Basal and Stimulated Protein S-Nitrosylation in Multiple Cell Types and Tissues*

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There is substantial evidence that protein S-nitrosylation provides a significant route through which nitric oxide (NO)-derived bioactivity is conveyed. However, most examples of S-nitrosylation have been characterized on the basis of analysis in vitro, and relatively little progress has been made in assessing the participant forms of nitric-oxide synthase (NOS) or the dynamics of protein S-nitrosylation in situ. Here we utilize antibodies specific for the nitrosothiol (SNO) moiety to provide an immunohistochemical demonstration that protein S-nitrosylation is coupled to the activity of each of the major forms of NOS. In cultured endothelial cells, SNO-protein immunoreactivity increases in response to Ca²⁺-stimulated endothelial NOS (eNOS) activity, and in aortic rings, endothelium-derived and eNOS-mediated relaxation (EDRF) is coupled to increased protein S-nitrosylation in both endothelial and associated smooth muscle cells. In cultured macrophages, SNO-protein levels increase upon cytokine induction of induced NOS (iNOS), and in PC12 cells, increased protein S-nitrosylation is linked to nerve growth factor induction of neuronal NOS (nNOS). In addition, we describe developmental and pathophysiological increases in SNO-protein immunoreactivity within human lung. These results, which demonstrate Ca²⁺-, neurotransmitter, growth factor, cytokine, and developmental regulation of protein S-nitrosylation that is coupled to NOS expression and activity, provide unique evidence for the proposition that this ubiquitous NO-derived post-translational protein modification serves as a major effector of NO-related bioactivity.

Nitric oxide (NO),¹ generated by cell type-specific NO synthases (NOSs), has classically been characterized as a freely diffusible intercellular messenger that functions in target cells to subserve NOS-dependent signaling, which includes generation of endothelium-derived relaxing factor (EDRF) via eNOS, synaptic transmission and plasticity via nNOS, and antimicrobial activity via iNOS (see Ref. 1 for review). More recently, it has been proposed that S-nitrosylation of cysteine thiols may constitute a major route of NO trafficking through which NO-related bioactivity is effected, serving as a ubiquitous post-translational modification that regulates dynamically a broad functional spectrum of proteins (2–4). However, the analysis of protein S-nitrosylation in situ, originating with endogenous NOS activity, has been impeded by substantial technical barriers, and there is little direct evidence for cellular protein S-nitrosylation that can be ascribed specifically to the activity of any NOS isoform (4–7). Here we show that S-nitrosylated proteins can be identified, on membrane blots and with immunohistochemistry, by antibodies that recognize the SNO moiety. We exploit this capacity to demonstrate ongoing and physiologically regulated protein S-nitrosylation that is coupled to the activity of each of the major forms of NOS in NO-generating cells and (in the case of endothelial NOS) their functional cellular partners (vascular smooth muscle). These results support the proposition that S-nitrosylation serves as an important mediator of NO-related bioactivity, both in NOS-containing cells and during NO-derived intercellular signaling.

EXPERIMENTAL PROCEDURES

Enzyme-linked Immunosorbent Assay and IEF—We used a primary antiserum raised in rabbit to a gluteraldehyde conjugate of bovine serum albumin (BSA) and cysteine, which was nitrosated with acidified nitrite (HCNO₂⁻) prior to immunization (8–10). The avidity and specificity of the antiserum for S-nitroso protein was evaluated with a solid phase enzyme-linked immunosorbent assay employing as substrate BSA nitrosated with HCl/NO₂⁻ at a 1:1 molar ratio with regard to nitrite (see Fig. 1).

Samples were transferred onto a 0.45-μm nitrocellulose membrane by vacuum for dot blots or by semi-dry electrophoretic transfer for Western blots of isoelectric focusing (IEF) gels, and membranes were blocked with 10% powdered milk before exposure to antiserum at a dilution of 1:1000. Solutions in this and all other procedures (including immunohistochemistry) contained 0.1 mM diethylenetriaminepentaacetate; SNO, nitrosothiol; HRP, horseradish peroxidase.

Nitric oxide synthase (NOS) was activated by L-arginine tetrahydrochloride (L-arginine; 50 μM) and L-NAME, N⁵-monomethyl-L-arginine; NGF, nerve growth factor; nNOS, neuronal or type 1 nitric-oxide synthase; pCMB, p-chloromercuribenzenesulfonate; SNO, nitrosothiol; HRP, horseradish peroxidase.

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§ The abbreviations used are: NO, nitric oxide; NOS, nitric-oxide synthase; Ach, acetylcholine; BSA, bovine serum albumin; EDRF, endothelial-derived relaxing factor; eNOS, endothelial or type 3 nitric-oxide synthase; IEF, isoelectric focusing; iNOS, induced or type 2 nitric-oxide synthase; L-NAME, N⁵-nitro-L-arginine methyl ester; L-NMMA, N⁵-monomethyl-L-arginine; NGF, nerve growth factor; nNOS, neuronal or type 1 nitric-oxide synthase; pCMB, p-chloromercuribenzenesulfonate; SNO, nitrosothiol; HRP, horseradish peroxidase.

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Fig. 1. Specificity and sensitivity of anti-SNO antibody binding to immobilized SNO-BSA. A, quantitation of dot blots shows that treatment with pCMiB prior to or with mercury after nitrosation of BSA greatly attenuates reactivity, and unmodified BSA is unreactive. (Signal is given as photomultiplier counts from phosphorimaging of 125I-labeled secondary antibody). B, a representative dot blot (diaminobenzidine reaction product) illustrates that S-nitrosogluthathione (GSNO; 1 mm) but not an N-nitroso "NONOate" (NNO, 1 mm) competes effectively with SNO-BSA for antibody binding. Unmodified BSA is unreactive. C, on a Western blot following isoelectric focusing (diaminobenzidine reaction product), BSA nitrosated with a 1.5-fold (SNO-BSA) or 3-fold (SNO-BSA+) molar excess of acidified NO3 separates into immunoreactive isoforms that reflect degree of S-nitrosylation.

RESULTS AND DISCUSSION

Unreduced fatty acid-free BSA nitrosated with HCl/NO2 at a 1:1 molar ratio with regard to nitrite incorporates about one mercury-labile NO group per BSA. As illustrated in Fig. 1 (A and B), as little as 0.8 nmol of S-nitrosylated BSA (SNO-BSA) could be detected on dot blots, whereas unmodified BSA was not recognized. Reactivity was greatly reduced by treating BSA with the sulfhydryl-specific organic mercuiral pCMiB prior to nitrosation (to block cysteine thiols) or with either mercuric chloride or dithiothreitol (1 mm; not shown) following nitrosation (to cleave the SNO linkage) (Fig. 1A). Similar results were obtained using nitrosated glyceraldehyde phosphate dehydrogenase as substrate (not shown). In addition, antigen competition experiments demonstrated that incubation of antiserum with S-nitrosylated glutathione eliminated subsequent antibody binding to SNO-BSA, whereas incubation with an N-nitroso compound, PAPA-NONOate, had no effect (Fig. 1B).

Finally, we employed the SNO antiserum to detect SNO-BSA on Western blots following IEF in polyacrylamide gels (Fig. 1C). Whereas dithiothreitol-reduced but otherwise unmodified BSA was not detected, nitrosation with 1.5 mol of acidified nitrite/mol of BSA led to the appearance of two prominent immunoreactive bands, and treatment with a 3-fold molar excess of NO3 resulted in increased abundance of those isoforms and the appearance of a third, more acidic species. These results reveal the nitrosation-dependent loss of charge groups of basic pKs, and in combination with the dot-blot analysis indicate that SNO-BSA separates in IEF as immunoreactive isoforms due to loss of the influence of thiolate anions on isoelectric point following addition of NO groups to cysteine sulfhydryl groups. In addition, we have recently employed anti-SNO antibodies with Western blotting following non-reducing electrophoresis to demonstrate S-nitrosylation of the ryanodine receptor protein from mammalian skeletal muscle (11), although the general utility of anti-SNO antiserum in detecting varied SNO-proteins on Western blots remains to be determined.

Overall, our analysis indicates that SNO-directed antibodies will provide a uniquely useful marker for S-nitrosylated proteins, if specificity is verified by the criterion of mercury and thiol sensitivity as well as, optimally, NOS dependence as described below. To apply this immunological tool to the analysis of S-nitrosylation in situ, we employed immunohistochemistry to examine SNO-proteins in cultured cells and tissue sections. Specificity of SNO-protein detection was demonstrated by mercury reversibility and NOS dependence of staining and by parallel measurements of protein-SNO by photolysis/chemiluminescence. In addition, control samples of all cells and tissues were processed with non-immune serum.

Endothelial and smooth muscle cells in tangential sections of pig aorta, and bovine pulmonary artery endothelial cells in primary culture, exhibited a low basal level of immunoreactivity, which was increased substantially by nitrosation with HCl/NO2 prior to fixation (Fig. 2, A and B). Both basal and induced staining was largely eliminated by treatment with either mercuric chloride or pCMiB before incubation with antiserum (Fig. 2, A and B). Mercury treatment had no effect on nitrotyrosine immunohistochemistry in parallel sections (not shown). These findings extend the results with membrane-bound proteins to demonstrate that SNO-proteins can be visualized specifically by immunohistochemistry. We then examined endogenous protein S-nitrosylation in intact cells in which individual NOS species could be activated selectively, focusing first on vascular tissue.

Type 3 NOS (eNOS) is the predominant form in vascular endothelial cells, where it is activated by stimuli that increase intracellular Ca2+ (12, 13). In both primary bovine pulmonary artery endothelial cells and rat CPA-47 endothelial cells, incubation with the Ca2+ ionophore A23187 (1 mM, 10 min) resulted in a large increase in immunoreactivity, which was greatly reduced by treatment with mercury prior to staining and by treatment prior to A23187 exposure with the NOS inhibitor N³-monomethyl-L-arginine (L-NMMA, 1 mM) (Fig. 2B). In addition, we examined the SNO-protein content of resting and A23187-treated CPA-47 cells by photolysis/chemiluminescence of cellular extracts. Lysates of resting cells contained a low level of SNO (15 ± 1.1 nmol/g of protein), consistent with the presence of basal mercury-labile endothelial immunoreactivity, and treatment with A23187 resulted in a significant increase in SNO content (43 ± 6.8 nmol/g of protein; p < 0.05). Thus, chemical measurement confirms the immunohistochemical demonstration that, in cultured arterial endothelial cells, stimulation of eNOS by ionophore-mediated Ca2+ influx is coupled to increased protein S-nitrosylation.
Fig. 2. Immunohistochemical detection of protein S-nitrosylation coupled to eNOS activity. Reaction product is brown (diaminobenzidine). A, in tangential sections of pig aorta, basal SNO immunoreactivity is increased by treatment with HCl/NO$_2^-$ , and both basal and induced staining is eliminated by treatment before staining with mercuric chloride or pCMB. B, mercury-sensitive immunoreactivity of cultured bovine pulmonary artery endothelial cells is increased by exogenous nitrosation (HCl/NO$_2^-$ ) and by L-NMMA-inhibitable activation of eNOS with a Ca$^{2+}$ ionophore (A23187). C, rings of mouse aorta, precontracted with phenylephrine (PE), relax upon addition of ACh (10$^{-6}$ M) due to activation of L-NAME-inhibitable eNOS (top panels depict strain gauge output). Exposure to ACh results in increased SNO-protein immunoreactivity (bottom panels) of endothelium (black arrow) and smooth muscle (red arrow), which is prevented by prior exposure to L-NAME. Rings were removed and immediately transferred to fixative at the point in time indicated by the end of the strain gauge tracings.

We next examined protein S-nitrosylation in intact vascular tissue in which eNOS could be activated physiologically. Type 3 NOS has been identified as the source of EDRF, and eNOS activity is modulated accordingly by vasoactive neurohormonal signals (1, 13). EDRF mediates vasodilation at least in part through activation in vascular smooth muscle of charybdo toxin-sensitive potassium channels and of Ca$^{2+}$-ATPase, possibly via S-nitrosylation (14, 15). Rings of mouse aorta, suspended in bioassy chambers and precontracted with phenylephrine, relaxed upon application of acetylcholine (ACh) (Fig. 2C). Immunoreactivity of both endothelium and smooth muscle was increased substantially in sections of rings that were removed from the bioassay shorty after exposure to ACh (Fig. 2C). Incubation (40 min) with the eNOS inhibitor N$^o$-nitro-L-arginine methyl ester (L-NAME, 1 mM) blocked ACh-induced relaxation and decreased immunoreactivity below basal levels in both endothelium and smooth muscle, demonstrating turnover of protein SNO (Fig. 2C). Thus, S-nitrosylation is a dynamic protein modification coupled to physiological activity of endothelial type 3 NOS, in both endothelium and associated smooth muscle of intact vascular tissue.

To examine S-nitrosylation linked to the additional forms of NOS, we employed cultured cell types in which type 1 NOS (nNOS) or type 2 NOS (iNOS) could be induced selectively by varying culture conditions.

Type 1 NOS is the predominant form in neurons (12), and adrenocarcinoma-derived PC-12 cells acquire a neuronal phenotype under the influence of nerve growth factor (NGF). We exposed PC-12 cells overnight to low levels of NGF (100 ng/ml), which increased nNOS expression without gross morphological differentiation. Untreated PC-12 cells contained moderate levels of nNOS and of SNO-protein, and induction of nNOS led to a large increase in protein S-nitrosylation as revealed by immunohistochemistry (Fig. 3A).

Type 2 NOS is the form detected in macrophages, and iNOS expression is greatly enhanced by exposure to cytokines (12). Treatment of macrophage-derived RAW 254.7 cells overnight with bacterial lipopolysaccharide (200 ng/ml) and γ-interferon (200 units/ml) led to a substantial increase in SNO-protein immunoreactivity, which accompanied the morphological differentiation characteristic of cytokine induction of macrophage/neutrophil cells (Fig. 3B). Increased immunoreactivity was correlated with an L-NMMA-inhibitable increase in SNO-protein levels from about 10 to 150 nmol of SNO/g of protein (p < 0.05), as assessed by photolysis/chemiluminescence of cellular extracts passed through Sephadex G-25 to eliminate low molecular weight nitrosothiols. However, it should be noted that staining of RAW 254.7 cells exhibited greater culture to culture variability than other cell types examined.

Finally, we extended our analysis to a more complex tissue, the human lung. The mature lung displays substantial levels of each of the three major forms of NOS, which are distributed discretely across a range of cell types that includes most prominently vascular endothelium (eNOS) and airway epithelium.
Fig. 4. SNO-protein immunoreactivity in human lung. Distribution of reaction product (brown) is depicted in sections of lung from preterm infants untreated (Basal) or following NO inhalation (NO) and from term neonates free of pulmonary disease (Basal) or with bronchopulmonary dysplasia (Dysplasia). Symbols indicate staining of airway smooth muscle (left arrow in PRE-TERM LUNG, NO and right arrow in TERM LUNG, Basal), airway epithelium (all other arrows), vascular endothelium (blue asterisk), and cartilage chondrocytes (red asterisk).

(iNOS), but NOS expression is greatly limited before birth (16). In sections from the lung of a pre-term infant, SNO-protein immunoreactivity was sparse and limited largely to the tracheal epithelium (Fig. 4). In contrast, sections from the lung of a full-term neonate (Fig. 4) exhibited extensive staining, which was most prominent in small artery endothelium, cartilage chondrocytes, and tracheal epithelium and muscle, whereas large artery endothelium was unstained. In addition, in sections from the lung of a pre-term infant administered NO therapeutically by inhalation (≤20 ppm), SNO-protein immunoreactivity was increased in a distribution that overlapped that seen in the neonate except that small artery endothelium remained unstained (Fig. 4). Increased S-nitrosylation was also evident throughout sections from the lung of a term infant with bronchopulmonary dysplasia (Fig. 4), consistent with the increased expression of iNOS by resident and infiltrating cells that accompanies lung pathophysiology. Basal, NO-induced, and bronchopulmonary dysplasia-associated immunoreactivity was eliminated by treatment with pCMB prior to staining (not shown). Thus, protein S-nitrosylation is developmentally regulated in multiple tissue compartments of the human lung and altered by both disease and NO therapy.

Our immunohistochemical findings, which are consistent with recent results obtained for nNOS with the “biotin-switch” method (7), demonstrate directly that protein S-nitrosylation is coupled to activity of all major forms of NOS, and reductions in basal SNO-protein levels by NOS inhibition emphasize that S-nitrosylation will be governed by a dynamic equilibrium between addition and removal of NO groups from cysteine thiols. Our findings for eNOS (EDRF) in particular emphasize further that S-nitrosylation will be regulated by NOS activity in both NOS-containing cells and their functional partners, in keeping with the proposition that the formation of SNO-protein serves as a major route through which NO-related bioactivity is expressed, both within NO-generating cells and during NO-derived intercellular signaling. Thus, because the various forms of NOS are expressed across a wide range of mammalian cell types, our results support the emerging view that S-nitrosylation is a ubiquitous post-translational protein modification with broad regulatory purview (2–4), analogous in that respect to protein O-phosphorylation.

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