Argininosuccinate Synthetase is Reversibly Inactivated by S-Nitrosylation in Vitro and in Vivo*

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Prior studies have demonstrated that the substrate for NO synthesis, L-arginine, can be regenerated from the NOS co-product L-citrulline. This requires the sequential action of two enzymes, argininosuccinate synthetase (AS) and argininosuccinate lyase (AL). AS activity has been shown to be rate-limiting for high output NO synthesis by immunostimulant-activated cells and represents a potential site for metabolic control of NO synthesis. We now demonstrate that NO mediates reversible S-nitrosylation and inactivation of AS in vitro and in lipopolysaccharide-treated cells and mice. Using a novel mass spectrometry-based method, we show that Cys-132 in human AS is the sole target for S-nitrosylation among five Cys residues. Mutagenesis studies confirm that S-nitrosylation of Cys-132 is both necessary and sufficient for the inhibition of AS by NO donors. S-nitrosoglutathione content is regulated by cellular glutathione levels and selectively influences NO production when citrulline is provided to cells as a protosubstrate of NO but not when L-arginine is provided. A phylogenetic comparison of AS sequences suggests that Cys-132 evolved as a site for post-translational regulation of activity in the AS in NOS-expressing species, endowing NO with the capacity to limit its own synthesis by restricting arginine availability.

Nitric oxide (NO)¹ is a cell signaling molecule with diverse and important biological functions (1, 2). The mammalian genome encodes three distinct isoforms of NO synthase (NOS) that catalyze identical reactions-conversion of L-arginine (Arg) to NO and L-citrulline (Cit) (3). The immunostimulant-induced isoform of nitric-oxide synthase (iNOS) produces cytotoxic quantities of NO and NO-derived species that are used to fend off tumor cells and various invading organisms (4) at the expense of possible injury to itself (5). Regulation of iNOS activity occurs principally at the level of transcription, endowing cells with a relatively slow means to adapt high output NO production to mammalian cell needs. Once iNOS protein is resident in a cell, however, the availability of substrate Arg is thought to be the key determinant of NO synthesis rate. Importantly, Arg is synthesized within NO-producing cells from the NOS co-product Cit (6) via the sequential action of two enzymes: argininosuccinate synthetase (AS) and argininosuccinate lyase (AL). AS catalyzes an ATP-dependent ligation of the NOS product Cit with Asp, yielding argininosuccinate, AMP, and pyrophosphate. Argininosuccinate is subsequently cleaved by argininosuccinate lyase (AL), yielding Arg and fumarate. Although AS and AL are generally considered in the context of their contribution to the urea cycle of the liver, where AS serves as the rate-limiting enzyme for ammonia detoxification (7), these enzymes also endow iNOS-expressing cells with an Arg/Cit cycle for continuous regeneration of Arg from Cit, providing iNOS with a sustained supply of substrate. Although AL was found to be constitutively expressed in NO-producing cells, AS mRNA, protein and activity are synergistically induced (along with iNOS) by immunostimulants in a wide variety of cells and tissues (8–12). Importantly, we previously demonstrated that AS activity is rate-limiting for immunostimulant-induced high output NO production in cultured vascular smooth muscle cells (8), and the Arg/Cit cycle provides the preferred source of Arg to iNOS (13). More recently, in transgenic mice that constitutively overexpress iNOS in a cardiac-specific manner, Arg/Cit recycling was found to profoundly limit iNOS activity (14). Thus, the level of AS activity can dictate the quantity of substrate presented to iNOS and therefore represents a potential site for regulation of high output NO production. In the present study, we questioned whether AS activity is down-regulated by levels of NO produced by iNOS-expressing cells. If operative, such a system would confer NO with the capacity to limit its own synthesis.

NO and NO-derived species can reversibly modulate protein activities by covalently adding to sulfur on critical Cys residues, a reaction termed S-nitrosylation (14). S-nitrosylation has been implicated in the physiological regulation of a broad spectrum of cellular and physiological activities, including regulation of blood flow (15), skeletal muscle contractility (16), neurotransmission (17), DNA repair, and apoptosis (18, 19). Interestingly, the thiol-reactive reagent 5,5'-dithiobis-2-nitrobenzoic acid was shown to modify a single undefined Cys residue in AS purified from bovine liver, inactivating catalytic function (20). It was also demonstrated that NO donors inhibit the urea cycle in cultured hepatocytes (21), and urea cycle activity can be increased when NOS is inhibited (22). Induction of iNOS activity in immunostimulant-treated cells is associated with a substantial increase in the overall level of protein S-nitrosylation (23). Although S-nitrosylation of AS is a potential mechanism for modulation of

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the urea cycle by NO donors and inhibitors, this possibility has not been previously considered.

In the present study, we demonstrated that NO down-regulates AS activity via S-nitrosylation and molecularly defines the Cys residue that is responsible for regulation of AS activity by NO. This modification provides a regulatory mechanism that can limit NO overproduction by governing Arg availability to iNOS.

EXPERIMENTAL PROCEDURES

AS Expression and Purification—Recombinant human AS was expressed in bacteria and purified as described previously (13). Briefly, AS cDNA was cloned into pMAL-P2 (New England Biolabs) and expressed as a C-terminal fusion protein with maltose-binding protein (MBP) in Escherichia coli. MBP-AS was purified by affinity chromatography using an amylose-agarose column. Free AS was prepared by cleavage of the MBP-AS protein using Factor Xa (1:200) and overnight incubation at 4 °C. AS was separated from MBP using an S-300 sephacryl gel-filtration column (Amerham Biosciences), and purified AS was kept at −20 °C in storage buffer (20 mM Tris-HCl, pH 7.4, 6 mM Asp, 2 mM MgCl2, 20 mM KCl, and 0.2 units of pyrophosphatase) to give a final volume of 200 μl. The samples were incubated at 37 °C in 96-well microtiter plates, and the reactions were terminated after 30 min by the addition of an equal volume of molybdate buffer (10 mM ascorbate acid, 2.5 mM ammonium molybdate, 2 mM sulfuric acid). Accumulation of phosphate in the samples was determined spectrophotometrically at A650, by comparison with inorganic phosphate standards.

The second method used to assay AS activity was based on the conversion of [15]Hraspartate to [15]Hargininosuccinate, as described previously (13). Reaction mixtures contained (final concentration): Asp (400 μM), [15]HAsp (250,000 counts/min), Cit (2 mM), Tris-HCl (10 mM, pH 7.5), ATP (2 mM), MgCl2 (6 mM), myokinase (20 mM), pyruvate kinase (4.5 units), myokinase (4 units), and pyrophosphatase (0.2 units) in a total volume of 150 μl. Reactions were incubated in glass test tubes and allowed to proceed for 1 h at 37 °C before enzyme activity was measured by subtraction of the total 50 μl of 1 M acetic acid and heating for 30 min at 90 °C. Following this procedure, 800 μl of distilled H2O was added to the incubates, and the contents were applied to 0.5 × 4 cm columns of Dowex 1-X8 (200–400 mesh, Bio-Rad) that had been pre-equilibrated with 0.05 M acetic acid. The columns were washed twice with 1 ml of 0.05 M acetic acid, and the flow-through containing [15]Hargininosuccinate was quantified by liquid scintillation spectrometry after the addition of In-Flow BD liquid scintillant (IN/US Systems Inc.).

Culture and Buthionine Sulfoximine (BSO)/Glutathione Monooethyl (GSE) Treatment of Rat Aortic Smooth Muscle Cells (RASM)—Native RASM were isolated from thoracic aorta explants of Fisher rats (26). RASM were grown to 70% confluence in T-75 culture flasks containing RPMI 1640 medium and 10% fetal bovine serum. The cells were studied at passages 8–12. To induce NO synthesis, the cells were treated for 12 h with a combination of 30 μM/LPS (E. coli serotype O111:B4) and 50 ng/ml of murine interferon (IFN)-γ. After LPS/IFN treatment, the cells were washed three times with Arg- and Cit-free RPMI 1640 culture medium and then incubated with the same medium containing either 1 mM Arg or 1 mM Cit. To the medium were added 1 μM BSO, a γ-glutamylcysteinyl synthetase inhibitor to deplete cellular GSH, or 1 mM membrane-permeable GSE to increase the cellular GSH level. After 24 h of incubation, the culture media were harvested for quantification of NO release as nitrite, and the cells were scraped harvested for analysis of SNO-AS and GSH content.

Assay of NO by the Griess Assay—NO accumulation in cell culture medium and content in SNO-AS solutions (following displacement of NO with mercuric ions) was quantified as nitrite using the Griess assay as described previously (26). The samples (100 μl) were treated in a microtiter plate with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthalenediamine in 5% O-phosphoric acid), and the resulting azo dye product was analyzed within 5 min at A540 using a spectrophotometer (Molecular Devices). Nitrite concentration was quantified by comparison with standard sodium nitrite standards.

Kinetics of NO Production by Ferroheme Myoglobin Capture Assay—Ferroheme myoglobin was employed to efficiently scavenging cell-derived NO and to quantify the rate of NO production. Quantification of NO production was based on the rate of progressive increase in A505, which results from oxidation of ferri- to ferroheme. Ferroheme myoglobin was prepared as described previously (27). Briefly, a PD-10 column (Amerham Biosciences) was pre-equilibrated with 50 mM Tris (pH 7.6) buffer. A solution of 2 mM horse heart myoglobin was prepared in 2.5 ml of 50 mM Tris buffer, and 0.5 mg of fresh sodium hydrosulfite (dithionite) was added to reduce ferri- to ferroheme. The reaction mixture was rapidly mixed with the PD-10 column, and the PD-10 column was washed with 50 mM Tris (pH 7.6). The eluted myoglobin was diluted in 50 mM Tris buffer to a final concentration of 400 μM and stored at −70 °C until use. RASM were pretreated in RPMI 1640 medium containing 10% newborn calf serum, LPS, and rat IFN-γ (30 mg/ml; 50 ng/ml) for 12 h.
followed by incubation in 100 μl of serum-free medium containing either 1 mM Arg or Cit for 24 h in the absence or presence of 3 mM NOS inhibitor NMA. Capture of cell-derived NO was initiated by the addition of 10 μl of the 400 μM ferrocene myoglobin to the Arg- or Cit-containing media, and after 5 min, the rate of NO production was monitored based on the rate of increase in A405 over a period of 10 min.

**GSH Assay**—Cell lysates (20 μl) were prepared and added to 100 μl of GSH assay buffer containing 100 mM sodium-phosphate (pH 7.5), 1 mM EDTA, 0.25 mM NADPH, 5 mM 5,5′-dithiobis-2-nitrobenzoic acid, and 0.5 unit of glutathione reductase. The reaction mixtures were incubated at 37 °C for 5 min and A412 was monitored. The GSH content of the cell lysates were quantified by comparison with GSH standards.

**Neutral Loss Mass Spectrometry to Identify S-Nitrosylation Sites in AS**—Purified huAS protein (100 μg) was treated with 100 μM S-nitrosoglutathione (GSNO) in AS storage buffer (excluding DTT) for 30 min at 37 °C. GSNO was removed, and the buffer was exchanged by 3 cycles of concentration-dilution into 20 mM ammonium bicarbonate buffer (pH 8.0) using a microcentrifugal concentration device with a 10-kDa cutoff membrane (Centrikon, Millipore). The protein was then digested at 37 °C for 4 h with trypsin (1:50). The peptide digest of AS was diluted with two volumes of 50:50:2 water:methanol:acetic acid and introduced into a triple quadrupole electrospray mass spectrometer (Quattro II, Micromass) by infusion at 10 μl/min with a source temperature of 80 °C. Mass spectra were acquired in neutral loss mode with a cone voltage of 25 V and collision energy of 15 V.

**Site-directed Mutagenesis**—An AS C132A mutant plasmid was constructed using the QuikChange site-directed mutagenesis kit according to the manufacturer’s instructions (Clontech). Wild type human AS cDNA in pMAL-P2 served as the template for mutagenesis with AS C132A mutant primers as follows: forward 33-mer, 5′-CGGTGGTGGC-TGAGGGCTTCTATCACTGGGCC-3′; reverse 33-mer, 5′-GGGGGCC-AATGAGCCGTAGCTCAACC-3′. Confirmation of the desired mutation was obtained by DNA sequencing.

**RESULTS AND DISCUSSION**

**Glutathione Depletion Selectively Attenuates NO Production from Cit**—Treatment of RASMC with LPS/IFN results in iNOS induction and commencement of high output NO synthesis (28). Although Arg is the sole physiological substrate for all mammalian NOS isoforms, Cit can be converted to Arg by the concerted action of AS and AL, allowing Cit to support high output NO synthesis. As shown in Fig. 1A, NO production by RASMC during a 24-h interval resulted in increased concentrations of nitrite in the cell culture medium. In the absence of Arg, Cit supported NO synthesis in a concentration-dependent manner, although 10-fold more Cit, relative to Arg, was needed for a comparable rate of NO synthesis. Treatment of RASMC with buthionine sulfoximine (BSO; 100 μM, 24 h), a selective inhibitor of GSH synthesis (29), depleted GSH by 75–80%, but did not alter 24-h LPS/IFN-induced NO synthesis from Arg. In contrast, this level of GSH depletion was associated with a 50% decrease in NO production when Cit was provided as a precursor of Arg.

Fig. 1B details the time course of NO production by LPS/IFN-treated RASMC provided with either Arg or Cit (1 mM) as a precursor. Results show a progressive decline over time in the efficacy of Cit utilization for NO synthesis relative to the use of Arg. Notably, GSH depletion did not influence the time course of NO synthesis from Arg, whereas it resulted in a decrease in the rate of NO synthesis from Cit (Fig. 1B). Together, these findings suggest that Arg and/or AL are subject to inhibition in association with nitrosative stress and thiol depletion. Because AS (20), but not AL (30), has been shown to contain a redox-sensitive thiol that inhibits activity when covalently modified, we considered whether this thiol may be a target for modification by NO. If so, we would predict that NO production from Cit would not fail in the presence of an efficient NO scavenger. The *inset* to Fig. 1B shows that following the addition of the efficient NO scavenger ferromyoglobin to the cells (24 h after treatment with LPS/IFN), the rate of NO production from Arg and Cit becomes identical. Moreover, in the presence of ferromyoglobin, BSO fails to suppress NO production from Cit. Notably, in this setting, NO production from Arg was not different from that detected by nitrite assay, whereas NO production from Cit was selectively accelerated by the NO scavenger. This finding indicates that NO production selectively impeded the generation of NO from the Arg precursor Cit.

**Nitric Oxide Reversibly Inactivates AS by S-Nitrosylation in Vitro**—Purified AS was incubated for 30 min with NO donors over a range of concentrations and then assayed for AS activity. Three NO donors were tested: S-nitro-N-acetyl-penicillamine (SNAP), sodium nitroprusside (SNP), and GSNO. None of the NO donors interfered with the assay used to measure AS activity (data not shown), however all elicited concentration-dependent inhibition of AS (Fig. 2A). The potency of tested NO donors for inhibiting AS activity followed the rank-order GSNO > SNAP > SNP, with IC50 values of 60, 120, and 800 μM, respectively. Once inhibited by GSNO treatment, AS was observed to undergo full and rapid reactivation upon the addition of 10 mM DTT (Fig. 2B). These observations are consistent with a mechanism whereby inhibition of AS is mediated by S-nitrosylation of one or more Cys residues, and reactivation occurs via denitrosylation of AS by NO group transfer to DTT (*i.e.* trans-nitrosylation).
Chemical analysis confirmed that AS is indeed susceptible to S-nitrosylation by GSNO. The extent of SNO-AS formation was determined by mercuric displacement of S-NO as nitrite, followed by quantification of nitrite using the Griess colorimetric assay. Exposure of AS to progressively increasing concentrations of GSNO followed by repurification and analysis of SNO content showed that GSNO elicits saturable and concentration-dependent S-nitrosylation (Fig. 2C). The upper limit of NO incorporation into AS was observed to be slightly in excess of 1 NO/AS monomer; saturation was obtained with 1 mM GSNO. Thus, stable S-nitrosylation of only a single Cys residue appears to occur in AS, and this is associated with thiol-reversible inhibition of AS activity by NO.

Having discerned that S-nitrosylation of a single thiol may inactivate AS, we tested whether AS substrates (ATP, Cit, or Asp) can protect against inactivation. Toward this end, AS was pre-incubated in a buffer containing NO donor (100 μM GSNO or 200 μM SNP) and the indicated concentrations of only one of the three substrates for 30 min. After 30 min, the other two substrates were added to the mixture at a fixed concentration of 2 mM, and after 30 min of incubation, AS was assayed for product formation. Double reciprocal plots of substrate concentration versus reaction velocity are depicted for ATP, Asp, and Cit. Values are means ± S.E. of 3–4 replicates. ATP protects AS from S-nitrosylation by GSNO. AS was incubated with 100 μM GSNO in the presence or absence of the indicated concentrations of ATP for 30 min. After GSNO removal, SNO content was quantified by mercury displacement and Griess assay as described in the legend to panel C.
Cys-132 Is the Only Site of Stable S-Nitrosylation on Human AS—To determine which of five cysteines in human AS is accessible to S-nitrosylation, we developed a novel “neutral loss” mass spectrometry-based method that selectively identifies SNO peptides in digests of GSNO-treated proteins. Following exposure of AS to GSNO, tryptic digestion was performed and peptides were analyzed using a triple quadrupole electrospray ionization mass spectrometer. Two quadrupole mass analyzers were used sequentially (designated M₁ and M₂, respectively), separated by an argon gas collision cell. The collision cell was set to impart sufficient energy to rupture the labile S–NO bond but not enough to fragment other more stable chemical bonds. The two mass analyzers were configured to monitor the neutral loss spectrum was acquired as described under “Experimental Procedures.” A, neutral loss of 15 Da from peptides derived from GSNO-treated AS reveals a single detectable parent ion peak of 764.3 Da corresponding to the doubly protonated peptide containing Cys-132. B, neutral loss of 30 Da reveals a single detectable parent ion peak, occurring at m/z 1527.8 and corresponding to the Cys-132-containing peptide in the singly protonated state. Upper traces in panels A and B demonstrate that neutral loss ions are not observed when AS is not pre-treated with GSNO. Theoretical SNO-peptide ions predicted by in silico tryptic digestion of all possible SNO-AS modifications are: AA₅₋₁₆, 2210.59 (+1), 1105.79 (+2); AA₁₆₋₃₀, 1563.87 (+1), 782.44 (+2); AA₃₀₋₃₄, 1528.77 (+1), 764.85 (+2); AA₃₄₋₃₈, 1100.54 (+1), 550.77 (+2).

Mutagenesis of Cys-132—If S-nitrosylation of Cys-132 is necessary for inhibition of AS by NO, then mutation of this site should afford protection. To test this prediction, we generated and purified a mutant AS protein in which Ala was substituted for Cys-132 (C132A huAS) and characterized both activity and sensitivity to inhibition by NO. Kinetic studies revealed that the specific activity of C132A huAS was indistinguishable from wild type huAS, confirming that Cys-132 does not contribute to AS catalysis (Fig. 4A). A 2–3-fold increase in Kₘ was observed for the substrates ATP and Asp, whereas the Kₘ for Cit was unaltered. Most notably, C132A mutant AS displayed a 10-fold reduction in sensitivity to inhibition by GSNO relative to wild type AS (Fig. 4B). Moreover, in contrast to wild type AS, GSNO treatment of the C132A mutant AS failed to result in detectable S-nitrosylation (Fig. 4B). These results confirm that Cys-132 is indeed the sole target for stable S-nitrosylation of AS.
and the relevant target for potent inhibition of AS activity. In contrast to the differential sensitivity of mutant and wild type huAS to inhibition by GSNO, both forms of AS were equipotently inhibited by hydrogen peroxide (Fig. 4C). These findings suggest that NO and peroxide (i.e. nitrosative and oxidative stress mediators) target distinct sites on AS that result in the inhibition of enzymatic activity.

It is notable that attenuation of C132A huAS activity was observed following treatment with GSNO, although at substantially greater GSNO concentrations than required for inhibition of wild type huAS. Accordingly, AS must contain at least one low affinity target site for modification by NO in addition to the relatively high affinity Cys-132 site. This low affinity site of NO modification may be either (a) an S-NO that is too labile for detection or (b) an NO-promoted Cys modification, other than S-nitrosylation, potentially requiring additional reactants (e.g. formation of an intra- or intermolecular disulfide bond, S-glutathionylation, or S-oxidation to sulfenic group). In any case, results suggest that Cys-132 is the only stable site for S-nitrosylation in huAS.

S-Nitrosylation of Endogenous AS Is Triggered in LPS-treated RASMC and Mice, Resulting in Inhibition of AS Activity—RASMC were analyzed for their SNO-AS content using the biotin-swap method (see “Experimental Procedures” for details). RASMC were either untreated or pretreated with LPS/IFN alone or in the presence of the NOS inhibitor NMA. Despite the Western blot finding that AS protein was equally abundant in both LPS/IFN-treated groups, SNO-AS was detected only in LPS/IFN-treated cells that produce NO, and not in untreated cells or iNOS-expressing cells in which NO production was inhibited by concurrent treatment with NMA (Fig. 5A, upper panel). These findings indicate that S-nitrosylation of AS occurs in vivo in immunostimulated RASMC, and iNOS-derived NO is a requirement.

Experiments were performed to determine whether SNO-AS accumulates in iNOS-expressing mouse tissues in vivo. Six h after injection with LPS (20 mg/kg, intraperitoneally) virtually all rodent tissues express iNOS (31) and AS (12). Western blotting of total SNO proteins isolated from the heart, liver, and brain of LPS-treated adult male mice confirmed the presence of SNO-AS in each of these tissues (Fig. 5A, lower panel). In contrast, SNO-AS could not be detected in tissues isolated from mice that either did not receive LPS (i.e. vehicle-injected controls) or that received both LPS and the iNOS inhibitor NMA ad libitum in their drinking water prior to and 6 h following LPS administration. These findings confirm that SNO-AS accumulates in mouse tissues in vivo via a mechanism that depends on iNOS-derived NO synthesis.

Although SNO-AS was evident in tissues of LPS-treated mice, we questioned whether the extent of this modification is sufficient to significantly dampen AS activity. To test this possibility, we examined AS protein abundance, activity, and
extent of S-nitrosylation in mouse lung tissue before and after in vitro exposure to the protein S-denitrosylating agent DTT (see Fig. 5B). Although AS activity was essentially undetectable in lung homogenates from untreated mice, activity was markedly induced 6 h after intraperitoneal treatment with LPS (20 mg/kg). When NO synthesis was inhibited in LPS-treated mice by concurrent treatment with NMA ad libitum in drinking water prior to and following LPS injection, Mice injected with an equivalent volume of phosphate-buffered saline, rather than LPS, served as vehicle-treated controls. SNO-proteins were isolated by the biotin switch method and visualized by immunoblotting with a specific anti-AS serum. AS was detected in the brain, heart, and liver of NO-producing (LPS-injected) mice. AS activity in each lung lysate was quantified in identical samples supplemented with either DTT (black bar) or an equivalent volume of vehicle (gray bar). Values are means ± S.E. from five animals. Lower panel, total AS and SNO-AS as determined by Western blot analysis. Inset, argininosuccinate production by AS in lung lysates from LPS-treated mice as a function of time in the presence of either DTT or an equivalent volume of vehicle.

**Fig. 5. S-nitrosylation of AS in rodent cells and tissues following LPS treatment; in vivo detection and evidence for SNO-dependent inactivation of AS catalytic function.**

**A. upper panel,** RASMC were treated with LPS/IFN for 12 h to induce expression of iNOS. SNO-proteins were isolated from cells using the biotin-switch method and subjected to SDS-PAGE and Western blot with anti-AS antibody. **Lower panel,** mice were injected with LPS and sacrificed after 6 h. One group of mice was allowed unlimited access to drinking water containing 5 mM NMA for 48 h prior to and following LPS injection. Mice injected with an equivalent volume of phosphate-buffered saline, rather than LPS, served as vehicle-treated controls. SNO-proteins were isolated by the biotin switch method and visualized by immunoblotting with a specific anti-AS serum. AS was detected in the brain, heart, and liver of NO-producing (LPS-injected) mice. AS activity in each lung lysate was quantified in identical samples supplemented with either DTT (black bar) or an equivalent volume of vehicle (gray bar). Values are means ± S.E. from five animals. **B.** LPS-treatment of mice induces AS protein but causes NO-mediated, DTT-reversible inhibition of activity. **Upper panel,** lung lysates were prepared from mice treated with LPS or vehicle with or without NMA. AS activity in each lung lysate was quantified in identical samples supplemented with either DTT (black bar) or an equivalent volume of vehicle (gray bar). Values are means ± S.E. from five animals. **Lower panel,** total AS and SNO-AS as determined by Western blot analysis. Inset, argininosuccinate production by AS in lung lysates from LPS-treated mice as a function of time in the presence of either DTT or an equivalent volume of vehicle.
Effect of intracellular GSH levels on SNO-AS content and NO production in RASMC. Expression of iNOS and AS were induced by exposure to LPS/IFN (30 μg/ml/50 ng/ml) for 12 h. Culture medium was replaced with Arg/Cit-free RPMI 1640 medium supplemented with either 1 mM Arg or Cit as a precursor for NO synthesis. To the medium were also added either 100 μM BSO to deplete cellular GSH, or 1 mM GSE to replete GSH, or vehicle control. After 24 h of incubation, the culture media were harvested for SNO-proteins and AS protein after SDS-PAGE of SNO-proteins prepared by the biotin-switch method from control, BSO-, and GSE-treated cells. Western blot for AS protein after SDS-PAGE of SNO proteins prepared by the biotin-switch method from control, BSO-, and GSE-treated cells. Western blot shows total AS, and the mean GSH content is also depicted. The results indicate that SNO-AS content is modulated by intracellular GSH levels and that SNO-AS inversely correlates with NO production from Cit in cytokine-induced RASMC. SNO proteins via a trans-nitrosylation reaction. Experiments were performed to test whether SNO-AS content can be modulated by changing levels of cellular GSH and whether SNO-AS levels, in turn, impact on NO production. RASMC, pre-induced with LPS/INF, were incubated in culture medium containing either Arg or Cit as a precursor for NO production. In some wells, the culture medium was modified by the addition of either the γ-glutamylcysteine synthase inhibitor BSO to deplete cellular GSH or GSE (a well established membrane-permeable GSH donor that is hydrolyzed into GSH and ethanol after entering the cells) (33), thereby increasing GSH levels. Nitrite production after 24 h was compared for BSO-treated, GSE-treated, and control cells, using either Arg (AS-independent) or Cit (AS-dependent) as precursors for NO synthesis. As shown at the bottom of Fig. 6B, GSH levels decreased to ~25% of control levels following BSO treatment and increased to ~180% of control following GSE treatment. NO production from Arg was unaffected by BSO or GSE treatment. In contrast, NO production from Cit showed significant sensitivity to changes in the level of intracellular GSH (Fig. 6A). Indeed, GSH depletion attenuated nitrite production from Cit by ~50%, and GSH repletion enhanced nitrite production by ~40%, i.e. to the same level as Arg-supplemented cells. As shown in Fig. 6B, BSO and GSE treatment markedly increased and decreased SNO-AS content, respectively. Western blotting revealed no effect of treatments on total AS expression. Together, these findings indicate a significant role for S-nitrosylation of AS in the regulation of NO production.

Implications for Control of AS by S-Nitrosylation—The present study demonstrated a novel feedback mechanism whereby NO can limit its own synthesis by governing substrate regeneration for high output NO synthesis. Intracellular Arg levels range from 0.1 to 1 mM (24), whereas the K_m for NO is only 5–10 μM (3). This knowledge would predict that, over the entire range of intracellular Arg concentration, iNOS activity should be maximal, and Arg should never limit NO production. Paradoxically, the provision of extracellular Arg has often been shown to increase NO production by cells and tissues. This observation has been termed the “arginine paradox” (32), and it has been suggested that Arg is sequestered in discrete intracellular pools that are at least partially inaccessible to NO. We have reported previously that Arg regeneration by the Arg/Cit cycle provides a preferred substrate source for iNOS, relative to Arg, that is newly taken up from the extracellular milieu (9, 13). The preferential usage of regenerated Arg by iNOS could arise from co-localization of enzymes of the Arg/Cit cycle, limiting the mixture of total cellular Arg with the Arg pool that resides within the cycle. Co-localization of iNOS with AS would also facilitate S-nitrosylation of AS so that cells are endowed with the capability to efficiently sense an excess of NO and acutely respond by down-regulating the provision of NO substrate. This paradigm resembles the known interaction of N-methyl-d-aspartate receptors with nNOS, proteins that interact through PDZ domain associations facilitating S-nitrosylation of the receptor, inactivation of Ca^2+ influx, and down-regulation of nNOS activity under conditions of sustained NMDA receptor activation (33).

The Arg/Cit cycle has been found to limit NO production by eNOS and nNOS under some experimental conditions (6, 34, 35); hence, S-nitrosylation of AS might similarly contribute to regulation of NO synthesis by these NO isoforms. Our failure to detect significant quantities of basal SNO-AS in the tissues of untreated mice could arise from a relative inefficiency of the “biotin switch” method used for detection of SNO-AS (estimated to be 1–5% efficient) and relatively low levels of AS in most tissues prior to immunostimulant exposure. Notably, AS has been reported to localize in caveolae with eNOS in endothelial cells (36) and with nNOS in neuronal cells (37). AS also associates in the outer membranes of mitochondria (38) where NOs have been identified (39–41). Association of NO isoforms with AS may engender efficient recycling of Arg substrate while additionally providing for the tight governance of NO production via NO-mediated control of AS activity.

Homology modeling of huAS based on crystal structures of two bacterial AS proteins revealed that Cys-132 is located on a highly conserved helix, which scaffolds the binding of two substrates, Asp and Cit. Addition of NO can lead to unfavorable steric interactions that perturb the conformation of this helix and provide a molecular explanation for the loss of AS catalytic function. The huAS model suggests that GSNO accesses Cys-132 from the opposite side of the ATB binding site, thus the protection provided by ATB against S-nitrosylation and inactivation (Fig. 2, D and E) may not be mediated by direct blocking of GSNO access. However, it has been reported that ATB binding elicits a conformational change in the active site region of E. coli AS, with the potential to limit Cys-132 access and thereby afford protection. Alignment of AS sequences from more than 100 species revealed that Cys-132 of huAS is conserved in all mammals and NOS isoforms. Association of NOS isoforms with AS may engender efficient recycling of Arg substrate while additionally providing for the tight governance of NO production via NO-mediated control of AS activity.

Together, our findings demonstrated a novel defense mechanism of immunoregulated cells that can prevent autoimmunity arising from NO overproduction. Assessment of the biological importance of AS S-nitrosylation on Cys-132 for regulation of

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the Arg/Cit cycle in mammalian tissues awaits the future analysis of genetic knock-in mice and cells bearing the C132A mutant AS in lieu of wild type AS.

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