Argininosuccinate Synthetase Overexpression in Vascular Smooth Muscle Cells Potentiates Immunostimulant-induced NO Production*

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Immunostimulants trigger vascular smooth muscle cells (VSMC) to express both the inducible isoform of NO synthase (iNOS) and argininosuccinate synthetase (AS). With constitutively expressed argininosuccinate lyase (AL), AS confers cells with an Arg/Cit cycle that can sustain NO production via continuous regeneration of the NOS substrate, L-arginine (Arg), from the NOS co-product, L-citrulline (Cit). To assess whether NO synthesis can be rate-limited by Arg recycling, we tested whether AS-overexpressing cells have an enhanced capacity for immunostimulant-induced NO synthesis. Rat VSMC were stably transfected with human AS cDNA in a eukaryotic cell expression vector, driven by a strong viral promoter. AS activity in transfected VSMC exceeded that induced in untransfected cells treated for 24 h with a combination of bacterial lipopolysaccharide and interferon-γ (LPS/IFN). AS activity was predominantly associated with membranes but was also found in cytosol. Recombinant AS was purified from cytosol and possessed a specific activity exceeding that reported for native AS. Western blotting verified the basal expression of AS antigen in membranes from untransfected AS-transfected VSMC and from untransfected VSMC after 24 h exposure to LPS/IFN. Epifluorescence histochemistry revealed a punctate distribution of AS antigen in transfected cells, consistent with a predominant membrane localization. Remarkably, on a per cell basis, LPS/IFN-induced NO production was 3–4-fold greater in AS-transfected cells than untransfected VSMC. In untransfected VSMC, maximal NO production during 48 h required millimolar Arg; notably, Cit was needed at lower concentrations; 100 μM of either precursor supported a maximal rate of NO synthesis for 48 h. The enhanced ability of AS-transfected cells to produce NO, compared with untransfected cells, could not be ascribed to differences in iNOS protein content or LPS/IFN potency for immunostimulation. We conclude that transfaction with AS provides a continuous flux of Arg which drives NO synthesis in immunoactivated VSMC. Arg regeneration by AS is rate-limiting to NO synthesis and apparently provides iNOS with a preferred cellular source of Arg. In accord with the reported “channeling” of substrates by urea cycle enzymes, we hypothesize that the Arg/Cit cycle sequesters a discrete pool of reusable substrate that sustains high-output NO synthesis.

Nitric oxide is a cell signaling gas with diverse functions and global importance to mammalian cell physiology (1). NO synthases (NOS,1 EC 1.14.13.39) catalyze two sequential oxygenations of L-arginine (Arg), producing stoichiometric amounts of NO and L-citrulline (Cit) (2, 3). All nucleated mammalian cells have the capacity for high-output NO production upon transcription of the gene that encodes inducible NOS (iNOS). While the iNOS gene lies dormant in cells at rest, iNOS transcription can be initiated by various immunostimulants, cytokines, and growth factors (3). In macrophages, iNOS-derived NO appears to be a major weapon in the arsenal of molecules that mediate host defense (1). On the other hand, inappropriate expression of iNOS mediates a variety of pathophysiological conditions caused by NO excess (4), e.g. iNOS induction in vascular smooth muscle cells (VSMC) has been implicated in the genesis of lethal septic shock (5). Appreciation of the factors that control iNOS activity should provide a rationale for the design of therapeutics that effectively limit pathophysiological NO overproduction.

Inasmuch as high-output NO synthesis requires a continuous supply of substrate, it is important to identify where and how this Arg originates. Possible sources of cellular Arg include uptake from plasma, intracellular protein degradation, and de novo biosynthesis from Cit. Although de novo biosynthesis of Arg in ureotelic organisms is accomplished principally by the kidney, and to a lesser extent the small intestine (6, 7), it has become clear that other tissues can also produce Arg from Cit. Biosynthesis of Arg requires two enzymes, argininosuccinate synthetase (AS, EC 6.3.4.5) and argininosuccinate lyase (AL, EC 4.3.2.1), that together catalyze the conversion of Cit, L-aspartate, and ATP to Arg, fumarate, AMP, and pyrophosphate. AS and AL are typically considered in the context of their contribution to the five-enzyme urea cycle of liver; however, in conjunction with NOS, these enzymes may also provide NO from Arg/Cit cycles that can continually regenerate Arg from Cit for sustained NO production (8). Accordingly, Arg production is NO synthesis has been observed in endothelial cells, which basally produce NO, and has been shown to increase as a function of NO synthesis rate (9).

We reported earlier that along with iNOS, immunostimu-
lants synergistically induce VSMC to express AS mRNA and activity (8), allowing for NO synthesis from Cit in the absence of extracellular arginine. Induction of AS is rate-limiting to Arg in vivo synthesis in VSMC, since AL mRNA and activity were found to be constitutively expressed. Notably, AS is also the rate-limiting enzyme of the urea cycle (6). Similar induction by immunostimulants of AS mRNA and activity, and constitutive expression of AL, were also found in murine macrophages (10) and human pancreatic β-cells (11) in culture. Coinduction of AS and iNOS has more recently been shown in vivo and vitro to be rate-limiting to high-output NO production.

The present study was performed to investigate whether the availability of AS may limit NO synthesis in VSMC. Toward this end, we engineered VSMC to overexpress human AS and examined how this impacted on immunostimulated-induced NO synthase activity and substrate preference. While it is predictable that AS-overexpressing cells would more efficiently produce NO from Cit, it is remarkable that maximal NO production from Arg was also increased 3-4 fold on a per cell basis. This suggests that the Arg/Cit cycle provides the preferred source of Arg for NO synthesis by VSMC and can be rate-limiting to high-output NO production.

**EXPERIMENTAL PROCEDURES**

**Expression of Human AS cDNA in E. coli and Purification of AS Fusion Protein**—Human AS cDNA, kindly provided by Drs. William O’Brien and Gerald Petajenius (Baylor College of Medicine), served as template for PCR amplification. Primers used were as follows: AS forward 24-mer (created to contain an EcoRI site), 5'-ATATCCAGCAGAAGGGC-3'; AS reverse 23-mer (created to contain a SalI site), 5'-CTTCTTGGCAGGCTCTTGTG-3'. Thirty cycles of PCR were performed according to the following schedule: denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and elongation for 1 min at 72 °C. PCR products were electrophoresed on a 1% agarose gel containing ethidium bromide and visualized by UV-induced fluorescence. The predicted size for human AS (1266 base pairs) that was ligated into the TA3 pCR™ vector (Invitrogen) and transformed into competent DH5α Escherichia coli. Successful subcloning of the PCR product was confirmed by restriction analysis of purified plasmid DNA (Wizard Mini- prep, Promega). AS cDNA was then subcloned from the TA3 pCR™ plasmid (New England Biolabs) to enable high-level expression into the bacterial expression of AS as a fusion with maltose-binding protein. The pMAL-P2 plasmid was cut with both XmnI and SalI, and the AS DNA/TaA3 pCR™ plasmid was cut with both EcoR and SalI. Cut plasmids were subjected to electrophoresis on 1% agarose, purified, ligated overnight at 16 °C, and transformed into DH5α E. coli. pMAL-P2/AS plasmid DNA was purified (Wizard Miniprep, Promega), sequenced by the dye-deoxyribonucleotide chain termination method, and found to be >99% identical to that for human AS cDNA (22). Observed nucleotide differences from the reported human cDNA were three, an A to G substitution at nucleotide 410 and substitution of CT for AC at nucleotides 1050-1051. A clone harboring the pMAL-P2/AS plasmid was inoculated into 1 liter of LB medium containing 100 mg/ml ampicillin and grown at 37 °C until A600 reached 0.5. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM, and cultures were incubated for an additional 3 h at 37 °C. Bacteria were harvested by centrifugation at 4,000 rpm for 10 min, and pellets were stored at −20 °C until use. For AS purification, the stored pellets were resuspended in 50 ml of TEND buffer (20 mM Tris-Cl (pH 7.4), 1 mM EDTA, 200 mM NaCl, and 1 mM dithiothreitol) which was supplemented with 1 mM citrulline, 1 mM aspartate, and a mixture of protease inhibitors (10 μg/ml pepstatin, 10 μg/ml leupeptin, and 100 μg phenylmeth- ylsulfonyl fluoride) and then subjected to two cycles of freezing at −70 °C and thawing at 37 °C. Sodium chloride was added to final concentration 1 M, and samples were lysed using a Branson sonicator. Lysates were centrifuged at 15,000 × g for 30 min at 4 °C, and supernatants were applied to a 5-ml column of amylase resin (New England Biolabs) that had been previously equilibrated with TEND buffer. The column was washed with 25 ml of TEND buffer two times. Finally, the column was eluted with 50 ml of 10 mM maltose in TEND buffer containing 1 mM citrulline and 1 mM aspartate. SDS-PAGE with Coomassie Blue staining revealed a predominant protein band which was of predicted size for the AS-fusion protein; this identity was confirmed by fact that cleavage of 4 C terminus using factor Xa (New England Biolabs with fusion protein), yielding two fragments of the predicted sizes. The products of factor Xa cleavage were separated by ion exchange fast protein liquid chromatography using a Mono Q column (Pharmacia Biotech Inc.) and a NaCl gradient as follows: 100% buffer A (20 mM Tris (pH 7.4), 1 mM aspartate, 1 mM citrulline) for 0–5 min, followed by a linear gradient B buffer A containing 25 mM NaCl to 35 min. Purified AS eluted as a discrete peak at 24 min and gave a single protein band of ∼46 kDa on Coomassie Blue-stained SDS-PAGE.

**Expression of Human AS cDNA in Vascular Smooth Muscle Cells and Purification of His₅-AS Fusion Protein**—Rat aortic smooth muscle cells were isolated from thoracic aorta explants of Fisher rats and grown as described previously (14). Cells in passage 8–12 were seeded and grown to confluence in 96-well plates for nitrite assay or in 75-cm² culture flasks for preparation of extracts for AS activity measurements. For transfection, cells in passage 4–6 were seeded and grown to 50% confluence in 6-well plates. Human AS cDNA was used as a template for PCR amplification of a cDNA encoding AS preceded by six N-terminal histidine residues and containing convenient restriction sites for ligation into the cytomegalovirus promoter-driven mammalian expression vector, pcDNA3 (Invitrogen). Primers for PCR were as follows: AS forward 49-mer (containing a BamHI site and His-Tag sequence), 5'-GGGATCCACGATGCACCACCACCACCACATGTCCAGCA- AAGGCTC-3'; AS reverse 25-mer (containing an EcoRV site), 5'- CGGCGGCGGATATCATCGCACAAT-3'. Both the PCR product and pcDNA3 plasmid were digested with BamHI and EcoRV, purified, and ligated at 16 °C overnight. The resulting AS/pcDNA3 was transformed into DH5α E. coli. Plasmid DNA was purified from a positive clone for nucleotide sequencing and transfection into rat aortic smooth muscle cells. Sequencing revealed identity of the insert with that in our AS-pMAL-P2 plasmid (described above), encoding a predicted protein having >99% amino acid identity to human AS (22). Transfection of VSMC, was performed using 15 μg of AS/pcDNA3, 52.5 μl of LipofectAMINE (Life Technologies, Inc.), and 500 μl of Opti-MEM medium (Life Technologies, Inc.). While mixing the plasmid/LipofectAMINE mixture by inversion for 30 min, a plate of cells was twice washed with Opti-MEM and incubated with an additional 5 ml of Opti-MEM. The plasmid/ LipofectAMINE mixture was then added to the cells and incubated for 6 h, after which the cells were replaced with culture medium. After 48 h, G418 was added at a concentration of 500 μg/ml to select stable transfectant-resistant cells; culture medium was replaced at 2–3-day intervals. Approximately 3 weeks later, G418-resistant clones were isolated using an 8 × 8-mm cloning cylinder and analyzed individually for expression of AS activity. His₅-AS protein was purified from a cell pellet prepared from 60 confluent T-75 flasks of a VSMC clone that was found to be positive for AS activity. The purification was performed using His-bind resin, according to the manufacturer’s (Novagen) protocol.

**Preparation of Rabbit Antibodies to AS Fusion Protein**—The purified and concentrated AS/maltose-binding protein fusion was used to develop polyclonal antibodies in each of two rabbits. Fusion protein in Freund’s complete adjuvant was injected intradermally into four dorsal sites (50 μg/injection) in each rabbit. After 28 days the animals were boosted by intradermal injection of an additional 100 μg of fusion protein in incomplete adjuvant and thereafter boosted by subcutaneous injection of 50 μg of protein at 14-day intervals. Commencing at week 8, animals were bled every 2 weeks from a marginal ear vein. Serum was separated from the clotted blood and stored at −20 °C until use. Antibody titer was determined by enzyme-linked immunosorbent assay, and specificity for AS was established by Western blot analysis.

**Preparation of Smooth Muscle Cell Cytosol and Membrane Fractions**—Cells were washed twice with 10 ml of ice-cold phosphate-buffered saline and harvested with a Teflon cell scraper into an additional 10 ml of ice-cold phosphate-buffered saline. Cell suspensions were centrifuged at 500 × g for 10 min and pelleted cells frozen in 1 ml of DMEM culture flask were resuspended in 1 ml of cold dissected H2O containing a mixture of protease inhibitors (10 μg/ml pepstatin, 10 μg/ml leupeptin, and 100 μg phenylmethanesulfonyl fluoride) and lysed by three cycles of freezing in liquid nitrogen and thawing in a 37 °C water bath. Lysates were centrifuged at 500 × g for 5 min, and the pellets were discarded. The resulting supernatants were recentrifugated at 15,000 × g for 5 min, and this pellet provided the membrane fraction, and the supernatant, after
a further centrifugation at 100,000 \( \times g \) for 1 h, provided the cytosolic fraction. Both fractions were immediately assayed for AS activity.

**Protein Assay**—Protein was measured by the Bio-Rad dye binding assay (Bio-Rad), using bovine serum albumin as standard.

**Western Blotting**—After the protein concentrations of both cell cytosol and membrane samples were determined, they were added 1:1 with 2 \( \times \) SDS-PAGE sample loading buffer and incubated for 10 min at 95 °C (Novex). Samples were then normalized for protein content and applied to lanes for SDS-PAGE on 8–16% gradient gels (Novex). Proteins were transferred by electroelution onto polyvinylidene difluoride membrane (0.2 \( \mu \)m, Trans-Blot medium, Bio-Rad). Western blot analysis and autoradiography was performed using polyclonal AS antisera from rabbit, at a dilution of 1: 1,000. Immunoreactive bands were detected by sequential incubation in biotinylated goat anti-rabbit IgG (1:3,000), streptavidin-alkaline phosphatase (1:5,000, Life Technologies, Inc.), and the chemiluminescent 1,2-dioxetane substrate, CSPD® (Tropix), and visualized using a chemiluminescent detector.

**Rat recombinant IFN-\( \gamma \) (50 U/ml), PGE\(_2\) (10 nM), and pyrophosphatase (0.2 units) in a final volume of 150 \( \mu l \). Reactions were incubated in glass test tubes and allowed to proceed for 60 min at 37 °C, before enzyme activity was terminated with addition of 50 \( \mu l \) of 1 M acetic acid and heating for 30 min at 90 °C. Following this procedure, 800 \( \mu l \) of distilled H\(_2\)O was added to incubates, and the contents were applied to 0.5 \( \times \) 4-cm columns of Dowex 1-X8 (200–400 mesh, Bio-Rad) in 0.05 M acetic acid. An additional 2 \( \times \) 1 ml of 0.05 M acetic acid was then applied to the columns. Radioactivity in the column flow-through, reflecting [\(^{3}H\)Aspartate], was added to In-Flow BD liquid scintillant (IN/US Systems Inc.) and quantified by liquid scintillation spectrometry. Additionally, purified AS activity was assayed by measuring the formation of inorganic phosphate by the method of Fiske and Subbarow (36), as previously adapted to the detection of alkaline phosphatase-coupled second antibody was visualized using a chromogenic substrate (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; Life Technologies, Inc.).

**Immunofluorescence**—Cells were permeabilized and nonspecific binding was blocked by treatment for 2 h with 0.075% saponin in HBS containing 0.2% bovine serum albumin. After an additional wash in HBS, cells were incubated for 1 h at room temperature in 1:500 AS antiserum, diluted in HBS containing 0.2% bovine serum albumin and 0.075% saponin. Cells were washed 3 times in HBS and then incubated for 30 min at room temperature with fluorescein isothiocyanate-conjugated secondary antibody, at 1:200, Vector Labs, diluted in HBS/bovine serum albumin. After a final 3 washes in HBS, cells were washed in distilled H\(_2\)O and coverslipped with glycerol-based mountant (Vectorshield, Vector Laboratories, Inc.).

**Chemicals**—Rat recombinant IFN-\( \gamma \), RPMI culture medium, and cell culture reagents were from Life Technologies, Inc. [\(^{3}H\)Aspartic acid was purchased from Amer sham Life Sciences, Inc. (specific activity 40 Ci/mmol). Factor Xa was from New England Biolabs. Enzymes, LPS (E. coli serotype 0111:B4), and all other chemicals were obtained from Sigma or Calbiochem.

**RESULTS AND DISCUSSION**

**Expression in E. coli of Functional Human AS/MBP Fusion Protein**—AS protein was previously isolated from human (15, 17), bovine (18), and rat (19) and found to be homotetrameric. The human gene that encodes AS comprises 16 exons which span 63 kilobases (20) and map to chromosome 9q34 (21). Based on cDNA cloning, the predicted polypeptide sequence is 422 amino acids and 46.4 kDa (22). To obtain large quantities of human AS for antibody development and to confirm functionality of the recombinant protein, we sought to express AS in bacteria. Although AS has previously been purified after overexpression in bacteria, it was not recovered in a catalytically active state (23).

AS was directionally cloned into pMAL-P2 in an orientation that would result in the N terminus of AS fused to the C terminus of maltose-binding protein. Relative to the reported human AS cDNA, the plasmid we constructed was found to contain an A to G substitution at nucleotide 410 and a substitution of CT for AC at nucleotides 1050–1051. These nucleotide changes are non-conservative, resulting in predicted differences in amino acid sequence from that reported for human AS: Lys\(^{112}\) to Glu and Leu\(^{232}\)-Arg\(^{235}\) to Phe-Trp. Although Lys\(^{112}\) is conserved in human, rat, murine, and bovine AS cDNA, it is notable that Saccharomyces cerevisiae, Streptomyces lavendulae, and E. coli each have a different amino acid at the corresponding position (Phe, Gin, and Asn, respectively). Thus, although conserved in mammals, a basic residue in this position is not a requirement for AS activity. Substitution in human AS of Phe-Trp for Leu\(^{235}\)-Arg\(^{238}\) is also consistent with enzymatic activity. Indeed, sequence alignment reveals that rat, mouse, and bovine AS sequences each have Phe-Trp at the corresponding sites. Therefore, the reported Leu\(^{232}\)-Arg\(^{235}\) sequence in human AS cDNA is the exception, rather than the rule.

The Phe-Trp is partially conserved in AS cDNAs from S. cerevisiae, S. lavendulae, and E. coli AS cDNAs, which encode Phe-Leu, Arg-Trp, and Arg-Trp, respectively. Thus, the sequence we observed at this site makes our predicted enzyme identical with that from three other mammalian isoforms and more closely in alignment with more evolutionarily divergent species. Since all other nucleotides in our human AS-containing plasmids were common to human rather than rodent AS cDNA, we are confident that we have not inadvertently amplified from rodent template cDNA. This substitution could conceivably represent a correction to the previously reported sequence or perhaps results from human gene allelotype.

E. coli harboring the AS/pMAL-P2 plasmid expressed the predicted 89.1-kDa fusion protein to an extent of 10–20% total cellular protein (estimated from Coomassie staining of SDS-PAGE). A single step purification on amylose resin gave homogeneous AS/MBP (>90% purity; see Fig. 1A, lane 1), the most prominent contaminant being free MBP. Yields of 10–15 mg of purified fusion protein were obtained per liter of culture broth. The purified AS/MBP fusion protein was found to be catalytically active, as indicated by Fig. 1B. While the specific activity of the preparation described in Fig. 1B was 5.4 \( \mu \)mol/mg/h, other preparations ranged in activity to 11.9 \( \mu \)mol/mg/h (based on the initial rate of AS formation). Considering that the fusion protein is ~50% AS by weight, the specific activity of AS/MBP fusion protein was 20–40% that observed with AS purified from a human lymphoblast cell line (17). As shown in lane 2 of Fig. 1A, cleavage of AS/MBP by treatment with factor Xa yielded a mixture of free AS (46.4 kDa) and MBP (42.7 kDa). Subsequent purification of free AS on Mono Q resin was per-
overexpression of functional AS in vascular smooth muscle cells (VSMC) Basal AS activity was barely detectable in rat aortic smooth muscle cell cytosol (100,000 x g supernatant) or membranes (15,000 x g pellet). However, substantial AS activity was observed in each of these fractions from three individual cell lines selected for stable expression of AS/pcDNA3, a plasmid in which the cytomegalovirus promoter drives transcription of His6-AS. The nucleotide sequence of this plasmid predicts the identical AS protein product to that encoded by AS/pMAL-P2. Shown for the AS9 cell line (Fig. 2), expressed AS activity was ~3-fold enriched in membranes versus cytosol. In accordance with our previous report (8), treatment of untransfected VSMC with a combination of LPS and IFN-γ significantly induced AS activity (Fig. 2). Again, AS activity in membranes predominated 3-fold over that measured in cytosol. Although the magnitude of AS induction by immunostimulants was substantial, it was less than that expressed basally by the AS-transfected AS9 cell line (used in all studies described below). Treatment of AS9 cells with immunostimulants caused a further increase in AS activity, more than doubling that which was basally expressed in both membrane and cytosolic fractions.

His6-AS was purified to homogeneity from the 100,000 x g supernatant of 60 T-75 flasks of LPS/IFN-treated VSMC by affinity chromatography on nickel-chelating resin. Similar to AS purified from E. coli, the inclusion of 1 mM aspartate and 1 mM citrulline was required in all buffers used for cell lysis and AS purification to recover catalytic activity of the purified enzyme. When substrates were present, purified His6-AS was found to have a specific activity of 90 μmol/min/mg protein, which is 7.5-fold greater than the most active preparation of bacterially expressed AS and 1.5-fold greater than that reported for purified AS from human lymphoblasts (17). Whether this enhanced activity is due to reduced losses during purification or perhaps some post-translational protein modification remains to be ascertained.

Consistent with AS activity measurements, AS antigen was undetectable by Western blot analysis in untransfected VSMC but evident in AS-transfected cells at the predicted molecular mass of 46 kDa (Fig. 3). Also in agreement with activity measurements, AS antigen was found to be inducible by LPS (in both untransfected and AS-transfected VSMC) and more robustly expressed in membrane versus cytosolic fractions (Fig. 3).

From the above findings, it is apparent that VSMC have been successfully engineered to basally express high levels of...
Fig. 3. Western blot demonstrating argininosuccinate synthetase (AS) protein in rat aortic smooth muscle cells that were either untransfected or stably transfected with AS; influence of LPS/IFN treatment. AS protein mass was assessed in supernatant (supnt) and membrane (memb) fractions from cells before (−) and after (+) 24 h treatment with a combination of LPS (30 μg; E. coli serotype 0111:B4) and rat IFN-γ (50 ng/ml). Equivalent protein loads of 7.5 μg were applied to all lanes. Visualization is with a chemiluminescent substrate of alkaline phosphatase (CSPD).

Functional AS protein. The preliminary localization of AS to membranes of immunostimulant-treated control cells and AS-overexpressing cells offers a potential explanation to a puzzling dilemma regarding the role of AS in the urea cycle. While two of five urea cycle enzymes in liver are intramitochondrial, the remaining three enzymes (AS included) have previously been considered to be cytosolic. Nonetheless, the reported "channeling" of substrates through urea cycle enzymes (24, 25) (i.e. maintenance of a discrete pool of urea cycle intermediates which do not freely mix with intracellular substrates) necessitates that all five enzymes of the urea cycle assemble into a functional complex. Thus, AS may be associated with the outer membrane of mitochondria (26), where it links with other "cytosolic" urea cycle enzymes, AL and arginase, and receives Cit from intramitochondrial ornithine transcarbamylase. Consistent with this view, epifluorescence immunohistochemical localization of AS antigen in AS-transfected VSMC reveals a punctate distribution (Fig. 4). This intracellular pattern of AS argues against homogeneous cytosolic expression but is consistent with a mitochondrial membrane association of AS.

Impact of AS Overexpression on the Magnitude of Immunostimulant-induced NO Synthesis—Treatment of VSMC with a combination of LPS and IFN-γ induced the progressive accumulation over time of nitrite, a stable oxidation product of NO. When expressed on a per cell basis, nitrite accumulation by AS-transfected cells was substantially greater than that by untransfected cells, at all times examined (Fig. 5). This increased capacity for NO synthesis of AS-transfected VSMC is further manifest by a 3.6-fold increase in slope of the relation between cell number and nitrite accumulation during a 48-h period, relative to untransfected cells (Inset to Fig. 5). It is notable that potentiation of nitrite production afforded by AS overexpression increased progressively with time. While nitrite accumulation by untransfected cells waned during the interval 24–48 h following LPS/IFN treatment, AS-transfected cells continued to produce nitrite at a linear pace for this interval. The fold increase in nitrite production by AS-transfected VSMC, relative to untransfected cells, was 2.6, 3.0, and 3.8 at 12, 24, and 48 h, respectively. Continued regeneration of Arg from Cit offers a likely explanation for why NO synthesis by AS-transfected cells does not taper with time; in contrast, untransfected VSMC becomes progressively more limited by the availability of Arg.

Alternative explanations were considered for why AS-transfected cells possess an increased capacity for NO synthesis. One possibility is that VSMC are non-homogeneous, and we happened to propagate a cell line that produces more NO than the "average" cell. Arguing against this view, NO synthesis was found to be up-regulated to a similar extent in each of two stably-transfected AS clones that we examined. Moreover, such a mechanism would imply that AS-transfected cells produce more NO by virtue of a greater iNOS protein content. Western blot analyses indicated indistinguishable amounts of LPS/IFN-induced iNOS antigen in cytosol from AS-transfected and untransfected VSMC (Fig. 6).

We also examined whether AS-transfected cells may produce more NO by virtue of a greater inherent sensitivity to immunostimulants. To simplify the interpretation of experiments designed to test this possibility, cell number was adjusted to give near-equivalent nitrite accumulation by both AS-transfected and untransfected VSMC, during a 48-h exposure to immunostimulants. Results presented in Fig. 7 demonstrate that induction of NO synthesis by LPS alone is concentration-
ability of VSMC to use Cit as the sole source of substrate for NO. We previously reported that the induction of AS by LPS/IFN accounted for the identical concentration dependence which must be supplied to sustain this maximal rate. We therefore conclude that the increase in NO synthesis seen with AS-transfected VSMC cannot be attributed to an enhanced sensitivity of VSMC to use Cit as the sole source of substrate for NO production. Although Cit was able to support the maximal level of NO synthesis obtained with Arg as substrate, a minimum 10-fold higher concentration of Cit was necessary to achieve this maximum.

Fig. 8 depicts the relationship between substrate concentration and LPS/IFN-elicited nitrite accumulation, over a 48-h period of VSMC treatment. Provision of Arg, over the range of 0.01 to 3 mM, led to a progressive and non-saturating increase in NO production by untransfected VSMC. This indicates that during a 48-h period, even millimolar concentrations of Arg were insufficient to sustain a maximal rate of NO production by VSMC. Similarly, Cit was found to support NO production in the absence of Arg but was less effective; the concentration-response relation for Cit utilization was displaced ~3-fold to the right of that for Arg. In sharp contrast, AS-transfected cells were able to use either Arg or Cit equi-effectively for NO synthesis and at comparatively low concentrations. With either Arg, or its precursor Cit, maximal NO synthesis was observed with a concentration of 100 μM and an EC_{50} of ~40 μM (see Fig. 8). While it might be anticipated that AS-overexpressing VSMC would use lower concentrations of Cit for support of NO synthesis, due to more efficient recycling to substrate Arg, the increased conversion of Arg to NO was unanticipated.

The Arg/Cit Cycle and High-output NO Production by VSMC—It is well established that a guanidino-nitrogen of Arg is the origin of the NO formed by the NOS enzyme family (2). Nonetheless, the in vivo source of Arg used by NOBs has not been defined and is likely to vary with the magnitude and duration of NO synthesis. Our findings reveal that recycling of Arg via the combined action of AS and AL is needed to sustain maximal rates of NO production by immunostimulant-activated VSMC. Thus, we found that overexpression of AS increased both the maximal rate at which VSMC can produce NO (on a per cell basis) and reduced the concentration of Arg or Cit which must be supplied to sustain this maximal rate. We surmise that formation of Arg from Cit must be exceedingly rapid in the AS-transfected VSMC to account for the identical concentration dependence we observed with each substrate for support of NO synthesis.
While it is predictable that reliance of NO synthesis on Arg regeneration from Cit would predominate as Arg in the culture medium becomes progressively consumed, the profound effect of AS at early times, when Arg in the culture medium is abundant (~1 mM, while $K_{\text{m}}$ for iNOS utilization = 5–10 $\mu$M), demands an alternative explanation. One possibility is that VSMC, and perhaps other cell types, possesses discrete pools of Arg which subserve distinct functions. Conceivably, the Arg that is repleted from Cit, via the combined action of AS and AL, is more available to iNOS than Arg which newly enters the cell via transport from the extracellular milieu. Maintenance of an Arg pool which is used preferentially by iNOS could arise from a common localization of enzymes of the Arg/Cit cycle, limiting the mixture of Arg and Cit that reside within the cycle with total cellular pools of Arg and Cit. In this context, it is intriguing that evidence presented herein suggests that both native and overexpressed recombinant forms of AS are predominantly membrane-associated. Support for such a view has previously been provided (26), contrasting with the common belief that AS and AL are cytosolic enzymes (6). Similarly, ~50% iNOS in activated primary mouse macrophages has been shown to associate with intracellular membranes (27); although membrane-bound and cytosolic iNOS appear to derive from a common precursor, it is possible that they subserve distinct roles. It is tempting to speculate that the specific localization of iNOS will influence the source and quantity of Arg available to it for NO synthesis. The possibility therefore exists that the functional association of enzymes comprising the Arg/Cit cycle is complemented by a physical organization of these enzymes which is responsible for sequestration of a discrete pool of Arg. Inasmuch as substrate channeling through AS and AL to the other three urea cycle enzymes is established (24, 25), extension of this concept to iNOS seems plausible.

A functional linkage between Arg synthesis via AS and the production of NO is implicit in numerous earlier reports. Unlike the VSMC which we study here, cells that constitutively express isoforms of NO, such as vascular endothelial cells and certain populations of neurons, have a basal capacity to recycle Cit from Arg (9, 28). Indeed, Arg regeneration from Cit may be a common feature of all NO producing cells. It is significant that Cit formation by endothelial cells was shown to increase when NO production is stimulated (9, 29) and diminish when subjected by chronic hypoxia (30), a condition that attenuates NO synthesis (31). It is also noteworthy that hypoxia reduced Cit production without a change in intracellular Arg levels (30). These observations extend to endothelial cells our speculation that a discrete pool of Arg and Cit is utilized for the Arg/Cit cycle. Recent case studies have connected a deficiency in endothelium-derived NO synthesis in neonates with a genetic dysfunction in AS expression (33); these babies presented with hypertension that was reversed by administering L-arginine (34, 35) and a deficiency in NO production is inversely correlated with NO synthase (33); these babies presented with hypertension that was reversed by administering L-arginine (34, 35).

The lack of a selective AS inhibitor impedes examination of the extent to which AS provides Arg for low-output NO synthesis by endothelial cells.

It is now well-established that high-output NO synthesis by immunostimulant-activated cells occurs concomitantly with the induction of AS activity and mRNA in vitro (8, 10, 11) and in vivo (12, 13). We conclude from the present study that, at least in vitro, Arg is provided for NO synthesis via recycling of the NOS-derived co-product Cit and that the rate of Arg regeneration is limited by AS availability. Thus, AS is the rate-limiting enzyme for high-output NO synthesis by immunostimulant-activated VSMC. Several important questions await answers: 1) Is AS rate-limiting to NO synthesis in all cells engaged in high-output NO production or is this a property restricted to particular cell types? 2) Does AS limit NO synthesis in vitro? 3) What is the molecular basis for the functional coupling of enzymes comprising the Arg/Cit cycle?

Inhibiting immunostimulant-activated NO expression affords a potential therapeutic opportunity for limiting NO production in pathophysiological conditions arising from NO excess. The challenge will be to develop specific inhibitors of AS or AL that can be targeted to specific cell types.

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