Regulation of intracellular polyamine biosynthesis and transport by NO and cytokines TNF-α and IFN-γ

JOSEPH SATRIANO,1,2 SHUNJI ISHIZUKA,1 D. CLAY ARCHER,1 ROLAND C. BLANTZ,1,2 AND CAROLYN J. KELLY1

1Division of Nephrology-Hypertension and 2Program in Molecular Pathology, Department of Medicine, University of California, San Diego, and Veterans Affairs Medical Center, La Jolla, California 92161

Satriano, Joseph, Shunji Ishizuka, D. Clay Archer, Roland C. Blantz, and Carolyn J. Kelly. Regulation of intracellular polyamine biosynthesis and transport by NO and cytokines TNF-α and IFN-γ. Am. J. Physiol. 276 (Cell Physiol. 45): C892–C899, 1999.—Nitric oxide (NO) has been documented as a cytostatic agent (1, 12, 26, 37, 45, 46, 48). Ornithine is the precursor of polyamines through the induction of ornithine decarboxylase (ODC). Polyamines (putrescine, spermine, and spermidine) are required components for entry into and progression of the cell cycle (3) and as such play an important role in proliferation. In inflammatory models of experimental glomerulonephritis and wound healing these two arginine-based pathways are temporally regulated (2, 11, 25). The production of NO from arginine by inducible NOS (iNOS) is an early phase response, whereas arginine is diverted to proliferative and extracellular matrix production pathways in the later repair phase response. Administering a NOS inhibitor, N6-monomethyl-L-arginine, in experimental glomerulonephritis increases both the magnitude and rapid onset of the repair phase response (11). Thus models of inflammation suggest an interrelationship of arginine metabolic pathways to maintain the correct temporal relationships between such pathways. As NO is not an effective arginase inhibitor (for review, see Ref. 24), we speculated that NO may modulate the proliferative response in the early phase of inflammation by suppressing ODC. ODC requires a cysteine in its active center for full enzymatic activity. NO has been shown to modulate the activity of several enzymes through nitrosylation of cysteines (14–16, 36, 43, 44).

Along with polyamine biosynthesis by ODC, cells can also acquire polyamines from their external milieu. Induction of polyamine transport by arginine deprivation, but not ornithine deprivation (8), strengthens the importance of arginine at the crux of both the polyamine and NO pathways. Polyamine transporters are stimulated by many of the same factors that induce ODC activity. In addition, polyamine transport can substitute for de novo polyamine biosynthesis under conditions such as NO inhibition by difluoromethylornithine (DFMO) (8). Polyamine autoregulation occurs through the induction of antizyme, a protein that inhibits both ODC and polyamine transport, exemplifying the importance of polyamine transport in vivo. Taken together, these results illustrate the need to examine polyamine transport as well as biosynthesis when examining cellular access to polyamines.

In this study we have demonstrated that NO and the iNOS-inducing cytokines tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) can negatively regulate ODC activity and cellular polyamine uptake.

MATERIALS AND METHODS

Chemicals and supplies. Sodium nitroprusside (SNP), S-nitroso-N-acetyl-L,L-penicillamine (SNAP), S-nitroso-L-glutathione (GSNO), (Z)-1-[2-(2-aminoethyl)-N-(2-aminoethyl)amino]diazene-1-ium-1,2-diolate (DETA NONOate), (Z)-1-[N-methyl-L-[6-(N-methylammoniohexyl)amino]diazene-1-ium-1,2-diolate (MAHMA NONOate), 3-morpholinosydnonimine (SIN-1), and L-Nω-(1-iminoethyl)lysine (L-NIL) were purchased from Alexis Biochemicals (San Diego, CA). TNF-α...
and IFN-γ were purchased from Boehringer Mannheim (Indianapolis, IN). DFMO was kindly supplied by Dr. E. Bohme, Hoehst Marion Roussel (Cincinnati, OH). All other chemicals were purchased from Sigma (St. Louis, MO), unless otherwise noted.

Cell preparations. A transformed proximal tubule cell line, MCT (20), was used for all experiments, except where noted. Other cell lines included J774 (monocyte/macrophage), mMCM (mouse glomerular mesangial) (50), ENDO (rat glomerular endothelial) (27), NIH/3T3 (fibroblast like), Ras/3T3 (Ras-transformed NIH/3T3) (49), and HT-1080 (fibrosarcoma). All cells were plated and allowed to grow to near confluence in DMEM (GIBCO/BRL, Grand Island, NY) supplemented with 5% calf serum (Gemini Bio-Products, Calabasas, CA). Cell lines are from American Type Culture Collection (ATCC; Manassas, VA), unless otherwise referenced.

ODC activity. Cells were grown on 10-cm culture plates, harvested by first washing with 10 ml ice-cold PBS, placed in ice-cold ODC reaction buffer [10 mM Tris, pH 7.4, 2.5 mM dithiothreitol (DTT), 0.3 mM pyridoxyl-5-phosphate, and 0.1 mM EDTA], and scraped, collected, and homogenized. Cell preparations were then centrifuged at 30,000 × g for 40 min at 4°C. The supernatant was collected and assayed for ODC activity as previously described (35). Briefly, samples were aliquoted at 250 µl in triplicate to large-bore glass tubes; activity was measured as above, except the labeled [3H]putrescine addition was determined as above, except the labeled [3H]putrescine addition was immediately terminated by aspiration and PBS washes.

Statistical evaluations. Variations between samples within groups were analyzed by ANOVA, with significance determined by Fisher’s protected least-significant difference post hoc test. StatView software was used for these analyses.

RESULTS

SNP suppresses ODC activity. We tested the possibility that the NO donor SNP could suppress ODC activity in the transformed kidney proximal tubule cell line MCT. The proximal tubule is the primary source of arginine synthesis in the kidney (28) and is known to have locally elevated arginase activity (29). These transformed cells also display constitutively elevated ODC activity. MCT cells were prepared for the ODC activity assay as described in MATERIALS AND METHODS. SNP was added to the homogenized cell preparation in the concentrations indicated in Fig. 1A at the start of the ODC assay reaction. SNP suppressed ODC activity in a dose-dependent fashion (Fig. 1A).

We measured ODC activity in a number of established and transformed cell lines in the absence or presence of SNP (Fig. 1B). Primary cultures were not used because of their low inherent ODC activity. SNP markedly suppressed ODC activity in all cell lines examined.

NO donors suppress ODC activity. SNP suppression of ODC activity was previously described by Blachier et al. (7) in a colon carcinoma cell line. To confirm that the suppressive effects of SNP are due to NO rather than other by-products of SNP, we examined the effects of several other NO donors on ODC activity in MCT cells. Three different redox groups of NO donors were examined and compared to SNP (Fig. 1B). Primary cultures were not used because of their low inherent ODC activity. SNP markedly suppressed ODC activity in all cell lines examined.

SNP suppression of ODC activity by sodium nitroprusside (SNP). A: varying concentrations of SNP were added directly to the high-speed spin supernatant fraction of lysed MCT cell preparations in labeled ODC activity reaction. B: J774 (monocyte/macrophage), mMCM (mouse glomerular mesangial), ENDO (endothelial), NIH/3T3 (fibroblast like), Ras/3T3 (Ras-transformed NIH/3T3), and HT-1080 (fibrosarcoma) cell lines were collected, homogenized, and incubated for 1 h in the absence (control) or presence of 0.5 mM SNP before a high-speed spin and the labeled ODC activity reaction. Values are means ± SD of 3 observations. For A and B, all SNP-treated samples were significantly different from control groups (P < 0.001).
represented: NONOates as NO· donors, S-nitrosothiols (SNAP is a less toxic alternative to SNP, GSNO a physiological S-nitrosothiol), which release NO· and transfer nitrosonium (NO⁺) to sulfhydryl centers (44), and SIN-1 as a peroxynitrite (OONO⁻) generator. Cell lysates were incubated for 1 h in the absence or presence of NO donors, as indicated in Fig. 2A, before the ODC assay. All NO donors displayed the capacity to suppress ODC activity. Comparative NO end product generation by the various NO donors is shown in Fig. 2B.

Cytokine generation of NO suppresses ODC activity. To determine if NO suppression of ODC activity was of biological significance, we induced iNOS generation of NO by TNF-α (2.5 ng/ml) and IFN-γ (50 U/ml) in MCT cells. Cytokine stimulation significantly suppressed ODC activity (Fig. 3A). Incubating cells with L-NIL (50 mM), an iNOS selective inhibitor, markedly reduced NO generation in response to cytokines (Fig. 3B) and consequently reduced the suppressive effects of NO induction on ODC activity (Fig. 3A). Similar results were observed in the J774 cell line (not shown) or if lipopolysaccharide (20 ng/ml) and IFN-γ were used to induce NO generation in MCT cells (not shown).

Reversibility of ODC activity after NO treatment. To determine if NO inhibition of ODC activity involves nitrosylation of a sulfhydryl group, the effects of the thiol reductant DTT were evaluated. Cells were harvested and centrifuged as per MATERIALS AND METHODS, except that DTT was omitted from the ODC reaction buffer. Each sample was split and incubated in either the absence (control) or presence of 0.5 mM MAHMA NONOate for 10 min at room temperature. Varying concentrations of DTT, as shown in Fig. 4A, were then added to the control and NO-treated samples for 10 min before determination of ODC activity. MAHMA NONOate has a 1 min half-life, thereby alleviating the need to separate the NO donor from the sample by column purification before DTT addition. Increasing concentrations of DTT resulted in increased enzymatic activity of ODC in samples exposed to NO, relative to control samples in the presence of DTT alone (Fig. 4A).

The ability of DTT to reverse TNF-α- and IFN-γ-mediated ODC suppression was also examined. Cyto-
kines were administered to the cells for varying lengths of time, as shown in Fig. 4B. Cells were then washed in PBS and harvested in ODC reaction buffer without DTT and frozen. Thawed samples were centrifuged, and the supernatants were aliquoted into reaction tubes containing either 0.005 or 10 mM DTT for 15 min at room temperature before assessment of ODC activity. The percent increase of ODC activity from 0.005 to 10 mM DTT, relative to non-cytokine-treated samples (0-h cytokine stimulation), is shown in Fig. 4B for each time point. DTT reversal of ODC activity in cells stimulated for 24 h with TNF-α and IFN-γ was ineffective (not shown). However, there was a significant effect of increased DTT concentration in cytokine-treated cells at 8, 10, and 12 h (Fig. 4B). A corresponding increase in NO end product generation was observed at these time points (Fig. 4C).

Polyamine transport is suppressed in the presence of NO. Administration of DFMO, a potent specific inhibitor of ODC, results in a compensatory increase in polyamine uptake (8). We evaluated the effects of NO suppression of ODC activity on polyamine transport to determine if non-antizyme-mediated inhibition of ODC consequently results in compensatory induction of transport. Such an effect could reestablish intracellular polyamine levels, negating polyamine limitation as a mechanism by which NO could inhibit proliferation. Although exceptions are known, most cells use a single transporter for putrescine, spermidine, and spermine (41). Competition studies in MCT cells suggest a single polyamine transporter (not shown). [3H]Putrescine uptake by MCT cells was therefore used as an indicator of polyamine transport in these studies. Transformed cells commonly display increased polyamine uptake, and Fig. 5 demonstrates rapid transport of [3H]putrescine into MCT cells. The effects of DFMO require time to evolve. The presence of DFMO did not change putrescine uptake relative to control values at 6 h, but increases were observed at 24 (105% increase over control) and 48 h (270% increase over control; not shown). In the presence of NO donors a dose- and time-dependent suppression of putrescine uptake was observed (Fig. 5). ENDO cells treated with SIN-1 gave similar results (not shown).

Fig. 4. Assessment of nitrosylation of ODC by dithiothreitol (DTT) reversal of NO effects on ODC activity. A: MCT cells were prepared for ODC activity determination, except for exclusion of DTT in ODC reaction buffer. Cytosolic extracts were split and incubated in the absence (control) or presence of 0.5 mM (Z)-1-N-methyl-N-[6-(N-methylammoniohexylamino)diazen-1-ium-1,2-diolate (MAHMA NONOate) for 10 min. This was followed by a 10-min DTT incubation of control and NONOate-treated samples at the concentrations indicated. ODC activity was then evaluated. B: DTT reversal of TNF-α- and IFN-γ-mediated ODC suppression. MCT cells were incubated with TNF-α and IFN-γ for indicated times and then harvested in DTT-free ODC reaction buffer. After preparation, samples were split and incubated for 15 min in either 0.005 or 10 mM DTT before ODC activity assay. Nos. represent percent increase in ODC activity from 0.005 to 10 mM DTT, relative to non-cytokine-treated control group (set to 0%). C: NO2 determination was performed on media from B. Values are means ± SE of 3 observations. For B and C, *P < 0.01, **P < 0.001 compared with control group.

Fig. 5. Effect of difluoromethylornithine (DFMO) and NO donors on polyamine transport. MCT cells were grown in 6-well plates to ~80–90% confluent. Cells were then incubated in absence (Control) or presence of DFMO (5 mM), a synthetic suicide inhibitor of ODC, or NO donors S-nitroso-L-glutathione (GSNO; 0.1 and 0.5 mM), (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA NONOate; 0.1 and 0.5 mM), or 3-morpholinosydnonimine (SIN-1; 0.5 mM) for 6 or 24 h before a 5-min uptake period of [3H]Putrescine. Values are means ± SD of 3 observations. *P < 0.05, **P < 0.001, ***P < 0.0001 compared with control group.
Cytokine administration suppresses polyamine transport. To determine the biological significance of NO suppression of polyamine transport we used TNF-α and IFN-γ to induce iNOS generation of NO in MCT cells, as in Fig. 3. Cytokine stimulation significantly suppressed [3H]putrescine uptake in MCT cells (Fig. 6A). Incubating cells with L-NIL (Fig. 6), an iNOS selective inhibitor, or 2,4-diamino-6-hydroxy-pyrimidine (DAHP) (not shown), an inhibitor of the synthesis of the NOS cofactor tetrahydrobiopterin (BH₄), markedly reduced NO generation in response to cytokines (Fig. 6B), but had little effect in arresting cytokine-mediated suppression of [3H]putrescine uptake (Fig. 6A). TNF-α and IFN-γ appear to act additively in suppressing polyamine transport (Fig. 6A).

DISCUSSION

Limiting intracellular polyamine stores required for entry and progression through the cell cycle may prove a viable way of inhibiting proliferation. We have recently observed suppression of polyamine biosynthesis and transport in a transformed cell line through polyamine-independent induction of antizyme (39). Rapid depletion of putrescine and spermidine was observed with consequent inhibition of proliferation. Here we demonstrate that NO is capable of suppressing both polyamine biosynthesis and transport.

NO donors suppress the activity of the polyamine biosynthetic enzyme ODC in MCT cell extracts and in extracts of all cell lines examined (Fig. 1B), suggesting this effect is not cell type specific. NO donors differed in their ability to suppress ODC activity (Fig. 2A). Varying cellular responses to different redox forms of NO are previously noted (13). NO generated through cytokine induction of iNOS is also capable of suppressing ODC activity. This suppression is diminished in the presence of L-NIL, a selective inhibitor of iNOS (Fig. 3A). These data suggest that the effect on the enzyme is NO mediated and that cytokine-inducible levels of NO are capable of suppressing ODC activity.

Intracellular polyamine levels are autoregulated through translational frameshift induction of a unique regulatory protein, antizyme (31). Inhibition of polyamine biosynthesis occurs by antizyme binding to ODC and suppressing ODC activity as well as rendering the enzyme susceptible to proteolysis by the 26S proteasome in a ubiquitin-independent manner (21, 34). A 30- to 60-min lag phase is prerequisite to the onset of antizyme inhibition, with maximum inhibition observed by 4 h in MCT cells (39). Suppression of ODC activity by NO is rapid, as demonstrated in Fig. 1A, where the NO donor SNP is added directly to the enzymatic reaction. These results are not temporally compatible with those of antizyme. However, a rapid transition of enzymatic activity could result from cysteine nitrosylation, which has been shown to modulate the activity of several enzymes (14–16, 36, 43, 44). Active ODC is a homodimer containing a cysteine within the active site at the dimer interface (10). A C360A mutation of this cysteine in ODC has revealed this residue to be an absolute requirement for full activity (9, 10). Because of the rapid interchange of enzymatic subunits under physiological conditions (10), this cysteine may be subject to nitrosylation. DTT reversal of NO's inhibitory effects on ODC activity (Fig. 4) is consistent with nitrosylation as the mechanism of inhibition (19, 32). In our hands, TNF-α and IFN-γ stimulation of MCT cells results in an observable increase in iNOS mRNA, as determined by Northern blotting (unpublished data), and an increase in NO end product generation by 6 h (Fig. 4C). DTT reversal of cytokine-mediated ODC suppression at 8, 10, and 12 h (Fig. 4B) is in temporal accord with these observations. The ability of DTT to reverse ODC inhibition by TNF-α and IFN-γ shortly after induction of NO generation suggests nitrosylation as an early event in this response. The ineffective reversal of ODC activity by DTT in cells stimulated for 24 h (not shown) implies that other cytokine-mediated mechanisms suppress ODC activity by this later time point. We show here that
cytokine-mediated suppression of ODC activity in cells treated for 24 h can be largely averted in the presence of the iNOS inhibitor L-NIL (Fig. 3), suggesting that NO may be required for induction of some of these other mechanisms. Cytokines may additionally suppress ODC activity through NO-independent mechanisms. The time frame and level(s) of cytokine regulation of ODC activity require further investigation.

Suppression of polyamine transporters is a second function in the regulation of intracellular polyamines ascribed to antizyme (33, 47). It is this two-pronged mechanism of antizyme that distinguishes it from synthetic ODC inhibitors, such as DFMO. DFMO inhibition of ODC causes a compensatory induction of polyamine transporters, allowing polyamine uptake to substitute for de novo biosynthesis (8) (Fig. 5). In vivo, tumor cells can access polyamines released into the circulation by normal cells, wasting cells, gut flora, and dietary sources. A compensatory increase in polyamine transport can explain why drugs targeted exclusively at inhibition of ODC often did not result in the expected levels of polyamine depletion (6, 23, 30), thus complicating experimental interpretation and yielding less than anticipated results in clinical trials (22). However, coadministration of DFMO with a polyamine-free diet (38, 42) or a polyamine transport inhibitor (4) was beneficial in experimental cancer models in vivo and in vitro, respectively. We therefore addressed the effects of NO donors on polyamine transport as well as polyamine biosynthesis. Administration of NO donors does not result in the compensatory increase observed with DFMO, but rather a suppression of polyamine uptake (Fig. 5). At 6 h, only the high concentrations of NO donors demonstrate an effect on polyamine uptake. By 24 h the effects of the NO donors, as well as DFMO, are more apparent (Fig. 5). Thus NO modulation of polyamine transport is a gradual process. The time course for the inhibition of polyamine transporters by antizyme is similar to that for ODC, that is, it occurs rapidly after a short lag phase of 1 h (39, 47). Therefore, the slow onset of polyamine transport suppression by NO occurs in a manner that appears temporally distinct from a directly mediated event, such as nitrosylation, or that described for antizyme. Whether NO indirectly affects antizyme induction has yet to be investigated.

The iNOS-inducing cytokines TNF-α and IFN-γ also suppressed polyamine uptake in MCT cells. However, neither L-NIL (Fig. 6A) nor DAHP, a NOS cofactor inhibitor (not shown), was able to substantially attenuate these effects. It should be noted that neither L-NIL (Fig. 6B) nor DAHP (not shown) was able to completely inhibit cytokine-stimulated NO generation. Therefore we cannot unequivocally state that the effects of IFN-γ on polyamine transport are NO independent. However, as shown in Fig. 6B, inhibition of polyamine transport by TNF-α cannot be attributed to NO generation. Although our data demonstrate that NO donors suppress polyamine transport (Fig. 5), the presence of cytokines establishes a complex biological setting where polyamine transport may be affected by a variety of mediators (for review, see Refs. 18, 40). Furthermore, the inability of DTT to effectively reverse cytokine inhibition of ODC activity at 24 h (not shown) supports a complex and redundant regulation of intracellular polyamines during inflammation.

These data present NO as the first described endogenous molecule with the capacity to inhibit both polyamine biosynthesis and transport in a manner that appears independent of antizyme. Direct inhibition of ODC by NO, without a compensatory increase in polyamine uptake, could allow depletion of intracellular polyamine levels required for replication. NO inhibition of ODC activity could also provide a direct mechanism for the temporal interregulation of these two arginine pathways during inflammation, as has been observed in wound healing and experimental glomerulonephritis (see Fig. 7) (2, 11, 25). The capacity of the cytokines TNF-α and IFN-γ to suppress polyamine uptake could effectively contribute to the inhibitory effects of NO on polyamine biosynthesis in regulating intracellular polyamines.

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Address for reprint requests and other correspondence: J. Satriano, UCSD/VAMC, Div. Nephrology-Hypertension, mail code 9111H, 3350 La Jolla Village Dr., San Diego, CA 92161 (E-mail: jsatriano@ucsd.edu).

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