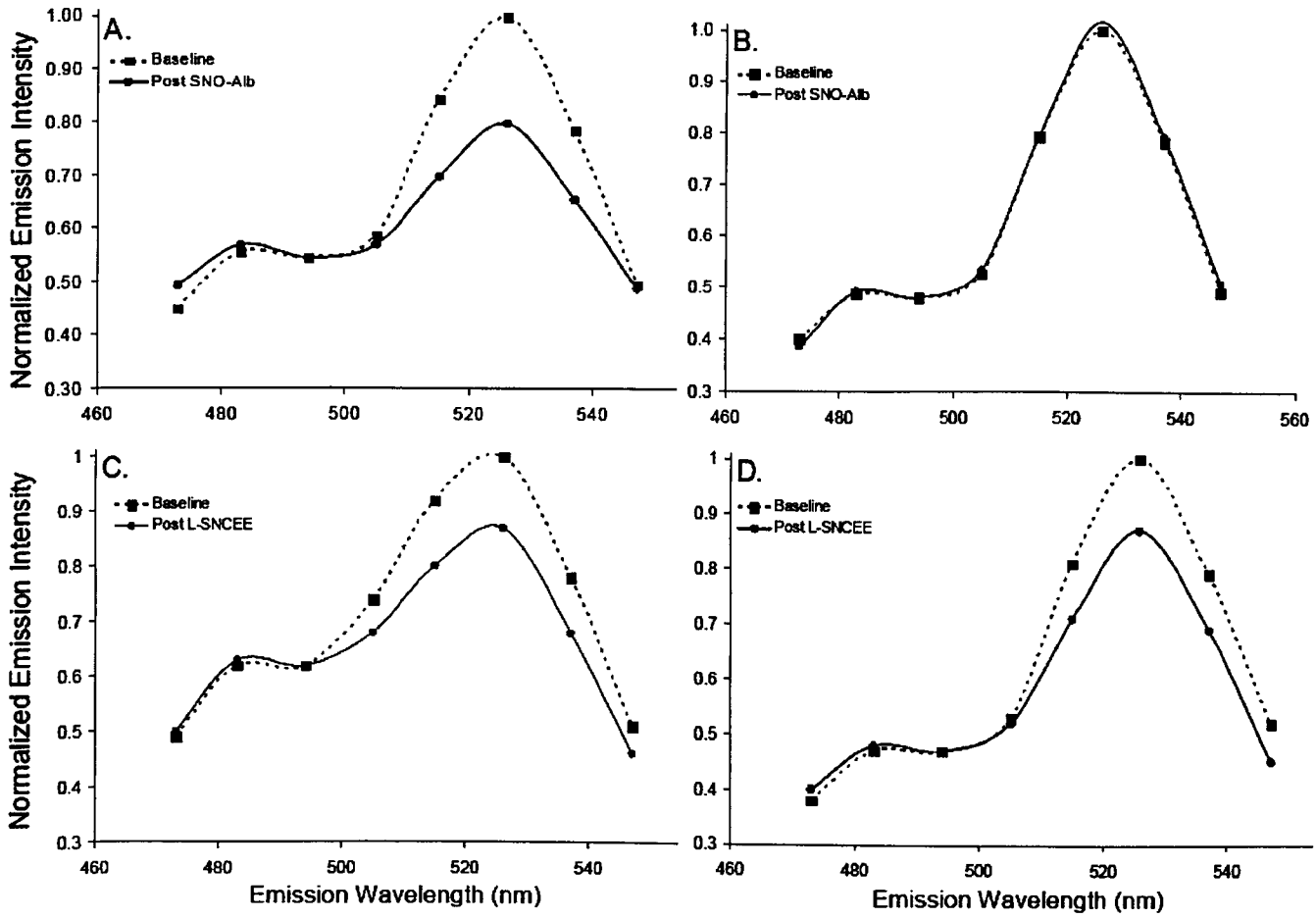

Figure 6. Spectral FRET-MT changes after exposure of RPMEC to S-nitrosothiols in real time. RPMEC were infected with an adenovirus (MOI: 50:1) that carries cDNA that encodes a chimeric fluorescent protein consisting of the 6 kDa heavy metal binding protein, MT, sandwiched between enhanced cyan and yellow fluorescent proteins (FRET-MT). Full spectral reports of FRET-MT were recorded by laser scanning confocal microscopy. (A) The effect of SNO-albumin in control cells. (B) The effect of SNO-albumin in cells treated with 1.2 μM antisense ODN to PDI. (C) The effect of L-SNCEE in control cells. (D) The effect of L-SNCEE in cells treated with 1.2 μM antisense ODN to PDI.

(8). The α-domain of MT is more facile than the β-domain in releasing metals in response to S-nitrosation (7), and MT-III appears more sensitive to metal release after NO exposure than either MT-I or MT-II (6). Metal occupancy is critical in that >10 moles of copper per mole MT are required for NO to release a sufficient amount of copper from MT to regenerate the activity of apo-superoxide dismutase (30). Based on studies with cells from MT-null mice, a critical role for MT has been recognized for eNOS-derived NO and cytoplasmic zinc release (1) and iNOS-derived NO and nuclear zinc release (2). Other important implications for a role of MT S-nitrosation in cell biology include NO-mediated cadmium (31), zinc (32), and copper (4) toxicity, NO-mediated expression of MT (33, 34), and NO-mediated zinc release and reduced sensitivity to lipopolysaccharide (LPS) in pulmonary artery endothelial cells (35).

The chemical biology and synthesis of S-nitrosated compounds remains largely conjectural. Indeed, in spite of the common use of S-nitrosothiols as NO donors, relatively little information is available on their metabolic fate in various cell types and physiological conditions. The chemistry of S-nitrosothiols in biological systems remains ill-defined (36), but critical requirements for the formation of low molecular weight thiols include the presence of extracellular cysteine and cysteine transporters and intracellular glutathione and formaldehyde dehydrogenase (11). Although direct interaction of the free radical, NO, with thiols is possible, most current research efforts have focused on the formation of S-nitrosothiols from nitrogen oxide intermediates such as nitrosonium ion and dinitrogen trioxide (N_2O_3; 37). Accordingly, transnitrosation is likely to involve multiple chemical steps and intense interest has been directed toward revealing enzymes, accessory proteins, and other molecules and redox active components to account for the sensitivity, specificity, and spatiotemporal regulation of this posttranslational modification. In this regard, csPDI has emerged as a candidate enzyme in catalyzing transnitrosation and regulating the entry of NO into cells (38). PDI is primarily located in the endoplasmic reticulum but also is present elsewhere, including the plasma membrane (39). The low pKa of its active site and

its well known function in thiol-disulfide exchange led Zai *et al.* (38) to hypothesize its involvement in transnitrosation. Indeed they reported that entry into human erythroleukemia cells of NO derived from S-nitroso-glutathione, which leads to activation of intracellular soluble guanylyl cyclase, involves csPDI. Subsequently, Ramachandran *et al.* (12) utilized the fluorescent properties of N-dansylhomocysteine to reveal nitrosation and denitrosation in intact cells and to demonstrate a role for csPDI in the transfer of NO from extracellular S-nitrosothiols, including SNO-albumin, into cultured endothelial cells. They went on to modify membrane redox status with α-tocopherol and from these experiments postulated that csPDI catalyzes the removal of NO from SNO-albumin at the surface of cells, which leads to the formation of N_2O_3 (in the presence of molecular oxygen) and subsequent NO entry and intracellular transnitrosation. Our results are consistent with their findings and provide the first demonstration of a role for csPDI in facilitating the entry of NO derived from large molecular weight protein S-nitrosothiols and the transnitrosation of a critical endogenous target, MT.

Although the absolute level of plasma SNO-albumin is a subject of considerable debate (10), it is apparent that it increases under various pathological conditions (9, 16), can deliver NO from the lungs to the systemic circulation (40), and act as a potent venodilator in rats (41), among other effects. Reduced native albumin has been shown to be removed from the circulation by a process of endothelial cell receptor–mediated endocytosis (42), and nitrated albumin may be a substrate for similar processes (43). Because of the complexities of its metabolism, the fluorescent approaches we utilized to follow the translocation of SNO-albumin in cell culture do not allow us to distinguish between its transcytosis or denitrosation at the membrane surface followed by transcytosis of the remaining reduced albumin precursor. The similarities between SNO-albumin-Cy3 and Alexa 488–albumin translocation (Fig. 1) and temperature sensitivity (Fig. 2) suggest common pathways of uptake, but cannot distinguish if the NO adduct remains on albumin following translocation into cells or is effectively removed beforehand. The fact that downregulation of csPDI by antisense ODN (Figs. 3 and 4) inhibited SNO-albumin transnitrosation of MT (Figs. 5 and 6) is consistent with the latter possibility and is in line with the schema presented by Ramachandran *et al.* (12). This is further supported by the lack of effect of inhibition of csPDI on the transnitrosation of MT upon exposure of RPAEC (Fig. 5) and RPMEC (Fig. 6) to the membrane permeant, low molecular weight S-nitrosothiol, L-SNCEE (8, 13). Nonetheless, without knowing the chemical intermediates that result from csPDI-mediated denitrosation of SNO-albumin, it is difficult to assess the impact of csPDI on SNO-albumin–mediated intracellular events *in situ*. It is possible, as suggested by Ramanchandran *et al.* (12), that reduction of SNO-albumin by csPDI liberates NO that reacts with molecular oxygen and facilitates transnitrosation through the formation of an N_2O_3 intermediate. Alternatively, as we recently reported (44) for thioredoxin as a nitrosothiol reductase, it is possible that csPDI-mediated denitrosation of SNO-albumin to its reduced state results in concomitant generation of nitroxyl, a nitrogen monoxide with considerably different biochemical properties than NO. In this context, it is possible that csPDI actually limits the capacity of SNO-albumin to transnitrosate intracellular targets and thereby minimizes promiscuous transnitrosation events that might occur if SNO-albumin is readily taken up (as is the case for native reduced albumin) by endothelial cells (42). Regardless, it is apparent that the extracellular large molecular weight protein-nitrosothiol, SNO-albumin, in conjunction with csPDI, can potentially have significant biological effects in the endothelium, including nitrosation of MT and potential alterations in labile zinc.

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